ROLES OF CONFINED PLACENTAL MOSAICISM (CPM) AND *H19/IGF2* IMPRINTING IN PREGNANCIES DERIVED FROM INTRACYTOPLASMIC SPERM INJECTION (ICSI)

by

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Abstract

In contrast to the success of ICSI in treating male infertility, concerns have been raised about the health outcomes of the children conceived through this procedure. Cohort studies have shown that the ICSI population has an increase in low birth weight (LBW), birth defects, chromosomal abnormalities, and imprinting disorders. However, the underlying causes remain unknown. Two potential risk factors for these negative pregnancy outcomes, particularly for LBW, were investigated in this study – Confined Placental Mosaicism (CPM) and epigenetic defects at the differentially methylated region (DMR) of *H19/IGF2*. CPM was examined in villi from thirty post-delivery placentas derived from ICSI after confirming a normal karyotype in cord blood. Subsequently, the parental origin was determined in detected CPM as well as in non-mosaic chromosomal abnormalities ascertained through spontaneous abortions. When a paternal origin was confirmed, aneuploidy in sperm from the father was investigated. Finally, methylation pattern at two CpG sites from the DMR of *H19/IGF2* was quantitatively analyzed in placentas from ICSI pregnancies with LBW (n=10) and with normal BW (n=12). Placentas from natural conceptions (n=14) served as controls.

Among the thirty placentas, one monosomy X case was detected from a conceptus with a normal blood karyotype and pregnancy outcomes. Thus, the incidence of CPM was 3.3% in the study population, which is not significantly different from the rate observed in the general population (6.0%). A paternal origin was revealed in one out of four cases, which include chromosome abnormalities derived from CPM (n=2) and spontaneous abortions (n=2). In the paternally inherited t(13;21) case, 88.39% of the sperm were normal or balanced, and 7.29% were nullisomic or disomic for chromosome 13 and 21. In methylation analysis, differences were not found among the three groups; however, hypomethylation (<33%) was exclusively detected from the ICSI-LBW group. Taken together, the roles of CPM and epigenetic alteration at the DMR of *H19/IGF2* were not apparent in ICSI pregnancies studied, regardless of the pregnancy outcomes. However, due to the limited sample size, we cannot exclude the possibility that these factors may play a role in certain cases that were not included in the present study.

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List of Abbreviations

ADP	Adenosine diphosphate
AR	Androgen receptor
ART	Assisted reproductive technologies
AS	Angelman Syndrome
AZF	Azoospermia factor region
BW	Birth weight
BWS	Beckwith-Wiedemann Syndrome
C	Cytosine
CBAVD	Congenital bilateral absence of vas deferens
CCD	Charge-coupled device
CF	Cystic fibrosis
CFTR	Cystic fibrosis trans-membrane conductance regulator
CGH	Comparative genomic hybridization
CPM	Confined placental mosaicism
CTCF	CCCTC-binding factor
CVS	Chorionic villus sampling
DAPI	4',6-Diamidine-2'-phenylindole
DMR	Differentially methylated region
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
DSA	3,5-Di-iodosalicytic acid
DTT	Dithiothreitol
EDTA	Ethylenediamine tetra-acetic acid
EEM	Extraembryonic mesoderm
EM	Embryonic lineage
EX	Extraembryonic lineage
F	Forward
FISH	fluorescent in situ hybridization
FITC	fluorescein-12-dUTP
FSH	Follicle stimulating hormone
GnRH	Gonadotropin-releasing hormone
HBSS	Hank's Balanced Salt Solution
hCG	Human chorionic gonadotropin
HDAC	histone deacetylase
HTF	Human tubal fluid
IC	ICSI controls
ICE	Interchromosomal effects
ICSI	Intracytoplasmic sperm injection
IGF2	Insulin-like growth factor 2
IL	ICSI-LBW
IUD	Intrauterine death
IUGR	Intrauterine growth restriction
IVF	In vitro fertilization
IVM	In vitro maturation
LH	Luteinizing hormone

LTC	Long term culture
MESA	Microsurgical epididymal sperm aspiration
Mesen	Mesenchyme
MI	Meiosis I
MII	Meiosis II
MPI	Maintenance of paternal imprint
Ms-SNuPE	Methylation sensitive Single Nucleotide Primer Extension
NA	Not available
NC	Natural conceptions
No.	Number
NOA	Non-obstructive azoospermia
NS	Not significant
OA	Obstructive azoospermia
OAT	Oligoasthenoteratozoospermia
OMIM	Online Mendelian Inheritance in Man
P	Short chromosome arm
PAGE	Polyacrylamide
PAR	Pseudoautosomal region
PBS	Phosphate Buffered Saline
PCR	polymerase chain reaction
PHA	phytohaemagglutinin
PP	Placental protein
PZD	Partial zona dissection
q	Long chromosome arm
R	Reverse
RNA	Ribonucleic acid
RT	Robertsonian translocation
SA	Spontaneous abortion
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SGA	Small for gestational age
SRS	Silver-Russell syndrome
SRY	Sex-determining region Y
SSC	Sodium chloride sodium citrate
STC	Short term culture
SUZI	Subzonal insemination
TE	Tris-EDTA
TESE	Testicular sperm extraction
TRITC	Tetramethylrhodamine-5-dUTP
Troph	Trophoblast
UBE3A	Ubiquitin protein ligase E3A
UPD	Uniparental disomy
US	United States
WHO	World Health Organization
WK	Week

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CHAPTER 1. Introduction

1.1 Human reproduction

1.1.1. Basics of cell divisions and possible errors

Human reproduction involves two major processes – gametogenesis and fertilization/implantation (Carslon, 2004). These events comprise a series of cell divisions, namely, mitosis and meiosis. Somatic cell divisions and multiplication of the germinal stem cells are facilitated through mitosis. Meiosis, unique to gametogenesis, reduces the number of the chromosome complement from diploid to haploid in the gametes. Both mitosis and meiosis are subjected to formation of aneuploidy if proper segregation of chromosomes fails.

1.1.1.1. Mitosis

Mitosis is the basic mechanism of cell proliferation, by which a single parent cell gives rise to two genetically identical daughter cells. There are four stages in mitosis – prophase, metaphase, anaphase, and telophase. During prophase, the chromatin, which was duplicated prior to mitosis, condenses into chromosomes, each consisting of two identical chromatids held together by a centromere. As the nuclear envelope dissolves, centriosomes migrate to opposite sides of the nucleus and begin to develop the mitotic spindle. At metaphase, following prophase, the growing spindle microtubules start to interact with chromosomes and align them on the equator of the spindle. Anaphase begins with the separation of centromeres, which leads to a splitting of sister chromatids into individual daughter chromosomes. These daughter chromosomes move toward opposite poles of the spindle as the kinetochore microtubules shorten. Once chromosomes reach the poles, the cell enters telophase, when the nuclear envelop reforms and chromosomes uncoil back into chromatin. Simultaneously, cytokinesis occurs, which splits the cytoplasm into two compartments and completes the formation of two daughter cells (Miller and Therman, 2001).

1.1.1.2 Meiosis

Meiosis is a process of cell division by which chromosome number is reduced from diploid (2n) to haploid (n). It comprises of two successive nuclear divisions, meiosis one (MI) and meiosis two (MII). Prior to meiosis, each chromosome is replicated to form two identical sister chromatids in interphase. MI then begins, and consists of four stages: prophase, metaphase, anaphase, and telophase (Alberts *et al.*, 1983). MI is also called reductive division because the chromosome complement is reduced from 2n to n. At prophase of MI, replicated homologous chromosomes pair, mediated by a protein structure known as the synaptonemal complex, and recombination occurs between non-sister (from different parents) chromatids. The recombination sites, also known as chiasmata, play an additional role of keeping the two homologous chromosomes together throughout prophase I. After Prophase I, homologous chromosomes are aligned at the equatorial plate in metaphase I, and then separate during anaphase I. Subsequently at telophase I, two daughter cells are formed, each containing a haploid (n) number of chromosomes (Miller and Therman, 2001).

In MII, the two chromatids in each chromosome are further separated, without an intervening round of DNA replication. The end product of meiosis is four haploid (n) daughter cells (Miller and Therman, 2001). Meiosis in spermatogenesis gives rise to four functional spermatozoa; whereas in oogenesis, only one daughter cell becomes a functional oocyte and the rest degrades eventually (Elder and Dale, 2001).

1.1.1.3 Aneuploidy formation

Aneuploidy arises from segregation errors during mitosis and meiosis. The predominant mechanism for aneuploidy is non-disjunction, in which homologous chromosomes or chromatids migrate to the same nuclear pole (Hassold and Hunt, 2001). Alternatively, anaphase lag, which is a failure to incorporate a chromosome into the daughter cells, can also generate aneuploidy, specifically monosomy (Warburton 1987).

Mitotic non-disjunction, occurring in post-zygotic somatic cells, results from sister chromatids failing to separate at anaphase. Mitotic non-disjunction leads to a mixed population of trisomic and monosomic cells (Figure 1.1). Meiotic non-disjunction occurs

during gametogenesis at either anaphase I or II. When a resulting nullisomic or disomic gamete (gametes being normally monosomic for each chromosome) is involved in fertilization, an aneuploid zygote is produced (Figure 1.2). MI non-disjunction has been suggested to result from reduced recombination (Hassold and Hunt, 2001). It has been reported that 85% of 47, XXY cases result from failure of recombination between the pseudoautosomal region of the X and Y chromosomes (Hassold *et al.*, 1991). The effect of reduced recombination on aneuploidy is also evidenced in small chromosomes, which contain fewer recombination sites (Thomas *et al.*, 2000; Cupisti *et al.*, 2003). In addition to the reduction or absence of recombination, a position shift of chiasmata toward the centromere has also been suggested to cause a MI non-disjunction (Hassold *et al.*, 1995). MII non-disjunction occurs through a mechanism similar to that of mitotic non-disjunction: by chromatid separation failure. Interestingly, recombination errors is also thought to be responsible for MII non-disjunction if the pericentromeric exchange is present, as seen in trisomy 21 cases (Lamb *et al.*, 1996).

The most common origin for autosomal trisomy in human, detected predominantly in spontaneous abortions, is a maternal MI non-disjunction. This becomes more prevalent with age; thus, suggesting that the spindle or chiasmata holding the chromosomes in place may deteriorate during the prolonged MI arrest in females (Hawley *et al.*, 1994; Lamb *et al.*, 1996). Nevertheless, sex chromosome aneuploidy has been linked to paternal errors, presumably due to limited pairing regions between the X and Y chromosomes (Ma *et al.*, 2006). A paternal age effect on segregation errors has yet to be determined (Nicolaidis and Petersen, 1998).

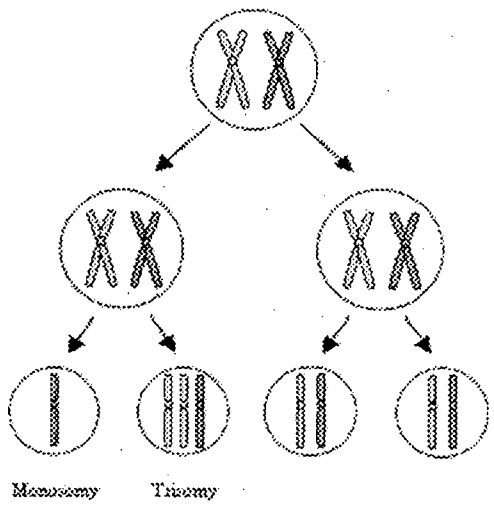
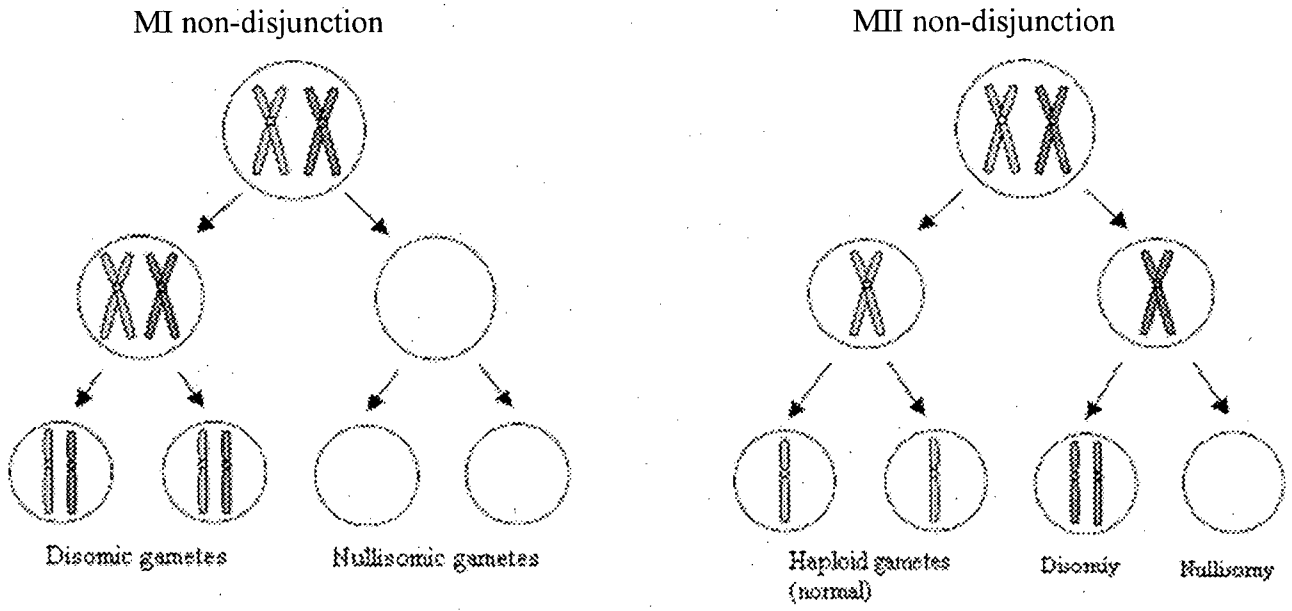


Figure 1.1 Aneuploidy due to mitotic non-disjunction. Failure of sister chromatid separation leads to a monosomic and a trisomic cell. (www.medgen.ubc.ca/wrobinson/mosaic/mos_how.htm)

a) Generation of aneuploid gametes due to meiotic non-disjunction



b) Generation of aneuploid zygotes upon fertilization

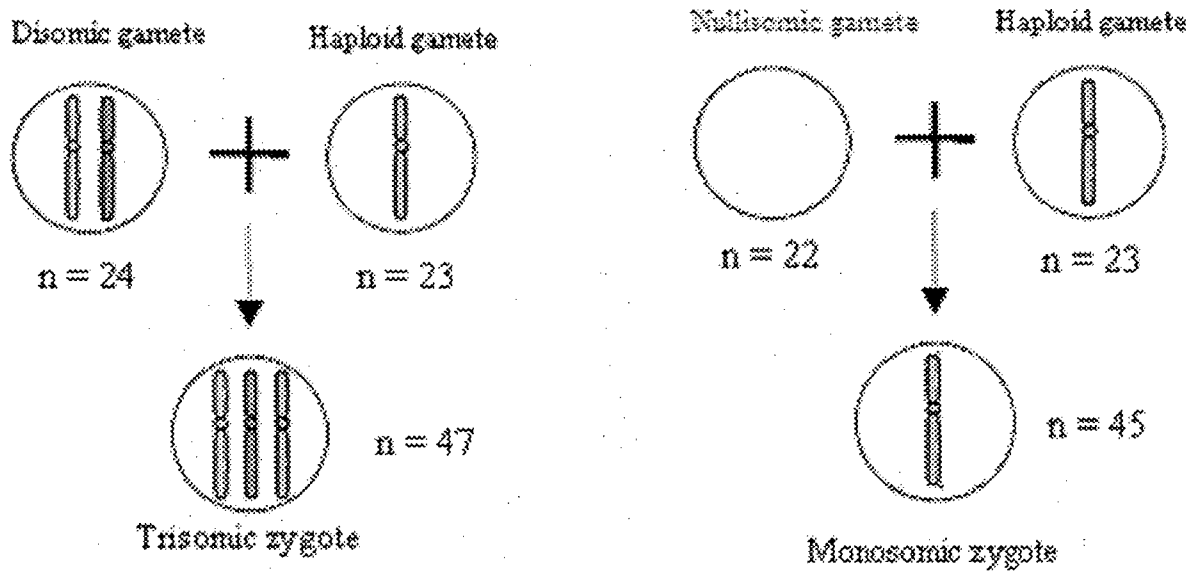


Figure 1.2 MI non-disjunction leads to disomic and nullisomic gametes, whereas MII non-disjunction results in normal haploid, hyperploid, and hypoploid gametes. b) Fertilization of a disomic gamete with a normal gamete results in a trisomic zygote; fertilization of a nullisomic gamete with a normal gamete results in a monosomy zygote. (Modified from www.medgen.ubc.ca/wrobinson/mosaic/mos_how.htm).

1.1.2 Gametogenesis

Gametogenesis is a series of changes by which diploid germ cells transform into specialized haploid gametes, i.e. oogenesis in the female and spermatogenesis in the male (Figure 1.3). Gametogenesis consists of four typical phases: (1)migration of primordial germ cells, the precursors of gametes, to the gonad (2) mitotic division of germinal stem cells (3) reduction to haploid by meiosis (4) Structural and functional maturation (Carlson, 2004).

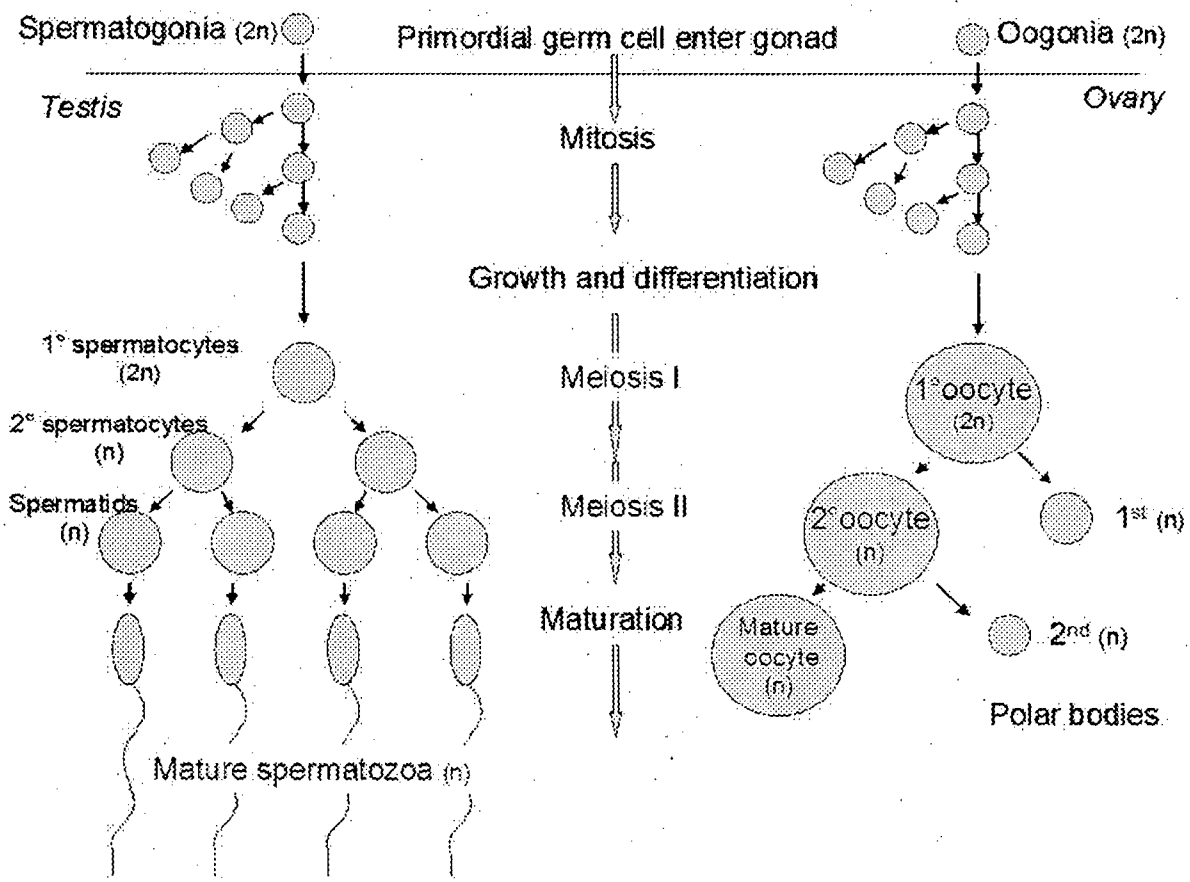


Figure 1.3 Basic scheme of gametogenesis in males and females.

1.1.2.1 Oogenesis

Oogenesis begins in the developing ovary at approximately the eighth week of gestation. Primordial germ cells become oogonia as they enter the ovary. Oogonia undergo a period of intensive mitotic division from the second to the fifth month of gestation, increasing their number from a few thousand to about seven million. However, most of them degenerate through a process known as atresia, which continuously decrease the number of germ cells until menopause. By the fifth month of gestation, all the surviving oogonia develop into primary oocytes as they enter the first meiotic division (Elder and Dale, 2001). Meiosis, however, halts in the diplotene stage of prophase I until just prior to ovulation at puberty. During this extended period, the primary oocytes become surrounded by zona pellucida and cortical granules, and actively synthesize and store bio-molecules required for development. As primary oocytes grow, they become further enveloped by layers of follicle cells such as granulosa and theca cells. The primary oocytes together with their surrounding follicle cells become primary follicles. Nutrients and meiosis inhibiting substances enter the primary follicles, and allow the oocytes to grow in size while remaining at prophase of MI (Alberts *et al.*, 1983).

At puberty, follicular maturation and ovulation are regulated by pituitary gonadotropins, namely follicle stimulating hormone (FSH) and luteinizing hormone (LH), which are in turn controlled by gonadotropin hormone releasing hormone (GnRH) produced from the hypothalamus. At the beginning of each menstrual cycle, the low level of circulating estrogen and progesterone leads to an increased release of FSH and LH as part of the negative feedback system. FSH enhances the production of granulosa cells and the development of follicles to fluid-filled secondary follicles. LH stimulates the theca cells to secret androgens, which are subsequently converted to estrogen by the granulosa cells. In response to the peak of estrogen production around the time that follicles reach maturity, a surge of LH is induced, which initiates ovulation by disrupting the supplement of nutrients and meiosis inhibiting factors to the ovulating follicles. This allows the oocyte to resume meiosis, and complete MI with extrusion of the first polar body. However, the oocyte is arrested again at metaphase of MII until fertilization (Alberts *et al.*, 1983).

1.1.2.2 Spermatogenesis

Testicular development begins in male embryos after the arrival of primordial germ cells to the genital ridge and expression of the sex-determining region Y (SRY) gene is initiated. However, unlike oogenesis, the onset of spermatogenesis comes at puberty when the level of FSH and LH is elevated (Elder and Dale, 2001). Also, in contrast to the prolonged process of oogenesis, each cycle of spermatogenesis is estimated to take approximately 64 days (Carlson 2004).

Spermatogenesis occurs in the seminiferous tubules of the testis and consists of three major stages – spermatogoniogenesis, meiosis, and spermiogenesis. Spermatogoniogenesis begins with successive mitotic divisions of type A spermatogonia at the basal compartment of the seminiferous epithelium. In contrast to oogenesis, this mitotic proliferation continues throughout life in males. During each wave of mitosis, about 40% of the undifferentiated type A spermatogonia cells succumb to apoptotic cell death, while the remaining continue to proliferate. Some of the surviving type A spermatogonia develop into type B spermatogonia, which differentiate into meiotic preleptotene primary spermatocytes. These primary spermatocytes move into the adluminal compartment and enter into meiosis. After the first meiotic division, two secondary spermatocytes are produced from each primary spermatocyte. Subsequently, the two secondary spermatocytes divide into four immature haploid spermatids by the second meiotic division (Alberts *et al.* 1983; Carlson, 2004). Meiosis of spermatocytes is thought to possess a more stringent selection over meiotic errors than in oogenesis. Two additional checkpoints, pachytene checkpoint and spindle assembly checkpoint, assure DNA quality, chromosome alignment, and spindle integrity (Hunt and Hassold, 2002). Upon the completion of meiosis, spermatids undergo a differentiation process called spermiogenesis to become mature spermatozoa. Spermiogenesis involves a series of morphological changes such as condensation of chromatin by replacing histones with protamines, acrosome development, formation of middle piece and tail, and removal of majority of the cytoplasm. The mature spermatozoa migrate from the seminiferous tubules to the epididymis, where they obtain the last modification, a glycoprotein coat, at which point, the spermatozoa are finally ready to fertilize oocytes (Carlson, 2004).

1.1.3 Fertilization and implantation

Fertilization takes place in the fallopian tube one or two days after an ovum is released (Norwitz *et al.*, 2001). Prior to fertilization, sperm are further capacitated by secretions in the female genital tract including the removal of the epididymal and seminal plasma protein coating, and an alteration in the glycoproteins in the sperm plasma membrane. As the first sperm attaches to the zona pellucida, the contents of its acrosome, mainly enzymes, are released to assist in the penetration of the ovum and to initiate a series of chemical reactions. Depolarization was induced by activating potassium channels by the spermatozoon, resulting in an outward current in the oocyte plasma membrane. Meanwhile, a cortical reaction occurs, which induces a calcium influx into the ooplasm and a structural change in the surface glycoprotein. Both the depolarization and cortical reactions prevent other sperm from fertilizing the ovum (Elder and Dale, 2001). As mentioned earlier, fertilization triggers the completion of meiosis and the formation of an ovum containing the haploid female pronucleus and a second polar body, which eventually degenerates. Fertilization also transforms the sperm nucleus to a male pronucleus by breaking down the sperm nuclear envelop, decondensing the chromatin, and reforming a pronuclear envelop. The completion of fertilization is marked by the fusion of the two pronuclei and the formation of a zygote (fertilized ovum). After fertilization, the zygote increases its mass by mitotic divisions and develops into a morula in approximately three days. The morula travels down the fallopian tube to the uterine cavity, and further develops into a blastocyst, which implants into the uterine lining (Carlson, 2004). After implantation, the different cell lineages in the blastocyst develop into embryo and placenta, which grow dependent on the maternal nourishment and eventually lead to a birth (Elder and Dale, 2001).

1.2 Male infertility

1.2.1 Overview of male infertility

The chance for a fertile couple to achieve a pregnancy is expected to be 25% for each month, and 90% for a year under unprotected intercourse (Spira, 1986). Those who fail to conceive after a year of regular unprotected intercourse are categorized as infertile. It has been estimated that 15% of couples are suffering from infertility, of which about one third is linked to solely male factors, and another third of the cases are caused by a combination of male and female factors. While 30-50% of those cases remain idiopathic, male factors contribute significantly to infertility (Bhasin *et al*, 1994). The World Health Organization (WHO) established a guideline to identify the population of males who have a reduced ability to conceive naturally and thus are sub-fertile by evaluating sperm according to three parameters: sperm concentration, motility, and morphology (WHO, 1999). *Oligozoospermia* is diagnosed when the sperm concentration is lower than 20 million sperm per milliliter of semen; *asthenozoospermia* describes the condition that less than 50% of the sperm are motile; and *teratozoospermia* is defined as less than 70% of the sperm with normal morphology. Low sperm concentration, and abnormal levels of motile and amorphous sperm can be present in isolation or in combination in a given infertile patient. The condition where the patient has no sperm in ejaculated semen sample is known as *azoospermia*. Azoospermia can be further sub-divided into non-obstructive azoospermia (NOA) and obstructive azoospermia (OA) (Chow and Cheung, 2006).

1.2.2 Genetic causes for male infertility

The etiology of male infertility is multi-factorial, primarily involving defects in sperm production, sperm dysfunction, and transport. These defective functions are thought to result from pituitary disorders, varicocele, cryptorchidism, anti-sperm antibodies, testicular cancer, and other unknown pathogenic factors (Sigman and Howards, 1998). However, it is almost impossible to exclude genetic contributions from these physical, endocrinal, and immunological explanations (Chow and Cheung, 2006). In this chapter, the genetic causes

for male fertility are reviewed with focus on chromosome abnormalities. Other important contributing factors such as cystic fibrosis (CF), Y-chromosome microdeletions, and sperm DNA fragmentation are also discussed.

1.2.2.1 Chromosomal abnormalities

The common types of chromosome abnormalities associated with male infertility are: somatic abnormalities, which primarily include sex chromosome aneuploidy and structural rearrangement, and germ cell specific chromosomal abnormalities.

1.2.2.1.1 Somatic chromosome abnormalities

The incidence of chromosomal abnormalities in infertile men is 7.1% on average, ranging from 2.2% to 14.3%. This is 10 to 20 fold higher than the general male population in which the rate of chromosomal abnormalities is 0.7-1% (Retief *et al.*, 1984; Matsuda *et al.*, 1992). Specifically, a karyotype abnormality is detected in approximately 14.3% of azoospermic and 6.5 % of oligozoospermic patients (Nagvenkar *et al.*, 2005). Men with constitutional chromosome abnormalities have an increased risk to produce chromosomally abnormal sperm and offspring (Shi and Martin, 2000; Ma *et al.*, 2003). Sex chromosome aneuploidy and balanced structural rearrangement have been predominantly identified in infertile men, however, other aberrations such as supernumerary marker chromosomes and ring chromosomes have also been observed (Gekas *et al.*, 2001).

Sex chromosome aneuploidy is present in 3.7% of infertile men, but only in 0.3% in the general population (Gekas *et al.*, 2001). Individuals with Klinefelter's syndrome (47, XXY karyotype) typically have testicular atrophy and frequently have non-obstructive azoospermia. In some cases, sperm can be found in the semen; however, the rate of sex chromosome disomy (2-25%) in the sperm is significantly higher than that in the general population. A less common sex chromosome aberration is 47, XYY, which is present in 0.32 % in the infertile population (Gekas *et al.*, 2001). Individuals with this aberration have also been reported to have a higher incidence of sperm aneuploidy (0.3-15%) (Shi and Martin, 2000).

Translocations are the structural chromosomal rearrangements detected most frequently in infertile men. Gekas *et al.* performed cytogenetic investigation in 2,196 men undergoing intracytoplasmic sperm injection (ICSI), and identified reciprocal translocation, Robertsonian translocation, and inversion in 1.2%, 0.8%, and 0.1% on the study population respectively. Men carrying a balanced reciprocal or a Robertsonian (translocation between acrocentric chromosomes) translocation often have problems with fertility but are otherwise phenotypically normal (Morel *et al.*, 2004; Scriven *et al.*, 2001; Hatakeyama *et al.*, 2006). Structurally rearranged chromosomes may produce chromosomally unbalanced gametes depending on the segregation pattern of quadrivalent (for reciprocal) or trivalent (for Robertsonian) during meiosis. Consequently, abnormal germ cells are arrested at cell cycle check-points before maturation or escape the check points, producing chromosomally abnormal sperm.

Since fluorescent in situ hybridization (FISH) became available, a number of studies have been performed to investigate meiotic segregation patterns in sperm from men carrying translocations using this technique. The majority of sperm from reciprocal translocation carriers were found to be normal or balanced, with individual values ranging from 19% to above 80% (Benet *et al.*, 2005). In contrast, investigations of meiotic segregation in Robertsonian translocation carriers have been carried out in fewer cases, with t(13;14) and t(14;21) being most frequently studied. According to available data, the prevalence of normal or balanced gametes has been found to range from 73.5% to 96.6% (Morel *et al.*, 2004). This high incidence of normal balanced segregations may result from the preference of a *cis* configuration of the trivalent during meiosis which promotes the alternate segregation pattern leading to normal or balanced chromosomal complement (Sybenga, 1975).

1.2.2.1.2 Germ line specific chromosomal abnormalities

Men with a normal somatic karyotype of 46, XY can produce chromosomally abnormal sperm if segregation errors occur during meiosis. The rate of sperm aneuploidy in infertile men has been reported to be higher than in the fertile controls (Shi and Martin, 2001). Most studies in the literature agree with the association between abnormal sperm parameters

and an increased rate of sperm aneuploidy (Rives. 2005).

It is now well established that males with reduced sperm concentration have a higher incidence of sperm aneuploidy (Miharu, 2005). While the aneuploidy rate varies dependent on the chromosome, the incidence of sex chromosome aneuploidy has been reported to be particularly pronounced by several studies (Nishikawa *et al*, 2000; Ohashi *et al.*, 2001; Martin *et al.*, 2003). This observation may be explained by an abnormal pairing of the pseudoautosomal region (PAR) between the X and Y chromosomes in infertile men. A reduced recombination in PAR has been associated with sex chromosome aneuploidy (Shi *et al*, 2001; Ma *et al.*, 2006).

The relationship between other abnormal semen parameters (motility and morphology) and sperm aneuploidy remains controversial. A few studies have reported an increased rate of sperm aneuploidy in asthenozoospermic patients (those with immotile sperm), although most other studies have shown no such correlation (Vegetti *et al.*, 2000; Hristova *et al.*, 2002). Asthenozoospermia induced by flagella deformities, however, was found to have a significantly higher risk for sperm aneuploidy (Rives *et al.*, 2005). The relationship between sperm morphology and aneuploidy has been investigated in (karyotypically normal) infertile men with different sperm morphological abnormalities – polymorphic teratozoospermia, globozoospermia, and enlarged head teratozoospermia. "Enlarged head teratozoospermia" has a particularly high risk of aneuploidy, ranging from 1.5% to 62.4% depending on the percentage of enlarged heads. The other two forms also had increased rates of aneuploid sperm, but the increases were moderate (Machev *et al.*, 2005).

It is worth noting that there is a considerable variability in results from studies investigating the correlation between sperm parameter and aneuploidy rate. The inconsistency probably results from 1) the variability of laboratory conditions such as sample size and number of sperm included for analysis; (2) the difficulty to isolate abnormal sperm parameters. Infertile men frequently possess abnormal values in more than one parameter; (3) the complexity of infertility pathogenesis. Other genetic factors may contribute to abnormal sperm parameters besides chromosomal abnormalities.

1.2.2.2 Other genetic causes for male infertility

1.2.2.2.1 Cystic fibrosis

Cystic fibrosis (CF) is a common autosomal recessive disorder among western European and Ashkenazi Jewish population. CF is caused by mutations in the cystic fibrosis transmembrane regulator (*CFTR*) gene, which maps to chromosome 7q31.2 (Stern, 1997). To date, more than one thousand different mutations have been identified with the ΔF508 deletion (a 3bp deletion responsible for the absence of phenylalanine at position 508) being most common. The CFTR protein is involved in regulation of airway chloride transport as well as sodium and water transport across the respiratory epithelium and sweat ducts. Its malfunction causes typical clinical phenotypes of CF including dehydrated airway mucus, pancreatic insufficiency, and high chloride concentration in sweat (Jarzabek *et al.*, 2004).

CF has also been closely associated with male infertility, accounting for 10% of obstructive azoospermic (OA) cases (Gazvani and Lewis-Jones, 2004). Up to 99% of adult men with CFTR mutation have Congenital Bilateral Absence of Vas Deferens (CBAVD) with which spermatozoa cannot be transported to the ejaculate, thus leading to OA (Jarzabek *et al.*, 2004). However, CBAVD does not always concur with other clinical phenotypes of CF and may not even have identifiable *CFTR* mutations. In fact, the majority of patients with CBAVD have only a single allele mutation (>50%), fewer patients have double allele mutations, and no mutation is detected in about one third of the cases (Chillon *et al.*, 1995). Intracytoplasmic sperm injection (ICSI) using sperm retrieved from the epididymis has accomplished a high pregnancy rate for CF-related OA patients (McCallum *et al.*, 2000). It is important that the underlying CFTR mutation can be passed to the offspring through the procedure. Therefore, genetic screening for the CFTR mutation is recommended to couples undergoing ICSI treatments (Gazvani and Lewis-Jones, 2004).

1.2.2.2.2 Y-chromosome microdeletion

The Y chromosome is important in sexual development and spermatogenesis. Deletions of the long arm of the Y chromosome can impair spermatogenesis, and is thought to affect about 10-15% of men with severe oligozoospermia or azoospermia (Pryor *et al.*,

1997). The Azoospermia factor (AZF) region, mapped to Yq11.23, has been identified as an essential genetic component for spermatogenesis (Tiepolo and Zuffardi 1976). The proximal, middle, and distal domains AZF are designated AZFa, AZFb, and AZFc, respectively. Microdeletions in each region have been associated with different types of infertility. Microdeletions in AZFa have been associated with the absence of germ cells; AZFb deletions cause meiotic arrest at spermatocytes; and AZFc deletions affect the maturation process of the post-meiotic germ cells (Vogt *et al.*, 1996). Krausz *et al.* reported that the most frequently deleted region is AZFc, accounting for approximately 60% of all AZF deletions, followed by 35% of the cases in AZFb, AZFb+AZFc, or AZFa+AZFb+AZFc. Deletions in the AZFa region account for only 5% of the cases (Krausz *et al.*, 2003).

Y-chromosome microdeletion in at least one of the AZF regions occurs in 3.5% of azoospermic or severe oligozoospermic patients (Vogt *et al.*, 1996). The most common AZFc deletions have been identified in 20% of azoospermic patients, of whom 50% had sperm in their testis. The same deletion was identified in 13% of men with severe oligozoospermia. Pregnancies were achieved in 56% of the patients aided by assisted reproductive technologies; however the deletion was transmitted to their sons (Silber *et al.*, 1998). No sperm was found from the testis of patients with deletions extending beyond the AZFc region (Silber, 1998); therefore, screening for Y deletions is recommended to infertile patients before they undergo the invasive testicular sperm extraction procedure (Brandell *et al.*, 1998).

1.2.2.2.3 Sperm DNA fragmentation

Sperm DNA integrity has been associated with male fertility potential. Abnormal sperm chromatin/DNA structure is thought to arise from three potential sources: 1) incomplete maturation of sperm due to diminished topoisomerase II activity (Bianchi *et al.*, 1996; Manicardi *et al.*, 1995); 2) incomplete apoptosis (Richburg, 2000); and 3) oxidative stress (Aitken *et al.*, 2003).

Damaged sperm DNA is increased in subfertile men, in spite of normal sperm parameters (Spano *et al.*, 2000). Thus, sperm integrity may provide an explanation for some idiopathic infertility and serve as an independent indicator. The probability of natural

fertilization becomes extremely low if the level of DNA damage exceeds 30% as detected by the sperm chromatin structure assay (SCSA) (Evenson *et al.*, 1999). Several studies have also linked abnormal sperm concentration, morphology and mobility with increased DNA fragmentation (Zini *et al.*, 2000, Host *et al.*, 1999; Gandini *et al.*, 2000). Although ART, particularly ICSI, may improve fertilization efficiency, studies have suggested that sperm DNA damage is associated with poor ART outcome, affecting embryo cleavage, blastocyst development, and post-implantation embryo development. Hence, the assessment of chromatin organization and DNA integrity has been suggested in the clinical practice (Erenpreiss *et al.*, 2006).

1.3 Intracytoplasmic sperm injection (ICSI)

1.3.1 Development of assisted reproductive technologies (ART) preceding ICSI

Currently, one in six couples worldwide experiences infertility (Anderson et al., 2004). A number of assisted reproductive technologies have been developed to fulfill the infertile couples' wishes to have their own biological children.

The beginning of ART can be traced back to two centuries ago. The first birth after artificial insemination, by which sperm is placed into the uterus or cervix using artificial means, was carried out in 1785. However, there was no further development until artificial insemination with frozen semen was successfully performed in 1954. About two decades later, the birth of Louise Brown was successfully facilitated by *in vitro* fertilization (IVF) followed by embryo transfer in 1978 (Edwards et al., 1980). Since then, there has been an remarkable development in this field, and more than one million of children are born world-wide using assisted reproductive technologies (ARTs) (Anderson et al., 2004).

IVF consists of ovarian stimulation, oocyte retrieval, fertilization *in vitro*, and embryo culture followed by embryo transfer to the uterus (Elder and Dale, 2001). It has proven to be effective in treating a variety of infertility including tubal dysfunctions, endometriosis, unexplained infertility and fertility involving male factors. However, the

success rate was extremely low when IVF was used to treat couples with severe male factor infertility. Thus, patients with less than 500,000 progressive motile sperm could not be included in the IVF treatment (Devroey and Van Steirteghem, 2004). Several techniques were developed to improve the conventional IVF. Partial zona dissection (PZD), which introduces a small opening in the zona pellucida to improve the access of sperm to the ooplasm, was first developed, but the results were inconsistent. Subsequently, subzonal insemination (SUZI) was invented, with which a few motile sperm were microinjected into the perivitelline space between the oocyte and zona pellucida. Similar to PZD, SUZI also could not provide a satisfying fertilization rate. In 1992, the first successful birth facilitated by ICSI was reported. In ICSI, a single viable sperm is drawn up into a micropipette and injected directly into the ooplasm (Palermo *et al.*, 1992). ICSI has improved fertilization rate and implantation rate compared to the previous technologies, and has been used world-wide to treat male factor infertility (Van Steirteghem et al., 2002).

1.3.2 Clinical practice of ICSI

ICSI is a breakthrough in the field of male infertility treatment as it requires only a single viable sperm. Ovarian stimulation and oocyte retrieval are similar to that for IVF. The female partner of a couple pursuing treatment with ICSI is given hormonal medications to stimulate the ovary to mature several oocytes simultaneously. Approximately twelve cumulus-oocytes are retrieved, but only MII oocytes, identified by the extrusion of the first polar body, will be used for ICSI. A single sperm is drawn up into a micropipette and injected directly into the cytoplasm of the oocyte with the use of a micromanipulator. Once fertilization is accomplished with ICSI, embryos are cultured *in vitro* (as with conventional IVF), and transferred to the uterine cavity (Palermo *et al.*, 1992). Embryo transfer is performed on day 2 at the four-cell stage in most centres. Day-3 transfer and blastocyst transfer on day 5 are carried out less frequently. Preferentially, two embryos with good quality are transferred; however the number may be increased depending on maternal age and rank of trial. Limiting the number of embryo transferred is thought to effectively prevent multiple birth (Van Steirteghem, 1999).

In general, ICSI is offered to infertile couples with severely defective semen parameters or with previously failed IVF cycles (Lewis and Klonoff-Cohen, 2005). ICSI can be used to treat some azoospermic patients, who have been considered sterile until ICSI became available, so long as a single viable sperm can be retrieved (Devroey and Van Steirteghem., 2004; Craft *et al.*, 1995; Schoysman *et al.*, 1993). Microsurgical epididymal sperm aspiration (MESA) is used to extract sperm from the epididymis in OA patients with blockages in the ductal system such as CBAVD in men affected by CF (Tournarye *et al.*, 1994; Schlegel *et al.*, 2004). Testicular sperm extraction (TESE) can be used to obtain sperm from the testis when no sperm is present in even the epididymis. Consequently, TESE can be performed on patients with abnormal spermatogenesis, endocrine defects, maturation arrest, hypogonadism, and hypospermatogenesis (Schelgel *et al.*, 2004, Silber *et al.*, 1995, Tournaye *et al.*, 1996).

1.3.3 Pregnancy outcomes of ICSI

In contrast to the remarkable success of ICSI in treating male infertility, concerns have been raised regarding the short- and long- term effects of this technology. When compared with natural conceptions, a higher incidence of low birth weight, multiple-birth, pregnancy loss, chromosomal abnormality, and congenital and developmental abnormalities in ICSI pregnancies have been repeatedly reported (Bounduelle *et al.*, 1999, Schieve *et al.*, 2002, Aytoz *et al.*, 1999). More recently, increased rates of imprinting disorders have also been documented in the literature (DeBaun *et al.*, 2003; Halliday *et al.*, 2004).

1.3.3.1 Reduced birth weight in ICSI

There are several ways to define reduced birth weight. Low birth weight (LBW) and very low birth weight (VLBW) describe birth weight below 2500g and 1500g, respectively. Small for gestational age (SGA) describes a birth weight below a specific cut-off, normally the 10^{th} or the 5^{th} percentile, at particular gestational age. Intrauterine growth retardation (IUGR) is thought to best describe the pathological state of reduced birth weight, defined as failure of a fetus to reach their genetic potential. Because the detection of IUGR requires ultrasound measurements of in utero growth, which is not readily available in every study,

IUGR is commonly used interchangeably with SGA to describe birth weight below the 5th or 10th percentile (Kingdom *et al.*, 2000; Cetin *et al.*, 2003). Although these terms are not synonymous, there is a considerable overlap among them (Monk and Moore, 2004).

Infants with abnormal fetal growth may be predisposed to health problems at all stages of life. Infants have increased rates of perinatal mortality and morbidity (Jarvis et al., 2003). Children with lower birth weights were also found to have abnormal neurodevelopment and cerebral palsy (Blair, 2000). According to Barker's hypothesis suggests, size at birth may even play a role in developing cardiovascular disease, hypertension, and type II diabetes later in adulthood (Barker *et al.*, 1998).

Many studies have reported that the rate of LBW and VLBW is higher in ICSI pregnancies compared to those conceived naturally. Although the difference was originally thought to be attributed to the higher rate of multiple-births in ICSI pregnancies, significantly higher rates of LBW have been observed in singletons born after ICSI (Table 1.1). Katalinic *et al.* (2004) investigated 2055 ICSI babies and found a 2-fold increase in the incidence of LBW in ICSI singletons compared to the natural conceived controls. While not limited to ICSI, Schieve *et al.* (2002) studied 18398 ART newborns and estimated a 2.6-fold increase in LBW singletons conceived with ART compared to the controls, although the type of ART used was not specified. Similarly, Wang *et al.* (2005) concluded that ART derived pregnancies (including 7174 ICSI newborns), in general, are 2.1 times more likely to have LBW than natural conceptions, with no significant difference between the ICSI and IVF groups. In contrast, one study found a weaker correlation between reduced birth weight and ICSI. Ombelet *et al.* (2005) conducted a large scale retrospective cohort study including 1655 singletons and 1102 twin ICSI births. The naturally conceived control group was selected from a regional registry and was matched for maternal age, parity, place of delivery, year of delivery and fetal sex. Interestingly, the rates of LBW and VLBW in the ICSI and control singleton pregnancies were comparable.

Table 1.1 Rates of Low Birth Weight (LBW) and Very Low Birth Weight (VLBW) in ICSI and natural pregnancies for singletons and twins

Singleton				
	LBW		VLBW	
	ICSI	Natural conceptions	ICSI	Natural conceptions
Wang *et al.*, 2005[a]	9.1%	-	-	-
Ombelet *et al.*, 2005	132/1655(7.9)	231/3278 (7.0)	32/1655 (1.9)	51/3278 (1.5)
Katalinic *et al.*, 2004	224/2055 (10.9)*	417/7861 (5.3)	66/2055(3.2)*	86/7861(1.1)
Vernaeve *et al.*, 2003[b]	16/142 (11.3)	-	-	-
Bounduelle *et al.*, 2002	106/1493 (7.1)	-	22/1493 (1.5)	-
Schieve *et al.*, 2002c	2723/18398 (13.2)*	1339/18398 (7.3)d	480/18398 (2.6)*	263/18398 (1.4)
Wennerholm *et al.*, 2000	59/773 (7.6)	-	11/773 (1.4)	-
Loft *et al.*, 1999	32/476 (6.7)	-	8/476 (1.7)	-
Govaerts *et al.*, 1998	23/121 (19)	-	4/121 (3.3)	-
Twins				
	LBW		VLBW	
	ICSI	Natural conceptions	ICSI	Natural conceptions
Ombelet *et al.*, 2005	652/1102 (59.2)	1290/2163 (59.6)	97/1102 (8.8)	218/2163 (10.1)
Katalinic *et al.*, 2004	656/1158 (56.7)	79/152 (52.3)	11/1158 (10)	21/152 (13.9)
Vernaeve *et al.*, 2003[b]	42/88 (47.7)	-	-	-
Bounduelle *et al.*, 2002	593/1233 (48.1)	-	64/1233 (5.2)	-
Schieve *et al.*, 2002c	10156/18399(55.2)	-	-	-
Wennerholm *et al.*, 2000	164/416 (39.4)	-	35/416 (8.4)	-
Loft *et al.*, 1999	44/118 (37.6)	-	6/118 (5.1)	-
Govaerts *et al.*, 1998	114/170(67.1)	-	20/170 (11.8)	-

a. Numbers were not available. The total number of infants born after ICSI was 7174
b. Non-obstructive and obstructive azoospermia only
b. Data includes both ICSI and IVF
c. The expected values were calculated according to the LBW from the 1997 U.S> birth-certificate data. Values were adjusted to the difference in the distribution of maternal age was adjusted
* Significantly different from the control values

Several studies also investigated birth weight from ICSI pregnancies derived through variable conditions. Aytoz *et al.* (1999) investigated the effect of cryopreservation of embryos on birth weight and concluded that the incidence of LBW is higher in ICSI pregnancies using frozen embryos compared to fresh embryos (12.1% for fresh vs 32.7% for frozen), although the difference was only significant in twin pregnancies. Wennerholm *et al.* (2000) compared the birth weights according to sperm origins and quality, and found comparable results in the subgroups with different sperm concentration and sperm sources (i.e. ejaculate, epididymal, and testicular). Interestingly, the authors also reported that

cryopreservation led to a higher birth weight; however, significant difference was found in the singleton group.

Since reduced birth weight is a general concern of ICSI and other ARTs, the neonatal outcomes of VLBW infants born after ART were studied (Schimmel *et al.*, 2006). The risks for congenital abnormality, postnatal morbidity or mortality in the ART conceptions were not higher than that of natural conceptions, after adjusting for plurality. However, this study did not provide information on the specific types of ART used or report the pregnancy loss rates due to fetal malformations.

1.3.3.2 Spontaneous abortions

Spontaneous abortion (SA) occurs in approximately 10-20 % of clinically detected pregnancies in the general population (Wilcox *et al.*, 1981; Nybo Andersen *et al.*, 2004). The incidence of SA in pregnancies conceived by ICSI has been investigated by multiple studies. However, an accurate comparison of SA rates between ICSI and natural conceptions is difficult because the incidence is strongly influenced by maternal age, which tends to be higher in the ICSI population. In addition, the detection of pregnancy is more accurate in ART cycles than natural conceptions due to closer surveillance. The general belief is that the rate of SA is slightly higher in ICSI conceptions compared to natural conceptions because of the inherent insufficiencies found in the infertile couples undergoing ICSI (Schieve *et al.* 2004). The overall rate of spontaneous loss in ICSI conceptions ranges from 11% to 26% (Aytoz *et al.*, 1999; Wennerholm *et al.*, 2000; Van der Westerlaken *et al.*, 2001; Vernaeve *et al.*, 2003), however, these rates did not fully adjust for important factors such as maternal age, pregnancy plurality, and pregnancy history. In studies with appropriate adjustments, the relative risk of SA in ICSI pregnancies was determined to be 1.03 fold (Schieve *et al.* 2003) and 1.20 fold increase (including both ICSI and IVF, Wang *et al.*, 2004).

To investigate the potential factors that may contribute to pregnancy loss in ICSI conceptions, several studies attempted to correlate the risk of SA with types of infertility and sperm source. Some studies found that SA rate was not affected by underlying infertility (Bahceci and Ulung, 2005, Wang *et al.*, 2004, Vernaeve *et al.*, 2003) or by the sperm types

(e.g. ejaculate or testicular sperm). (Palermo *et al.*, 2000; Wennerholm *et al.*, 2000; Bahceci and Ulung, 2005). Others found a higher rate of SA in cases with severely defective sperm in the ejaculate, but no such increase in cases where sperm was obtained from epididymis and testis (Aytoz *et al.*, 1998a, b). The impact of hormonal ovarian stimulation on ICSI or IVF has been suggested as one risk factor for SA. Schieve *et al.* (2003) found that SA rate increased in pregnancies using clomiphene for stimulation. Raziel *et al.* (2002) also found that the risk of SA was significantly increased in IVF patients with severe ovarian hyperstimulation syndrome. A few studies indicated that transferring thawed embryos or poor quality embryos could increase the risk for SA (Winter *et al.*, 2002; Schieve *et al.*, 2003). Thus, the etiology of SA in ICSI or IVF conceptions has yet to be fully elucidated.

1.3.3.3 Chromosomal abnormalities

ICSI conception assumes several risk factors that may lead to chromosomal abnormalities - advanced maternal age, male infertility, and the invasiveness of the procedure. Firstly, the correlation between maternal age and chromosome anomaly has been well established. It is thought that as women age, the spindles that organize and facilitate chromosomal segregation in oocytes tend to deteriorate and cell division becomes more susceptible to chromosomal non-disjunction (Hawley *et al.*, 1994; Lamb *et al.*, 1996). Secondly, chromosomal abnormalities have also been associated to male infertility. The incidence of chromosomal abnormalities in infertile men is 10 to 20 fold higher than in the general male population (Retief *et al.*, 1984; Matsuda *et al.*, 1992). Hence, there may be an elevated risk that ICSI can transmit a chromosomal abnormality to the conception from the selection of an abnormal sperm. Finally, as demonstrated in animal models, the injection procedure has been shown to result in perturbations of spindle apparatus, cytoskeleton, and chromatin configurations. These damages delivered on the oocyte may cause meiotic II or mitotic segregation errors (Hewitson *et al.*, 1996; Terada *et al.*, 2000).

The incidence of chromosomal abnormality in ICSI-derived offspring detected by prenatal diagnosis ranges from 1.5% to 4.2% (Table 1.2), which is significantly higher than the 0.9% reported in natural conceptions (Jacobs *et al.*, 1992; Nielsen and Wohlert, 1991). Several studies reported *de novo* abnormalities in ICSI derived offspring involving both

autosomal and sex chromosomes; while other studies with relatively small sample size could detect only autosomal abnormalities (Wennerholm *et al.*, 2000; Vernaeve *et al.*, 2003.). Comparing with the incidence of chromosomal abnormalities detected in the general population, Bounduelle *et al.* (2002) suggested that the increase is primarily attributed to the higher incidence of sex chromosome abnormalities, estimated to be 3-fold higher than that of the controls. Similarly, a higher incidence of *de novo* gonosomal than autosomal abnormalities were observed in some studies (Van Opstal *et al.*, 1997; Samli *et al.*, 2003), confirming the preponderance of sex chromosomal abnormalities in the ICSI pregnancies.

Inherited abnormalities, mostly balanced structural rearrangement, also present more frequently in the ICSI population. These inherited structural abnormalities were found to be mostly of paternal origin (Table 2). This is thought to be a direct consequence of the chromosomal abnormalities in the underlying male-factor infertility (Bonduelle *et al.*, 2002). However, an effect due to increased maternal age is also widely considered to exist in the ICSI population. Advanced maternal age is present in up to 49% of the infertile couples undergoing ICSI (SART, 2000). Loft *et al.* (1999) reported about a half of the chromosomal abnormalities (1.9% out of 3.4%) detected in ICSI conceptions were due to advanced maternal age, which incidentally encompassed 29% of the couples studied.

Table 1.2. Prenatal diagnosis in ICSI pregnancies.

conception	Reference	n	*de novo* (%)		inherited	total	origin
			Gonosomal	Autosomal	%	%	
ICSI	Jozwiak *et al.*, 2004	1136	0.6 (n=7)	0.6 (n=7)	0.2 (n=3)	1.5 (n=17)	2/3 pat
	Samli *et al.*, 2003	142	2.8 (n=4)	1.4 (n=2)	0	4.2 (n=6)	--
	Veraeve *et al.*, 2003	85	0	2.35 (n=2)	1.18 (n=1)	3.53 (n=3)	--
	Bonduelle *et al.*, 2002	1586	0.63 (n=10)	0.95 (n=15)	1.39 (n=22)	2.96 (n=47)	17/22 pat
	Wennerholm *et al.*, 2000	149	0	1.34 (n=2)	1.34 (n=2)	2.68 (n=4)	2/2 pat
	Loft *et al.*, 1999	209	0	2.9 (n=6)	0.5 (n=1)	3.35 (n=7)	1/1 pat
	Van Opstal *et al.*, 1997	71	8.4 (n=6)	4.2 (n=3)	--	12.7 (n=9)	6/8 pat
Natural	Jacobs *et al.*, 1992	56952	0.19	0.26	0.47	0.92	--
	Nielsen and Wolhlert, 1991	34910	0.23	0.61	--	0.84	--

Several studies have correlated *de novo* chromosomal abnormality in ICSI conceptions to sperm parameters. Bonduelle *et al.* (2002) found correlation between low sperm concentration ($<10x10^6$/ml) and abnormal sperm motility to abnormal karyotypes detected through prenatal testing. Similarly, Samli *et al.* (2003) found an association between very low sperm count ($<10^6$/ml) with abnormal karyotypes in the conceptions. These findings suggest that chromosomal abnormalities may be associated with some types of male infertility and can be passed on from the fathers to the offspring.

Case reports have provided further evidence for the direct association between aneuploidy in sperm and that in the fetus. Moosani *et al.* (1999) reported a 47, XXY pregnancy conceived with sperm from a man with elevated level of XY disomy in his gametes (1.39% vs. 0.16% in controls). Carrell *et al.* (2001) also reported a trisomy 15 pregnancy with a paternal origin. A significantly higher level of disomy 15 (4.03% vs. 0.4% in controls) was observed in sperm from the father, who was affected by the round-headed sperm syndrome. A slight increase in aneuploidy involving sex chromosomes was also observed. This finding is in agreement with the interchromosomal effect reported in many studies (reviewed by Douet-Guilbert et al., 2005). More recently, Tang *et al.* (2004) conducted a series of investigations on a 45, X case which was missing the paternal X chromosome. FISH analysis on the father's sperm revealed a roughly 1:1 ratio of sex chromosome nullisomy (19.6%) and XY disomies (18.6%), both higher than the controls (0.3% and 0.1%). Therefore, a meiotic II non-disjunction was speculated to be responsible for the abnormality. A moderate but statistically significant elevation of aneuploidy involving chromosome 13, 18, and 21 was also detected, suggesting an interchromosomal effect. Furthermore, an immunofluorescent assay was used to investigate the synaptonemal complex and recombination foci in spermatocytes in pachytene stage. No recombination between sex chromosomes was observed in this man, while in controls (n=2), the recombination rate involving the sex chromosomes was about 80%. Reduced recombination was also observed on chromosomes 13 and 21 (Ma *et al.*, 2006). These thorough analyses provided insight into the etiology of aneuploidy in ICSI conceptions, further linking chromosomal abnormalities in the father's sperm to chromosome pairing and recombination.

The incidence of chromosomal abnormality in spontaneously aborted conceptions has also been investigated. Studying spontaneous abortions allows for a wider investigation of abnormalities than what can be detectable at prenatal testing. The rate of chromosomal abnormalities detected in abortuses from ICSI has been suggested to be comparable to that from IVF and natural conceptions (Causio *et al.*, 2002; Ma *et al.*, 2006). In the general population, the incidence of chromosomal abnormality in spontaneous abortions is estimated to be about 65% though it is strongly influenced by maternal age (Ohno *et al.*, 1991; Sanchez *et al.*, 1999). As per ART pregnancies, Causio *et al.* (2002) found that chromosomal abnormality was present in 48% of ICSI and 43% of IVF abortions, respectively. Ma *et al.* (2006) found that 59% of ICSI and 71% of IVF abortuses were chromosomally abnormal. Another study found a significant higher rate of aneuploidy in abortuses from ICSI (76%) than those from IVF (41%) (Lathi and Milki, 2004). However, the number of cases included in the study was relatively small, with only 21 abortion cases from ICSI. Cytogenetic analysis of spontaneous abortions in the general population showed that the predominant abnormality was autosomal trisomy (64%; 62%), followed by polyploidy (9%; 20%), monosomy X (7%; 11%), structural rearrangements (6%; 5%), mosaicism (6%), double trisomy (4%), and double chromosome anomaly (3%) (Ohno *et al.*, 1991; Eiben et al., 1991). Data from ICSI studies consistently reported a higher rate of monosomy 45, X cases with a frequency of up to 33.3% (Causio *et al.*, 2002). This may be associated with the higher level of sex chromosome aneuploidy observed in infertile men. Also, the rate of triploidy and tetraploidy is lower in ICSI abortuses than those from IVF and even natural conceptions. This can probably be explained by the nature of the ICSI procedure which prevents possibility of more than one sperm fertilizing an oocyte (Causio *et al.*, 2002).

1.3.3.4 Imprinting disorders

Recently, increasing interest has focused on the association between ART and imprinting disorders, such as Beckwith-Wiedemann Syndrome (BWS) (DeBaun *et al.*, 2003; Maher *et al.*, 2003, Gicquel *et al.*, 2003; Halliday *et al.*, 2004), and Angelman Syndrome (AS) (Cox *et al.*, 2002; Orstavik *et al.*, 2003) (Table1.3).

BWS occurs in one in 14,500 live births in the general population. It is characterized by macroglossia, pre- or/and postnatal overgrowth, and anterior abdominal wall defects. Pregnancy affected by BWS may have large and thickened placenta, polyhydramnios, long umbilical cord, and a large fetus for gestational age. Additional clinical features include hemihypertrophy, ear pits and creases, neonatal hypoglycemia, and facial nevus flammeus. BWS patients are also at risk of developing embryonic tumors such as Wilms tumor (Elliott, 1994). Most sporadic cases are caused by mutations or epimutations affecting the imprinted genes located on chromosome 11p15.5. Up to 45% of the sporadic cases are due to epigenetic alterations at imprinting centers (IC), which regulate imprinted genes such as maternally expressed *H19* and *CDKN1C* and paternally expressed *IGF2* and *KCNQ1*. Other etiologies include uniparental disomy (20%) and mutations of *CDKN1C* gene (~10%) (BWS [OMIM 130650]). It has been suggested that BWS is overrepresented in the ART population. Among a total of 33 ART derived BWS cases, 11 cases underwent ICSI. The relative risk for ART derived children to develop BWS has been estimated to be about 4 fold higher than the general population in two retrospective studies (Maher *et al.* 2003; Gicquel *et al.*, 2003), whereas, two other groups reported even more alarming numbers (up to 9 fold higher risk) (DeBaun *et al.*, 2003; Halliday *et al.*, 2004). Molecular analyses have demonstrated that 13 out 14 cases were caused by hypomethylation at the *KCNQ1OT* gene; one case was also affected by hypermethylation at the *H19* (Table 1.3). Thus, the prevalence of epigenetic disturbance in the ART derived BWS cases (93%) is much higher than the expect rate of 45% in the sporadic cases.

These findings mirror studies that linked Angelman Syndrome (AS) and ICSI. AS is characterized by mental retardation, delayed motor development, poor balance, speech impairment, and happy disposition. The majority of the AS cases (70%) is caused by deletion of the maternal chromosome 15 followed by *UBE3A* mutation (5-10%) (OMIM 105830). Merely 2% of the sporadic AS cases are caused by epigenetic defects at the imprinting centre. However, 3/3 cases of AS reported in children born after ICSI were found to be due to this infrequent epigenetic error. Although the fathers of the two AS children reported by Cox *et al.* (2002) had abnormal sperm parameters, the third case indicated that infertility was related to maternal factors (Orstavik *et al.*, 2003). Thus, authors suggested a possible correlation

between the ICSI procedure and AS. However, a recent study reported that 4/16 (25%) of AS children born to subfertile couples had a sporadic imprinting defect. Some of these patients underwent ICSI, but the relative risk of AS in ICSI population was identical to those who were untreated but had more than two years of infertility history. Also, hormonal treatment was found to increase the relative risk of AS (Ludwig *et al.*, 2005). Thus, these observations extended the possible causes for imprinting defects to other factors related to infertility rather than ICSI procedure.

Table 1.3 Summary of imprinting disorders in the ART population.

Syndrome	Reference	No. of ICSI	No. analyzed	Molecular analysis results
BWS	DeBaun *et al.*, 2003	5/7	6	4/6 *KCNQ1OT* hypomethylation
				1/6 *KCNQ1OT* hypomethylation and *H19* hypermethylation
	Maher *et al.*, 2003	3/6	2	2/2 *KCNQ1OT* hypomethylation
	Gicquel *et al.*, 2003	2/6	6	6/6 *KCNQ1OT* hypomethylation
	Halliday *et al.*, 2004	1/4	3	3/3 *KCNQ1OT* hypomethylation
AS	Cox *et al.* 2002	2/2	2	2/2 imprinting defects at IC
	Orstavik *et al.*, 2003	1/1	1	imprinting defects at IC

IC: imprinting centre.

1.4 Confined placental mosaicism

Since the first birth facilitated by ICSI in 1992, a number of cohort studies have revealed an elevated incidence of prenatal and perinatal abnormalities in children born through this treatment, as described above. However, the underlying causes have not been identified. In the current study, we hypothesized that confined placental mosaicism (CPM) might be one of the factors contributing to these abnormalities, particularly for low birth weight (LBW). CPM is defined as a feto-placental chromosomal discrepancy where abnormality is found only in the placenta but not in the embryo proper (Kalousek and Dill, 1983).

1.4.1 Normal placental formation and functions

The placenta has a short life span as it develops only during the nine months of gestation; however it contributes significantly to the development of the fetus. In order to understand the etiology and clinical relevance of CPM on pregnancies, normal development and functions of the placenta are reviewed here.

1.4.1.1 Development of the placenta

A blastocyst, which develops about four days after fertilization, consists of a fluid-filled inner cavity (blastocele), an inner cell mass, and trophoectoderm on its surface. These different cell types define the developmental lineages. Only a few cells in the inner cell mass are destined to become the embryo, while the remaining cells in the inner cell mass and the trophoectoderm give rise to the extraembryonic tissues (e.g. the placenta) (Norwitz *et al.*, 2001) (Figure 1.4).

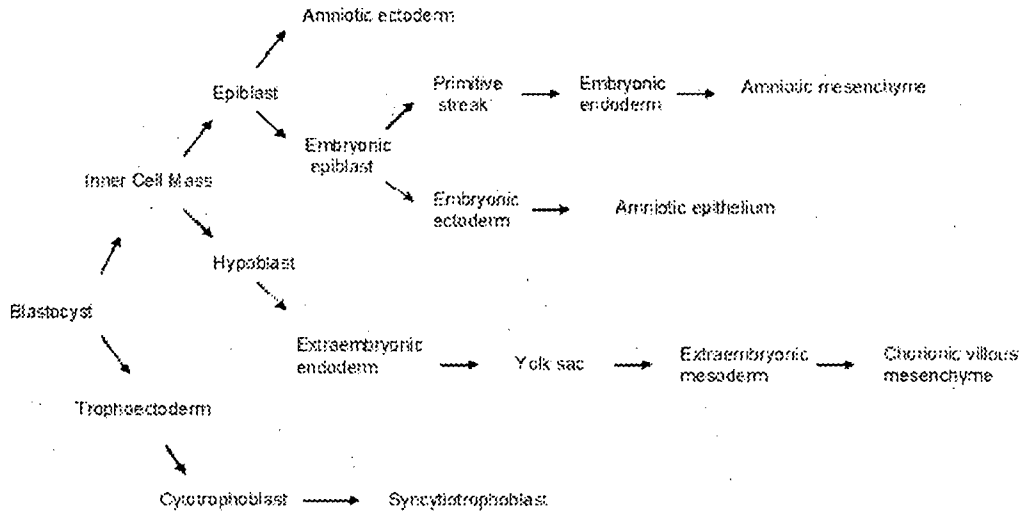


Figure 1.4 Development of placenta tissues. (Modified from Carlson 2004; Minor, 2005)

The trophoblast, the main constituent of the placenta, derives from the trophoectoderm and contains two layers: the inner cytotrophoblast and the outer syncytiotrophoblast. Trophoblast plays an important role in implantation by enhancing the interaction and invasion into the uterine lining (Carlson 2004). Upon the invasion of syncytiotrophoblast into uterine epithelium, lacunae (later called intervillous space) begin to form among trophoblast

cells, and maternal blood enters to provide a source of nutrients. Concurrently, a series of differentiation steps takes place in the inner cell mass of the blastocyst. The hypoblast begins to give rise to the yolk sac, some of which will transform into extraembryonic mesoderm. The extraembryonic mesoderm, in turn, gives rise to the mesodermal core of the villi and the allantois, and forms the chorion when fused with the trophoblast endoderm. The dorsal cells of the epiblast (extraembryonic ectoderm) spread across the mesoderm and give rise to the mesenchymal layer of amniotic membrane, whereas, the amniotic epithelium arises from embryonic ectoderm. The amniotic membrane provides a fluid-filled cavity to protect the embryo. As the blastocyst continues to invade, the fibroblast-like stromal cells of the maternal endometrium begin to enlarge and accumulate glycogen and lipid droplets. These cells, now called decidua, surround the implanting embryo and form the maternal compartment of the placenta (Carlson, 2004).

By the 10^{th} day after fertilization, the blastocyst becomes completely implanted into the uterine wall (Norwitz *et al.*, 2001). Upon implantation, primary villi begin to form as the cytotrophoblast invades into the syncytiotrophoblast. Once mesenchymal core appears inside an expanding villus, secondary villus is formed. Tertiary villi develop when blood vessels penetrate into the mesenchymal core. This occurs at the end of the third week of the pregnancy. The invasive nature of the cytotrophoblast is limited to the first trimester when photolytic enzymes are expressed. Cytotrophoblast invasion goes beyond the syncytiotrophoblast, and into the maternal endometrium, myometrium, and uterine vasculature. As trophoblast erodes maternal endometrial blood vessels (e.g. the spiral arteries), maternal blood seeps into the intervillous space and bathes the chorionic villi. The feto-maternal exchange of gases and molecules takes place across the villus capillaries that separate the maternal and fetal blood (Carlson, 2004).

1.4.1.2 Placental functions

The placenta has three important functions for the survival and development of the embryo: (1) allows the fetus to acquire oxygen and nutrients and to extrude waste; (2) serves as a barrier from rejection of the embryo by the maternal immune system; (3) synthesizes a

number of hormones and growth factors that are required for the progress of the pregnancy.

Gas exchange between the maternal and the fetal blood occurs by diffusion across the villous capillaries. Despite similarities to gas exchange in the adult lung, diffusion across the placenta is not as efficient because of greater diffusion distances and smaller surface area available for diffusion. To compensate, fetal hemoglobin is more concentrated and has higher affinity to oxygen. Fetal cardiac output is also much higher than that in adults to increase the delivery of oxygen to fetal tissues. Finally, maternal blood flow to the uterus is increased during pregnancy (Rurak, 2001). The nutrition from the mother to the fetus is transferred through active transporters. The main nutrients, glucose and amino acids, are transported via specific transporters (Bell *et al.*, 1990; Cetin *et al.*, 2003). In addition, fatty acids, cholesterols, steroid hormones are also transported across the placenta to the fetus (Rurak, 2001). The placenta also serves as a barrier to protect the fetus from the maternal immune system. The trophoblast, specifically the syncytiotrophoblast and the non-villous cytotrophoblast cells, do not produce two major histo-compatibility antigens that can trigger an immune response, as seen in tissue transplantations (Carlson, 2004). In addition, the placenta is thought to produce compounds with immunosuppressive properties. These include human chorionic gonadotropin (hCG), progesterone, prostaglandins, and placental proteins such as PP-5, PP-12, and PP-14 (Rurak, 2001).

The placenta, particularly the syncytiotrophoblast, produces a number of protein and steroid hormones that are important for the pregnancy. hCG is the first protein hormone produced by the embryo even before implantation (Carlson, 2004). hCG stimulates the differentiation of cytotrophoblast into syncytiotrophoblast (Kliman *et al.*, 1986) and maintains the corpus luteum and its production of progesterone and estrogen. The production of hCG peaks at around the 8^{th} week of gestation, and declines as the placenta begins to produce enough progesterone and estrogen by itself. Human placental lactogen, also produced by the syncytiotrophoblast, enhances fetal growth, lactation, and lipid and carbohydrate metabolism (Carlson, 2004). In addition, several hormones produced by the syncytiotrophoblast are thought to influence exclusively the maternal metabolism during pregnancy. Some examples are human placental growth hormone, chorionic thyrotropin and

chorionic corticotrophin. Cytotrophoblast cells produce a homologue of gonadotropin-releasing hormone (GnRH), which regulates the production of hCG and progesterone by the syncytiotrophoblast (Carlson, 2004).

1.4.2 Pathogenesis of CPM

As described above, the placenta plays essential roles for the development of the fetus. Therefore, when the placenta is pathologically affected, the pregnancy outcome can be adversely influenced. One of such examples is confined placental mosaicism (CPM) in which a chromosomal abnormality is limited to the extra-embryonic tissues and does not affect the embryo proper. There are three categories of CPM according to the cell type involved. The type I CPM is the most common and affects trophoblast cells only. Type II CPM is limited to the mesenchymal core cells. In type III CPM, abnormality is present in both trophoblast and mesenchymal cells (Figure 1.5) (Kalousek and Vekemans, 1996).

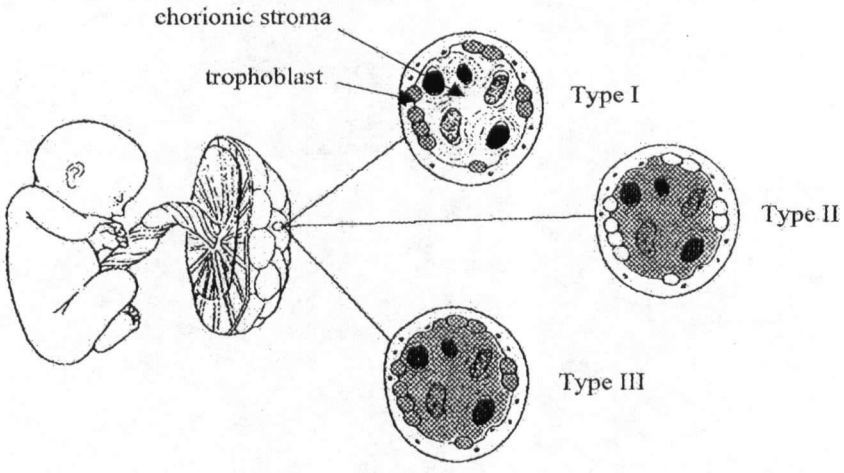


Figure 1.5. Three types of confined placental mosaicism (CPM). Shaded tissues are chromosomally abnormal. In all three types, the fetus is unaffected. (from Kalousek and Vekemans, 1996).

CPM can have a mitotic or a meiotic origin. With mitotic origin, CPM results from chromosomal non-disjunction in a post-zygotic cell division event in an originally normal zygote, leading to a chromosomal abnormality in the extra-embryonic tissues. Mitotic errors give rise to all three types of CPM, depending on the timing of the error (Simoni and

Sirchia, 1994). Type I (trophoblast only) and type II (mesenchymal cells only) CPM derive from the propagation of a chromosomal mal-segregation event in a small portion of cells in a developing morula destined to become extraembryonic mesoderm (EEM) (Wolstenholme, 1996). Type III CPM derives from an error occurring early in an undifferentiated blastomere prior to the blastocyst stage. In addition to timing, the mosaic pattern of CPM is also influenced by the number of cells affected by the error, viability of the error, and the level of developmental selection occurring after the erroneous event (Robinson *et al.*, 1997).

CPM can have a meiotic origin if an aneuploid gamete (derived from chromosomal non-disjunction in Meiosis I or II) produces an aneuploid zygote upon fertilization and the chromosomal abnormal zygote subsequently undergoes a 'rescue' process such that abnormal cells are selected against, promoting the development of any normal cells. Because the zygote starts off abnormal, a larger proportion of chromosomally abnormal cells is expected (Wolstenholme, 1996). Meiotic CPM may lead to a particularly high level of trisomy in trophoblasts as a large proportion of an early blastocyst gives rise to the trophoblast lineage. However, if the 'rescue' event affects cell lineages specifically excluding EEM cells that develop into mesenchymal stroma, then type II CPM can result. Because such specific requirements for type II CPM are less likely, meiotic origin is more often associated with type I and type III CPM (Wolstenholme, 1996). Robinson *et al.* (1997) have, however, identified three exceptional cases of meiotically-derived CPM: one had no detectable abnormality in the placenta at term despite a non-mosaic abnormality detected by CVS; another case had no abnormality in trophoblasts at term but a high level of abnormality in stroma cells; a third case had trisomy 22 in 100% cultured mesenchyme but 0% in trophoblast (direct CVS culture). However, the sensitivity of mosaicism detection can be affected by the number of cells analyzed, site-to-site variability in the distribution of trisomic cells.

The origin of CPM, whether it is meiotic or mitotic, also appears to differentially involve specific chromosomes. The majority of the CPM cases involving chromosomes 2, 3, 7, 8, 10, and 12 have a mitotic origin; whereas, CPM 9, 16, and 22 more frequently have a meiotic origin. CPM 16 almost exclusively evolves from a maternal meiotic error. This

chromosome specific association with origin of CPM has been suggested to involve the selection and viability of certain trisomic cells (Wolstenholme, 1996; Robinson *et al.*, 1997).

1.4.3 Ascertainment of CPM

CPM is typically diagnosed through prenatal diagnosis by chorionic villus sampling (CVS) at 10-12 weeks of gestation, with which a biopsy of chorionic villli is taken and subsequently cultured to examine the chromosomal complement. Discrepant results can be observed in short and long term culture methods because the cell populations are different. The direct or short-term culture selectively grows the rapidly dividing cytotrophoblast cells, and the long-term culture promotes the growth of mesenchymal core cells (Phillips *et al.*, 1997). The mesenchymal core cells are thought to be more representative of the fetal cells (Phillips *et al.*, 1997), presumably because mesenchymal cells and the embryo proper both originate from the progenitor cells in the inner cell mass (Crane and Cheung, 1988).

CPM is identified in 1-2% of CVS when a different cytogenetic result is obtained from amniocentesis or fetal blood karyotyping, both of which are representative of the fetal chromosome constitution (Grati *et al.*, 2006). In contrast, mosaicism found exclusively in the fetal cells is about ten times less common than CPM (Stetten *et al.*, 2004). It has been suggested that a higher rate of placental abnormality is either due to error-prone cell divisions in the extraembryonic cells, or due to a less stringent selection against aneuploidy in the extraembryonic cells compared to the fetal cells (Schreck *et al.* 1990).

As pregnancy progresses, CPM detected by CVS may disappear, decrease or persist. Approximately 30-50% of CPM found by CVS is not detectable at term. However, when abnormalities persist, CPM has been associated with negative pregnancy outcomes such as spontaneous abortion, IUGR, or congenital abnormalities (Schwinger *et al.*, 1989; Kalousek *et al.*, 1991; Miny *et al.*, 1991). CPM has been ascertained through various pregnancy complications, although ascertainment through IUGR cases is the most frequent.

1.4.4 Outcomes of pregnancies affected by CPM

The majority of pregnancies affected by CPM have normal pregnancy course and fetal development. In fact, an abnormal CVS result is often disregarded when the abnormality is not detectable in the subsequent amniotic fluid cell culture and there is normal fetal development in ultrasound examinations (Simoni and Sirchia, 1994). Also, CPM involving chromosomes 2, 3, 7, 8 and sex chromosomes is usually associated with a normal fetal development (Wolstenholme, 1996; Farra *et al.*, 2000). However, CPM involving chromosomes carrying imprinted genes may have more severe outcomes due to UPD (Wolstenholme *et al.*, 1994). In addition, several prenatal and perinatal complications have been associated with CPM, such as spontaneous abortions, intrauterine growth restriction (IUGR), and congenital abnormalities. It has been suggested that the outcome of CPM is chromosome specific and largely influenced by the origin (Robinson *et al.*, 1997).

1.4.4.3 Uniparental disomy and CPM

Uniparental disomy (UPD), a possible consequence of the 'trisomic rescue', has been associated with meiotic CPM (Robinson *et al.*, 1997). If a trisomic conception loses the duplicated chromosome, it leads to a normal constitution with biparental chromosomes. However, if two chromosomes from the same parent are left after "rescue", the result is a UPD. In theory, the probability of UPD resulting from trisomic rescue is one in three, derived from a random loss of one chromosome out of three. However, UPD is present in 14.2% of CPM and in 49% of the meiotically derived cases (reviewed by Kotzot, 2002). The deviation from the expected one-third ratio can possibly be explained by ascertainment bias toward IUGR and other pregnancy complications (Robinson *et al.*, 1997). In agreement with the high incidence of UPD in meiotic CPM, UPD occurs with high levels of mosaicism, particularly in the trophoblast (Robinson *et al.*, 1997; Kotzot, 2002). UPD has been correlated to negative pregnancy outcomes including IUGR, malformations, and fetal death, presumably due either to altered expression of imprinted genes that are developmentally important or to homozygosity of recessive traits (Wolstenholme *et al.*, 2001, Robinson *et al.*, 1997).

1.4.4.1 Pregnancy loss and CPM

A positive correlation has been suggested between CPM and fetal losses including spontaneous abortion (SA), intrauterine death (IUD), neonatal death, and stillbirth, partially due to ascertainment bias (Table 1.4). It has been suggested that these losses may result from abnormal placental development and function caused by the presence of chromosomally abnormal cells in the placenta (Kalousek *et al.*, 1992). The reported SA rates in pregnancies affected by CPM range between 5% and 33% (Eiben *et al.*, 1990; Warburton *et al.*, 1978; Qumsiyeh, 1998; Griffin *et al.*, 1997; Johnson *et al.*, 1990; Hogge *et al.*, 1986). CPM has also been associated with stillbirth and neonatal death in 4.8% and 2.4% of cases, respectively (Johnson *et al.*, 1990). However, the CPM rate is often underestimated in many studies because only one tissue type and low number of cells are examined (Kalousek *et al.*, 1992; Wolstenholme, 1996).

Table 1.4 Fetal loss and CPM

Reference	Abnormality	Ascertained through	Cytogenetic results		pregnancy outcomes
			Troph (%)	Stroma(%)	
Kalousek *et al.*, 1992	Trisomy2	SA	100	73	SA
	Trisomy 3	SA	27	100	SA
	Trisomy 4	SA	0	100	SA
	Trisomy 7	SA	100	60	SA
	Trisomy 16	SA	58	100	SA
	Trisomy 16	SA	100	84	SA
	Trisomy 16	SA	100	-	SA
	Tetraploidy	SA	0	100	SA
	Tetraploidy	SA	0	100	SA
	Tetraploidy	SA	13	100	SA
Kennerknecht *et al.*, 1993	trisomy 18	CVS	positive		IUD at 31wks
Leschot *et al.*, 1996	tetraploidy	CVS	100	--	neonatal death, micrognathia
	trisomy 10	CVS	63	--	infant death 7wks, heart defect
Griffin *et al.*, 1997	trisomy 16	CVS	75	100	SA
	trisomy 13	CVS	0	100	SA
	iso (8q)	CVS	0	63	SA
	trisomy 15	CVS	0	100	SA
Qumsiyeh, 1998*	monosomy X	SA	0	100	SA
	trisomy 16	SA	0	100	SA
Farra *et al.*, 2000	Trisomy 9	CVS	30	--	SA
	trisomy 13	CVS	10	--	SA
	trisomy 16	CVS	80	--	SA

1.4.4.2 Intrauterine growth restriction (IUGR) and CPM

IUGR, birth weight below the 10th percentile for gestational age, has also been linked to CPM (Leschot *et al.*, 1996). The incidence of CPM in pregnancies ascertained through IUGR has been reported to range from 6.5% to 16%, which is significantly higher than the background rate (1-2%, detected by CVS) (Kennerknecht *et al.*, 1993; Wolstenholme *et al.*, 1994; Leschot *et al.*, 1996). The chromosomes most commonly involved in IUGR-related CPM are (in order of prevalence) chromosome 16, followed by chromosomes 22, 2, 7, and 8 (Table 1.5).

Table 1.5 CPM ascertained from abnormal CVS in fetuses affected by IUGR

Reference	abnormality	% abnormalities detected by CVS	
		Direct (trophoblast)	Cultured (mesenchyme)
Kalousek *et al.*, 1991	Trisomy 2	2	7
	Trisomy 7	53	96
	Trisomy 7	24	73
	Trisomy 15	10	0
	Tetraploidy	33	0
Schring-Blom *et al.*, 1993	Trisomy 8	100	--
	Monosomy X	100	--
Kennerknecht *et al.*, 1993	Trisomy 18	100	--
	Tetraploidy	78	--
	Triple trisomy 6, 21, 22	--	3.2
Wolstenholme *et al.*, 1994	Double trisomy 2, 15	70	--
	Trisomy 9	100	100
	Trisomy 16	--	100
	Trisomy 16	30	--
	Trisomy 16	100	--
	del (13) (q13)	100	--
	Trisomy 22	100	--
Leschot *et al.*, 1996	Trisomy 3	31	--
	Trisomy 8	100	--
	Trisomy 13	54	--
	Trisomy 13	13	--
	Trisomy 16	100	--
	Trisomy 22	95	--
	Monosomy X	89	--
	Monosomy X	100	--
	double trisomy 20, 21	53	--
	double trisomy 5, 13	100	--
	46, XX, der(5)	13	--

Reference	abnormality	% abnormalities detected by CVS	
		Direct (trophoblast)	Cultured (mesenchyme)
Robinson *et al.*, 1997*	Trisomy 2	100	43
	Trisomy 7	100	--
	Trisomy 8	0	100
	Trisomy 16	100	100
	Trisomy 16	100	100
	Trisomy 16	100	100
	Trisomy 16	100	--
	Trisomy 16	100	100
	Trisomy 16	--	100
	Trisomy 22	100	--
	Trisomy 22	70	66
Farra *et al.*, 2000	Trisomy 2	40	--
	Trisomy 2	30	--
	Double Trisomy 13, 16	48	--

* includes data from Hansen *et al.* (1993) and Kalousek *et al.* (1996)

The investigation of CPM in term placentas has been conducted by many groups (Table 1.6). The CPM rate at term in IUGR pregnancies has been estimated to be approximately 15%, which is significantly higher than that detected in pregnancies with normal outcomes (about 1.5%) (Wilkins-Haug, *et al.*, 2006; Minor *et al.*, 2006). Only two studies failed to identify CPM in any of the IUGR pregnancies studied (Kennerknecht *et al.*, 1993; Verp and Unger, 1990). However, only one tissue type was analyzed in these studies and therefore, the rate is presumably underestimated.

Table 1.6 CPM in term placentas

Reference	IUGR n (%)	Non-IUGR	Abnormality detected (% aneuploidy)	Methods
Kalousek and Dill, 1983	2/9 (22.2)	0/9	(1) trisomy 2 (1) trisomy X	culture chorion (100 cells)
Verp and Unger, 1990	0/11	0/2		culture chorion (10 cells)
Kennerknecht *et al.*, 1993	0/71	0/24		STC/LTC (20 cells)
Krishnomoorthy *et al.*, 1995	4/26 (15.4)	0/30	(2) monosomy 21 (>15) (1) monosomy 3 (>15) (1) multiple aneuploidy (>15)	Culture villi-FISH (30 cells)
Artan *et al.*, 1995	6/10 (60)	0/115	(1) trisomy 14 (17) (1) trisomy 18 (52) (2) trisomy 21 (17, 54) (1) monosomy X (59) (1) tetraploidy (54)	culture chorion, villi (10-15 cells)
Cowles *et al.*, 1996	1/20 (5)	0/20	(1) tetraploidy	culture villi (20 cells)
Amiel *et al.*, 2002	8/16 (50)	0/6	(1) Trisomy 8, XXY (1) XXY (1) monosomy 16, 17 (1) monosomy 17 (3) XXX (1) XYY	CGH on villi
Matsuzaki *et al.*, 2004	9/50	n.a	(1) trisomy 22 (80) (1) trisomy 2 (84) (1) trisomy 7 (68) other cases n.a	culture villi (50 cells)
Wilkins-Haug *et al.*, 1995	11/70 (15.7)	1/70 (1.4)	(10) tetraploidy (1) double trisomy 17, 21	LTC chorion (>10 cells)
Barrett *et al.* 2001 (background rate)	0	5/219 (2.3)	(1) trisomy 2 (21) (1) trisomy 4 (17) (1) trisomy 12 (15) (1) trisomy 13 (13) (1) trisomy 18 (77)	CGH - chrorion, villi, trophoblast
Total	**43/303 (14.2)**	**6/515 (1.2)**		

The mechanism by which CPM causes fetal growth abnormalities remains unknown; however, several studies have characterized IUGR-related CPM. It has been reported that IUGR is more prevalent in pregnancies with type III CPM (Johnson *et al.*, 1990; Kalousek *et al* 1991), particularly when the high level of abnormality persisted to term (Miny *et al.*, 1991). It has also been suggested that CPM with meiotic origin is associated with a higher risk of IUGR (Robinson *et al.*, 1997). Meiotic CPM is intrinsically related to higher level of aneuploidy and UPD, thus it is difficult to distinguish the effects by the high-level mosaicism, UPD, or undetected fetal trisomy (Kalousek *et al.*, 1993; Robinson *et al.*, 1997). However, evidence from UPD16 indicates that UPD may cause abnormal fetal growth independently of fetal trisomy (Yong *et al.*, 2002). It has also been suggested that certain types of UPD may have placenta-specific imprinting effects that influence placental function and thus, cause abnormal fetal growth (Robinson *et al.*, 1997).

1.5 Genomic imprinting

The other possible contributing factor for the elevated incidence of abnormal pregnancies outcomes derived from ICSI may be epigenetic alteration in imprinted genes. Genomic imprinting is the differential gene expression of alleles from different parents.

1.5.1 Imprinted genes and human health

One to two hundred genes in human are estimated to be imprinted (Lucifero, 2004). Imprinted genes tend to be clustered within the same chromosomal domain, resulting in a highly coordinated regulatory system. Several imprinting centres have been identified, which can affect the expression of imprinted genes kilobases away (Spahn and Barlow, 2003). Another hallmark of imprinted genes is the abundance of CpG islands. A mouse study has suggested that approximately 88% of imprinted genes have CpG islands compared to the average of 47% observed in the whole genome (Paulsen *et al.*, 2000). Tandem repeats are also abundant in the vicinity of the CpG islands and considered important for the maintenance of their imprints (Reinhart *et al.*, 2002). In addition, asynchronous replication timing (Kitsberg *et al.*, 1993) and sex-specific meiotic recombination frequency (Paldi *et al.*, 1995; Robinson and Lalande, 1995) are two other features of imprinted genes.

Current understanding on genomic imprinting suggests that the regulation of expression of imprinted genes is rather intricate. A primary imprint by means of methylation is established in the germ line. However, a secondary imprint may be acquired during post-implantation development to stabilize the imprints either through DNA methylation and/or histone modifications (Gabory *et al.*, 2006). For instance, a secondary imprint is placed on the differentially methylated region 1 (DMR1) of the *Igf2*r (insulin-like growth factor 2 receptor) promoter region (Fournier *et al.*, 2002; Yang *et al.*, 2000). Finally, tissue-specific transcription factors control the expression of imprinted genes (Gabory *et al.*, 2006).

Imprinted genes are highly expressed in fetal and placental tissues during development; thus, their importance in early development is speculated. Disruptions of imprinted genes such as *Igf2* in mice, , have been shown to lead to placental insufficiency and IUGR (Constancia *et al.*, 2002). Aberrant expression of imprinted genes have also been associated with many imprinting disorders such as Beckwith-Weidmann syndrome (BWS), Prader-Willi syndrome (PWS), Angelman syndrome (AS) and Silver-Russell Syndrome (SRS) in human (Table 1.7). The phenotypes of these imprinting disorders include developmental delay, neurological disorders, hormonal and metabolic dysfunctions, and certain types of cancers. The pathogenesis seems to also be distinctive for each disease and presumably depends on the chromosome involved. Imprinting disorders can be caused by epimutations, uniparental disomies (UPD), translocations, deletions of the imprinting regulatory region, and even microdeletions or point mutations that interrupt the imprints (reviewed by Walter and Paulsen, 2003).

Table 1.7 Common imprinting disorders in human.

Disorder	Main phenotype (s)	Loci	Main genes involved	Pathogenesis	OMIM
Beckwith-Wiedemann Syndrome (BWS)	Over-growth, Wilm's cancer	11p15.5	*IGF2, CDKN1C*	Imprinting defects; UPD; Duplication; translocation	130650
Prader-Willi Syndrome (PWS)	Obesity, muscular hypotonia	15q11-13	*SNRPN*	Deletion; matUPD; Imprinting defects	176270
Angelman Syndrome (AS)	Neurological disorder	15q11-13	*UBE3A*	Deletion; patUPD; Imprinting defects	105830
Silver-Russell Syndrome (SRS)	Pre- and postnatal growth retardation	7p11.2 7q32	*GRB10, PEG1*	UPD; Duplication; Translocation; Inversion	180860

1.5.2 Epigenetic regulation of imprinted genes

Imprinting is regulated by epigenetic modifications such as histone modifications and DNA methylation (Li, 2002). Histone modifications including acetylation, phosphorylation, methylation, and ubiquination have been suggested to play important roles on imprinted genes. These modifications may not only regulate accessibility of DNA binding proteins, but also serve as landmarks for effector proteins (Morgan et al., 2005). However, understanding of the mechanism and functions of histone modification is still limited.

DNA methylation is the best characterized epigenetic modulator in the literature. Approximately 70 imprinted genes have been identified to be regulated by DNA methylation at differentially methylated regions (DMRs) (Holmes and Soloway, 2006). DNA methylation is the attachment of methyl groups (-CH3) to cytosine bases located at the 5'side of guanosines (CpG). DNA methylation is facilitated by DNA methyltransferases (DNMTs) and use of S-adenosylmethionine as the methyl donor (Bestor, 1988). The role of DNMT1 is to maintain methylation patterns in replicated DNA sequences with its ability to recognize hemi-methylated DNA sequences. DNMT 3a/3b and 3L function on *de novo* methylation and are particularly important in the establishment of imprints (Suetake *et al.*, 2004). Although DNMT3L lacks methyltransferase activity, it regulates the catalytic activity of other enzymes in the DNMT3 family both *in vitro* and *in vivo* (Hata *et al.*, 2002; Suetake *et al.*, 2004). The function of DNMT2 has not been fully elucidated. Liu *et al.* (2003) confirmed DNMT2 activity for methylation of the endogenous genomic sequence *in vivo*, although biallelic deletion of Dnmt2 does not cause any obvious methylation defects in mice (Okano *et al.*, 1998).

The mechanism of DNA methylation has been extensively studied using the murine model. There are three main components in the life cycle of primary methylation imprints: erasure, establishment, and maintenance. Around the time of implantation (10.5 to 12.5 days post coitum), DNA demethylation takes place in primordial germ cells (PGCs) up until their arrival to the genital ridge (Hajkova *et al.*, 2002). As a result, the old imprints from the parents are erased so that a new set of imprints can be established according to the sex of the embryo (Brandeis *et al.*, 1993; Tada *et al.*, 1998). Demethylation in male and female PGCs

appears to occur around the same time for most genes and is completed by day 12 -13 of embryonic development. The mechanism of demethylation remains unknown; however, it has been suggested to be an active process because it is completed within one day (Hajkova *et al.*, 2002).

Subsequent to erasure, new imprints are established by *de novo* methylation during gametogenesis. It is well documented that this process occurs at different times in the male and the female. In the male, remethylation occurs earlier, starting before spermatogonia stage in the embryo and is completed by the pachytene stage of meiosis I at puberty (Davis *et al.*, 2000). In contrast, oocytes begin to acquire methylation postnatally while still arrested at prophase I, and methylation is completed by metaphase II for most genes (Obata *et al.*, 1998). The establishment of imprints in spermatocytes is mainly conducted by DNMT3a/3b, DNMT3L, and histone modification enzymes such as Suv39h and HDACs (Lucifero *et al.*, 2002; Obata and Kono, 2002). In oocytes, *de novo* methylation is also mediated by DNMT3a/3b and DNMT3L, but no histone modification is involved (Suetake *et al.*, 2004). An oocyte-specific DNMT1 isoform has been identified during oocyte growth; however, it does not appear to be involved in *de novo* methylation (Howell *et al.*, 2001). Interestingly, there seems to be an allele-dependent difference in the timing of re-methylation in some imprinted genes. As seen in the paternally methylated *H19* and maternally methylated *Snrpn*, studies using mice suggested that the originally methylated alleles acquired *de novo* methylation earlier than the originally unmethylated ones, as if the alleles retained epigenetic memory of their origin (Davis *et al.*, 1999; Lucifero *et al.*, 2004). These finding suggested that other types of epigenetic markings, perhaps histone modifications, may persist after complete erasure of methylation in those genes and provide signals for earlier remethylation (Morgan *et al.*, 2005).

Once the imprints are established in the gametes, they are maintained through fertilization and development, even during a wave of genome-wide demethylation occurring in zygotes after the fertilization (Figure 1.6) (Reik and Walter, 2001). The maternal genome is demethylated by a passive mechanism that depends on the absence of DMNT1 function during DNA replication; whereas, the paternal genome is presumably demethylated by an

active mechanism as it occurs before DNA replication. Although it is not understood how imprinted genes escape the active and passive genome-wide demethylation in early embryos, a specialized chromatin structure has been speculated to preserve methylation at those genes (Reik and Walter, 2001). Several cis-acting DNA sequences have been suggested to bring about the specialized chromatin structure. For instance, these cis-acting signals include the tandem repeats seen with *Rasgrf 1* (Yoon *et al.*, 2002), the 'maintenance of paternal imprint' (MPI) sequences in Snrpn (Kantor *et al.*, 2004), and the differentially methylated region (DMR) of *H19*. Similarly, the unmethylated alleles are also thought to be maintained by distinct chromatin structure from *de novo* re-methylation around the time of implantation (Reik and Walter, 2001). DNMTs are suggested to be trans-acting factors that affect the maintenance of imprinted methylation. Okano *et al.* (1999) reported that DNMT 1 and DNMT 3a/3b are required for the maintenance of methylation at the DMR 2 of *IGF2*.

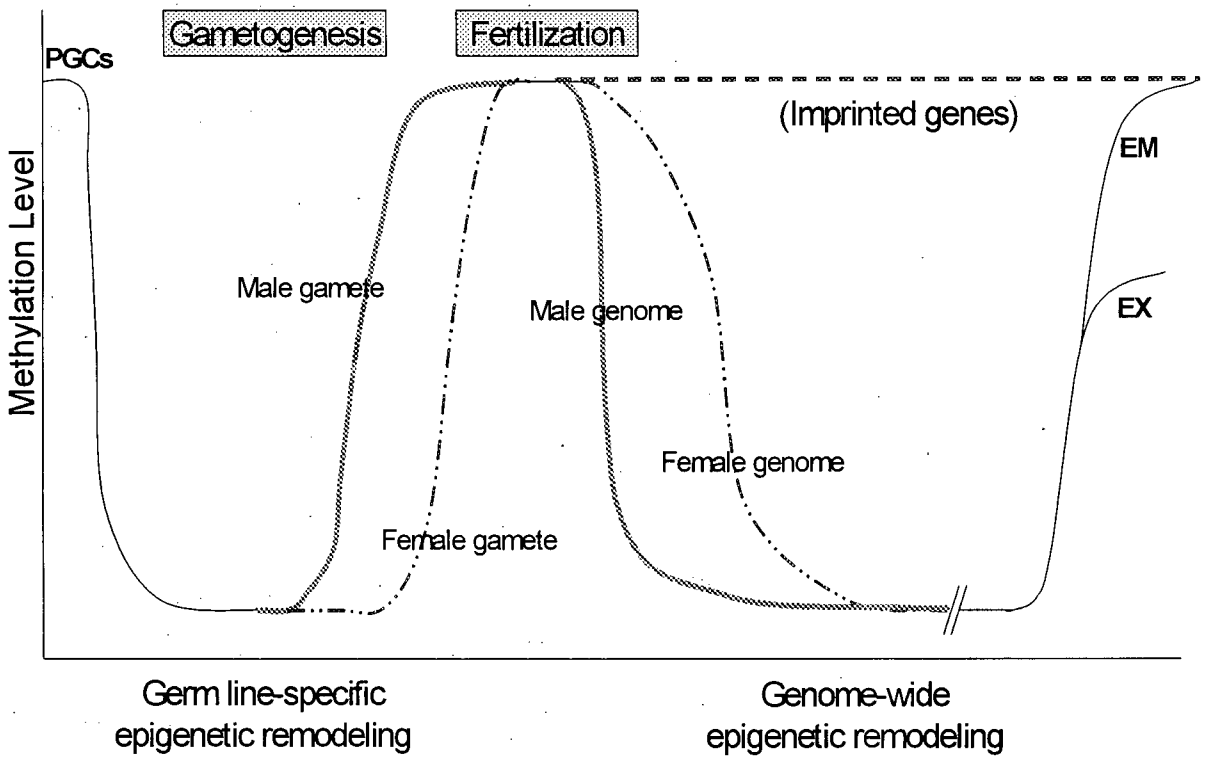


Figure 1.6 Methylation reprogramming in germ lines and in preimplantation embryos. Imprinted genes and some repeat sequences (green dashed line) do not become demethylated after fertilization. Unmethylated imprinted genes do not become methylated in somatic cells during development. PGCs: Primordial germ cells; EM: embryonic lineage; EX: extraembryonic lineage.

1.5.3 Genomic imprinting in the placenta

A genetic conflict model has been proposed to explain the evolution of imprinted genes which suggests that the paternal genome promotes growth of the placenta as the nutritive source for the fetus whereas the maternal genome is inhibitory with respect to the placental development to preserve resources for her own survival (Haig, 1996). Nuclear transplantation experiments that generated parthenogenetic embryos demonstrated such contrasting contributions of the maternal and paternal genome to the embryonic and extraembryonic development (McGrath and Solter, 1984). Similar parental conflicts in human physiological development also include hydatidiform moles and ovarian teratoma (de Grouchy, 1980). Indeed, functional discrepancy is apparent in placental development for many imprinted genes. Data from knockout and transgenic mice suggest that, in general, the paternally active genes tend to enhance the placental growth and the maternally active genes suppress placental growth and trophoblastic invasion (Table 1.8). For instance, knockout of paternally expressed *Igf2, Peg1,* and *Peg3* led to restricted growth of the labyrinthine trophoblast, spongiotrophoblast, and labyrinthine blood vessels (Lefebvre *et al.*, 1998; Li *et al.*, 1999; Constanticia *et al.*, 2002); whereas deletions of maternally expressed *Igf2r* and *p57Kip2* resulted in placental hyperplasia in all layers (Ludwig *et al.*, 1996; Takahashi *et al.*, 2000).

Table 1.8 Examples of imprinted genes and their functions

Imprinted genes	active allele	function on placenta growth	protein product	Reference
IGF2	pat	positive	Insulin-like growth factor II	Constancia et al., 2002
Peg1/Mest	pat	positive	Paternally expressed gene 1	Lefebvre *et al.*, 1998
Peg3	pat	positive	Paternally expressed gene 3	Li *et al.*, 1999
Ascl2	mat	? (lethal if deleted)	Achaete-scute homolog 2	Tanaka, *et al.*, 1997
IGF2r	mat	negative	Insulin-like growth factor II receptor	Ludwig *et al.*, 1996
Phlda2	mat	negative	pleckstrin homology-like domain, family A, member 2	Frank *et al.*, 2004
p57Kip2	mat	negative	a cyclin-dependent kinase inhibitor	Takahashi *et al.*, 2003
Dcn	mat	invasion suppressor	Decorin	Mizuno *et al.*, 2002
Stox1	mat	invasion suppressor	Storkhead box 1	Van Dijk *et al.*, 2005
Ctnna3	mat	invasion suppressor	Catenin (cadherin-association protain) α3	Van Dijk *et al.*, 2004

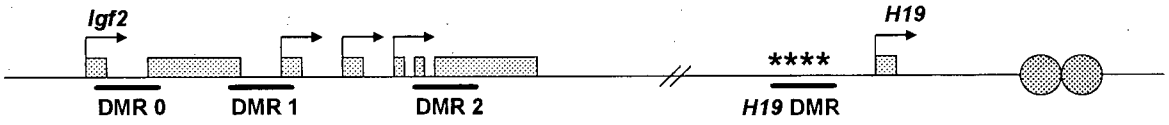
It is well documented that imprinted genes are highly expressed in extraembryonic tissues. Some imprinted genes even have placenta-specific promoters such as *IGF2* P0 (Constancia *et al.*, 2002). In addition, placenta-specific expression has been discovered in some imprinted genes. To date, a number of these genes have been identified in mice that are located on chromosomes 2, 6, 7, 10, and 17. While human data is still limited, several genes on chromosomes 10 and 11 have been found (Wagschal and Feil, 2006). Consistently, the paternal allele is suppressed and the maternal allele is expressed for these placenta-specific imprinted genes. Although functions of these genes have not been fully determined, some have been suggested to exhibit suppressive regulation of placental growth (*Phlda2*) and trophoblastic invasion (*Dcn, Ctnna3, and Stox1*) (Table1.8).

While the maintenance of DNA methylation by DMNT1 seems to be essential for proper expression of imprinted genes in the embryo (Li *et al.*, 1993), it has been demonstrated that the placental imprinting is maintained independently from DNA methylation (Tanaka *et al.*, 1999; Lewis *et al.*, 2004). Lewis *et al.* (2004) identified several imprinted genes clustered at the distal end of mouse chromosome 7 (*Ascl2, Cd81, Tssc4 and Osbpl5*) that exhibit this property. These genes are imprinted exclusively in the placenta and maternally expressed. Also, inactivation of the paternal allele of these genes is not mediated by DNA methylation because the promoter sequences of these genes were un-methylated. Similar findings have also been observed for the promoters of other placenta-specific imprinted genes such as *Slc22a2, Slc22a3, Gatm, Ppp1r9a, Pon2, and Pon3* (Sleutels *et al.*, 2002; Sandell *et al.*, 2003; Ono *et al.*, 2003). Instead of DNA methylation, the inactive allele is possibly suppressed by histone modification. For instance, histone methylation of K9 and K27 was observed at those genes on the distal chromosome 7. Furthermore, *Kcnq1ot1* non-coding antisense RNA seems to play a role in the recruitment of such histone modifications (Fitzpatrick *et al.*, 2002). Lewis *et al.* (2004) also provided an evolutionary explanation for the histone-based mechanism for placenta-specific expression of imprinted genes: histone modification is a less stable but evolutionarily older imprinting mechanism, but such a mechanism is sufficient for the placenta, a temporary organ that develops only during gestation.

1.5.4 *H19* and *IGF2*

Maternally expressed *H19* and paternally expressed *IGF2* are known as two paradigms of imprinted genes. These two genes are adjacently located on chromosome 11p15 in humans and chromosome 7 in mice, with about 100kb of intervening sequence (Gabory et al., 2006). The proximity of the two genes allows for the sharing of several regulatory elements, including enhancers lying downstream of *H19* and a differentially methylated region (DMR) 2 kb upstream of *H19*, which contains 25 CpGs methylated only on the paternal allele in human (Vu *et al.*, 2000). In mice, in addition to the *H19* DMR that controls expression of both genes, *Igf2* exclusively has several additional DMRs, of which DMR1 and DMR2 are suggested to have silencing and activating functions on *Igf2* expression, respectively (Constancia *et al.*, 2000; Murrell *et al.*, 2001). Human *H19/IGF2* has been suggested to have DMRs corresponding to the mouse H19 DMR and DMR2 (Vu et al., 2000). The chromatin structure and the interactions of the regulatory factors between *H19* and *IGF2* have been studied extensively using targeted deletions and transgenic mice. The latest model in the mouse proposes that chromatin loops separating an active and an inactive nuclear domain are formed distinctively on maternal and paternal chromosomes; thus, regulate the allele-specific expression of *Igf2* (Murrell *et al.*, 2004) (Figure 1.7)

(a) Schematics of *H19/Igf2*



(b) The chromatin loop model

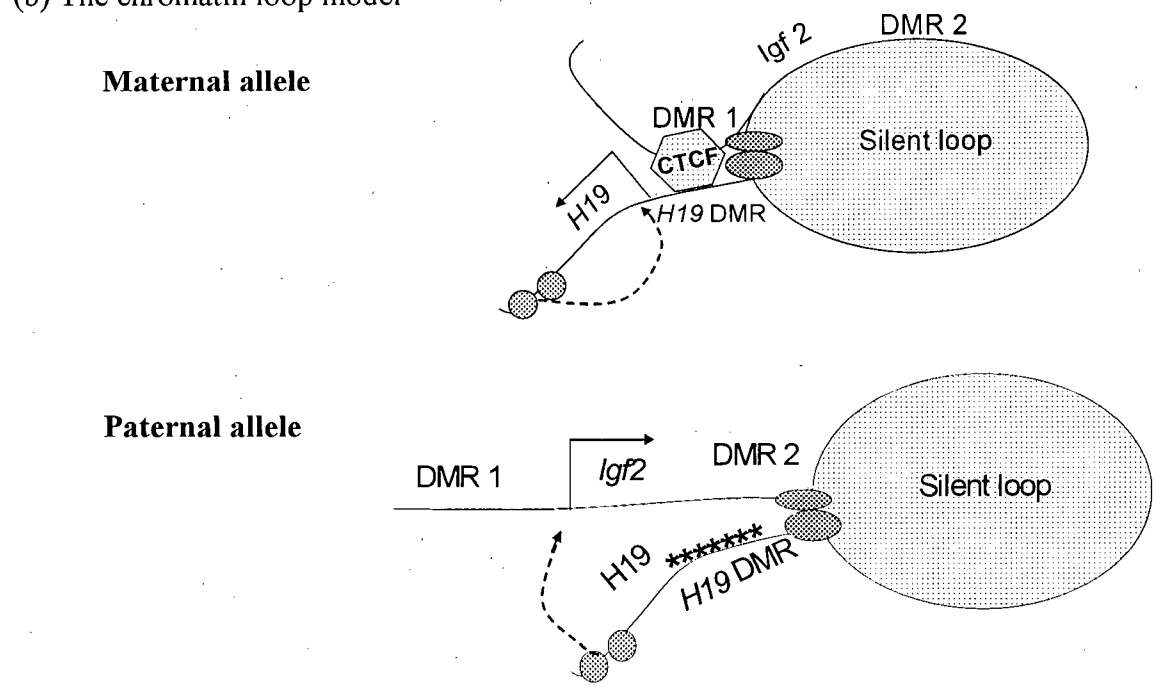


Figure 1.7 Schematic structures of *H19* and *Igf2* (a) and the chromatin loop model (b). ****: methylation.

The *H19* DMR has been suggested to play a particularly important role in allele-specific expression of *H19* and *IGF2*, as it regulates the interaction between enhancers and promoters of the two genes The *H19* DMR is methylated exclusively on the paternal chromosome and contains a CCCTC-binding factor (CTCF) binding domain (Figure 1.8). Methylation suppresses the *H19* promoter of the paternal allele; in contrast, *H19* expression occurs on the unmethylated maternal allele. Regulation of *IGF2* involves the binding of CTCF, which is a zinc finger protein that binds to the unmethylated maternal allele and functions as an insulator, blocking the interaction of enhancers with the promoter region of *IGF2*. As a result, *IGF2* expression is suppressed on the maternal allele, but occurs on the

methylated paternal allele (Fedoriw *et al.*, 2004).. Recent evidence indicates that a post-translational poly ADP-ribosylation of CTCF is important for its proper function. When poly ADP-ribosylation is interrupted, maternal *IGF2* is expressed, regardless of CTCF binding (Yu *et al.*, 2004). In addition to regulation of *IGF2* expression, the CTCF binding domain within the DMR has been suggested to be involved in establishment of methylation during gametogenesis. Point mutations in the CTCF binding site led to inappropriate methylation at the maternal allele, suggesting that methylation may be a default state in the *H19* DMR unless negatively regulated by CTCF binding site (Pant *et al.*, 2004).

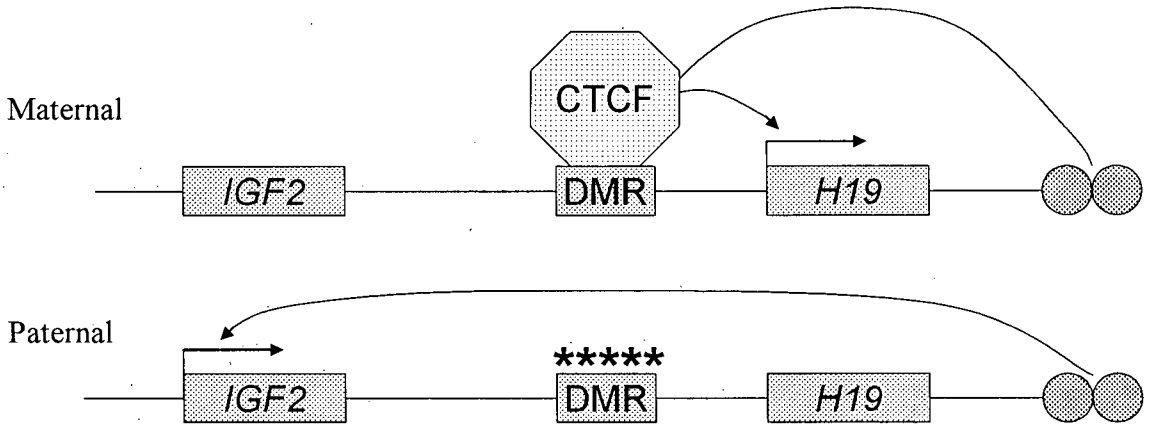


Figure 1.8 Differentially methylated domain at *H19/IGF2*. Imprinting regulation at *H19* and *IGF2* involving maternally methylated DMR domain.

H19 and *IGF2* play important roles in fetal and placental development, as does many other imprinted genes. Hypermethylation at the *H19* DMR, which in turn leads to over-expression of *IGF2* and reduced expression of *H19*, is responsible for the fetal overgrowth observed in BWS patients (OMIM130650). Hypomethylation at the *H19* DMR, on the other hand, has been recently associated with the Silver-Russell Syndrome, characterized by IUGR, postnatal growth retardation, and asymmetry (OMIM 103280) (Gicquel *et al.*, 2005; Bliek *et al.*, 2006).

H19 is highly expressed throughout fetal development in extraembryonic and embryonic tissues mostly derived from endoderm and mesoderm. Postnatal expression of *H19* is significantly down-regulated in all tissues except for skeletal muscles (Weber *et al.*, 2001). However, mice with targeted deletion of *H19* were basically viable and fertile,

although some deletions within the upstream region and transcription unit resulted in overgrowth of fetuses (Leighton *et al.*, 1995). As indicated above, interruptions in the *H19* DMR, which decrease *H19* expression and increase *IGF2* expression, can lead to fetal overgrowth. However, intriguingly a mouse model showed that maternal *IGF2* expression was also induced by a deletion within the *H19* transcription unit. This implies that in addition to the *H19* DMR, the *H19* gene product itself may have a regulatory function on *IGF2* expression.

Because the open reading frames that *H19* encodes contain multiple stop codons shortly after the initiators and *H19* mRNA does not associate with ribosomes, *H19* likely encodes for untranslated RNA (Brannan *et al.*, 1990). *In vitro* and *in vivo* studies also suggested that *H19* RNA may affect *IGF2* expression in a trans-acting manner (Forne *et al.*, 1997; Wilkin *et al.*, 2000). These findings may explain the coordinated expression of *H19* and *IGF2* in the same tissue and at the same developmental stage (Lee *et al.*, 1990). *H19* has also been suggested to be a tumor suppressor gene (Hao *et al.*, 1993; Juan *et al.*, 2000). Hao *et al.* (1993) demonstrated tumor suppressing activity in two embryonic tumor cell lines when transfected with *H19* expression. Thus, disruption of *H19* expression may provide an explanation to the high incidence of Wilms tumor observed in BWS (Gabory *et al.*, 2006).

IGF2 is also expressed in the fetus and extraembryonic tissues. An abundant expression of *Igf2* was observed in the placenta during early development in mice (Reynolds *et al.*, 1997) and was suggested to be related to the early invasion of trophoblast (Hamilton *et al.*, 1998). IGF2, along with other IGFs, have been suggested to positively affect nutrient transport in the placenta (Kniss *et al.*, 1994). A recent murine model demonstrated that targeted deletion of *Igf2* led to absence of a placental specific expression of the P0 transcript and caused reduced growth of the placenta and the fetus. The passive permeability for nutrients across the placenta was found to be significantly decreased. In contrast, active transport mechanisms were initially unrestrained to compensate for the reduced transport; however, this compensation could not supply enough nutrients for later gestational stages and IUGR resulted (Constancia *et al.*, 2002).

1.5.6 Epigenetic aberration and ICSI

Imprinting disorders casused by epigenetic alterations are over-represented in children conceived through ARTs including ICSI. Normal methylation was detected in 92 children born after ICSI at chromosome 15q11-13, which is associated with Angelman syndrome (AS) and Prader-Willi syndrome (PWS) (Manning *et al.*, 2000). However, the sample size was small and the number of methylation sites analyzed was limited. Thus, the link between imprinting error and ART has yet to be determined. Moreover, conclusions should not be drawn before the investigation of individual risk factors such as embryo culture, oocyte maturation, immature sperm, and abnormal semen parameters.

Animal studies have demonstrated that *in vitro* culture may have effects on epigenetic changes and subsequently, fetal growth and development. Large offspring syndrome (LOS), characterized by increased birth weight and perinatal morbidity, has been reported in ruminants after nuclear transfer and *in vitro* culture (Sinclair *et al.*, 2000). LOS has been linked to reduced methylation and expression of *Igf2r* (Young *et al.*, 2001). However, IUGR or LBW appears to be more prevalent in children born after ART, and epigenetic alteration of *IGF2r* has not been correlated to human growth disorders (Maher *et al.*, 2005).

Mouse models have provided further insights into the effects of preimplantation culture on expression and epigenetic alterations of different imprinted genes. Growth deficiency was observed in fetuses derived from embryos cultured in media complemented with fetal calf serum. Expression level of imprinted genes such as *H19, Igf2, Grb7,* and *Grb10* was altered in those fetuses, whereas *Mest* expression was not affected (Khosla *et al.*, 2001). Doherty *et al.* (2000) observed increased *H19* expression and reduced methylation at the *H19* DMR region in embryos cultured in Whitten's medium but not in those cultured in KSOM media with amino acids. *Snrpn* expression was not affected under the same conditions. Conversely, Li *et al.* (2005) compared mouse blastocysts and morulas cultured in human tubal fluid (HTF) with those derived *in vivo*, and detected abnormal *H19* expression that possibly resulted from a gain of DNA methylation and histone methylation at the CTCF binding site within the *H19* DMR region. The expression of other imprinted genes, *Cdkn1c* and *Slc22IL*, were unaffected. It has been proposed that *H19* is particularly vulnerable to

the environmental stress compared to other imprinted genes based on overtly observed *H19/IGF2* epigenetic alterations under different culture conditions (Doherty *et al.* 2001). In addition to a gene-dependent methylation change, Mann *et al.* (2004) proposed a tissue type (embryonic vs. extraembryonic) dependent methylation reduction based on their finding of a particularly high level of epigenetic alteration in the placenta after *in vitro* culture. Although these hypotheses remain to be tested, it is clear that *in vitro* culture of embryos indeed affects gene expression epigenetically, possibly in a gene dependent or tissue dependent manner.

Epigenetic alterations observed in a conceptus can be potentially inherited from the parents. An error can originate from a failure to erase or acquire methylation during gametogenesis. With regard to ART conceptions, manipulation of gametes may also give rise to epigenetically abnormal germ cells. The current belief is that the oocyte may be more prone to imprinting errors than sperm. In contrast to the early reset of paternal imprints (David *et al.*, 2000), acquisition of maternal imprints occurs after birth, and it is mostly completed by metaphase II with some exceptions. For instance, the maternal methylation in chromosome 15q11-q13 is established after fertilization (El-Maarri *et al.*, 2001). *In vitro* oocyte maturation (IVM), by which immature germinal vesicle stage oocytes are cultured until metaphase II, may pose a risk as the oocyte undergoes the epigenetic reprogramming *in vitro*. Mouse data suggested that DNA methylation is altered during IVM at the *IGF2r*, *Mest/Peg1*, and *H19* loci with the alteration at *IGF2r* being most frequent (Kerjean *et al.*, 2003). Borghol *et al.* (2006) detected similar findings in human oocytes retrieved from women who underwent ICSI. A gain of methylation at the CTCF binding domain within the *H19* DMR was found in the majority of MI-arrested oocytes and some of MII-arrested oocytes that were matured *in vitro*. In addition to IVM, hormonal treatment may also cause perturbation on maternal imprints. A two-fold higher incidence of abnormal global methylation pattern was observed in two-cell embryos derived from superovulated female mice compared to those from non-superovulated mice. This is consistent with the subsequent finding that embryos derived from superovulation fail to develop to the blastocyst stage at a higher rate (Shi and Haaf, 2002).

To date, only three genes - *H19, RASGRF1, and GTL2* - are known to be paternally

methylated through primary imprints. Despite this small number of genes known to acquire methylation in spermatogenesis, those genes may play essential role in fetal development. For instance, the importance of *H19* has been demonstrated by the creation of parthenogenetic mice using *H19* $^{-/-}$ females as donors for non-growing oocytes to mimic the paternal genome (Kono *et al.*, 2004). Thus, paternal contribution to the epigenetic regulation should not be overlooked. A low level of global methylation in ejaculated sperm has been associated with lower pregnancy rates in IVF (8.3% in the low methylation group vs. 33.3% in the high methylation group), while no correlation to fertilization rates was observed (Benchaib *et al.*, 2005). When the specific region of 15q11-13 imprinting center was examined, Manning *et al.* (2001) did not find significant difference in methylation of sperm from men with normal semen parameters compared to that with abnormal parameters. On the contrary, Marques *et al.* (2004) reported incomplete methylation at *H19* in oligozoospermic patients, and even more so in severe oligozoospermia, but not in the normozoospermic controls. In ICSI, sperm from infertile men with various abnormal semen parameters are routinely used. Immature spermatids have also been used in cases where ejaculate sperm is not available. In theory, both erasure and re-establishment of primary imprints should be completed by spermatid stage (Reik and Walter, 2001). Shamanski *et al.* (1999) suggested that imprinting is not affected in mouse embryos derived from ICSI using spermatids. A similar finding was reported by Miki *et al.* (2004), who examined mouse embryos derived from round spermatids and detected normal monoallelic expression of *H19, IGF2, Meg3,* and *IGF2r*. Thus, the potential risk regarding epigenetic alteration with the use of ICSI and the accompanying infertility factors is not related to the use of immature male germ cells. However, taking into account that chemical substances or oxidative stress during spermatogenesis have been suggested to alter the chromatin structure and cause DNA damage in sperm (Aitken *et al.*, 2003; Hales *et al.*, 2005), the epigenetic profile in sperm from infertile men requires further investigation.

1.6 Hypothesis and objectives

Pregnancies derived from Intracytoplasmic Sperm Injection (ICSI) have been associated with an increased incidence of low birth weight (LBW) (Schieve *et al.*, 2002,

Katalinic *et al.*, 2004), birth defects (Hansen *et al.*, 2002), chromosomal abnormalities particularly involving sex chromosomes (Bonduelle *et al.*, 2002), and imprinting disease (DeBaun *et al.*, 2003; Cox *et al.*, 2002). Nevertheless, the underlying causes for these adverse outcomes remain unknown.

One contributing factor to some of the negative pregnancy outcomes may be Confined Placenta Mosaicism (CPM). Although the prenatal and perinatal complications reported in ICSI pregnancies are reminiscent of clinical outcomes of CPM, few studies have determined the incidence and parental origin of CPM in ICSI pregnancies. Considering the higher rate of chromosomal abnormalities reported in ICSI newborns (Bonduelle *et al.*, 2002; Lam *et al.*, 2001), it would not be surprising to see a higher rate of placental mosaicism in the ICSI population. If a higher rate of CPM was indeed present in ICSI pregnancies, it may provide an explanation for the increased rate of LBW and SA detected in the ICSI conceptions. In the regard, we speculate a higher rate of CPM in the ICSI pregnancies compared to the general population and expect particularly more profound effects in the LBW group. The ICSI population is subjected to three risk factors that may lead to chromosomal abnormalities - advanced maternal age, male infertility, and the invasiveness of the procedure. According to the limited data on origin, the origin for chromosomal abnormality is primarily paternal, although most abnormality was inherited instead of de novo (Van Opstal *et al.*, 1997; Jozwiak *et al.*, 2004; Boundualle *et al.*, 2002; Tang *et al.*, 2004). Thus, we speculate that the origin for CPM detected in ICSI pregnancies may differ from that observed in the general population. If a paternal bias was present in chromosomal abnormalities found in ICSI conceptus, further investigation in sperm would help us to understand the etiology and to assess the risk. Therefore, the rate of chromosomal abnormality was investigated in cases determined with paternal origins.

Another possible cause for poor pregnancy outcome may be epigenetic defects in imprinted genes important for fetal and placental growth. Assisted reproductive technologies including ICSI has been linked with a higher rate of imprinting disorders (Beckwith-Wiedemann Syndrome and Angelman Syndrome) predominantly caused by an epigenetic alteration. This is not commonly found in the general population. Furthermore, studies in

mice have suggested that the differentially methylated domain (DMR) of *H19/IGF2* is particularly vulnerable to lose methylation in response to an unfavorable media condition. Such effects appear to be most apparent in the placenta (Doherty *et al.*, 2000). Because *H19* and *IGF2* are two important developmental genes, loss of methylation at *H19/IGF2* may subsequently leads to poor placental and fetal development. Taking into account the ICSI-related risk factors for epigenetic alterations such as *in vitro* oocyte maturation, embryo culture, and the use of immature sperm and sperm with abnormal semen parameters, we speculate that altered methylation pattern at the DMR of *H19/IGF2* may be associated with LBW observed in the ICSI population.

In summary, we hypothesize that (1) CPM may be more frequently detected in placentas derived from ICSI pregnancies, especially from those with LBW. 2) The origin of CPM in ICSI pregnancies may differ from the commonly observed mitotic origin in the general population; (3) Epigenetic alteration may be present at *H19/IGF2* in placentas derived from ICSI pregnancies with LBW.

Objective 1: To investigate the incidence of CPM in placentas derived from ICSI (Chapter II)

Objective 2: To determine the parental origin of CPM and non-mosaic chromosomal abnormalities in ICSI conceptions (Chapter III).

Objective 3: To determine the aneuploidy rate in sperm from the paternally derived chromosomal abnormalities (Chapter IV).

Objective 4: To investigate the methylation status at two CpGs from the DMR of *H19/IGF2* in placentas from ICSI pregnancies with LBW (Chapter V).

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CHAPTER2. Confined placental mosaicism in term placentas derived from ICSI pregnancies

2.1 Introduction

Cohort studies have consistently reported that the ICSI population has an increased incidence of low birth weight (LBW) in singleton births (Schieve *et al.*, 2002, Katalinic *et al.*, 2004), birth defects (Hansen *et al.*, 2002), and chromosomal abnormalities particularly involving sex chromosomes (Bonduelle *et al.*, 2002). One contributing factor to some negative pregnancy outcomes may be confined placenta mosaicism (CPM), which describes a feto-placental chromosomal discrepancy such that an abnormality is limited to the placenta. CPM has often been associated with intrauterine growth restriction (IUGR) (Wolstenholme *et al.*, 1996; Lestou and Kalousek, 1998), but is also thought to be responsible for a number of other pregnancy complications such as pregnancy loss (Johnson *et al.*, 1990), congenital abnormalities (Leschot *et al.*, 1996; Ferra *et al.*, 2000), premature labour (Schuring-Blom *et al.* 1993), and stillbirth (Kalousek and Barrett, 1994).

The incidence of CPM has been well established in the general population. It accounts for about 1-2 % of viable pregnancies detected by chorionic villus sampling (CVS) (Simoni *et al.*, 1986; Johnson *et al.*, 1990). Although the majority have a normal pregnancy outcome, CPM has been associated with fetal mortality and morbidity. CPM has been detected in 5-33% of spontaneous abortions (Kalousek *et al.*, 1992; Johnson *et al.*, 1990; Griffin *et al.*, 1997; Qumsiyeh, 1998), and in about 20% of the idiopathic IUGR cases (Leschot *et al.*, 1996; Lestou and Kalousek, 1998). When compared to the total incidence of 6.0% in the general population (Table 1.6), the role of CPM in IUGR cases is evident.

Taking into account the elevated rate of chromosomal abnormalities and other pregnancy anomalies in ICSI conceptions that mirror the clinical phenotypes of CPM, we hypothesized that CPM may be prevalent in the ICSI pregnancies and may explain for some of those frequently observed perinatal complications. The study of CPM in the ICSI population is still limited. Our previous study found that the incidence of CPM in the ICSI population was not statistically different from that in the general population (Minor *et al.* 2006). However, due to a relatively small sample size (n=51), further study was required

to more accurately elucidate the prevalence of CPM in ICSI pregnancies. Furthermore, it is still unknown whether CPM accounts for the higher incidence of low birth weight and other congenital abnormalities found in ICSI pregnancies.

2.2 Materials and methods

2.2.1 Sample collection

Thirty term placentas and cord blood from pregnancies derived from ICSI were collected from patients who gave informed consent. These patients underwent ICSI treatments at the UBC affiliated fertility clinic at Vancouver General Hospital from 2004 to 2006. Attending physicians were requested to provide us with clinical information including birth weight, maternal age, gestational age, pregnancy complications, and congenital abnormalities. LBW involved birth weights below 2500g, while IUGR defined birth weight below the 10^{th} percentile, after adjusting for the gestational age, gender, and plurality. The adjustments were based on the methods described by Hoffman *et al.* (1974) for singletons, and Min *et al.* (2004) for twins. Only pregnancies in which newborns were confirmed to have a normal karyotype in cord blood were included in the current study as the focus of this study is on abnormalities confined to the placenta. Ethics approval was obtained from the University of British Columbia Ethics Committee.

2.2.2 Tissue preparation and DNA extraction

Each placenta was measured, weighed and examined for any morphological abnormalities. Amnion and chorionic villi were collected from ten sites from each placenta. Maternal decidua was also sampled for maternal DNA in the case that genotyping for parental origin was required. DNA was extracted from whole villi at three random sites using standard salt extraction protocols. Specifically, a small piece of chorionic villi (about 2 cm^3) was washed in Phosphate Buffered Saline (PBS) to remove blood and stored at 4°C overnight in minimum essential medium complemented with 4% penicillin streptomycin. Clean villi were then resuspended in 3ml of 2x tissue lysis buffer (100mM Tris; 40mM EDTA; 500mM NaCl) mixed with 50 µL of Proteinase K (5mg/ml), 300 µL of 10% sodium dodecyl sulphate (SDS), and incubated at 55°C overnight. One-third volume (3ml) of 6M

NaCl was added, and the solution was shaken vigorously for about 30 seconds until stiff foam was formed. The mixture was then centrifuged at 4000 rpm at 4°C for 20 minutes. The clear supernatant was transferred to a test tube containing two volumes (8ml) of ice-cold 100% ethanol to precipitate DNA. The DNA was taken out with a pipette tip and washed briefly in 1mL of ice-cold 70% ethanol. The pellet was air dried and dissolved in an adequate amount of TE buffer (10mM Tris-HCl; 1mM EDTA, pH8.0). DNA concentration was measured with a UV spectrophotometer before being used in subsequent molecular analysis.

2.2.3 Karyotyping

Cord blood was cultured for 72 hours using standard protocols. Each flask contains 8.3 mL of culture Medium; 1.7mL of calf serum; 0.25mL of phytohaemagglutinin (PHA); 0.025mL of heparin; 0.083mL of Penicillin and Streptomycin and 0.5 mL of fresh cord blood. 5-Fluoro-deoxy-uridine (FUDR) was added eighteen hours before, thymidine was added four hours before, and colcemid was added 45 minutes before harvesting. Harvested cells were fixed in 3:1 methanol: acetic acid and dropped to slides. Cells spread on slides were stained with Leishman's staining (5ml) and Giemsa (3ml). Twenty-five G-banded metaphases were analyzed in karyotyping analysis including three metaphases fully analyzed by a certified technologist. In the event of culture failure, comparative genomic hybridization (CGH) was performed on DNA from amnion (representative of fetal chromosomal constitution).

2.2.4 Comparative genomic hybridization (CGH)

Comparative genomic hybridization (CGH) is a molecular cytogenetic technique that detects chromosomal imbalances at a resolution of 3 Mb (Kallioniemi *et al.*, 1993). In the current study, CGH was performed following a previously described protocol by Minor (2004) with minor modifications (Figure 2.1). 2μg of test DNA from whole villi was labeled with 4μL of dNTPs containing fluorescein-12-dUTP (FITC) (Roche Diagnostic, Penzberg, Germany) using 1.5μl of Nick Translation Enzyme Mix (Roche Diagnostic, Penzberg, Germany) in combination with 0.8μL of DNA polymerase I (New England Biolabs, Ipswich, MA), in a total 20μl reaction mixture. Nick translation products with optimal fragments lengths of 600bp to 3kb were obtained after incubation at 25°C for about 2 hours and 45

minutes. Reference DNA from a diploid genome (46, XX) was similarly labeled with tetramethylrhodamine-5-dUTP (TRITC) (Roche Diagnostic, Penzberg, Germany). The size and concentration of labeled fragments in each sample was estimated by gel electrophoresis on a 1.2% agarose gel using a Hind III digested Lambda DNA (Sigma, St. Louis, Mo) as a size marker. Test and reference DNA were mixed in a roughly 1:1 ratio, and then co-precipitated with 20 μg of highly repetitive human Cot-1 DNA (Sigma St. Louis, Mo) at -20 °C overnight in 200 μL of ethanol and 5μL of 5M LiCl. The probe pellet was reconstituted in 14μL of hybridization buffer (50% formamide/10% dextransulfate/2x sodium chloride sodium citrate (SSC)). The probe mixture was then denatured at 78°C for 5 minutes and pre-annealed at 37°C for 1 hour before it was applied to a target slide with metaphases from a male control with a confirmed chromosomal constitution of 46, XY. The metaphase slides were prepared with 450-500 band resolution, a high mitotic index, and minimal cytoplasm. Before hybridization, slides were pre-treated with RNase (0.125mg/ml; Sigma, St. Louis, MO) in 2x SSC, fixed in 10% buffered formalin for 10 minutes, and finally denatured in 70% formamide/2x SSC for 5 minutes at 73-75°C on a hot plate. After the probe mixture was applied to a slide, a glass coverslip was applied, sealed with rubber cement and allowed to hybridize in a humid chamber for at least three days. Post-hybridization washes included 0.4xSSC/0.3% NP-40 at 72°C for 2 minutes and subsequently 2xSSC/0.1% NP-40 at room temperature for 30 seconds. Lastly, the slides were counterstained in 4',6-Diamidine-2'-phenylindole (DAPI) (0.2ug/ml; Sigma, St. Louis, MO) and mounted with anti-fade reagent, Vectashield (Vector Laboratories, Burlingame, CA). The slides were stabilized at 4°C for several hours before analysis.

For each case, ten evenly hybridized metaphases with minimum background were captured with a CCD camera attached to a Zeiss Axioplan epifluorescent microscope. Digital images were captured for DAPI, FITC, and TRITC fluorescent wavelengths and analyzed with Cytovision Image Analysis software (Applied Imaging International, Santa Clara, CA). At least six captured metaphases were analyzed for each case. A profile is generated and illustrates a fluorescence ratio between the test and referenced DNA, with the centre line representing the 1:1 proportion. A shift in the profile to the left indicates a loss, and a shift to the right represents a gain of genetic material in the test sample. Abnormal

cases were re-analyzed with CGH before confirmation by fluorescent in situ hybridization (FISH) or polymerase chain reaction (PCR).

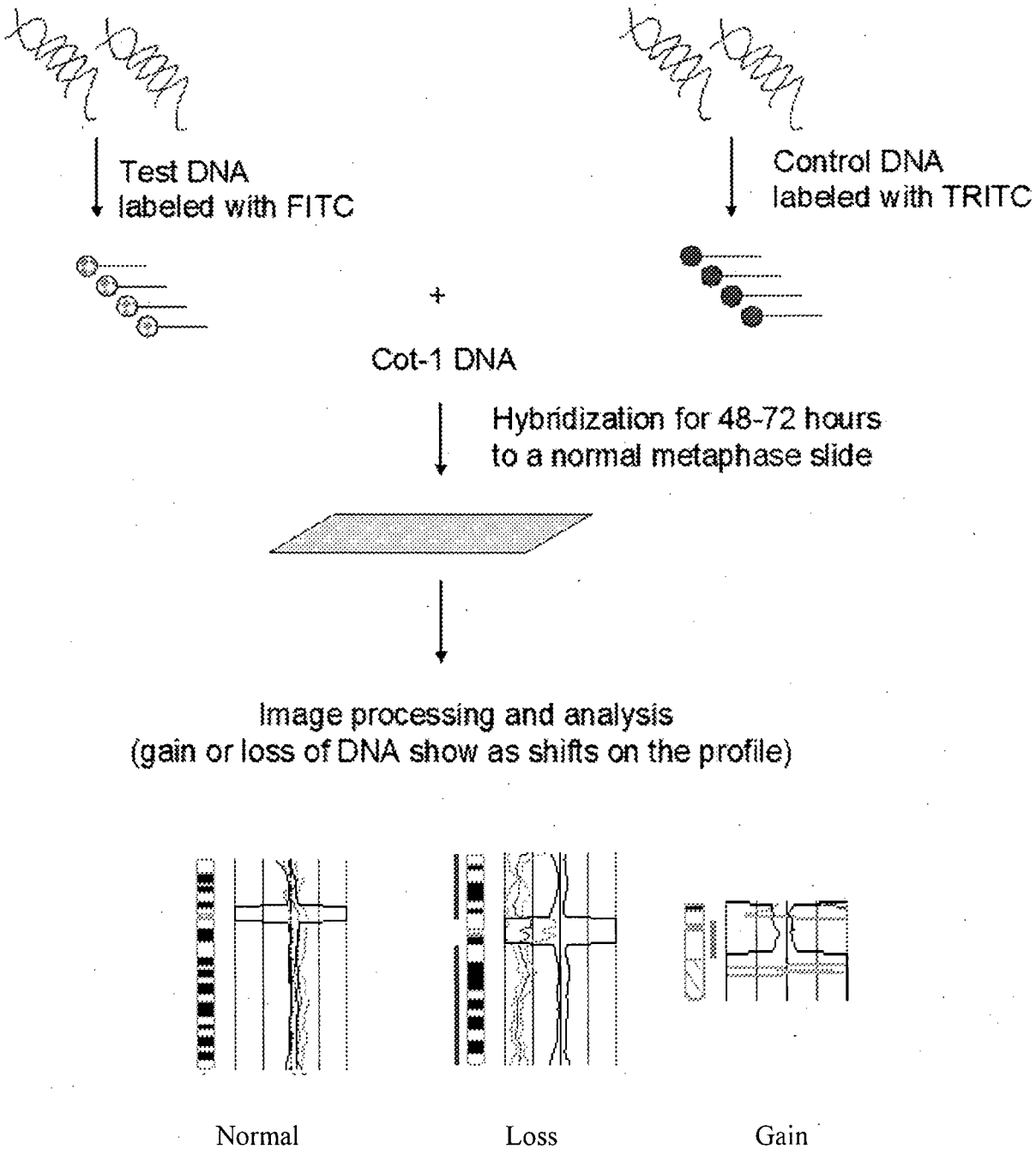


Figure 2.1 Schematics of comparative genomic hybridization (CGH).

2.2.5 Flow cytometry

One of the limitations of CGH is its inability to detect balanced polyploidy; therefore, flow cytometry was used to determine the ploidy level. The analysis was done on mesenchymal cells only, because in the presence of multi-nucleated syncytiotrophoblast cells in the trophoblast samples may inappropriately influence the measurement of ploidy. Preparation and analysis followed protocols described by Minor *et al.* (2006). Villi were digested in collagenase 1A (1mg/mL) (Sigma, Oakville, Canada) at 37°C for 20 minutes with intermittent vortexing. Once the enzyme digestion was complete, 3mL of Hank's Balanced Salt Solution (HBSS) with Ca and Mg was added to each sample to stop the digestion. The supernatant containing the trophoblast cells discarded, and the precipitated mesenchymal cells were washed in 0.9% NaCl solution (pH 1.5) and then digested with 0.5% pepsin (Sigma, St. Louis, MO) for 10 to 15 minutes in a 37°C water bath with constant agitation. The cells were then washed in PBS and filtered through a cell strainer (40μm, Becton Dickson, Franklin Lakes, NJ). The single cells were then fixed in 70% ethanol overnight at -20°C. The cells were resuspended in PBS and the concentration was determined with a haemocytometer. A total volume of about 500μL of single cell suspension with a concentration of 1 million cells per milliliter was prepared. The cells were stained with propidium iodide (PI; 40 μg/mL) (Sigma, St. Louis, MO) and pretreated with RNase (20ug/mL; Sigma, St. Louis, Mo). The analyses of stained cells were performed with the FACScan flow cytometer (Becton Dickson, Franklin Lakes, NJ) at Dr. Hmama's lab in the Department of Infectious Disease located at Vancouver General Hospital. The instrument was calibrated with a diploid control sample and the accuracy was confirmed with a polyploidy (triploidy or tetraploidy) control sample. Twenty thousand cells were included in the analysis for each case. The data recording was facilitated by the CellQuest software, and data analysis was performed with computer software called FlowJo (Tree Star, Inc. Ashland, OR). Diploidy range was determined by a peak area that shifted within ±10% of the control diploid peak (G1), and a G2 peak that contains lower than or equal to 15% of the cell population (Rua *et al.*, 1995). Abnormal cases were repeated before confirmation by FISH.

2.2.6 Fluorescent in situ hybridization (FISH)

Abnormalities detected by CGH or flow cytometry in villi samples were confirmed by Fluorescent in situ Hybridization (FISH).

Preparation and pretreatment

Villi were separated into cytotrophoblast and mesenchymal stroma cells in order to determine the type and level of mosaicism in cases where presence of aneuploidy was suggested by CGH or flow cytometry. Tissue separation was performed as described by Henderson *et al.* (1996). Villi were first cleaned of blood and decidua under an inverted light microscope. The villi were then digested in collagenase 1A (1mg/mL; Sigma, Oakville, Canada) at 37°C for 20 minutes with intermittent vortexing. Once the enzyme digestion was complete, 3mL of Hank's Balanced Salt Solution (HBSS) with Ca and Mg was added to each sample to stop the digestion. The supernatant containing the trophoblast cells was collected in a different tube, and the precipitated mesenchymal cells were further washed in HBSS several times. Trophoblast cells were resuspended in pre-warmed 1% sodium citrate and incubated at 37°C for 20 minutes before being fixed in 3:1 methanol:acetic acid. The cells were then dropped onto glass slides, pretreated with 0.03% trypsin (Difco, Oakville, Canada) in PBS for 10 seconds and further fixed in 10% buffered formalin for 10 minutes. The mesenchymal cells were first washed in 0.9% NaCl solution (pH 1.5) and then digested with 0.5% pepsin (Sigma, St. Louis, MO) for 10 to 15 minutes in a 37°C water bath with constant agitation. The cells were then washed in PBS and filtered through a cell strainer (Becton Dickson, Franklin Lakes, NJ) to isolate single cells. Subsequently, the cells were dropped onto slides and fixed in 10% buffered formalin for 2 hours and 15 minutes.

Hybridization procedure

Dual-colour FISH was carried out using LSI 13/21 probes (13q14 LSI13, SpectrumGreen/21q22.13-q22.2 LSI21, SpectrumOrange; Vysis, Downers Grove, IL, USA). Triple-colour FISH was performed using α-satellite DNA probes for chromosome 18, X and Y (CEP18 SpectrumAqua / CEP X SpectrumGreen / CEP Y SpectrumOrange; Vysis, Downers Grove, IL, USA). Prior to hybridization, the slides were denatured in 70%

formamide/2x SSC (pH7.4-7.5) at 75°C for 5 minutes. The slides were dehydrated in an ice-cold ethanol series (70%, 80%, and 100%) for 2 minutes each and air-dried at room temperature. 10 μL of pre-denatured probe mixture was applied onto each slide, and a 22x22 mm coverslip was applied and sealed with rubber cement. Hybridization occurred in a humid chamber overnight at 37 °C. Post-hybridization wash was carried out as described for the CGH protocol.

Scoring and analysis

The analysis was carried out with an epifluorescent microscope (Nikon Elipse E600W) equipped with a triple bandpass filter (DAPI / FITC / Cy3), a dual bandpass filter (FITC/Cy3), and single bandpass filters for DAPI, Aqua, FITC, and Cy3. Scoring was performed in areas with consistent hybridization. At least 500 non-overlapped intact nuclei from each tissue type were scored for the analysis of mosaicism.

2.2.1 Molecular analysis

Additional sites of the placenta from which the abnormality was detected were investigated using microsatellite DNA markers. Villi were enzymatically separated into trophoblast and mesenchymal cells as described above, and DNA was subsequently extracted. PCR amplification was performed using fluorescently labeled primers that target the microsatellite repeats on the chromosomes involved. An automated genetic analyzer, ABI310 (ABI, Foster city, CA), was used to quantify the PCR products. The products were separated by the size of alleles, displayed as distinctive peaks on ABI310 if heterozygous. Because there is an allele amplification bias for some markers, especially for those with relatively short amplicons, estimation of dosage difference was made after adjusting for the bias.

2.3 Results

2.3.1 Clinical outcomes

A total of thirty post-delivery placentas from ICSI pregnancies were included. These consist of 22 singletons and 4 sets of twins (Table 2.1). The common observation of advanced maternal age and multiple births in the ART population was present in this group as well. The average maternal age was 36.24 ± 5.10 years, which is significantly higher than that in the general population [29.9 years in British Columbia (BC); $p<0.0001$, t-test]. The rate of twins was 13%, which is also significantly higher than the 2.8% observed in the BC population ($p<0.05$, Chi-square). Surprisingly, the average birth weights for singletons (3430.73g ± 413.27g) and twins (3045.25g ± 532.63g) were both higher than those conceived spontaneously (3405g in BC for all live births in 2004; 3,368g ±580g for singletons, 2,299g ±738g for twins in German data, Katalinic *et al.*, 2004), although the differences were not statistically significant ($p>0.05$, t-test). The mean gestational ages observed in the ICSI group were 40.2 ± 1.37 weeks for singletons and 37.32 ± 1.01 weeks for twins, which are significantly higher than the 39.2 ± 2.3 weeks and 35.0 ± 3.6 weeks, respectively, observed in the general German population [Katalinic *et al.*, 2004 (Germany); 38.5 weeks for all live births estimated in BC]. About 62% of births overall in this study population occurred after 40 weeks, which is significantly higher than the rate reported by Katalinic *et al.* (2004) (30.1% in ICSI and 22.2% in the controls, both $p<0.0001$, Chi-square; BC data not available). The perinatal outcomes were generally normal except that two twins were found with LBW (2497g and 2313g). The frequency of LBW in twins (25%) is not significantly different from that in twin spontaneous pregnancies (52.3%) [Katalinic *et al.* 2004 (German); BC data not available], however sample size was small (n=8). No LBW or IUGR cases were observed in the singleton births (Table 2.2).

Table 2.1 Clinical information for term pregnancies

ICSI newborns	n	%	Maternal age (yr) (mean ± SD)	Gestational age (wk) (mean ± SD)	Birth after >40WK ges. (%)	birth weight (g)
singletons	22	73	35.18 ± 4.58	40.2 ± 1.37*	16 /22 (73)	3430.73 ± 413.27 ^{NS}
twins	8	27	38.50 ± 6.35	37.32 ± 1.01*	0	3045.25 ± 532.63 ^{NS}
total	30	100	35.62 ± 5.02*	39.61 ± 1.62	16/26 (62)**	3380.96 ± 435.24

*Significantly higher than that in natural conceptions (BC and German data) (t-test: $p<0.05$)
**Significantly higher than the controls (German data) (Chi-square: $p<0.05$)
^{NS} Not significantly different from the natural conception data.

Table 2.2 LBW in singletons and twins in ICSI pregnancies compared with controls

ICSI newborns	NBW	IUGR	LBW	LBW rate in controls		Statistics [d]
	(n)		n (%)	German data [a]	BC data [b]	p-value
singleton	22	0	0	416/7861 (5.3%)	2260/40318 (5.6%) [c]	n.s
twins	6	0	2 (25)	80/152 (52.3%)		n.s

a. from Katalinic *et al.*, 2004
b. from BC Vital Statistics Agency
c. data includes both singletons (97.1%) and multiple births (2.9%)
d. n.s = not significant ($p>0.05$, Chi-square)

2.3.2 Detection of CPM in ICSI pregnancies

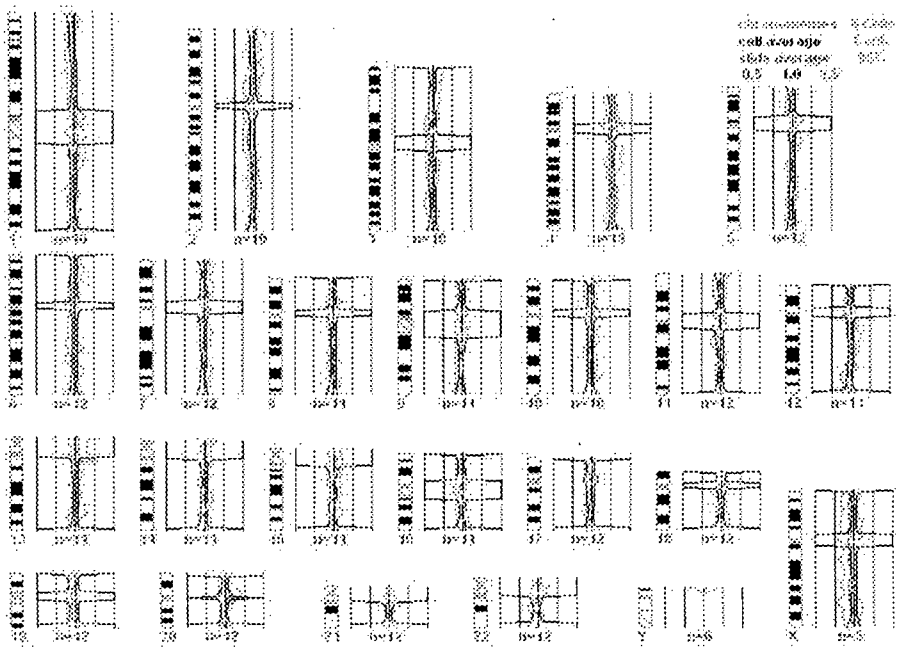
2.3.2.1 CPM detected by CGH

CGH was performed to detect gain or loss of genetic material in whole villi from the thirty placentas. In addition, amnion was analyzed by CGH when karyotype results were not available due to culture failure. Figure 2.2 illustrates typical CGH profiles for balanced chromosome constitution [Figure 2.2 (a) Balanced XX; Figure 2.2 (b) Balanced XY] at 95% confidence intervals. The CGH results are summarized in Table 2.3.

Table 2.3 Summary of CGH results

Fetal compartment		CGH on whole villi			number of cases
chromosome constitution	Tissue type	Site 1	Site 2	Site 3	
46,XX	Cord blood	bal, XX	bal, XX	bal, XX	12
46,XY	Cord blood	bal, XY	bal, XY	bal, XY	7
bal,XX	Amnion	bal, XX	bal, XX	bal, XX	6
bal,XY	Amnion	bal, XY	bal, XY	bal, XY	4
46,XX	Cord blood	bal, XX	bal, XX	**unbal, -X**	1

(a)



(b)

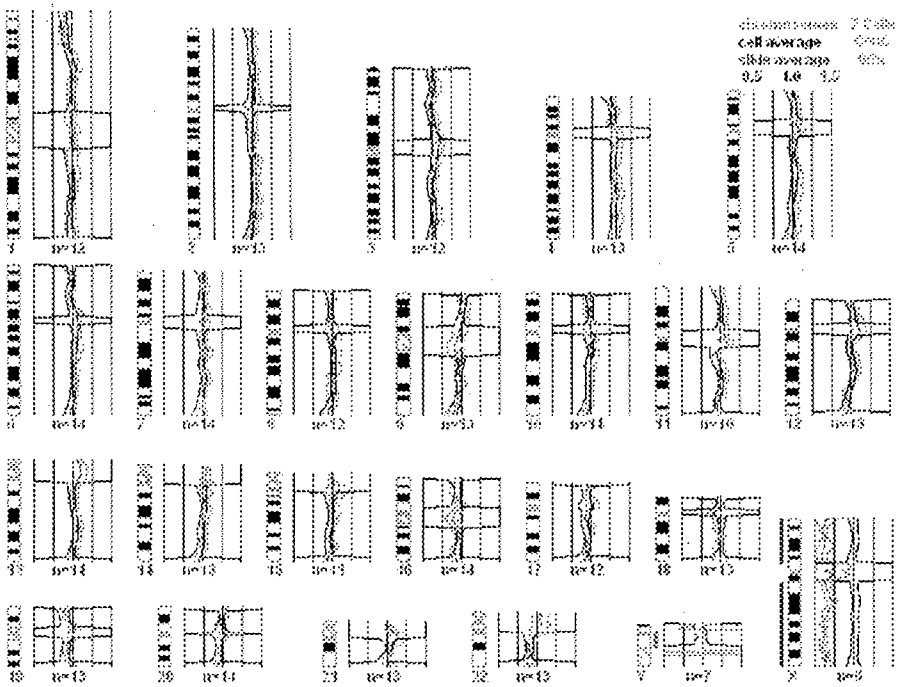


Figure 2.2 Balanced CGH profiles for (a) a normal female and (b) a normal male. Because normal female DNA was used as the reference, all chromosomes in the female profile (a) show a 1:1 ratio; whereas, the male profile (b) has a shift to the left on the X chromosome and to the right for the Y chromosome, representing a loss of X chromosome and gain of a Y chromosome compared with the female reference.

Sex chromosome monosomy (unbalanced X) was detected by CGH from one site of a placenta (SM04-69), while the other two placental sites sampled possessed balanced XX constitutions. The CGH profile of the abnormal cite from SM04-69 shows a shift to the left on the X chromosome but no shift on the Y chromosome (Figure 2.3), indicating monosomy of the whole X chromosome. The pregnancy outcome of this CPM case was normal with the birth weight being 4173g at 41weeks of gestation. Thus, the incidence of CPM in the present study is one in thirty cases (3.3%), which is lower than the background rate of 6.0 % (49/818) in the general population (Table 1.6). Moreover, CPM was not observed in either of the two LBW cases.

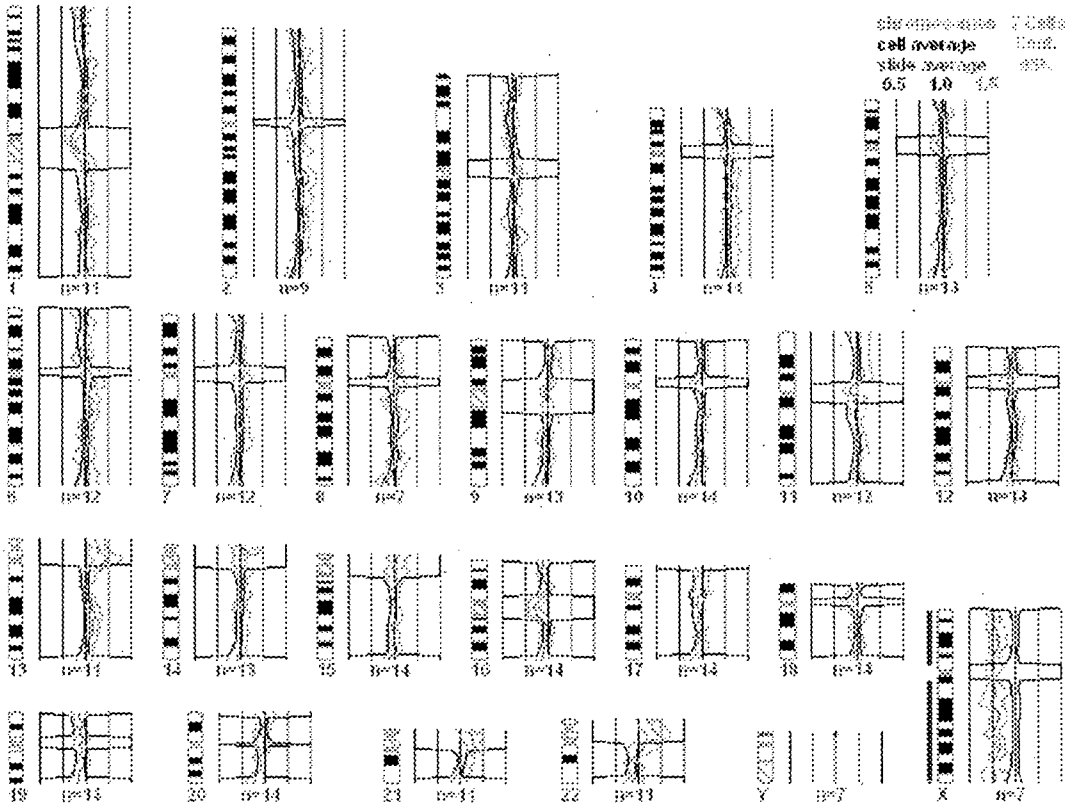


Figure 2.3 CGH profile of the abnormal site from SM04-69. A shift to the left indicates the loss of the whole X chromosome.

2.3.2.2 Confirmation of CPM by FISH

Trophoblast and mesenchymal core cells were separated from villi originally sampled from the site with the abnormality (SM04-69 site 10). FISH was performed on each tissue type with probes targeting chromosome 18, X and Y (Figure 2.4). The chromosome 18 probe serves as an internal control and provides a measure of hybridization efficiency (>99%). About 500 nuclei were scored for each tissue, and the results were compared to 200 nuclei isolated from mesenchymal core from control placentas with known normal karyotype. The results indicate that monosomy X (45,X) was present in the trophoblast (98.8%) but absent from the mesenchymal cells (5.1%) (Table 2.4).

(a) Trophoblast cells (b) Mesenchymal cells

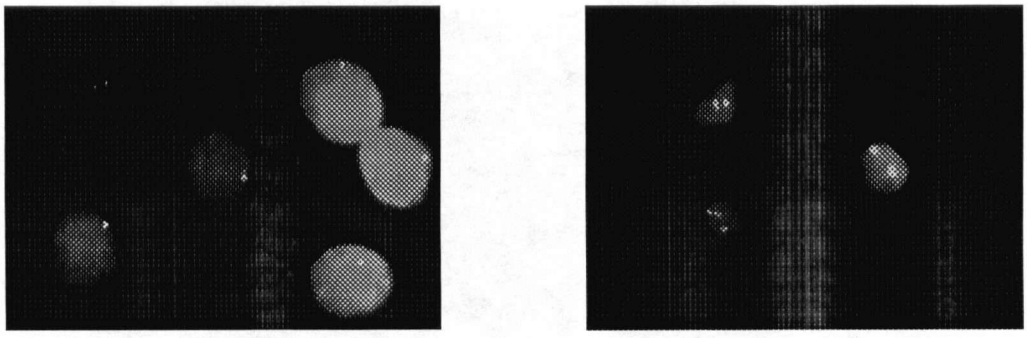


Figure 2.4 FISH confirmation of the cytogenetic abnormality (monosomy X). Images captured under DAPI and FITC filter were overlain to represent nuclei hybridized with the CEP X SpectrumGreen probe. (a) Trophoblast cells with a 45, X constitution (b) mesenchymal core cells with a 46, XX constitution.

Table 2.4 Confirmation of the 45, X abnormality ascertained thought CPM by FISH.

Chromosomal constitution	SM04-69 Troph 10 (n=507)	SM04-69 Mesen 10 (n=530)	Controls (n=205)
18, 18, X, X	4 (0.79%)*	503 (94.9%) ^{NS}	200 (93.5%)
18, 18, X	501 (98.8%)*	27 (5.1%) ^{NS}	5 (2.2%)
18, X	2 (0.4%) ^{NS}	0 ^{NS}	0

Controls are mesenchymal cells in control placentas with known normal karyotypes
*significantly different from the control ($p<0.05$ by Chi-square)
^{NS} Not significantly different from the control ($p>0.05$ by Chi-square)

2.3.2.3 Confirmation of CPM by molecular analysis

Trophoblast and mesenchymal cells from other sites sampled from the placenta with 45, X were analyzed by genotyping. PCR amplification was performed using fluorescently labeled primers that target the microsatellite repeats at the Androgen Receptor (AR) locus on the X chromosome. An allele ratio was calculated by P_{low}/P_{high}, where P_{low} represents the peak area of lower allele and P_{high} represents that of the higher allele. Due to an allele amplification bias, the allele ratio in a normal cell line is not exactly 1:1 (Table 2.5). The average ratio observed in our normal tissues was 1.42 ± 0.11. However, the ratio of 3.22 observed from trophoblast at site 9 is obviously higher and indicates mosaicism. If the allele ratio in the normal cells within the placenta with mosaicism is 1.42, the level of mosaicism is calculated to be 56% by the equation $(P_{high} - 1.42 \times P_{low}) / P_{high}$.. In addition, the abnormality was again confirmed at the site 10. Thus, the 45,X constitution was found to be confined to trophoblast from two sites of the placenta, sites 9 and 10, both of which are relatively close to the center of the placenta (Figure 2.5).

Table 2.5 Summary of peak ratios for all tissues tested by PCR

	site 1	site 2	site 3	site 4	site 5	site 6	site 7	site 8	site 9	site 10
Trophoblast	1.47	1.44	1.41	1.34	1.58	1.46	1.62	1.41	**3.22**	**complete loss of X**
Mesenchyme	1.41	1.41	1.32	1.23	1.4	1.23	1.37	1.54	1.32	1.56

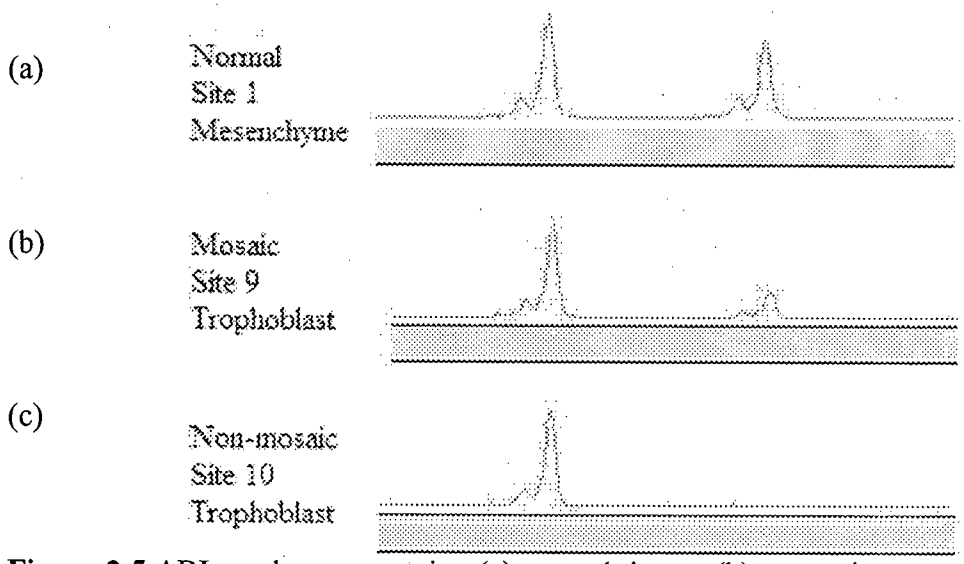


Figure 2.5 ABI results representing (a) normal tissues (b) a mosaic monosomy X and (c) a non-mosaic monosomy X.

2.3.2.4 Ploidy determination by flow cytometry

Because of CGH's inability to detect polyploidy, flow cytometry was used to examine the ploidy levels in all cases except SM04-69, in which the absence of polyploidy was confirmed by FISH analysis. A G1 peak represents the diploid cell population and a G2 peak represents the tetraploidy cell population. Triploidy is characterized by a peak positioned between the G1 and G2 peaks (Figure 2.6 and 2.7). Polyploidy detection is considered positive if triploid or tetraploid cell populations indicated by respective peaks represent more than 15% of the entire sample. Three samples were identified as tetraploidy (SM 05-100 S2, SM 06-131 S1, SM 06-135 S1) and one sample as triploidy (SM 05-94 S1) by the flow cytometry (Table 2.6). In spite of this, all abnormalities found were determined to be false positives by FISH analysis with chromosome 13 and 21 probes. About 200 nuclei were scored for each case (Table 2.7).

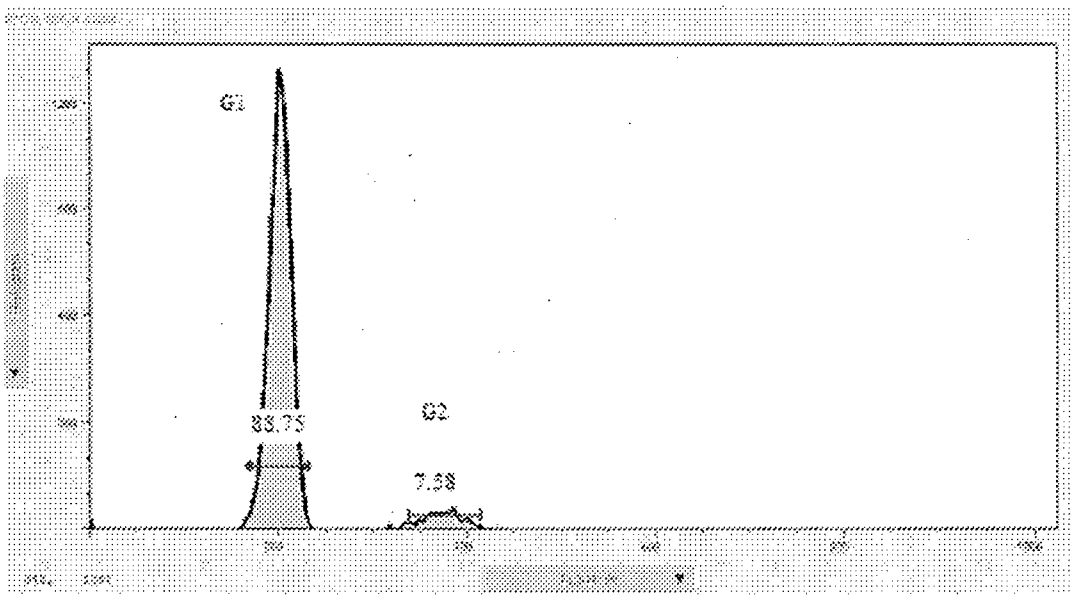
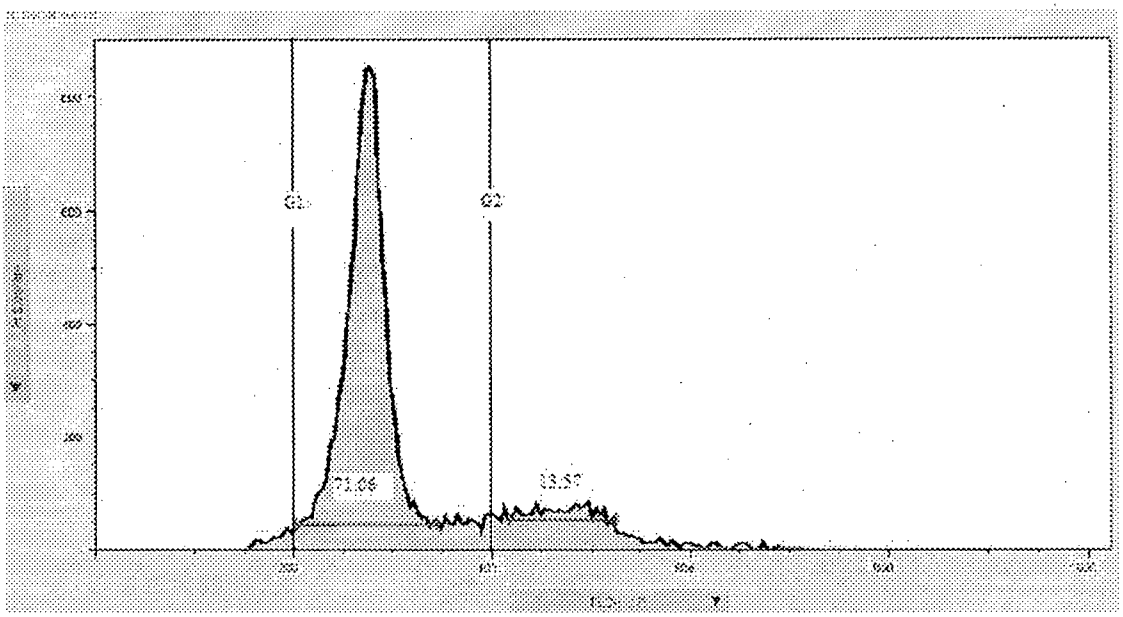


Figure 2.6 A typical flow cytometry result representing diploidy. The G1 and G2 peak represented 88.75% and 7.58% of the cells respectively. The G1 peak indicates a diploid cell population at the G1 phase. The G2 peak indicates the G2 phase or possible tetraploidy if higher than 15%.

(a) Triploidy



(b) Tetraploidy

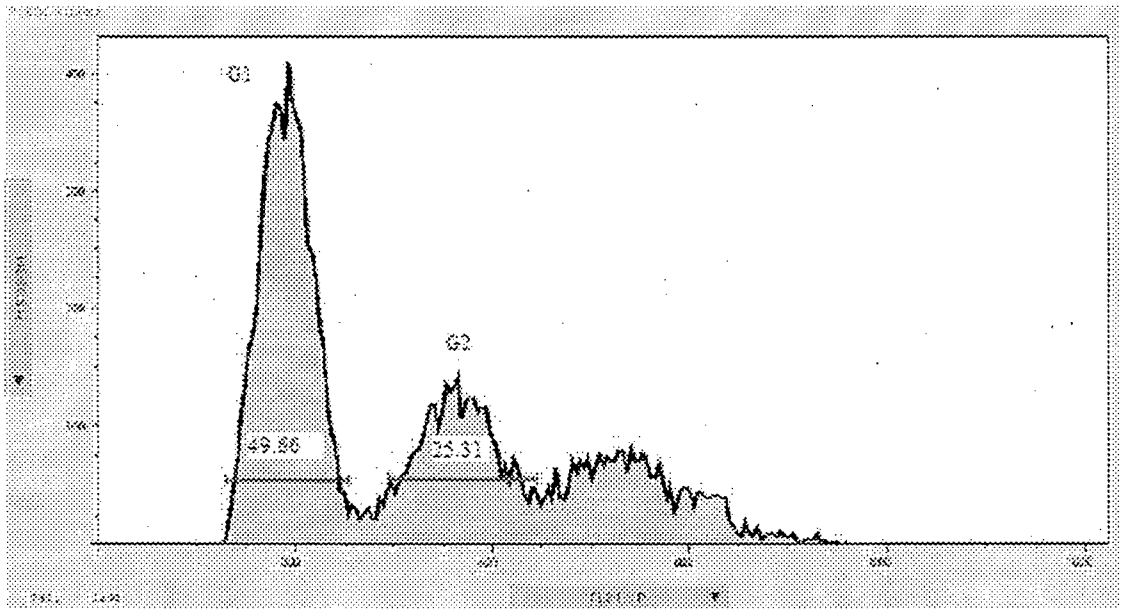


Figure 2.7 Flow cytometry results representing (a) triploidy and (b) tetraploidy. (a) 71.06% of the cells fall between the G1 and G2 peak, indicating a possible triploid cell population. (b) The G1 peak represents 49.66% of the cell population. The G2 peak was also higher than the threshold 15%, thus indicating a possible tetraploidy.

Table 2.6 Summary of polyploidy detected by flow cytometry

case number	Flow cytometry results			FISH confirmation
	Diploidy (%)	Triploidy (%)	Tetraploidy (%)	
SM 05-94 S1	-	71.06	-	Diploid
SM 05-100 S2	70.43	-	17.1	Diploid
SM 06-131 S1	49.66	-	25.31	Diploid
SM 06-135 S1	65.02	-	20.75	Diploid

Table 2.7 Confirmation of polyploidy by FISH

Case	total counts	Diploidy (%)	Polyploidy		P-value
			Triploidy (%)	Tetraploidy (%)	
SM 05-94 S1	216	216 (100)	0	0	ns
SM 05-100 S2	239	237 (99.1)	0	0	ns
SM 06-131 S1	208	205 (98.6)	0	2 (0.96)	ns
SM 06-135 S1	220	216 (98.2)	0	3 (1.36)	ns
control	214	200 (93.5)	0	0	

ns – Not significantly different from the rate in the control ($p>0.05$, Chi-square)

2.4 Conclusions and discussions

The current study included thirty placentas collected from twenty eight pregnancies facilitated by ICSI. Advanced maternal age and multiple-births (twins), frequently reported in the ART population, were observed. In contrast with most cohort studies that reported reduced birth weight in ICSI pregnancies (Schieve *et al.*, 2004; Helmerhorst *et al.*, 2004; Katalinic *et al.*, 2004), our study group found comparable birth weights with that from natural conceptions, in both singletons and twins. No LBW was present in the singletons, and two cases of LBW (<2500g) were observed out of eight twins (25%), which is less frequent than in other ICSI and control cohorts (40-60%) (Pinborg *et al.*, 2004; Katalinic *et al.*, 2004). These two cases could not be classified as IUGR because the birth weights were above the 10^{th} percentile, after adjusting for gestational age, plurality, and gender of the infants. The lack of reduced birth weight cases in our study group may be influenced by the effects of higher gestational age, with 62% of births occurring after 40 weeks. This is significantly higher than the 30.1% in ICSI and 22.2% in controls observed in a recent cohort study (Katalinic *et al.* 2004).

CGH was previously used to identify chromosomal abnormalities in the whole villi without separation of trophoblast and mesenchymal tissues (Minor *et al.*, 2006). Although it

has been suggested that CGH may not be sensitive enough to detect low level mosaicism (Barrett *et al.*, 2000), high resolution CGH used in the current study has proven to improve sensitivity in the detection of low level mosaicism (about 30%). In fact, the sensitivity may be better than reported values (Kallioniemi *et al.*, 1994; Ness *et al.*, 2002), as Minor *et al.* (2006) were able to detect mosaicism as low as 10.8%. To test the sensitivity of detecting aneuploidy in whole villi, CGH was performed on DNA from a combination of trophoblast and mesenchymal stroma from a case where abnormalities were confined to the trophoblast but absent from the mesenchymal layer (Minor *et al.*, 2006). Subsequently, CGH was sensitive enough to detect the trophoblast-confined abnormalities, gain of 7q31>qter (34% by FISH) and loss of Xp21 >pter (79% by FISH) (Figure 2.8) (Minor et al, 2006). Although an extremely low level of mosaicism may be overlooked, the clinical significance of low level mosaicism is expected to be less (Miny et al., 1991).

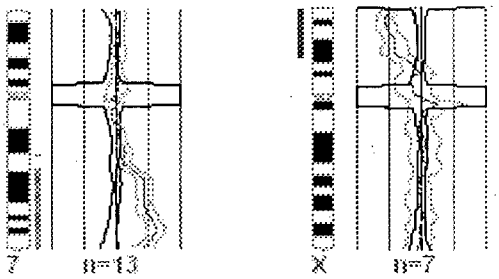


Figure 2.8 Confirmation of previously detected abnormal case with a gain of 7q31>qter and loss of Xp21 >pter by CGH using mixed trophoblast and mesenchymal DNA.

In combination with CGH, the incidence of CPM in term placentas derived from conceptions induced by ICSI was investigated by flow cytometry, FISH and microsatellite genotyping. Monosomy X (45,X) was identified from two sites of one placenta with approximately 56% and 99% mosaicism respectively, estimated by FISH and dosage ratio on genotyping . The abnormality was confined to the trophoblast tissue. This type I CPM has been associated with the least severe clinical outcomes (Kalousek et al 1991). It has also been suggested that CPM involving sex chromosomes is linked to normal fetal development (Farra *et al.*, 2000). In agreement with these reports, the pregnancy outcome of this placental 45, X karyotype was normal with the birth weight being 4173g at 41weeks of gestation.

Because only one tissue type was affected and the abnormality was limited to two out of ten sites, this cytogenetic abnormality is most likely derived from a post-zygotic error (for details on origin determination, refer to chapter 3).

Flow cytometry detected four polyploidy cases that could not be identified by CGH. However, the results were confirmed to be false positive by FISH with probes targeting two chromosomes (13 and 21). The possibility of mosaicism can be eliminated as cells from the exactly same sites were used for confirmation. False results from flow cytometry have been frequently reported in other studies at various rates (Jones *et al.*, 1991, Konchuba *et al.*, 1993; Zbieranowski *et al.*, 1993). It also has been suggested that flow cytometry has lower sensitivity than interphase FISH in the determination of aneuploidy (Cajulis *et al.*, 1995). The three false tetraploidy cases in the current study are presumably due to an incomplete isolation of single cells in a small fraction of cells since the G2 peaks were only 2 -10% higher than the threshold 15%. For future studies, finer cell strainers (<40 μm) should be used to improve separation of single cells. In contrast, the reason for the false triploidy case is more obscure since 71.06% of the cells appeared affected and the main peak was between G1 and G2 phases. We speculate that the diploid G1 peak shifted to the right either because of DNA degradation or sporadic instrumentation error from bubbles or voltage fluctuation, causing false readings.

Thus, only one case of CPM was identified in the thirty term placentas collected from ICSI-induced pregnancies (1/30 (3.33%)), which is not significantly different from that in the natural conceptions [49/818 (6.0%), Table 1.6; $p>0.05$, Chi-square]. This case had normal pregnancy outcomes including a normal birth weight; therefore we could not evaluate the role of CPM in LBW cases. Also, it is worth noting that the two LBW cases in this study were both from twins and did not fall into the IUGR category. Considering that twin pregnancies are intrinsically born smaller, the observed low birth weight may not be lower than their growth potential and is unlikely to be related to any pathology.

Due to the limited sample size (n=30), we combined our data with a previous study (n=51, including nine cases of IUGR) (Minor *et al.*, 2006) in order to increase the statistical power. With the combined data, the incidence of CPM is 4/81 (4.94%) in the ICSI population; however, it still does not differ significantly from the controls (6.0%). Although an increased aneuploidy rate has been observed in ICSI-derived pregnancies by CVS

(Bonduelle *et al.*, 2002), placental mosaicism does not appear to be prevalent in the ICSI population according to our findings. The discrepancy between the incidence of aneuploidy detected by CVS and that in term placentas may result from a selection against the abnormalities in early fetal development, by which a pregnancy may end in spontaneous abortion. In the general population, CPM has been associated with a number of prenatal and perinatal complications, particularly with IUGR. However, we did not observe an increased incidence of CPM in IUGR pregnancies derived from ICSI [0/9 (0%) in ICSI vs. 43/303 (14.2%) in naturally conceived controls (Table 1.6)]. Combining IUGR (n=9) and LBW (n=2) cases still does not reveal any cases associating CPM with reduced birth weight (0/11). Despite the extensively reported susceptibility of ICSI conceptions to LBW and chromosomal abnormalities, we observed a lower incidence of CPM in the ICSI-derived IUGR/LBW pregnancies (0%) compared to that in the spontaneous conceptions (14.2%).

Table 2.8 Summary of CPM rate in the previous study, the current study, and the combined data

Pregnancy outcomes	*CPM rate in the previous study (%)	CPM rate in the present study (%)	CPM rate in the combined data (%)	Controls (%)
normal	2/41(4.88)	1/28 (3.57)	3/69 (4.34) ^{NS}	6/515 (1.2) ^{NS}
Abnormal	1/10(10)	0/2	1/13 (7.69) ^{NS}	43/303 (14.2) ^{NS}
congenital abnormalities	1/2	0	1/2	-
IUGR	0/9	0	0/9	43/303 (14.2)
LBW	0	0/2	0/2	-
Total	3/51(5.88)	1/30(3.3)	4/81(4.94) ^{NS}	49/818 (6.0) ^{NS}

*Minor *et al.*, 2006
NS: The difference is not statistically significant ($p>0.05$ by Chi-square).

Although our findings suggested lack of correlation between CPM and reduced birth weight, more cases have to be examined in order to thoroughly evaluate this association. While both LBW and IUGR are used to describe reduced birth weight, they are significantly different in definition and etiology. Unfortunately, many clinical reports of perinatal outcomes of ICSI pregnancies indicate only the incidence of LBW and VLBW but not IUGR (or SGA), which requires a correction for gestational age and sex. This made it harder to pinpoint the cause for the generally observed reduced birth weight in the ICSI population. LBW is largely related to premature birth (delivery before the 37th week of gestation) and can be affected by maternal age (BC vital statistics). In addition, Gaudoin *et al.* (2003)

suggested that infertility, at least related to female factor, may also be responsible for LBW, because infertile females treated with ovarian induction/intrauterine insemination (OI/IUI) were associated with LBW pregnancies, while such a relation was not observed in fertile females treated by OI/IUI with donor sperm because of male factor infertility. On the other hand, the pathogenesis of IUGR appears to involve complicated interactions between the uterus, the placenta and the fetus. It has been well established that IUGR can result from a shallow trophoblast invasion at early gestation, adverse utero-placenta circulation, and inadequate placenta transport properties (Cetin *et al.*, 2004). The underlying genetic causes for IUGR have been suggested to include CPM, single gene disorders, and aberrant genomic imprinting, which has been evidenced by uniparental disomy cases and knockout mouse models. Thus, there are a number of contributing factors to consider as a cause for LBW and IUGR.

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CHAPTER 3. Origin of chromosomal abnormalities in conceptus derived from ICSI

3.1 Introduction

In the general population, most aneuploidies ascertained from spontaneous abortions (SA) have been attributed to maternal meiotic error, except trisomy 4, 5 and sex chromosome aneuploidy. Trisomy 4 and 5 primarily derive mitotically (Robinson *et al.*, 1999), and sex chromosome has a higher rate of paternal contribution especially in monosomy X (75% paternal, Hassold et al., 1992) and in cases involving the Y chromosome (Hassold and Hunt 2001; Robinson and Jacobs 1999). In contrast, confined placental mosaicism (CPM) is more frequently associated with mitotic origin, with the exception of trisomy 16 and 22, and possibly trisomy 9 (Wolstenholme, 1996; Robinson *et al.*, 1999). Trisomy 16 and 22 almost exclusively derive from a meiotic error in both non-mosaic and mosaic cases (Robinson *et al.*, 1999). While much remains to be understood regarding the etiology of aneuploidy, maternal age seems to be the only clear predisposing factor for aneuploidy in the general population (Hassold and Chiu, 1985).

Advanced maternal age is known to be prevalent in patients undergoing assisted reproductive technologies (ART); this may partially explain for the increase in chromosomal abnormality in the ART population. However, intracytoplasmic sperm injection (ICSI) appears to add additional risk for chromosomal anomalies, as a higher incidence of chromosomal abnormality has been detected in ICSI conceptions compared to those conceived through conventional in vitro fertilization (IVF) (Wennerholm *et al.*, 2000). Considering the fundamental difference between these two technologies, ICSI assumes two additional risk factors, the underlying male infertility and the invasiveness of the procedure. The incidence of somatic chromosomal abnormality in infertile men is 10-20 fold higher than the fertile population (Matsuda *et al.*, 1992), primarily involving sex chromosome aneuploidy and structural rearrangements (Gekas *et al.*, 2001). Also, a significantly higher rate of *de novo* aneuploidy has been found in sperm from infertile men with normal somatic karyotypes compared to the fertile controls (Shi and Martin, 2001). These paternal abnormalities can be more readily transmitted through ICSI as it bypasses many physiological selection barriers. ICSI is also a much more invasive by nature compared to other ARTs. The injection

procedure has been suggested to disrupt spindle apparatus, cytoskeleton, and chromatin configurations in animal models (Hewitson et al., 1996; Terada et al., 2000). This kind of damage on an oocyte may lead to a meiotic II error if the perturbation occurred early during the exclusion of the second polar body; or may lead to a mitotic segregation error if the damage takes effect later on. Because of these combined risk factors, origin of chromosomal abnormalities in ICSI conceptions may be distinctive from that found in other ART and the natural conceptions. In fact, not only did most of the inherited chromosome abnormalities have a paternal origin (17/22, Bonduelle et al., 2001), *de novo* cases were also predominantly resulted from a paternal error (6/8, Van Opstal et al., 1997). Although the available data predominantly points to a paternal origin, the number of cases studied is too small to eliminate other contributing factors. Thus, parental origin should be determined for every cytogenetically abnormal case in ICSI pregnancies, in order to clarify how much each risk factor – maternal age, male infertility, and mechanical stress - contributes to the elevated incidence of chromosomal abnormalities in the ICSI population. To date, studies of origin of chromosomal abnormalities in ICSI conceptions have been limited to non-mosaic cases. Studying the origin of mosaic and non-mosaic chromosomal abnormalities in ICSI conceptions and comparing it to that in the general population will contribute to our understanding of how the aforementioned factors are related to chromosomal abnormalities in the ICSI population. The study of parental origin in ICSI conceptions may also provide insight into the safety of the procedure as well as information for genetic counseling.

3.2 Material and methods

3.2.1 Clinical information

Two cases of CPM and three cases of spontaneous abortions were included in the study of origin of chromosomal abnormalities. The two CPM cases include the abnormal case (45, X) found in the current study and another case from previous study with a partial gain on chromosome 7 (7q31>qter) and a partial loss on the X chromosome (Xp21>pter) (Minor *et al.*, 2006). Three cases of spontaneous abortions (SAs) ascertained from Genesis, a local IVF clinic, all occurred during the first trimester (Table 3.1). CPM cases were identified by CGH, whereas chromosomal abnormalities in SAs were determined by routine

cytogenetic analysis at a clinical cytogenetic laboratory. Parents have given informed consent to provide us with placental tissues and peripheral blood for the study of parental origin.

Table 3.1 Clinical information

case	Ascertainment	Maternal age (years)	Gestational age (WK)	Abnormality
1	CPM	40	41	45, X (Troph)
2	CPM	31	40	Gain 7q31>qter; Loss Xp21>pter
3	SA	39	8	Balanced t(13;21)
4	SA	40	5	trisomy 2
5	SA	39	7+4/7	Analysis failed

3.2.2 Genotyping for the origin of chromosomal abnormality

Genotyping for the parental origin of a chromosomal abnormality was based on the inter-individual variation on the size of microsatellite repeats. Genomic DNA was amplified with primers that flank microsatellite repeat regions using polymerase chain reaction (PCR). A total of 10μL PCR reaction contained 1 x Rose Taq buffer [20 mM Tris HCl (pH8.0); 10mM KCl; 0.1% Triton X 100; 50μg / ml nuclease free BSA; 2mM MgCl2], 200μM dNTP, 300nM of each primer, 2.0U of Rose Taq and about 30ng DNA. Amplification was performed on an MJ research thermocycler with 35 cycles of 30s at 95°C for denaturation, 55s at 45°C for annealing, and 1 min 30sec at 72°C for elongation. Primers close to the centromeres are ideal for genotyping to distinguish Meiotic I and II errors because less recombination occurs between these sites and the centromere. DNA from abnormal tissues, maternal blood, and/or paternal blood was included in the analyses. In the event parental blood was unavailable, DNA was extracted from maternal decidua after removal of villi with the aid of a microscope.

The PCR products were separated by the size of amplicons. For products amplified with non-fluorescent primers, a 6% polyacrylamide (PAGE) gel (6% polyacrylamide/50% urea) was used. An equal amount of urea loading buffer (4.2% urea; 0.1% xylene cyanol; 0.1% bromophenol blue; 5mM EDTA) was added to each PCR product. The mixture was denatured at 95°C and set on ice-water shortly before loading onto the PAGE gel. Samples

were electrophoresed at 45W for 30-90 minutes depending on the size of the fragment. The gel was then stained with silver nitrate solution (0.6g in 300mL dH_2O) and developed in 300mL of 0.28M $NaCO_3$ and 0.05% formaldehyde. The gel was transferred to blotting paper and dried at 80°C for up to 2 hours.

The ABI310 automated genetic analyzer was used for analysis when fluorescently labeled PCR primers were used. A forward primer was labeled with fluorescent dyes, HEX (green) or 6-FAM (blue) (NAPS Oligonucleotide Synthesis Facility, UBC). In each tube, 9.5 μL of Hidi deionized formamide, 0.5μL of PCR products, and 0.2μL of ROX 500 size standards were mixed. The mixture was then denatured at 95°C for 5 minutes and then immediately cooled in ice water. The ABI prism data collection software and the GeneScan analysis software installed in the instrument converts the fluorescence signals to interpretable data. The peaks obtained from ABI data are equivalent to bands appearing on a silver stained PAGE gel. In a normal heterozygous biparental case, two differently sized peaks (bands) represent the maternal and paternal alleles. In cases of trisomy, an extra peak or a double-sized peak (band) may be present; whereas, in cases of monosomy, a single peak (band) is present.

3.3 Results

The results for origin determination are summarized in Table 3.2. Case 5 was not included because it did not have enough material for any cytogenetic analysis.

Table 3.2 Parent-of-origins for chromosomal abnormalities found in ICSI conceptuses.

Case#	Chr abnormality	ascertainment	Origin	FISH on sperm
1	45, X (Troph)	CPM	Mitotic, Missing paternal X,	n.a
2	Gain 7q31>qter Loss Xp21>pter	CPM (previous study)	Mitotic Chr7: N.A ; Chr X: missing maternal	n.a.
3	t(13;21)	SA	Paternal (by Karyotype)	Yes
4	Trisomy 2	SA	Maternal meiotic I	n.a

Case 1

Genotyping using microsatellite marker at the Androgen Receptor gene (AR), revealed an absence of paternal X chromosome in the 45, X case. However, our observation was not unique, as the missing X is paternal in most monosomy X cases (83% in SA, and 74% in livebirth, Hassold *et al.*, 1992; Jacobs *et al.*, 1997). The low level of mosaicism and the lack of UPD for the X chromosome in diploid cells implicate a mitotic error. As described in the previous chapter, two out of ten sites were affected exclusively in the trophoblast layer. One site is non-mosaic with 98.8% of trophoblast cells being 45,X, while the other abnormal site has lower level mosaicism with approximately half of the trophoblast being 45, X and the rest being 46,XX . Thus, the monosomic cells were probably derived from a diploid (46,XX) conception with a post-zygotic error in some progenitor cells of the trophoectoderm, which gives rise to the trophoblast (Carlson, 2004). Furthermore, based on the finding of only a monosomic cell line and no trisomic 47,XXX cell line (0/507), the error may be due to anaphase lag instead of nondisjunction. Mitotic nondisjunction theoretically gives rise to an equal number of monosomy and trisomy cells; whereas anaphase lag produces only monosomy. Nevertheless, we can not exclude the presence of the trisomic cell line in other parts of the placenta.

Case 2

Parental blood was not available for genotyping analysis; therefore DNA from decidua was used to determine the maternal genotype. However, the contamination of villi embedded in the decidua made the identification of parental origin for this case difficult. The partial monosomy X was determined to be a lack of maternal X chromosome contribution; whereas, the origin of partial trisomy 7 was not clear. CGH analysis revealed that this abnormality was present in the trophoblast at two out of ten sites (Minor *et al.*, 2006). Accordingly, the origin of this abnormality is probably a post-zygotic error resulting in an independent partial loss of chromosome X and a partial gain of chromosome 7.

Case 3

The origin of the abnormality found in the spontaneously aborted case, t(13;21), was determined from the cytogenetic analysis performed by a certified clinical technologist. Analysis of cultured chorion revealed a male karyotype with a balanced Robertsonian translocation with the breakpoint at the centromeres of both chromosomes [45,XY, t(13;21)(q10;q10)]. The same translocation was observed in his father, who presented with oligoasthenoteratozoospermia (OAT). In addition, the father's brother was found to have the same translocation; however, his fertility status is not clear because he underwent a vasectomy. The father's sister was not available for cytogenetic analysis although she reported a five-year history of infertility. Thus, this translocation appears to be familial involving at least two generations. Interestingly, the grandparents did not appear to have a problem with fertility. Unfortunately, they were not available for cytogenetic analysis or for providing pregnancy history.

Although the translocation was balanced, the pregnancy ended as a spontaneous abortion at 8th week of gestation. Since the fetus carried the same translocation as the father's and uncle's, the translocation is probably not responsible for the miscarriage. Female anatomical factors may also be excluded as being responsible for the pregnancy loss, as the mother did not have tubal, ovulatory or pelvic infertility factors. Thus, the cause for the pregnancy loss remains unexplained.

Case 4

The spontaneous abortion occurring at the 5th week of gestation was found to be trisomic for chromosome 2. Although the placental tissues appeared to be non-mosaic, the chromosomal constitution of the fetus is not known. The two informative markers located with great proximity to the centromeres support origin from a maternal meiotic I nondisjunction. In spontaneous abortions occurring after natural conception, a trisomy 2 was due to a maternal meiotic I error in 54% of the cases (n=18) (Hassold and Hunt, 2000). The couple had a previous ICSI cycle that ended in spontaneous abortion due to trisomy 21. Although its origin was not determined, considering trisomy 21 is associated with a maternal origin in 90% of the cases in the general population (Miller and Therman, 2001), we

speculate that the advanced maternal age (40 years old) in this case may have led to the trisomic conception and subsequent SA.

3.4 Discussion and conclusion

The 45,X confined to the placenta demonstrated a lack of paternal chromosome due to a post-zygotic error. Although CPM with monosomy X has been reported as the most common placental mosaicism involving sex chromosomes, origin has been not being frequently studied. Farra *et al.* (2000) reported two cases of CPM with monosomy X through CVS, of which one had 50% and the other had 100% monosomy cells in chorionic villi, but both cases had 0% abnormality in amniotic fluid. It is possible that the error occurred mitotically particularly in the case with 50% CVS result, as a small number of cells might have been affected. The investigation of origin for placental mosaicism with 45,X is difficult because meiotic and mitotic error cannot be distinguished, unless multiple sites from the affected placenta are examined. Moreover, many cases may be missed due to the lack of clinical phenotype in this type of CPM (Farra *et al.*, 2000)

Origin of monosomy X cases has been more frequently studied in non-mosaic cases (possibly including mosaic cases) ascertained through SA or CVS. In those cases, investigations were limited to the parental origin, i.e. mitotic errors were not looked. In the general population, 83% of SA cases showed an absence of the paternal X chromosome (n=47, Hassold *et al.*, 1992), and the rate is 74% in livebirths (n=93; Jabods *et al.*, 1997). It has been suggested that over 90% of the livebirth 45,X are mosaic (Fernandez-Garcia *et al.*, 2000). Although this data may not be representative of the placental mosaicism, it implies that the paternal chromosome is more susceptible to loss. Monosomy X found in ICSI conceptions including the present case, however, has been shown to be 42% (3/7) maternal and 57% (4/7) paternal in origin (Ma *et al.*, 2006; Van Opstal *et al.*, 1997; Lam *et al.*, 2001). Due to the extremely small sample size, it remains to be established whether the increase in monosomy X cases missing maternal chromosome is truly prevalent in the ICSI population.

The trisomy 2 case had a maternal meiotic I origin. Studies in the general population revealed that trisomy 2 is present in 1.1% of SA and does not lead to a livebirth (Jacobs and Hassold 1987). In about 54% of the cases the extra chromosome is derived from a maternal

MI error, and 28% of the cases are due to a paternal a MI error (Hassold and Hunt 2001). Prior to our study, only one case of trisomy 2 has been reported in ICSI conceptions (ascertained through SA); however, the origin was not determined (Causio et al., 2002)

Among the four abnormalities analyzed, only the Robertsonian translocation (13;21) was of paternal origin. Because this is a rare Robertsonian translocation involving two chromosomes that account for the majority of trisomies among livebirths, the meiotic segregation pattern in sperm should be investigated (chapter 4). It will add insight to the correlation between the chromosomal abnormality in sperm from infertile men and that in the resulting conception,

In summary, the chromosomal abnormalities in two CPM cases were mitotic in origin, and two SA cases were maternal and paternal in origin, respectively, in the ICSI group studied. Due to the limited sample size, we cannot draw any meaningful conclusion in terms of the distribution of origins of aneuploidy in ICSI conceptions. However, we emphasize the importance of the study of parental origin of aneuploidy in ICSI conceptions because it may provide answers for the increased incidence of chromosomal abnormality found in the ICSI population.

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CHAPTER 4. Meiotic segregation patterns and aneuploidy rate in sperm from a father of a Robertsonian translocation t(13;21)[1]

4.1 Introduction

Robertsonian translocations (RTs), fusions between two acrocentric chromosomes, are the most common structural chromosomal rearrangements in humans, occuring in approximately 1 in every 1000 newborns (Therman *et al.*, 1989). Among the possible combinations of the five acrocentric chromosomes (13, 14, 15, 21 and 22), translocations between chromosomes 13 and 14 and between chromosomes 14 and 21 are the most frequent, comprising about 75% and 10% of all RT cases, respectively. The t(13;21) is one of the rarest rare RTs, estimated to occur in approximately 2% of all RTs (Therman *et al.*, 1989).

Men carrying balanced RTs often have some degree of infertility, for instance oligozoospermia or azoospermia, but are otherwise phenotypically normal (Scriven *et al.*, 2001). The aetiology of infertility is related to synaptic abnormalities during meiosis leading to meiotic arrest. The incidence of RTs in infertile men is approximately 2–3% compared to 0.12% in the general population. (Nielsen and Wohlert, 1991; Baschat *et al.*, 1996; Testart *et al.*, 1996). With current advances in assisted reproductive technologies (ART), such patients have increasingly been given the opportunity to conceive their own biological children. Thus, it is of concern that chromosomally unbalanced gametes from RT carriers may be incorporated into the conceptus.

In addition, RTs may adversely affect the meiotic segregations of uninvolved chromosome pairs. The presence of such interchromosomal effects (ICE) is still an open debate. Supporting data have been provided by several studies using multicolour fluorescent in situ hybridization (FISH) to investigate sperm in infertile men (Rousseaux *et al.*, 1995; Morel *et al.*, 2001; Baccetti *et al.*, 2002; Anton *et al.*, 2004). Gianaroli *et al.* (2002) examined 111 in vitro-generated embryos from RT carriers and reported a considerable amount of ICE such that 31% of the embryos had abnormalities on RT non-related chromosomes and 36%

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had abnormalities in both RT-related and non-related chromosomes. Conversely, other authors reported lack of ICE in RTs (Blanco *et al.*, 2000; Pellestor *et al.*, 2001), and some suggest that ICE is restricted to sperm with poor quality (Vegetti *et al.*, 2000; Pellestor *et al.*, 2001). As most studies on ICE were done on common RTs, the investigation on the rare t(13;21) case will not only add information to the debate on the presence of ICE but also provide insight into the association between the types of translocated chromosomes and ICE, if it exists.

Although meiotic segregation and ICE in spermatozoa have been repeatedly studied in the t(13;14) and t(14;21) cases, t(13;21) cases have yet to be well documented. The aim of the current study was to investigate meiotic segregation pattern of involved chromosomes and possible ICE in a man with a balanced t(13;21), which was also transmitted to his ICSI-facilitated conception.

4.2 Materials and methods

4.2.1 Clinical information

A couple (39-years-old, female; 40-years-old male) presented with a 3-year history of primary infertility. The female partner displayed no evidence of tubal, ovulatory or pelvic infertility factors. The male partner (proband), however, was found to have oligoasthenoteratozoospermia from two consecutive semen analyses (sperm count, 7.4–8.4 x 10^6/ml; normal motility, 3–7%; and normal morphology, 2%). Subsequent karyotyping of the man's peripheral blood revealed a balanced RT involving chromosomes 13 and 21. The breakpoint appears to be at the centromeres of both chromosomes. This translocation karyotype, 45,XY,t(13;21)(q10;q10), appeared in all cells examined. The proband's brother was found to have the same translocation. However, the proband's parents and female sibling was not available for cytogenetic analysis. Both the proband's brother and sister had no children: his brother underwent a vasectomy in his early thirties and his sister had a 5-year history of infertility. Because of the severity of the proband's sperm parameters, IVF combined with ICSI was undertaken.

4.2.2 Fluorescent in situ hybridization (FISH)

Sperm preparations

Semen was washed in PBS and centrifuged at moderate speed (280g) for 5 minutes. The cell pellets were fixed in 3:1 methanol: acetic acid, and then spread and fixed onto slides. Prior to hybridization, sperm were decondensed as described by Palermo *et al.* (1999) with minor modifications. The slides were first washed in 2 x SSC solutions for 5 minutes. The slides were immersed in 10mM dithiothreitol (DTT) (Sigma, St. Louis, MO) / 100mM Tris (pH8.0; Fisher Scientific, NY) for 30 minutes, and then further treated with 10mM 3,5-Di-iodosalicytic acid (DSA) /1mM- DTT/100mM Tris (pH8.0) for another 30 minutes. The slides were then rinsed twice in 2x SSC and examined under a phase-contrast microscope while the slide was still wet. If sperm heads appeared to be larger and evenly darkened, the decondensation was complete. If decondensation was incomplete, the slide was further incubated in 10mM DTT / 100mM Tris and 10mM DSA / 1mM DDT/100mM Tris until the sperm were sufficiently decondensed while still retaining their tails.

Hybridization and Analysis

Dual-colour FISH was carried out using LSI 13/21 probes (13q14 LSI13, SpectrumGreen / 21q22.13-q22.2 LSI21, SpectrumOrange; Vysis, Downers Grove, IL, USA). Triple-colour FISH was performed using α-satellite DNA probes for chromosome 18, X and Y (CEP18 SpectrumAqua / CEP X SpectrumGreen / CEP Y SpectrumOrange; Vysis, Downers Grove, IL, USA). Prior to hybridization, the slides were denatured in 70% formamide / 2x SSC (pH7.4-7.5) at 75°C for 5 minutes. The slide was dehydrated in an ice-cold ethanol series (70%, 80%, and 100%) for 2 minutes each and air-dried at room temperature. pre-denatured probes (10 μL) were applied onto the slide and covered with a 22x22 mm coverslip and sealed with rubber cement. Hybridization occurred in a humid chamber overnight at 37 °C. Post-hybridization wash was carried out as described for CGH protocol.

The analysis was carried out with an epifluorescent microscope (Nikon Elipse E600W) equipped with a triple bandpass filter (DAPI / FITC / Cy3), a dual bandpass filter (FITC/Cy3), and single bandpass filters for DAPI, Aqua, FITC, and Cy3. Scoring was

performed in areas with consistent hybridization. Only morphologically intact sperm cells with tails were included to avoid scoring of other cell types or hybridization artifacts. At least ten thousand nuclei were scored for each probe set. The result was compared with data from infertile controls previously assessed in our laboratory under the same procedures.

4.3 Results

4.3.1 ICSI outcome

A summary of results from three ICSI cycles is given in Table I. Of the 17 oocytes retrieved in three consecutive ICSI cycles (over a 2-year interval), 12 metaphase II (MII) oocytes were used for ICSI. Of the 12 oocytes injected, 9 of them fertilized normally (75%). Four (4-, 6-, 7- and 8-cell stage), three (all 8-cell stage) and two (7- and 8-cell stage) embryos were transferred in three separate cycles. Among the nine transferred embryos, only two were of poor quality, while the others displayed normal development and good quality. In all three cycles, it was noted that sperm parameters had worsened at the time of the ICSI procedure compared to previous semen analyses: sperm concentrations were all less than 3 x 10^6/ml, and very few motile sperm were present.

Pregnancy was achieved only in the first ICSI cycle, in which a total of four embryos were transferred. At the eighth week of gestation, the pregnancy spontaneously aborted. Cytogenetic analysis of cultured chorion revealed a male karyotype with a balanced RT of paternal origin [45,XY,t(13;21)].

Table 4.1 ICSI clinical outcomes of the t(13;21) case .

Table 10.	Cycle one	Cycle two	Cycle three	Total
No. of injected oocytes	4	5	3	12
No. of fertilized oocytes	4	3	2	9
No. of clinical pregnancies	1	0	0	1
No. of spontaneous abortion	1	-	-	1

4.3.2 FISH on sperm

The meiotic segregation analysis on chromosomes 13 and 21 was performed on a total number of 10 223 sperm nuclei (Table 4.2). With respect to chromosomal constitutions of 13 and 21, the majority of spermatozoa (88.39%) were normal, 13q/21q, or balanced, der(13q;21q), both derived from alternate segregation in meiosis. Chromosomally unbalanced spermatozoa for chromosomes 13 and 21, derived from an adjacent segregation, account for 11.08%. This was significantly higher than in the six fertile controls (0.6%) ($p<0.05$). The rates of nullisomy for both chromosomes 13 and 21 were higher than the complementary disomy rates in the patient ($P < 0.05$); however, such discrepancy was not observed in the control group ($P > 0.05$). The rate for the 3:0 segregation and diploidy, indicated by two signals for each of 13 and 21, was 0.26%, whereas the control group had a frequency of 0.1%. In addition, there was 0.18% of 21q/21q and 0.09% of 13q/13q exclusively observed in the patient, which were categorized as 'other' modes of segregation in Table 4.2.

Table 4.2 Meiotic segregation analysis for chromosome 13 and 21 for t(13;21) case.

Segregation modes	Chromosomal constitution	Chromosomal status	FISH results (n=10223)		Controls (n=60975)
			n	%	%
Alternate	13q/21q or der(13q;21q)	Normal or balanced	9036	88.39	99.29
Adjacent	13q	Nullisomy 21	387	3.79 *	0.11
	21q/der(13q;21q)	Disomy 21	208	2.03 *	0.15
	21q	Nullisomy 13	399	3.9*	0.24
	13q/der(13q;21q)	Disomy 13	139	1.36*	0.1
			1133	**11.08***	**0.6**
3:0 or diploid	13q/21q/der(13q;21q) or 13q/13q/21q/21q	Diploidy	27	0.26	0.1
other			27	0.26	0

*P<0.05 by Chi-square test

The results of the ICE investigation are summarized in Table 4.3. A total of 10 172 sperm nuclei were analyzed with the 18, X, Y probe set, and 98.79% were normal haploid. The overall aneuploidy rate was not significantly higher in the patient compared to that of controls ($P > 0.05$). The rate of sex chromosome disomy (0.15%) was about five times as high as disomy 18 (0.03%), which was also observed in the control group (0.39 versus 0.07%). The diploidy rate was 0.05% in the patient and 0.04% in the controls.

Table 4.3 Analysis of ICE for chromosome 18, X and Y in the t13;21 case

Chromosomal number	FISH results (n=10172)		Controls (n=50740)	P-values
	n	%	%	
Haploid	10047	98.79	99.07	n.s.
Sex chromosome disomy	15	0.15	0.39	n.s
Disomy 18	3	0.03	0.07	n.s
Diploidy	5	0.05	0.04	n.s

n.s: Not significant (p>0.05 by Chi-square test).

4.4 Discussion and conclusion

Studies on meiotic segregation in RT carriers have been of great informative value in reproductive counseling. However, few reports of t(13;21) cases, along with other rare RTs, are available in the literature, despite the involvement of the two chromosomes accounting for the majority of trisomies among live births. To our knowledge, this is the first study that has investigated the meiotic segregation pattern and the possible ICE of t(13;21) in sperm. The clinical information regarding the ICSI treatment and the pregnancy outcome were also reported thoroughly in order to provide further information for the physicians and patients considering ART.

The segregation analysis of chromosomes 13 and 21 in the current study showed that 88.39% of the spermatozoa were normal or balanced. This percentage is in agreement with previous FISH studies on sperm for other types of RTs (Martin *et al.*, 1992; Mennicke *et al.*, 1997; Honda *et al.*, 2000; Ogawa *et al.*, 2000; Morel *et al.*, 2001). Normal or balanced sperm were present predominantly in all segregation studies, ranging from 60 to 96.6%,

regardless of different methodologies used for analyses (heterospecific IVF, sperm injection into mouse oocytes or FISH). Among the most well-studied t(13;14) cases, the percentage of normal or balanced constitutions also ranges from 73.5 to 92.3% (reviewed by Anton *et al.*, 2004). The high frequency of normal or balanced spermatozoa is presumably attributable to a selection in all RTs towards the cis-configuration of the trivalent during meiosis which leads to an alternate segregation (Sybenga, 1975).

The unbalanced chromosomal constitutions were comprised of nullisomies and disomies derived from adjacent segregations, 3:0 or diploidy, and unexpected combinations ('other' in Table II). There was an obvious deviation from the expected 1:1 ratio of disomic to its complementary nullisomic spermatozoa. For both chromosomes 13 and 21, the nullisomy rates were significantly higher than disomy rates ($p < 0.05$). Similar observations were made previously by other researchers (Honda *et al.*, 2000; Frydman *et al.*, 2001; Morel *et al.*, 2001; Anton *et al.*, 2004). The discrepancy possibly originates from two factors. First, hybridization artifacts can lead to a higher frequency of nullisomy. However, the high efficiency of hybridization makes hybridization artifacts an unlikely cause. Also, taking into consideration that such inconsistency was not seen in the controls and that a hybridization failure would affect all combinations of signals rather than one category, in our case the discrepancy is unlikely to be due to experimental errors. The second factor that may contribute to the observation is differential detection by meiotic checkpoints. As suggested by Honda *et al.* (2000), there may be a more stringent selection against disomic cells than nullisomic cells. Therefore, a higher frequency of nullisomy in the spermatozoa can be expected.

Two signals for each of chromosomes 13 and 21 were displayed in about 0.26% of spermatozoa, which is similar to that in studies on other RT types (0–0.8%; reviewed by Morel *et al.*, 2001). This phenomenon is caused by either diploidy or 3:0 segregation. The distinction was aided by the comparison with the diploidy rate found from the ICE analysis. The diploidy rate was 0.05% according to the investigation on chromosomes 18, X and Y. Hence, the rate of 3:0 segregation is estimated to be approximately 0.2%. This confirms that the occurrence of 3:0 segregation is a much rarer event compared to alternate and adjacent patterns.

The unexpected abnormalities observed in this study (13q/13q and 21q/21q) were perhaps attributable to non-disjunction events in meiosis II following an adjacent segregation pattern. In other words, a non-disjunction event at MII may have prevented the formation of nullisomy 13 or 21. Instead, cells with disomy for one chromosome and nullisomy for the other chromosome (13q/13q and 21q/21q) can be formed. However, the theoretical 'co-products' of the non-disjunction, cells missing both 13 and 21, were not detected in the study. Those cells may have been arrested at cell-cycle checkpoints, as they are genetically more intolerable forms. Investigating a larger number of nuclei may increase the chance to find such cells; however, in order to distinguish from those with hybridization failures, one would need to co-hybridize the slide with another chromosome marker as an internal control.

Although the existence of ICE remains controversial, a number of studies have supported its prevalence in patients with chromosomal arrangements including RTs. Gianaroli *et al.* (2002) suggested that ICE is more common in cases of RTs than other types of translocations. Several FISH studies on spermatozoa from RT patients have reported an increased frequency of sex chromosome and certain autosomal disomies (reviewed by Shi and Martin, 2001). The current case did not provide evidence for ICE on chromosome 18 and sex chromosomes. There was no significant difference in the aneuploidy rate of those chromosomes compared to the control group ($P > 0.05$). While Baccetti *et al.* (2002) observed ICE on sex chromosome disomy, disomy 18 and diploidy in a t(13;21) case, the disparity may depend on the region of chromosome involved, which may lead to certain meiotic configurations that enhance the formation of aneuploidies (Estop *et al.*, 2000). Thus, the same type of RT may have variable ICE during spermatogenesis.

Even though ICSI can possibly facilitate the transmission of the structural abnormality, in the current case, an unbalanced spermatozoon was not introduced to the conceptus. However, the pregnancy unfortunately ended as a spontaneous abortion. Although the fetus carried the same RT as in the father, the translocation is probably not directly responsible for the miscarriage because the breakpoint would likely be conserved; one may also exclude the female anatomical factors for the pregnancy loss, as the mother had no evidence of tubal, ovulatory or pelvic infertility factors. Thus, the cause for the loss remains unexplained with the available information at this point.

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CHAPTER 5. Methylation status at the differentially methylated domain of *H19/IGF2* in placentas derived from ICSI pregnancies

5.1 Introduction

Genomic imprinting is an epigenetic phenomenon that allows for differential expression of certain genes dependent on the parent-of-origin. Imprinting aberrations have been linked to abnormal fetal development and various diseases including cancers (Abu-Amero *et al.*, 2006; Arnaud and Feil, 2005). There is increasing evidence that assisted reproductive technologies (ARTs), including ICSI, are associated with an unexpectedly high incidence of imprinting disorders such as Beckwith-Wiedemann Syndrome (BWS) and Angelman Syndrome (AS) (Chang *et al.*, 2005; DeBaun *et al.*, 2003; Gicquel *et al.*, 2003; Maher *et al.*, 2003; Orstavik *et al.*, 2003). Interestingly, molecular studies have revealed that the cause for these diseases found in the ART population was predominantly epimutation, which is not common in the general population. Hypomethylation at the differentially methylated region (DMR) of *KCNQ1OT* caused 13/14 BWS cases, while the other case was due to epigenetic alteration at DMRs at both *KCNQ1OT* and *H19*. These types of epimutations are responsible for up to 45% of the sporadic BWS cases. Similarly, all three AS cases reported in the ICSI group were due to epigenetic defects at an imprinting center, while this etiology account for only an estimated 5% of the whole AS population (Cox *et al.*, 2002; Orstavik *et al.*, 2003). Notwithstanding the importance of epigenetic changes on specific imprinting disorders, they may also influence development and long-term health of ART children (Maher et al., 2003). Thus, there is a pressing need to investigate the relationship between ART and epigenetic dysregulation.

Maternally expressed *H19* and paternally expressed *IGF2* are known as two paradigms of imprinted genes. These genes are conversely regulated by a common DMR, which is methylated only on the paternal allele. *H19* encodes for untranslated mRNA that is expressed strongly during embryogenesis, and is important for embryonic development (Weber *et al.*, 2001; Kono *et al.*, 2004). *IGF2* also has been known to be important for embryogenesis, particularly for the placental development (Constancia *et al.*, 2002). Hypermethylation at the DMR down-regulates *H19* expression and is associated with a

increase in *IGF2* expressions. This is thought to result in placento-megaly and fetal overgrowth as seen in BWS cases. In contrast, hypomethylation at the DMR leads to over-expression of *H19* and reduced expression of *IGF2* (Fedoriw *et al.*, 2004). This may inversely suppress the placental development and lead to intrauterine growth restriction (IUGR) as seen in patients with maternal duplication of 11p15 (Fisher *et al.*, 2002), and some with Silver-Russell Syndrome (SRS) or SRS-like conditions (Gicquel *et al.*, 2005; Bliek *et al.*, 2006).

Mouse studies have suggested that the DMR at *H19/Igf2*, compared to other imprinted genes, was the most labile site to lose methylation when embryos were cultured in an unfavorable media condition (Mann *et al.*, 2004). Furthermore, the placenta appears to be particularly vulnerable to such environmental stress compared to the embryo proper. The reason for such a discrepancy has been suggested to be either due to vicinity of the trophoectoderm cells with the culture media, or due to a less robust restoring system in the placenta compared to the embryo (Mann *et al.*, 2004). ICSI involves not only *in vitro* culture of immature oocytes and embryos as in other ART, but also assumes additional risks that may be inherent in the use of sperm with abnormal parameters. Incomplete methylation at *H19* has been detected in mature spermatozoa in oligozoospermic patients (Marques *et al.* 2004). Hence, there is a concern that imprinted genes may not only be epigenetically altered during the process of ICSI, but also be passed on from the abnormal gamete to the offspring through the procedure. If hypomethylation at the *H19* DMR was somehow induced during ICSI, it would lead to over-expression of *H19* and reduced expression of *IGF2*, which in turn may cause poor placenta formation and restricted fetal development. Therefore, we speculated that this mechanism may account for some of the frequently observed but unexplained low birth weight (LBW) cases in ICSI pregnancies (Katalinic *et al.*, 2004). In the current study, the methylation pattern at the DMR of *H19/IGF2* was investigated in placentas from ICSI pregnancies with LBW. Comparisons were made between the ICSI-LBW placentas and those from normal-birth-weight pregnancies conceived naturally and through ICSI treatment.

5.2 Materials and methods

5.2.1 Clinical information

Placentas from ICSI pregnancies with reduced birth weight (9 IUGR and one LBW) were collected from patients who underwent an ICSI procedure at the University of British Columbia *in vitro* Fertilization (IVF) Program from 1997 to 2006. These cases were grouped as ICSI-LBW and included LBW (birth weight <2500g) and intrauterine growth restriction (IUGR) (birth weight below the 10th percentile). Each birth weight was also quantitatively converted to Z-score, which measures the standard deviation (SD) from the expected mean at the matched gestational age (Langlois et al., 2006). The Z value was obtained by (patient value-expected mean)/population SD, and the expected mean vague and SD for each gestational age was adopted from Usher and Mclean (1969). Placentas derived from ICSI pregnancies with normal birth weight (ICSI-NBW, n=12) were also collected from the same fertility center. DNA from placentas derived from natural conceptions was included in analysis as controls (control, n=14). All of these controls had normal birth weight and were the healthy controls in a preeclampsia study conducted in Dr. Robinson's laboratory at the BC Research Institute.

5.2.2 Methylation sensitive Single Nucleotide Primer Extension (Ms-SNuPE)

Chorionic villi were sampled from two sites from each placenta and DNA was extracted using standard protocols (Chapter 2). Methylation status of two CpG sites within the DMR of *H19/IGF2* was measured the using the SNuPE assay described by Sievers *et al.* (2005) with minor modifications (Figure 5.1). 300-500 μg of DNA was modified with bisulfite to convert unmethylated cytosine to uracil (EZ DNA Methylation-Gold Kit™, Zymo Research, Orange, CA). The converted DNA was first amplified by primers flanking the region of interest with F6005–R6326 followed by a semi-nested amplification with F6115–R6326 (GenBank accession no. AF087017: nucleotides 6005–6326; table 5.1) using Polymerase Chain Reaction (PCR). Each PCR reaction contained 1 x Rose Taq buffer [20 mM Tris HCl (pH8.0); 10mM KCl; 0.1% Triton X 100; 50μg / ml nuclease free BSA; 2mM MgCl2], 200μM dNTP, 300nM of each primers; 2.0U of Rose Taq. For the first round of amplification, a total of 10μL reaction was prepared with 2μL of bisulfite-converted DNA.

For the second round of amplification, 1μL of PCR product from the first amplification was added to a total of 20μL reaction. PCR amplification was performed on an MJ research thermocycler with 30 cycles of 45 s at 94 °C for denaturation, 45s at 61 °C for annealing, and 1 min at 72 °C for elongation. Products from the second round of PCR were cleaned (DNA Clean & Concentrator™-5, Zymo Research, Orange, CA) prior to the SNuPE reactions.

For the Ms-SNuPE assay, the Snapshot Multiple Kit (ABI, Foster city, CA) was used to amplify the differentially methylated CpGs with primers (C10 and C12) that target the sequences located one nucleotide upstream from a differentially methylated cytosine (Figure 5.2; table 5.1). Each primer was elongated by one of the four fluorescently labeled dideoxy nucleotides (ddNTP), depending on the methylation status at the CpG site. Specifically, ddCTPs targeting methylated sequences are labeled with dTAMRA (black) and ddTTPs targeting unmethylated sequences are labeled with dROX (red). Each assay was performed in a total of 10μL containing 5μL of SNaPshot Ready Reaction Premix, 0.2μM of primer (C10 or C12), and 2μL of cleaned PCR products. Amplification was done on the MJ Research thermocycler with 25 cycles of 10s at 96°C, 5s at 52 °C, and 30s at 60°C, followed by rapid cooling to 4°C. The extension reaction was terminated by treatment with 1U of calf intestinal phosphatase, which dephosphorylates the extending primers, at 37°C for 1 hour. The enzyme was then thermally deactivated at 72°C for 15 minutes. 0.5μL of the products was added to 9.5μL of HiDi formamide and denatured at 95°C for 5 minutes before processing by ABI Prism 310 automated capillary electrophoresis (ABI, Foster city, CA). Methylation status was visualized with the ABI310 and Genescan software. A typical result is shown in Figure 5.1 along with a completely methylated control obtained from sperm DNA. Peak areas were used to calculate the % methylation with the following formula: methylated peak/ (methylated + unmethylated peaks) x 100.

Exclusively in the ICSI-LBW group, chorionic villi were further separated to trophoblast and mesenchymal layers before the analysis, whereas whole villi were used in the ICSI-NBW and the control group. The methylation level in the trophoblast and the mesenchyme was averaged in the ICSI-LBW group when comparison was made with the control groups. Hypomethylation is defined by methylation below 33%, and such cases were repeated to ensure accurate measurements.

TGTATAGTATATGGGTATTTTTGGAGGTTTTTTTTTCGGTTTTATCGTTT

GGATGGTACGGAATTGGTTGTAGTTGTGGAATCGGAAGTGGTCGCGCGGCG

C12

C10

GTAGTGTAGGTTTATATATTATAGTTCGAGTTCGTTTTAATTGGGGTTCGTTCG

TTCGTGGAAACGTTTCGGGTTATTTAAGTTACGCGTCGTAGGGTTTA

CGGGGGTTATTTGGGAATAGGATATTTATAGGA

Figure 5.1 The sequence of the *H19/IGF2* DMR analyzed. The primers target the sequences within the DMR of the human *H19/IGF2*, located within the differentially methylated domain (Sievers *et al.*, 20005). The CTCF core binding domain is highlighted in the box. The primers used in semi-nested amplification with F6115–R6326 are underlined. The Ms-SNuPE primers (C10 and C12) are represented by arrows. The CpGs in boldface have been identified as consistently differentially methylated (Kerjean *et al.*, 2000).

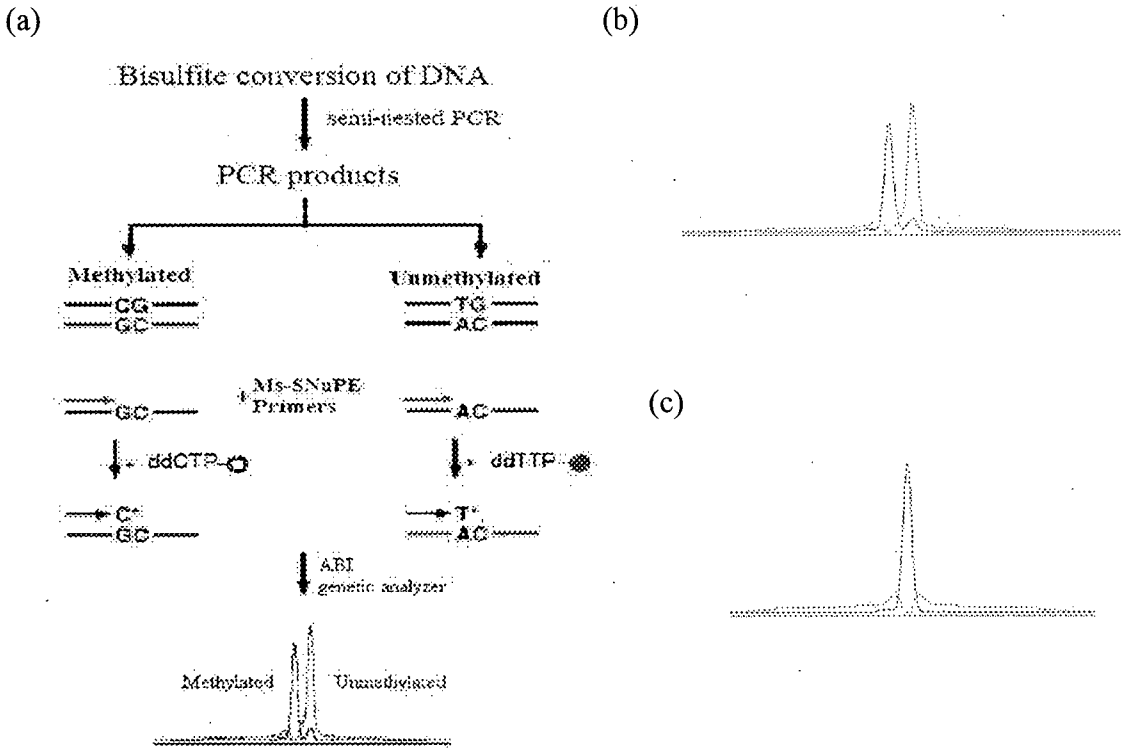


Figure 5.2 Schematics of SNuPE assay. (a) Schematic outline of Ms-SNuPE assay (b) a typical result obtained from ABI genetic analyzer. The black (left) peak represents the methylated sequences and the red (right) peak represents the unmethylated sequences. (c) Control using sperm DNA representing completely methylated sequences at the *H19/IGF2* DMR.

Table 5.1 Primer sequences

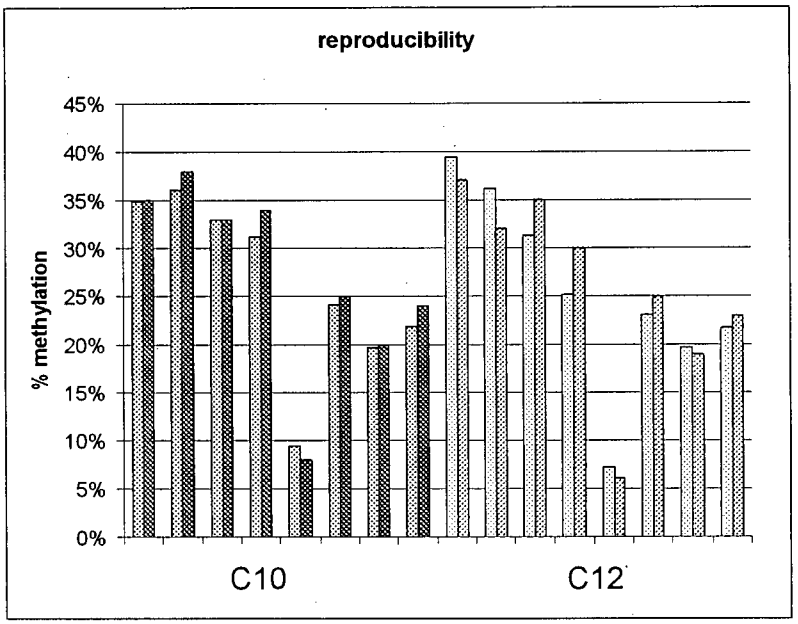
Primer	Sequence (5'-3')
F6005	AGG TGT TTT AGT TTT ATG GAT GAT GG
R6326	TCC TAT AAA TAT CCT ATT CCC AAA TAA CC
F6115	TGT ATA GTA TAT GGG TAT TTT TGG AGG TTT
C10	GTT GTG GAA T(C/T)G GAA GTG GT
C12	GAA TTG GTT GTA GTT GTG GAA T

Primer sequences for semi-nested PCR amplification (F6005; F6115; R6326) and Ms-SNuPE assays (C10 and C12).

5.3 Results

In order to assure an accurate measurement of DNA methylation, the reproducibility of the assay was first examined (Figure 5.3). Eight DNA samples were analyzed in two independent assays using both primers (C10 and C12). Similar results were obtained with the inter-assay difference ranges 0-5% (average 1% ± 2%; $p>0.05$ by Chi-square test). Assays were also repeated for several samples with purified DNA to test the effect of DNA purity on the validity of the assay, because DNA purity varies depending on the extraction procedure, and may affect the efficiency of certain molecular assays. Similar percentage of methylation was detected ($p>0.05$ by Chi-square test). The difference in % methylation ranges from 1-8% and was not statistically significant. Two primers, (C10 and C12) were used to amplify two differentially methylated CpG sites within the *H19/IGF2* DMR. Methylation assessed at both CpG sites were compared, and similar results were obtained from independent assays using the two primers ($r=0.92$; $p<0.001$; Figure 5.4).

(a)



(b)

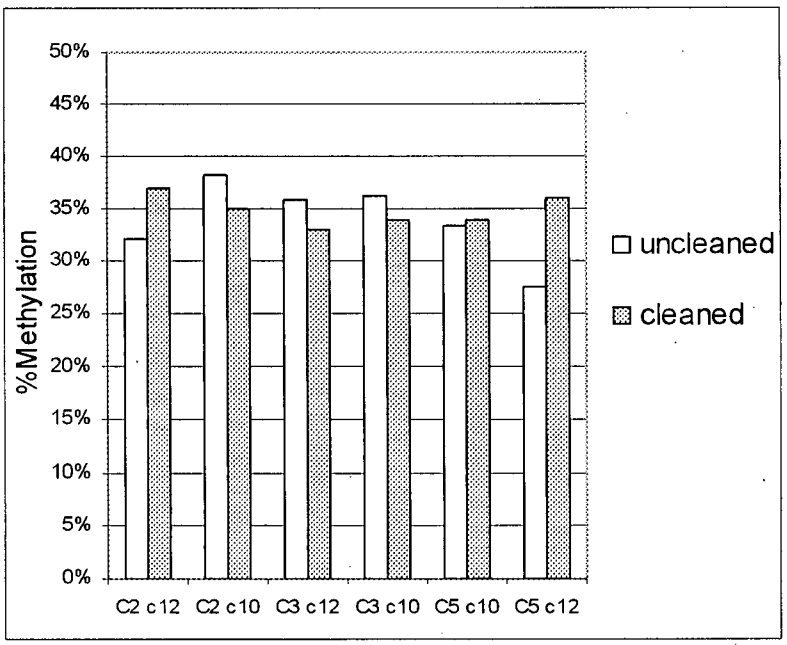


Figure 5.3 Reproducibility of the Ms-SNuPE assay and the effect of DNA purity on methylation assessment. (a) The reproducibility was tested using the same DNA repeated the entire process. The assay is reproducible as the results were not significantly different between independent experiments ($p>.05$, by Chi-square). Primer C10 (solid) and C12 (striped) were used in analysis. (b) DNA purity did not affect the measurement of methylation as uncleaned DNA (white) and cleaned DNA (shaded) yield similar results ($p>.05$, by Chi-square).

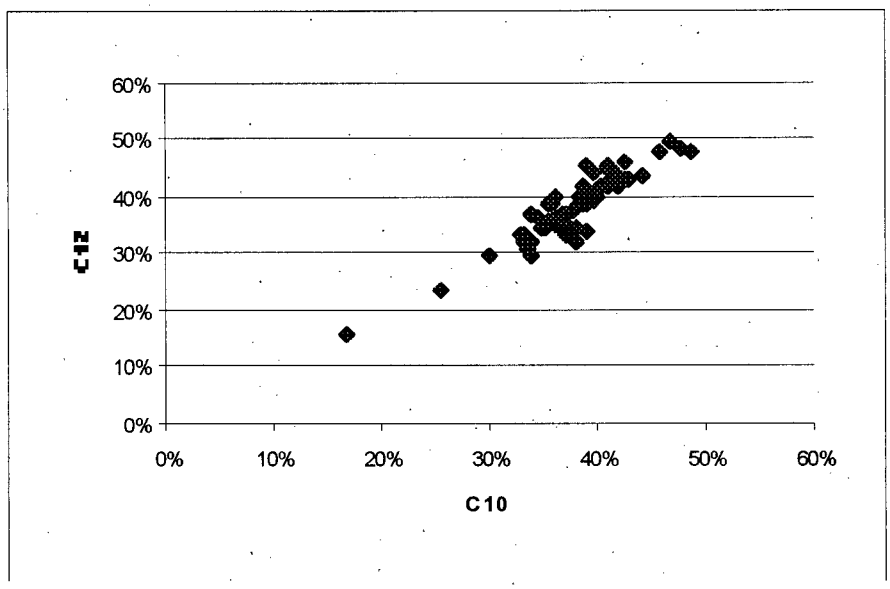


Figure 5.4 Correlations between the level of methylation at C10 and C12. Percent methylation measured in independent reactions ($r=0.92$; $p<0.001$)

The methylation patterns at the DMR of *H19* and *IGF2* were compared in placentas from ICSI pregnancies with LBW (ICSI-LBW; n=10), those with normal BW (ICSI-NBW; n=12), and placentas from natural conceptions (control; n=14) (Figure 5.5). The average percent methylation measured at C10 was 39 ± 7% in the ICSI-LBW group, 38 ± 2% in the ICSI-NBW group, and 36 ± 3% in the control group. C12 had similar results with the average % methylation being 40 ± 8% in the ICSI-LBW group, 38 ± 3% in the ICSI-NBW group, and 36 ± 3% in the control group (Figure 5.6). The Kruskal-Wallis Test yielded a non-significant result for the comparison of the means of the three groups ($p>0.05$). In more stringently defied IUGR cases [n=4, Z-score lower than -2SD (about 3rd percentile)], the mean %methylation was 41.7% ± 4.8% when values for C10 and C12 are averaged. This is slightly higher than those defined by less stringent criteria (BW<2500g; BW below 10th percentile; or by ultrasound diagnosis), which had a mean % methylation of 39.0% ± 4.8%.

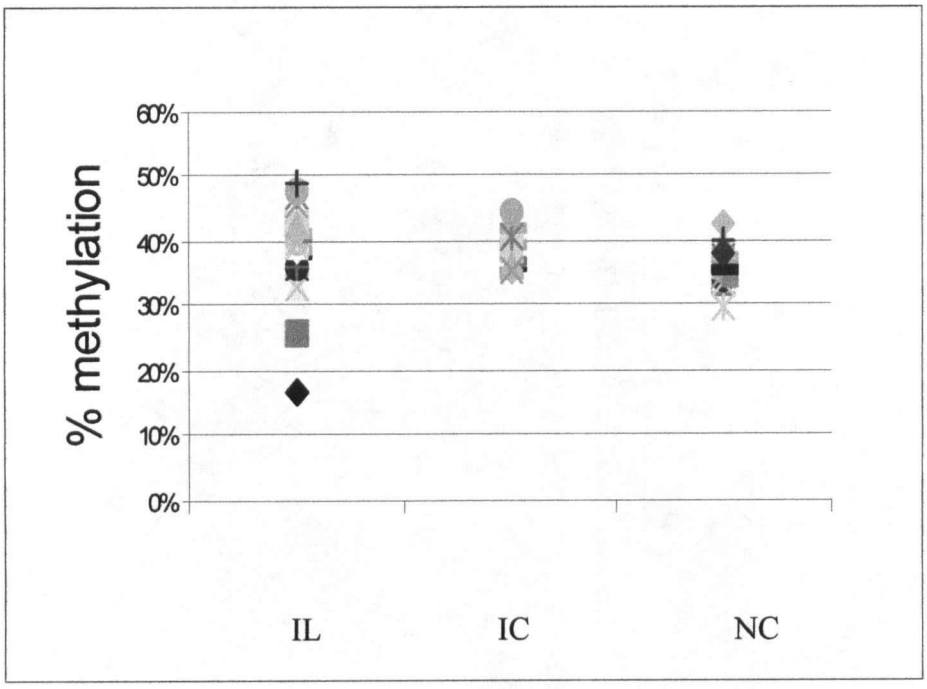


Figure 5.5 Methylation patterns measured at the DMR of *H19/IGF2*.Methylation was assessed using the Ms-SNuPE assay in the three study groups (IL: ICSI-LBW; IC: ICSI-NBW; NC: controls from natural conceptions).

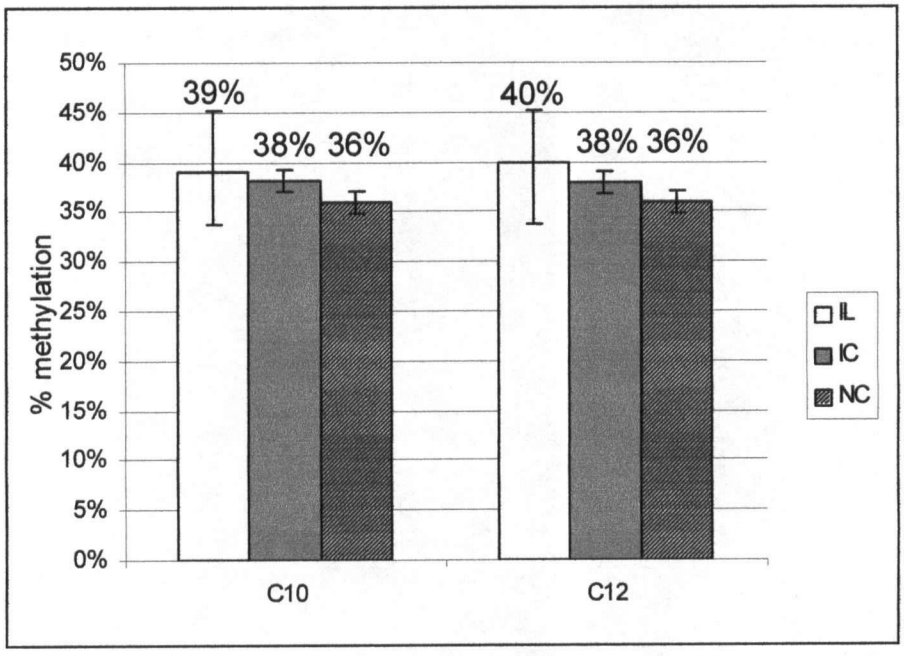


Figure 5.6 Average methylation at the DMR of *H19/IGF2*. Methylation patterns measured at differentially methylated CpGs, C10 and C12. (IL: ICSI-LBW; IC: ICSI-NBW; NC: controls from natural conceptions.

Using 33% as the cut-off for hypomethylation (Seivers *et al.*, 2005), hypomethylation (<33%) was present only in the ICSI-LBW group (Table 5.2). Case 1 (methylation: 16.6% at C10, 15.5% at C12) had a birth weight was below the 10th percentile of the average birth weight at the matched gestational age (2177g at 37WK, -1.51SD), and case 2 (methylation: 23.5% at C10, 22.5% at C12) was diagnosed by ultrasound examination (2088g at 36WK, -0.78SD). However, interestingly, hypomethylation was detected from only one out the two sites analyzed in each case. Correlation was not found between birth weights and the degree of methylation ($r = -0.0048$, $P > 0.05$) nor between the Z-scores and methylation ($r = -0.2054$, $p > 0.05$) in the ICSI-LBW cases.

Table 5.2 Summary of the incidence of hypomethylation in the study groups

	C10		C12	
	≥33%	<33%	≥33%	<33%
ICSI-LBW[ab]	92%	8% (n=2)	92%	8% (n=2)
ICSI-NBW[a]	100%	0%	100%	0%
Controls[b]	100%	0%	100%	0%

a,b: not significantly different by Chi-square test ($p > 0.05$). Hypomethylation was defined by less than 33%.

Our preliminary data showed that the trophoblast and mesenchymal cells presented similar rates of methylation ($r = 0.65$, $P < 0.05$) in placentas derived from ICSI pregnancies with normal outcomes (Figure 5.7). Therefore, analysis was performed on whole villi for the controls including the ICSI-NBW and the control group. However, as previously suggested by Mann *et al.* from studies in the mouse, the cells with trophoectoderm origin may be more vulnerable to *in vitro* culture effects than those derived from the inner cell mass. In order to test this hypothesis, villi were separated before the analysis of the ICSI-LBW group, which is more likely to be affected adversely by *in vitro* conditions. The results indicated that methylation pattern was comparable in most samples in the ICSI-LBW cases (Figure 5.8), with the average difference in % methylation being 2% lower in the trophoblast cells. In 64% of the cases, the trophoblast had a lower level of methylation but the difference ranges from 0% to 19%. Assuming the % methylation of the whole villi is represented by the average of the trophoblast and the mesenchyme, two IUGR cases were identified as hypomethylated (methylation ≤33%). Exclusively in these two cases, the methylation

level in the trophoblast was considerably lower than the mesenchyme (25.0% vs 7.0% in case 1; 26.5% vs 19.5% in case 2).

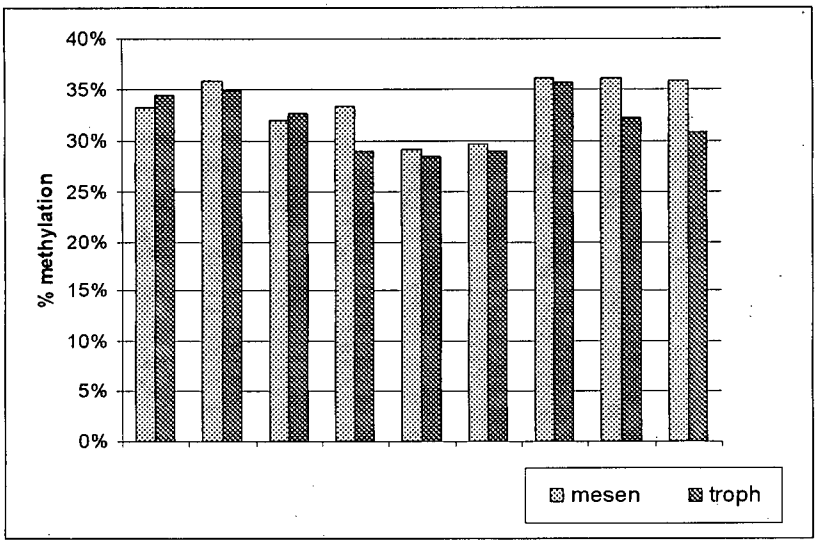


Figure 5.7 Methylation patterns of the trophoblast and the mesenchyme in placentas derived from ICSI pregnancies with normal outcomes. Methylation levels are similar in the trophoblast cells (dark) and the mesenchymal cells (light), measured in placentas derived from natural conceptions (n=9).

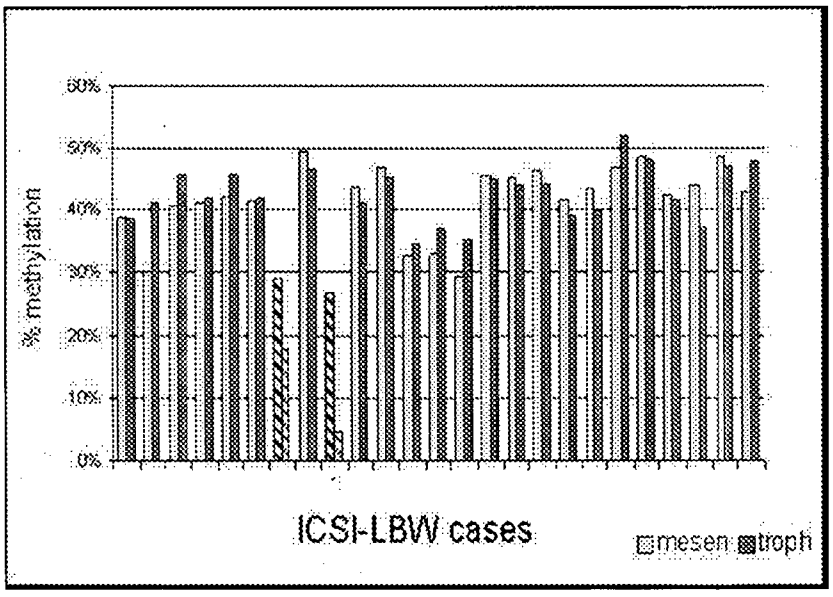


Figure 5.8 Methylation patterns of the trophoblast and the mesenchyme in the ICSI-LBW group. Methylation levels in the trophoblast cells (dark) and the mesenchymal cells (light), measured in the IL group, which comprises placentas derived from ICSI pregnancies with low birth weight. Level of methylation in the trophoblast is considerably lower than the mesenchyme in the hypomethylated cases (striped).

5.4. Discussion and Conclusion:

Several clinical studies have reported a considerably high incidence of imprinting disorders caused by epigenetic dysregulation in children conceived with ARTs (Cox *et al.*, 2002; DeBaun *et al.*, 2003; Maher *et al.*, 2003). Researchers are concerned that these imprinting disorders might be merely "the tip of an iceberg"; that is, epigenetic alterations may affect other imprinted genes, which may lead to less recognizable clinical features, but affect the health of ART children in long-term (Maher *et al.*, 2003). Today, up to 50% of ART treatments are performed with ICSI, which bypasses many physiological selection mechanisms that would be found in a natural conception. A number of prenatal and perinatal abnormalities have been associated with ICSI (Allen *et al.*, 2006); however, the causes remain unclear. This study was designed to investigate the methylation patterns at the DMR of two developmental genes, *H19* and *IGF2*, in placentas derived from ICSI pregnancies with focus on those with IUGR and LBW.

Comparing the three study groups: ICSI-LBW, ICSI-NBW, and controls, we did not detect a significant difference in average methylation level. Rodent models suggested that *in vitro* culture adversely affects the methylation pattern at certain imprinted genes, depending on the type of the media. Mann *et al.* reported that culturing in the Whitten's medium resulted in loss of methylation at the *H19* gene in mouse embryos (2004). In contrast, the same gene gained methylation when embryos were cultured in human tubal fluid (Li *et al.*, 2005). However, it should be noted that neither the medium used in these studies had the optimal composition for culturing mouse embryos. Based on our findings, *in vitro* culture with media used in clinical settings did not seem to significantly alter the methylation patterns at the DMR of *H19/IGF2* in the placentas derived from ICSI pregnancies. However, focusing on specific cases, we found two hypomethylated cases (<33%) exclusively from the ICSI-LBWL group. In both cases, the trophoblast appeared to be more hypomethylated than the mesenchymal cells. This observation is in agreement with the hypothesis proposed by Mann *et al.* that tissue with trophoectoderm origin is more vulnerable to external stress (2005). Curiously, only one site of these placentas was hypomethylated. Thus, it remains to be clarified whether partial hypomethylation at *H19/IGF2* in the placenta has a causal relationship with reduced birth weight, and whether these cases represent a subset of LBW/IUGR pregnancies after ICSI. In order to determine if methylation altered

specifically in ICSI derived LBW cases, we need to investigate methylation patterns in LBW cases not associated with ICSI.

There are several drawbacks in this study. First, the sample size was limited to ten cases, which included nine IUGR (birth weight below 10^{th} percentile) and one LBW (birth weight below 2500g). Many of these cases had borderline birth weight; therefore, may not truly represent the population of the pathological category. Also, because of the small sample size, we may have failed to identify a subgroup in which epigenetic defects play a role on LBW. Another shortcoming is that only two CpGs were examined out of the twenty-five differentially methylated CpGs present with in the DMR of *H19/IGF2*. Although similar approach was informative for analyzing tumor tissues (Sievers *et al.*, 2005; Nguyen *et al.*, 2001), a smaller number of CpGs may be perturbed in the LBW group as the phenotype is less severe. Furthermore, expression of *H19* and *IGF2* was not studied; therefore, understanding the effects of the ICSI procedure is limited to the very bottom of the biological pathway. The findings in the current study are preliminary; therefore, are insufficient to draw conclusions about the possibility of epigenetic dysregulation caused by the ICSI procedure.

For future studies, not only should these limitations be overcome, but a broader spectrum of the pathogenesis of fetal growth deficiency should be explored. Study of uniparental disomy (UPD) has revealed the association between IUGR and matUPD7, mat UPD14, mat UPD16, and matUPD20 (Kozot *et al.*, 2002). This suggests that many other candidate imprinted genes may be involved in fetal growth. Several placental imprinted genes are suggested to control the fetal growth rate in order to maintain the nutrient supplement to the placenta. These include Slc22a2, Slc22a3, Impt1/Slc22a11, and Ata3 (Dao *et al.*, 1998; Zwart *et al.*, 2001; Mizuno *et al.*, 2002). Furthermore, in order to pinpoint the step at which the epigenetic changes occurred, study of gametes and embryos at different stages should continue be conducted in animal models.

Also, there are several other risk factors for epigenetic alteration related to ICSI, in addition to embryo culture. Considering the relatively delayed acquisition of maternal imprints compared to paternal imprints, manipulation of oocytes has raised concerns for causing epigenetic dysfunction. Borghol *et al.* (2006) detected a gain of methylation at the CTCF binding domain with in the *H19/IGF2* DMR in MI-arrested and MII-arrested oocytes that were matured in vitro. Furthermore, superovulation has been suggested to increase

the incidence of abnormal global methylation by two folds in two-cell mice embryos (Shi and Haaf, 2002). Risk is not limited to the oocyte; sperm from infertile men with severe oligozoospermia have been demonstrated with incomplete methylation at *H19* (Marques *et al.*, 2004). While no supporting data has been published, the effect of germ cell and mechanical stress due to injection should also be considered.

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CHAPTER 6. Summary and conclusion

6.1 Summary

Utilizing only a single sperm to achieve fertilization, intracytoplasmic sperm injection (ICSI) has achieved remarkable success in treating male infertility since 1992. Today, ICSI accounts more than 50% of assisted reproductive technologies (ART) performed worldwide; however, health outcomes of children born from this technology remain a concern. An elevated rate of low birth weight (LBW), preterm delivery, birth defects and other pregnancy complications have been extensively reported in ICSI pregnancies (Loft *et al.*, 1999; Wennerholm *et al.*, 2000; Hansen *et al.*, 2002). The prevalence of chromosomal abnormalities, particularly those involving sex chromosomes, has also been suggested in ICSI conceptions (Bonduelle *et al.*, 2002). More recently, the unexpectedly high incidence of imprinting disorders, namely Beckwith-Weidman Syndrome and Angelman Syndrome, has been reported among children born after ART including ICSI (Cox *et al.*, 2002; DeBaun *et al.*, 2003). Despite the alarming data, little is understood regarding the etiology behind these findings. The current study was designed to investigate two potential risk factors in ICSI pregnancies that may lead to adverse pregnancy outcomes – confined placental mosaicism (CPM) and epigenetic alterations at imprinted genes.

CPM, defined as the presence of chromosomal abnormalities limited to the placenta, has been associated with intrauterine growth restriction (IUGR) and a number of other pregnancies complications (Lestou and Kalousek, 1998). Limited information is available in the literature regarding placental mosaicism in ICSI pregnancies and its correlation with the increased LBW rate. Taking into account that the increased rate of chromosomal abnormalities and other pregnancy anomalies that mirror the clinical phenotypes of CPM in ICSI conceptions, we hypothesized that the rate of CPM might be higher in ICSI pregnancies in general, and would be particularly high in those with LBW. The investigation of CPM was carried out in thirty term placentas at three random sites using comparative genomic hybridization (CGH) complemented with flow cytometry. One case of CPM (45, X) was identified out of the thirty placentas (3.33%), which is not significantly different from that in natural conceptions (5.88 %). The abnormality was detected from two out of ten sites in the

placenta, which was derived from a pregnancy with normal birth weight. In this study, no LBW case (n=2) presented with CPM.

As an extension to the study of CPM, origin of abnormalities was investigated using microsatellite markers in CPM cases as well as non-mosaic chromosomal abnormalities ascertained through spontaneous abortion (SA) in ICSI pregnancies. In the general population, maternal meiotic origin has been frequently found in non-mosaic trisomies (Robinson *et al.*, 1999) and post-zygotic origin has been suggested to predominate CPM cases (Wolstenholme, 1996; Robinson *et al.*, 1999). ICSI possesses three risk factors for chromosomal abnormalities - advanced maternal age, male infertility, and the invasiveness of the procedure. According to the limited data to date, the origin for chromosomal abnormality is primarily paternal, although most cases reported were inherited rather than *de novo* (Van Opstal *et al.*, 1997; Jozwiak *et al.*, 2004; Bonduelle *et al.*, 2002). Thus, we speculate that the origin of chromosomal abnormalities detected in ICSI pregnancies may differ from that in the general population. In this study, which included four cases, we identified that two CPM cases carried a mitotic origin, and the two SA cases were of maternal and paternal origin respectively. Due to the small sample size, the data lacks statistical power to draw conclusion as to whether there is a difference in origin of chromosomal abnormalities in ICSI conceptions compared to natural conceptions.

Subsequently, the meiotic segregation pattern and interchromosomal effect (ICE) were studied in sperm from the father of the paternally inherited cases [t(13;21)], using fluorescent in situ hybridization (FISH). Because t(13;21) is one of the rarest Robertsonian translocations (RTs), accounting for 2% of the RT cases, cytogenetic study of sperm in this case not only provide insight into the pathogenesis of this chromosomal abnormality, but also have great informative value in reproductive counseling. With respect to chromosomal constitutions of 13 and 21, 88.39% of the spermatozoa were normal or balanced, derived from alternative segregations, and nullisomy or disomy, as a result of adjacent segregations, account for 11.08%, which was significantly higher than that in the fertile controls (0.6%, $p<0.05$). However, for chromosome 18 and sex chromosomes, the proportion of normal haploid sperm was 98.79%. The rate of disomy was not significantly higher than the controls for either chromosome 18 or X/Y. Thus, the rare t(13;21) case exhibited similar pattern of meiotic segregation as in the common RTs, and ICE was not observed.

Finally, imprinting defect was studied as the other hypothesized risk factor for negative pregnancy outcomes in the ICSI population. The possible epigenetic dysregulation on ICSI conceptions has been brought to attention by the over-represented epimutations in the BWS and AS cases identified in children born after ICSI (Maher *et al.*, 2003). Animal studies have supported the hypothesis that epigenetic markings such as DNA methylation could be altered during *in vitro* culture (Doherty *et al.*, 2001; Mann *et al.*, 2005). In the present study, methylation patterns were investigated at the differentially methylated region (DMR) of *H19/IGF2* in placentas derived from ICSI pregnancies with IUGR or LBW (ICSI-LBW). Comparisons were made between the ICSI-LBW group and those from ICSI pregnancies with normal birth weight and the controls with normal birth weight. We did not detect a significant difference in average methylation level among the three groups. Interestingly, hypomethylated cases (<33%) were observed exclusively from the ICSI-LBW group in which the trophoblast lineage was more severely demethylated. We speculate that this could implicate a subset of LBW cases where methylation was reduced by *in vitro* culture particularly in the trophoectoderm that was in direct contact with media. Curiously though, the epigenetic change seems to affect the placenta unevenly; that is only one site of these placentas was hypomethylated. Nevertheless, the findings in the current study are preliminary and inconclusive due to the small sample size.

6.2 Conclusion

Based on our findings, the incidence of CPM in ICSI pregnancies was not higher than that in the general population, notwithstanding the reported increase in rate of chromosomal abnormality in ICSI conceptions detected through prenatal diagnosis. CPM was also not prevalent in ICSI pregnancies with LBW, suggesting that the cause for LBW may be other factors related to ICSI. Study of origin of chromosomal abnormality added information to the database; however, we could not conclude a general trend for origin of chromosomal abnormality in CPM and non-mosaic aneuploidy in ICSI conceptions. Meiotic segregation patterns were investigated in a paternally inherited t(13;21), which have not been previously reported in the literature. The incidence of abnormal segregation pattern in t(13;21) was significantly higher than in the infertile controls, however, was similar to other types of

Robertsonian translocations. Lastly, the methylation patterns at the DMR of *H19/IGF2* in placentas from ICSI pregnancies with LBW were found to be comparable with those with normal birth weight from ICSI pregnancies and natural conceptions. Hypomethylation (<33% methylation) was present in the two cases from the ICSI-LBW group, in which the trophoblast had considerably lower level of methylation compared to the mesenchymal lineage. Nevertheless, due to the limited sample size in this study, the mechanism responsible for the increased adverse pregnancy outcomes in ICSI is still unknown.

6.3 Bibliography

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