X-CHROMOSOME INACTIVATION AND FMR1 CGG REPEAT AND AGG INTERSPERSION NUMBER IN FEMALE NEWBORNS CONCEIVED BY ASSISTED REPRODUCTIVE TECHNOLOGIES (ARTs)

by

ELIZABETH XIANSHI WU

B.Sc., The University of British Columbia, 2010

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

(Reproductive and Developmental Sciences)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

JULY 2012

Abstract

Pregnancies derived from *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI) are associated with increased rates of chromosome abnormalities, congenital malformations, low birth weight, pre-term births, multiple gestations, and imprinting disorders. Assisted reproductive technologies (ARTs) enable sub-fertile individuals to circumvent the natural selective pressures involved in human reproduction. The risks of ARTs may be due to the underlying causes of subfertility in these individuals or to the artificial processes used to achieve pregnancy.

X-chromosome inactivation (XCI) may be at risk to perturbations in ARTs as it is thought to occur during the blastocyst stage when *in vitro* culturing would be taking place. We examined the XCI status in females conceived by ICSI (n=70), IVF (n=68), and naturally (NC, n=42). We found no significant differences between the populations in the frequency of mild skewing (≥75%) or extreme skewing (≥90%), or the mean level of skewing. Two extremely skewed cases were identified (1 IVF and 1 NC). It was determined that the maternal allele was preferentially inactivated in the cord blood of the extremely skewed IVF case. XCI status between placental sites varied in this case, but skewing values tended to correlate between different tissues within the same site.

Furthermore, it is possible that infertile individuals are passing on high repeat *FMR1* alleles associated with infertility or that repeat expansion could be occurring in subfertile parent's germlines or during *in vitro* culturing. We investigated *FMR1* CGG repeat and AGG interspersion number in the alleles of females conceived by ICSI (n=36), IVF (n=36), and NC (n=36). We did not find a significant difference between the populations in the frequency of intermediate alleles (45-54 repeats), premutation alleles (55-200 repeats), the mean allele

repeat number, the biallelic mean, the distribution of *FMR1* genotypes, or the distribution of total AGG interspersion number. No full mutation alleles were observed. A single premutation allele was found in a NC infant.

The results indicate that female newborns conceived through ICSI and IVF are not at a greater risk of having skewed XCI or of inheriting *FMR1* alleles with higher CGG repeat counts and more instability than female newborns conceived naturally.

Preface

This thesis was written with input and guidance from Dr. Sai Ma as well as my supervisory committee members: Dr. Carolyn Brown, Dr. Patrice Eydoux, and Dr. Dan Rurak. These projects were designed by Dr. Sai Ma and Elizabeth Wu. The experiments, data analysis, figures and tables were performed and prepared by Elizabeth Wu in exception of the capillary electrophoresis performed by Ruby Jiang (Robinson Lab) for Project 1 and Xiaohua Han (CMMT) for Project 2. All data collection and subsequent analyses was done by Elizabeth Wu. Preliminary results from cases included in Project 1 of this thesis were published in the abstract book and presented at the 27th Annual Meeting of the European Society of Human Reproduction & Embryology (ESHRE).

An ethics certificate of minimal risk approval was obtained from the University of British Columbia Clinical Research Board (Certificate numbers: CW07-0220/H06-70324).

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Genes are named following the rules established by the Human Genome Organization (HUGO). Human genes are in all capital letters while mice genes only have the first letter capitalized. Genes of both human and mice are italicized while proteins are non-italicized.

AGG	Adenine/guanine/guanine
AHC	Adrenal hypoplasia congenita
AIRE	Autoimmune regulator
AKAP 82	A-kinase anchor protein 82
ALD	Adrenoleukodystrophy
AMH	Anti- müllerian hormone
AMHR2	Anti- mullerian hormone receptor, type II
APECED	Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy
AR	Androgen receptor
ART	Assisted reproductive technologies
AS	Angelman syndrome
ATR-X	Alpha-thalassemia/mental retardation syndrome
AZF	Azoospermic factor
BWS	Beckwith-Wiedemann syndrome
c_0	Size correction factor
cDNA	Complementary DNA
CDY	Chromodomin Y
CE	Capillary electrophoresis
CENP-A	Centromere protein A
CFRI	Child and Family Research Institute
CFTR	Cystic fibrosis transmembrane conductance regulator
CGG	Cytosine/guanine/guanine
CGH	Comparative genomic hybridization
CMMT	Centre for Molecular Medicine and Therapeutics
CpG	Cytosine-phosphate-guanine
CPM	Confined placental mosaicism
CTCF	CCCTC-binding factor (zinc finger protein)
CYP11A1	Cytochrome P450, family 11, subfamily A, polypeptide 1
CYP17	Cytochrome P450, family 17, subfamily A, polypeptide 1
CYP19	Cytochrome P450, family 19, subfamily A, polypeptide 1
CYP21A2	Cytochrome P450, family 21, subfamily A, polypeptide 2
DAX1	Dosage-sensitive sex reversal adrenal hypoplasia congenital critical
region	on the X-chromosome gene-1
DAZ	Deleted in azoospermia
DBY	Dead box Y
DDP	Dystonia-deafness-optic neuronopathy syndrome
DHH	Desert hedgehog
Dhh-PTch1	Desert hedgehog/patched 1
DKC1	Dyskeratosis congenital

DMD	Duchenne muscular dystrophy
DMRT1	Doublesex and mab-3 related transcription factor 1
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DNAH5	Dynein, axonemal, heavy chain 5
DNMT	DNA methyltransferase
DNMT1	DNA (cytosine-5-)-methyltransferase 1
DNMT3	DNA (cytosine-5-)-methyltransferase 3
DNMT3A	DNA (cytosine-5-)-methyltransferase 3 alpha
DNMT3B	DNA (cytosine-5-)-methyltransferase 3 beta
DXS6673E	Zinc finger, MYM-type 3
ESR1	Estrogen receptor 1
ESR2	Estrogen receptor 2
FAM	6-carboxy-fluorescine
FGD1	FYVE, RhoGEF and PH domain containing 1
FMR1	Fragile X mental retardation 1 gene
FMRP	Fragile X mental retardation protein
FSH	Follicle stimulating hormone
FSHB	Follicle stimulating hormone, beta polypeptide
FSHR	Follicle stimulating hormone receptor
FXS	Fragile X syndrome
FXTAS	Fragile X-associated tremor/ataxia syndrome
GATA4	GATA binding protein 4
GnRH	Gonadotropin releasing hormone
GNRHR	Gonadotropin-releasing hormone receptor
hCG	Human chorionic gonadotrophin
hMGs	Human menopausal gonadotropins
HESX1	Homeobox gene expressed in ES cells
HEX	Hexachloro-6-carboxy-fluorescine
HSD17B3	Hydroxysteroid (17-beta) dehydrogenase 3
HSD3B2	Hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 2
ICF	Immunodeficiency, centromeric instability, and facial anomalies
ICM	Inner cell mass
ICSI	intracytoplasmic sperm injection
IHH	Idiopathic hypogonadotropic hypogonadism
IUI	Intrauterine insemination
IVF	*In vitro* fertilization
KAL1	Kallmann syndrome 1 sequence
KS	Kallmann syndrome
LEP	Leptin
LEPR	Leptin receptor
LH	Luteinizing hormone
LHB	Luteinizing hormone beta
LHR	Lutropin/choriogonadotropin receptor
LHX3	LIM homeobox 3
LINE-1	Long interspersed nucleotide element 1

m_0	Mobility correction factor
MCB	Macrochromatin body
MECP2	MECP2 duplication
MIDAS	Microphthalmia with linear skin-defects syndrome
mRNA	Messenger ribonucleic acid
MZ	Monozygotic
NEMO	Hypohidrotic ectodermal dysplasia with immunodeficiency
NR5A1	Nuclear receptor subfamily 5, group A, member 1
NROB1	Nuclear receptor subfamily 0, group B, member 1
PAR	Pseudoautosomal region
PCI	Phenol:chloroform:isoamyl alcoho*l*
PCR	Polymerase chain reaction
PGC	Primordial germ cell
PGD	Preimplantation genetic diagnosis
PIG-A	Phosphatidylinositol glycan anchor biosynthesis, class A
PNH	Paroxysmal nocturnal hemoglobinuria
POF	Premature ovarian failure
POP1	Processing of precursor 1, ribonuclease P/MRP subunit
PRY	PTP-BL related on the Y chromosome
PTPN11	Protein tyrosine phosphatase, non-receptor type 11
RBMY	RNA-binding motif on the Y chromosome
RNA	Ribonucleic acid
ROX	6-carboxy-X-rhodamine
RSA	Recurrent spontaneous abortion
RT	Robertsonian translocations
SAR	Nuclear receptor subfamily 1, group I, member 2
SDS	Sodium dodecyl sulphate
SF1	Steroidogenic factor 1
SHBG	Sex hormone-binding globulin
SOX9	SRY (sex determining region Y)-box 9
SRD5A2	Steroid-5-alpha-reductase, alpha polypeptide 2
SRY	Sex determining region Y
Tsix	X (inactive)-specific transcript, antisense
TSPY	Testis specific protein, Y-encoded
TSS	Transcriptional start site
USP9Y	Ubiquitin-specific protease 9 on the Y chromosome
UTR	Untranslated region
WASP	Wiskott-Aldrich syndrome
WHO	World health organization
WNT4	Wingless-type MMTV integration site family, member 4
WT1	Wilms tumor 1
Xa	Active X chromosome
XCI	X-chromosome inactivation
Xi	Inactive X chromosome
XIC	X inactivation centre
XIST	X-inactivate specific transcript gene
Xite	X-inactivation intergenic transcription element

XLA	Agammaglobulinemia
XLSCID	X-linked severe combined immunodeficiency syndrome
YY1	YY1 transcription factor

Acknowledgements

I would like to thank my graduate supervisor, Dr. Sai Ma for giving me the opportunity to work in her laboratory as an undergraduate student and for taking me on as a graduate student. I truly appreciate all the support and guidance that she has given me for my projects and my career. I would also like to thank my supervisory committee members; Dr. Dan Rurak, Dr. Carolyn Brown, and Dr. Patrice Eydoux. I am very grateful for the time all of you have taken out of your very busy schedules to offer helpful and constructive suggestions. Thank you for supporting and furthering the education of graduate students.

To previous and past members of Dr. Ma's lab; Dr. Sanuja Pitigalarachihi, Christa Darr, Kevin Ma, Kevin Dong, Kate Martelli, Sina Sakian, Chantelle Chand, Andrew Wilson, Tanya Vinning, and Daisy Cao; thanks for all the laughter and for making my graduate experience more enjoyable. I am especially indebted to Dr. Agata Minor and Edgar Chang Wong for training me and providing extensive assistance and encouragement in my work.

I am very appreciative for all the counsel and technical expertise that Dr. Maria Peñaherrera and Ruby Jiang provided me. I would like to thank Dr. Wendy Robinson for allowing me to run my samples for Project 1 on her ABI machine and for the use of her GeneScan and GeneMapper 4.0 software. I am also grateful to Xiaohua Han for all her efforts in troubleshooting on the CMMT's ABI machine for the samples for Project 2.

Finally, I would like to thank my friends and family for their love and support. Thank you to my father, Edmund Wu and mother, Marjorie Yeo for supporting me throughout my education and for instilling in me the importance of hard work. To my brother, Matthew Wu and grandmother, Annie Tan, thank you for everything that you do for me. Last but not least,

I am extremely grateful to Jordan Vellekoop for always keeping me sane and for being a constant support in my life.

The projects of this thesis were supported and funded by the Canadian Institute of Health Research (CIHR) grant issued to Dr. Sai Ma.

Chapter 1 : Introduction

1.1 Development

1.1.1 Early embryogenesis

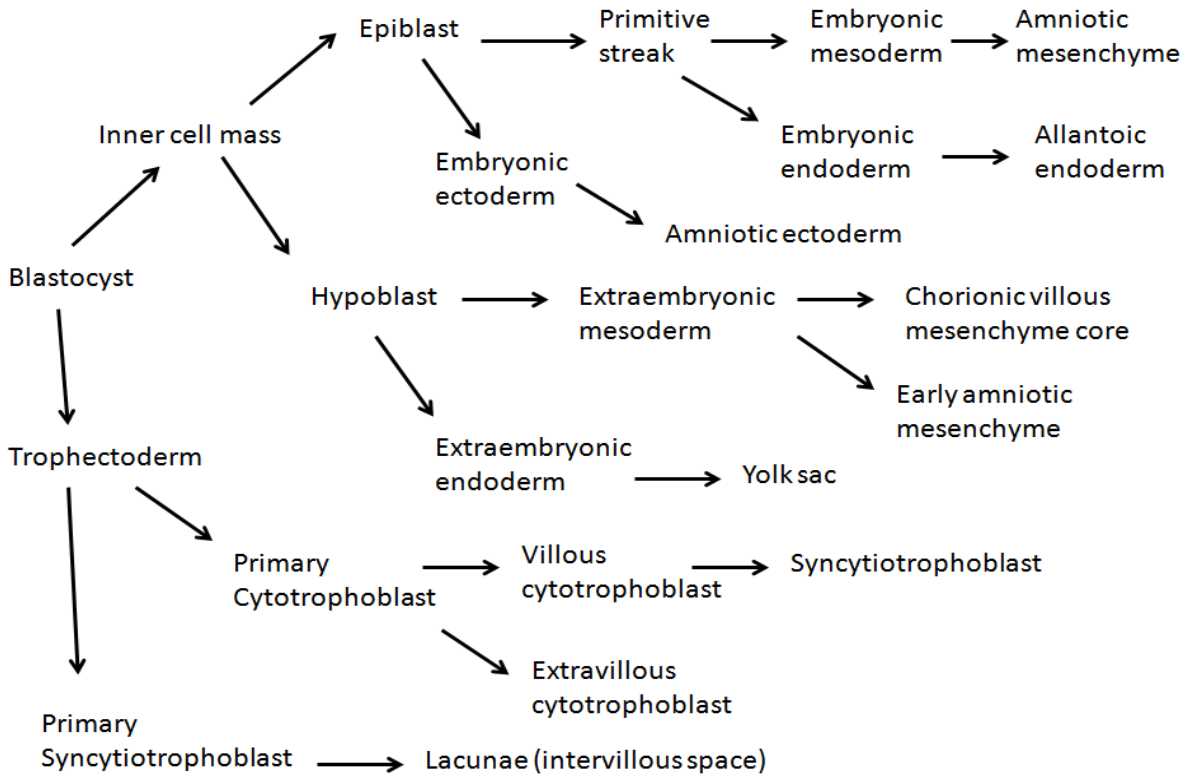
Following fertilization, one cleavage division occurs per day for the first 2 days (Carlson 2004). The cells in the embryo are called blastomeres (Carlson 1999). The embryo is called a morula when about 16 cells are present (Carlson 1999). By day 5, after several rounds of cleavage divisions, the outer blastomeres tightly adhere to each other through gap and tight junctions (Carlson 1999). Na^+, K^+-ATPase transport systems are used to move water across the outer blastomeres and into spaces around the inner blastomeres (Carlson 1999). The fluid filled space is known as the blastocoele and the embryo is now referred to as a blastocyst (Carlson 1999). The blastocyst is separated into two distinct cell lineages. The outer cell mass known as the trophectoderm surround cells referred to as the inner cell mass (ICM) (Carlson 1999). During the cleavage process the embryo is being transported down the fallopian tube, to the uterus. The embryo remains in the zona pellucida of the ovum until it reaches the uterus (Carlson 1999). When the embryo reaches the uterus, the trophectoderm cells secrete enzymes allowing the blastocyst to ``hatch`` from the zona pellucida (Carlson 1999). Six to seven days after fertilization, the embryo implants into the endometrial lining of the uterus (Carlson 1999). The uterine endometrium was prepared for implantation by the influence of hormones at the end of the previous menstrual cycle (Carlson 1999).

1.1.2 Origin of embryonic and placental tissues

The trophectoderm and ICM will give rise to distinct cell lineages (Figure 1.1). The cells of the ICM split into an upper layer of cells called the epiblast and a lower layer of cells called the hypoblast just before implantation (Carlson 2004). The epiblast contributes the cells

that will make up the embryonic ectoderm, embryonic mesoderm, and embryonic endoderm; the three embryonic germ layers that will give rise to the embryo (Carlson 2004). Extraembryonic tissues such as the amnion epithelium, amniotic mesenchyme, and allantois are also derived from these three germ layers, respectively (Carlson 2004, Robinson et al. 2002). The amnion is a membrane that encloses the embryo in fluid for protection and the allantois aids in the removal of embryonic waste and contributes to the umbilical cord (Carlson 2004). The hypoblast is thought to give rise to the extraembryonic mesoderm which will contribute to the chorionic villi mesenchyme (component of chorionic villi) and the chorionic plate ((Bianchi et al. 1993, Carlson 2004, Robinson et al. 2002). After implantation, the trophectoderm will differentiate into a primary inner cytotrophoblast layer and a primary outer syncytiotrophoblast layer (Carlson 2004). The primary syncytiotrophoblasts invade the maternal endometrial epithelium and will end up lining the lacunae or intervillous spaces through which maternal blood will flow (Gude et al. 2004). The primary cytotrophoblasts will differentiate into villous and extravillous cytotrophoblasts (Gude et al. 2004). The villous cytotrophoblast cells fuse to form the syncytiotrophoblasts and both will contribute to the inner and outer epithelial layer of the chorionic villi, respectively (Gude et al. 2004). The extravillous cytotrophoblasts will invade the uterine decidua interstitially to ensure a proper supply of maternal blood to the placenta (Gude et al. 2004).

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

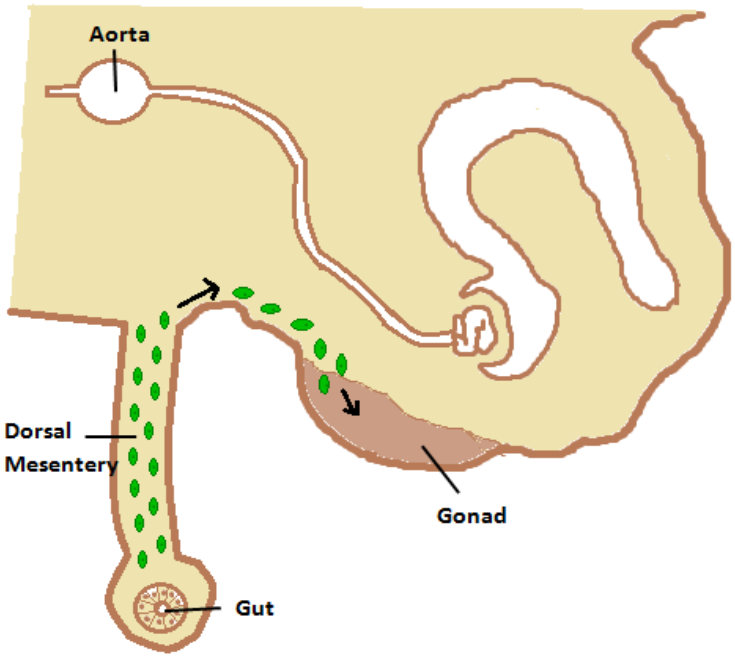


1.1.3 Germ cell migration and development

Human primordial germ cells (PGCs) can first be distinguished at approximately 24 days of development in the endodermal layer of the yolk sac (Carlson 1999). PGCs at this stage are distinguishable because they are large in size and have a high content of the enzyme alkaline phosphatase (Carlson 1999). The migration of PGCs occurs in three phases. The first is the separation phase. During this phase, PGCs will exit the posterior wall of the yolk sac into the hindgut epithelium and enter the mesenchyme of the dorsal mesentery (Carlson 1999, Pereda et al. 2006). The second phase is the migratory phase in which the PGCs travel by amoeboid movements through the mesenchymal cells of the dorsal mesentery and towards the genital ridges; the site of the future gonads (Figure 1.2) (Carlson 1999, Pereda et al. 2006). In the final phase, the colonization phase, about 5 weeks post fertilization, the PGCs reach the

gonadal crest, stop movement, and undergo rapid proliferation by mitosis (Carlson 1999, Pereda et al. 2006). During the whole migratory process, PGCs are multiplying by mitosis, but it is not until the PGCs arrive at the urogenital ridge that high rates of replication occur (Pereda et al. 2006).

Figure 1.2 Cross section showing the migration of primordial germ cells in the human embryo (adapted from Carlson 1999). PGCs exit the yolk sac into the hindgut. They then migrate through the dorsal mesentery towards the gonad.



Male and female fetuses are phenotypically identical prior to six weeks after fertilization, therefore the migration of PGCs is independent of the sex genotype of the fetus (Matzuk and Lamb 2008). Soon after XY germ cells reach the gonads, they arrest in the testis and remain in the stationary phase (G_0) of the cell cycle until birth (MacLaughlin and Donahoe 2004). An inhibitory factor produced from Sertoli or myoid cells is thought to be involved in this arrest (MacLaughlin and Donahoe 2004). After birth, male germ cells will resume the cell cycle (MacLaughlin and Donahoe 2004). Under the influence of gonadotropins, follicle stimulating hormone (FSH), and luteinizing hormone (LH) from the

pituitary, male germ cells will undergo spermatogenesis at puberty (MacLaughlin and Donahoe 2004). Unlike females, male germ cells maintain the ability to undergo mitosis throughout life (Carlson 1999).

XX germ cells will continue mitosis as they enter the gonads (MacLaughlin and Donahoe 2004). The female germ cells will undergo rapid proliferation through mitosis during the second through the fifth month of pregnancy (Carlson 1999). The number of germ cells increases from a few thousand to several million (Carlson 1999). These several million germ cells are the maximum number that will ever be present in the ovaries (Carlson 1999). Shortly after this period of mass replication, many female germ cells or oogonia will undergo atresia or natural degeneration (Carlson 1999). By birth, approximately one million are left in each ovary (Goswami and Conway 2005). Atresia of the oogonia continually occurs through the rest of the female lifespan until menopause (Carlson 1999). Only about 500 of the original seven million oocytes will be released during the reproductive lifespan of a woman (Goswami and Conway 2005). Oogonia will enter meiosis I and become arrested at the diplotene stage of prophase I by birth (MacLaughlin and Donahoe 2004). A single layer of granulose cells then surrounds the germ cells resulting in a primordial follicle (MacLaughlin and Donahoe 2004). After puberty, each month some of the primordial follicles are induced by follicle-stimulating hormone to grow into primary, secondary, and tertiary follicles (MacLaughlin and Donahoe 2004). The secondary oocyte within the tertiary follicle will arrest in metaphase of meiosis II until fertilization occurs (Carlson 1999). Chromosome aberrations and the improper segregation of chromosomes during meiosis can be detrimental to the offspring produced from these male and female gametes, not only in terms of morbidity and mortality, but also in terms of the ability to reproduce. Below, the human chromosomes will be discussed with a focus on chromosome abnormalities associated with infertility.

1.2 Human chromosomes

The human genome is organized into structures composed of DNA and proteins called chromosomes. Each somatic cell within the human body contains 23 pairs of chromosomes for a total of 46. Each of the pairs of chromosomes consists of a maternal and paternal copy.

1.2.1 Autosomal chromosomes

Of the 23 pairs of chromosomes, 22 of them are autosomal chromosomes. The autosomal chromosomes are numbered 1 through 22 from largest to smallest.

1.2.2 Sex chromosomes

One of the 23 pairs of chromosomes is the sex chromosomes. They are distinguished from autosomal chromosomes in that one of their functions is to determine the sex of an individual. The two sex chromosomes are the X chromosome and the Y chromosome.

1.2.2.1 Y chromosome

The Y chromosome is an acrocentric chromosome because its centromere is located near one end of the chromosome. The Y chromosome is small and contains around 300 genes (Payer and Lee 2008). The Y chromosome contains the sex determining region Y (*SRY*) (Brown and Robinson 2000). *SRY* is located close to the pseudoautosomal region (PAR) on the distal portion of the short arm of the Y chromosome (Yp) (Miller and Therman 2001). There is one PAR at both ends of the Y chromosome (Ross et al. 2005). Genes within the PARs are shared between the sex chromosomes (Ross et al. 2005). The Y chromosome also includes genes needed for spermatogenesis (Brown and Robinson 2000). Housekeeping genes located outside the PARs are also shared with the X chromosome (Ross et al. 2005).

1.2.2.2 X chromosome

The X chromosome is a submetacentric chromosome because its centromere is not midline, but is not as far to one end as in an acrocentric chromosome. The X chromosome is medium sized and contains about 1500 genes which make up about 5% of the genome (Brown and Robinson 2000, Payer and Lee 2008). The X chromosome contains genes for early spermatogenesis, oogenesis, brain development, and placental development. Housekeeping genes located outside the PARs are also shared with the Y chromosome (Ross et al. 2005).

1.3 Infertility

Infertility is defined as one year of unprotected intercourse without conception. Infertility affects about 15% of all couples (O'Flynn O'Brien et al. 2010). A World Health Organization study (WHO), published in 1987 attributed infertility to males in 20% of cases, females in 38% of cases, both partners in 27% of cases, and an unknown cause in 15% of cases (de Kretser 1997). Non-genetic causes of female infertility include tubal abnormalities, pelvic adhesions, endometriosis, and ovulatory dysfunction (Healy et al. 1994). Non-genetic causes of male infertility include tubal disorders and endocrine abnormalities (Adamson and Baker 2003). This thesis will mainly focus on the genetic causes of infertility.

1.3.1 Y-linked

Abnormalities on the Y chromosome can lead to infertility. Absence of or mutations in *SRY* impair the development of the testicles. Ten to fifteen percent of 46, XY individuals with abnormal gonadal development (Swyer syndrome) have mutations in *SRY* (Jager et al. 1990, Ostrer 2000). Individuals with Swyer syndrome have a female phenotype and characteristics include lack of breast development, very low estrogen levels, a normal vulva, vagina, and uterus, and streak gonads (Layman 2003). Streak gonads are non-functional and largely

comprised of fibrous tissue. The absence of anti-müllerian hormone (AMH) and testosterone in those with this condition allow normal uterine development and cause undermasculinizaiton, respectively (Layman 2003). Some individuals with Swyer syndrome can conceive through donor eggs and *in vitro* fertilization (IVF) (Layman 2003).

Microdeletions on the Y chromosome are the most common cause of male factor infertility. Genes involved in spermatogenesis have been mapped to a region on Yq11 referred to as the azoospermia factor (AZF) region (Layman 2003). The AZF region is divided into three subsections: AZFa, AZFb, and AZFc (from the area closest to the area farthest from the centromere). The following paragraph will describe abnormalities in these regions that can lead to male infertility. The spermatogenesis genes present in the AZFa region are *USP9Y* and *DBY* (Layman 2003). Individuals with both these genes deleted develop Sertoli cell-only syndrome in which there are Sertoli cells in the testes but there are no sperm in the ejaculate (O'Flynn O'Brien et al. 2010). *DBY* is found in the testes and is involved in pre-meiotic germ cell development (Vogt 2005). Deletions of *USP9Y* can result in azoospermia (absence of sperm), oligospermia (<20 million sperm/cm^3), and oligoasthenozoospermia (<20 million sperm/cm^3 and <50% sperm motility) (O'Flynn O'Brien et al. 2010). Deletions of *RBMY* and *PRY* found in the AZFb region cause spermatogenesis to arrest at the primary spermatocyte stage (Vogt 1998). Expression of *RBMY1* which codes for a testis-specific splicing factor is decreased in azoospermic men (Lavery et al. 2007). *PRY* is involved in the removal of abnormal sperm through regulation of apoptosis (Vogt 2005). Deletions within the AZF region are most often found in the subsection AZFc. It has been demonstrated that the AZFa and AZFb regions are needed to start spermatogenesis, but that spermatogenesis will not be normal without the AZFc region (Georgiou et al. 2006). Phenotypes of individuals with deletions in AZFc range from no sperm to reduced

spermatogenesis (Vogt 2005). The *DAZ* gene which is often deleted in azoospermia is found in the AZFc region. It is involved in regulating translation and meiosis, and in the maintenance of the PGC population (Reynolds and Cooke 2005). In addition, *DAZ* codes for germ-cell specific RNA binding proteins (O'Flynn O'Brien et al. 2010). Several spermatogenesis genes can be found in multiple copies on the Y chromosome while others are only found in a single copy. Fertile men have been observed to have deletions in spermatogenesis genes demonstrating the redundancy of some of these genes (O'Flynn O'Brien et al. 2010).

Deletions in *CDY* found on the long arm of the Y chromosome are also associated with male factor infertility. *CDY* codes for the chromodomain protein which is only expressed in the testis and controls histone substitution in spermatogenesis (O'Flynn O'Brien et al. 2010). Increases in copy number of the *TSPY* gene found on Yp have been shown to be present in infertile men (Vodicka et al. 2007). *TSPY* is thought to control the timing of spermatogenesis by stimulating spermatogonia to start meiosis (Nuti and Krausz 2008).

1.3.2 X-linked

There are many X chromosome abnormalities that are associated with infertility. Deletions on the short arm of the X chromosome at Xp11 cause ovarian failure in about half of infertile women (Layman 2003). The majority of women with deletions in the identified critical region from Xq13-Xq27 in the long arm of the X chromosome have primary amenorrhea and gonadal failure (Quilter et al. 2010). Fertility is thought to be affected by deletions on the X chromosome due to loss of ovarian determining genes or loss of genes needed in two copies for the development of the ovaries (Layman 2003).

Mutations in the Androgen Receptor (*AR*) gene located on the long arm of the X chromosome at Xq12 have been associated with infertility in men. *AR* is involved in spermatogenesis; in the maturation of spermatocytes into round spermatids (O'Flynn O'Brien et al. 2010). Mutations in *AR* can lead to androgen insensitivity syndrome which results from the inability of androgens to bind the androgen receptor. This syndrome is characterized by infertile individuals who are phenotypically female but whose genotype is 46, XY.

Mutations in *KAL1* located on Xp22 are responsible for about 30-70% of patients with Kallmann syndrome (KS) (O'Flynn O'Brien et al. 2010). KS syndrome is an X-linked recessive condition characterized by the absent or decreased function of male testes or female ovaries combined with the inability or reduced ability to smell (O'Flynn O'Brien et al. 2010). This disorder is caused by problems in the migration of gonadotropin releasing hormone (GnRH) neurons for which *KAL1* is involved in (Bhagavath and Layman 2007).

Mutations in the *NROB1* gene on Xp21 result in adrenal hypoplasia congenita (AHC) in men (Muscatelli et al. 1994, Zanaria et al. 1994). AHC is an X-linked recessive disorder in which there is abnormal development of the adrenal gland resulting in a lack of important steroids involved in reproduction such as glucocorticoids and mineralocorticoids (Muscatelli et al. 1994, Zanaria et al. 1994). *NROB1* codes for the protein DAX1 which is a transcription factor involved in pituitary gonadotropes and adrenal cortex development (Habiby et al. 1996). In addition, DAX1 is thought to regulate the release of gonadotropins from the pituitary and hypothalamus (Habiby et al. 1996).

Mutations of *AKAP 82* located at Xp11 have been associated with asthenozoospermia (low sperm motility) (Colledge and Scott 1999). *AKAP 82* codes for a protein that is localized to the sperm flagellum and thought to be involved in sperm motility (Colledge and Scott 1999).

1.3.2.1 Fragile X mental retardation 1 gene

The *Fragile X mental retardation 1 gene (FMR1*) is located at Xq27.3 and codes for the Fragile X mental retardation protein (FMRP) (Crawford et al. 2001). FMRP is a binding protein that is thought to transport certain mRNAs from the nucleus to the cytoplasm for translation (Crawford et al. 2001). In neurons, FMRP is proposed to bind RNA granules and travel along axons and dendrites to translocate mRNAs to specific locations in spine and growth cones (Davidovic et al. 2007). FMRP expression is observed in fetal ovaries suggesting its involvement in germ cell proliferation and hence, ovarian reserve (Bächner et al. 1993). At the 5' untranslated region (UTR) of *FMR1* is a polymorphic CGG repeat unit that is prone to expansions (Fu et al. 1991).

1.3.2.1.1 CGG repeat classifications

Based on the number of CGG repeats in the UTR of *FMR1*, alleles are arranged into categories. Six to forty-five CGG repeats is considered normal, 45-54 is intermediate, 55-200 is premutation, and >200 is full mutation (Nolin et al. 2003). Seventeen percent of male carriers aged 50-90, 38% of male carriers aged 60-69, 47% of male carriers aged 70-79, and 75% of male carriers over the age of 80 of the premutation allele display symptoms of the neurogenerative movement disorder known as Fragile X-associated tremor/ataxia syndrome (FXTAS) (Jacquemont et al. 2004). In one study, it was shown that 4.5% of women carriers (mean age 42.3 yrs) of premutation alleles had definite or probable FXTAS (Coffey et al. 2008). This disease is a progressive neurological condition which is characterized by multidimensional tremor, gain and limb ataxia, and Parkinsonism (Berry-Kravis et al. 2005, Hagerman et al. 2004). The onset of symptoms in men occurs at around the age of 50-70 years old. Women are less likely to develop FXTAS and if they do, the symptoms are shown to be milder due to the effects of X-chromosome inactivation (XCI) (Berry-Kravis et al. 2005).

Premutation alleles are also associated with premature ovarian failure (POF) as discussed below. Both FXTAS and POF are thought to be a result of cellular toxicity due to the elevated levels of *FMR1* mRNA produced from the premutated allele (Berry-Kravis et al. 2005, Murray 2000). Fragile X syndrome (FXS), the most common cause of inherited mental retardation in males results from expansion of the *FMR1* CGG repeat tract to >200 repeats (full mutation) (Fu et al. 1991). FXS is an X-linked dominant disorder with reduced penetrance (Crawford et al. 2001). The phenotype of individuals with the full mutation in terms of cognitive function, behaviour, and physicality varies (Crawford et al. 2001). FXS in males is characterised by macroorchidism (large testes), slightly increased head size, flat feet, arched palate, craniofacial abnormalities, large ears and jaw, hyperextensible joints, cardiac mitral valve prolapse, avoidance of gaze, repetitive mannerisms, repetitive speech, and hyperactivity (Heine-Suner et al. 2003, Miller and Therman 2001). Affected males show a more severe degree of mental retardation than females due to the disease's X-linked inheritance (Heine-Suner et al. 2003). Females usually display a milder phenotype with moderate mental retardation to normal intelligence (Heine-Suner et al. 2003). Females also generally lack the physical traits displayed in men with FXS (Heine-Suner et al. 2003).

1.3.2.1.2 Inheritance and expansion

Generally, the longer the *FMR1* CGG repeat tract the more unstable it is and hence, the more likely it is to expand in future generations. A model with four allelic states (N→S→Z→L) has been devised to show how a stable allele could expand to a full mutation allele (Morton and Macpherson 1992). The normal repeat allele (N) will acquire an initial mutation resulting in a susceptible repeat allele (S) (Chakravarti 1992). Susceptible or intermediate (grey zone) alleles can be either stable or unstable (Eichler et al. 1996). If the susceptible allele continues to expand through generations it can reach the premutation size

(Z) (Chakravarti 1992). Premutation alleles are very unstable and can easily expand to the full mutation allele (L) (Chakravarti 1992). Premutation alleles with as little as 59 repeats have been shown to expand to full mutation alleles within a single generation (Nolin et al. 2003).

FMR1 CGG repeat expansions can occur during meiosis and mitosis. Unequal crossing-over during meiosis or unequal sister chromatid exchange in the pre or post-zygotic stage can result in repeat expansion (Terracciano et al. 2004). Meiotic or mitotic replication strand slippage in which loop out structures are formed on the daughter strand can also result in an increased number of triplet repeats (Terracciano et al. 2004).

The gender of carrier parents determines how likely it is that the *FMR1* allele repeat size will expand in their children. Expansion of premutation to full mutation alleles will only occur through maternal transmission (Ashley-Koch et al. 1998). In addition, fathers with the full mutation never pass down full mutation alleles to their daughters (Nolin et al. 2003). It is thought that differences in meiosis between males and females can explain these phenomena. Mitotic divisions prior to meiosis only occur in females prenatally while mitotic divisions prior to meiosis occur in males throughout their lives (Sullivan et al. 2002). Long *FMR1* CGG repeats are thought to be unstable over numerous mitotic divisions resulting in the contraction of *FMR1* alleles and the selection against sperm with high repeat alleles (Sullivan et al. 2002). One study found only sperm with premutation alleles in the semen samples of four men that were carriers of full mutation alleles (Reyniers et al. 1993). There is also evidence that *FMR1* repeat expansion depends on the sex of the offspring. In 139 mother to offspring transmissions, *FMR1* repeat expansion is observed more in mother to son transmissions than in mother to daughter transmissions (Loesch et al. 1995). It is suggested that the presence of two *FMR1* alleles in female zygotes restricts expansion during replication (Loesch et al.

1995). However, in male zygotes, there is no *FMR1* homologue on the Y chromosome which might lead to replication slippage.

1.3.2.1.3 AGG interspersions

Loss of AGG repeat interspersions within the CGG repeat tracts are thought to increase repeat instability (Dombrowski et al. 2002, Eichler et al. 1996). One to three AGG interruptions are generally found in normal and intermediate alleles while 0-1 are found in premutation alleles (Yrigollen et al. 2011). An instability threshold has been identified in which uninterrupted CGG tracts of greater than 34-38 repeats can result in unstable transmission (Eichler et al. 1996). Loss of AGG interspersions can occur due to deletions or A to C transversions (Eichler et al. 1996).

1.3.2.1.4 *FMR1* and premature ovarian failure

The average age of menopause in females of the Western world is approximately 51 years (Goswami and Conway 2005). Premature ovarian failure (POF) occurs when there is amenorrhea, elevated gonadotropins and estrogen deficiency in women under the age of 40 years (Nippita and Baber 2007). The diagnosis of POF is based on elevated levels of FSH (> 40 UI/l) in the menopausal range (Goswami and Conway 2005). FSH is produced by the pituitary gland and causes ovarian follicles to grow and mature (Martini 2006). FSH will continue to be made and released until mature follicles release enough estrogen which will feed back negatively on FSH production (Martini 2006). Therefore, elevated levels of FSH are indicative of the depletion of the primordial follicle pool (Quilter et al. 2010). POF can result from follicles being irresponsive to hormone stimulation, a decreased number of follicles being present at the time of ovarian development, or an increased rate of follicle loss (Goswami and Conway 2005, Vujovic 2009). A large portion of POF cases are idiopathic, but

some are attributed to infections, autoimmune ovarian damage, anticancer treatments, genetic aberrations, and metabolic causes (Vujovic 2009).

Premutation *FMR1* alleles are found in approximately 5% of all POF cases, in 12% of familial POF cases, 3% of sporadic POF cases, but only 0.4% in the general population (Murray 2000). It has also been reported that approximately 21% of premutation carriers have POF compared to only 1% of the general population (Sherman 2000). In recent year, intermediate ranges of 41-58 repeats (Bodega et al. 2006) and normal ranges of 35-54 repeats (Bretherick et al. 2005) have been associated with POF. It has been observed that repeats below and above the range of 26-34 repeats are associated with a decreased ovarian reserve (Gleicher et al. 2011).

Increased levels of mRNA expression has been observed in premutation individuals (Murray 2000). It is suggested that the longer mRNA molecules transcribed from the premutated gene could aggregate and be processed incorrectly or bind proteins which could hinder *FMR1* expression or other genes involved in ovarian function (Murray 2000). A CpG (cytosine-phosphate-guanine) island near the 5' end of *FMR1* genes that are fully mutated is hypermethylated (Miller and Therman 2001). Therefore, full mutation alleles do not express any mRNA or generate any protein and hence, the full mutation is not associated with POF (Murray 2000). It has also been proposed that during oogenesis there is a particular isoform of FMRP that is critical and is produced less efficiently from premutation alleles (Davison et al. 1999). It is suggested that a back up protein provides the function of FMRP in full mutation cases (Davison et al. 1999). However, in premutations, *FMR1* is expressed and therefore this back up mechanism is not initiated and the abnormal effects of the premutation would be observed (Davison et al. 1999).

1.3.2.1.5 Methods to determine *FMR1* CGG repeat size

Southern blot analysis and polymerase chain reaction (PCR) are the two main methods used to size the trinucleotide repeat of *FMR1*. Southern blotting enables a crude measure of repeat size and the assessment of methylation status (Sherman et al. 2005). The use of methylation-sensitive restriction enzymes enable the methylated and unmethylated alleles to be distinguished (Sherman et al. 2005). Although Southern blotting enables the detection of alleles of all sizes, it requires large amounts of DNA, is very labor intensive, time consuming, often involves radioactive material, and precise sizing is not achievable (Nahhas et al. 2012, Sherman et al. 2005). PCR amplification using flanking primers to amplify a fragment of DNA containing the repeat region has only recently been optimized to enable the amplification of repeat ranges greater than 150 CGG repeats (Nahhas et al. 2012). PCR analysis enables accurate sizing of *FMR1* alleles, but does not provide information on *FMR1* methylation status (Sherman et al. 2005). The details of *FMR1* repeat size determination by PCR amplification will be discussed in detail in the materials and methods section of Chapter 3.

1.3.3 Autosomal chromosome abnormalities

There are many autosomal genes in males and females that have been associated with infertility (see Table 1.1 for a summary).

Table 1.1 Autosomal genes associated with infertility

Gene	Implicated Disorder	Location	Mode of Inheritance	Reference
LEP	Idiopathic hypogonadotropic hypogonadism (IHH)	7q31	Recessive	(Montague et al. 1997, Strobel et al. 1998)
LEPR	IHH	1p31	Recessive	(Clement et al. 1998)
GNRHR	IHH, GnRH resistance	4q12	Recessive	(Beranova et al. 2001, de Roux et al. 1997)

Table 1.1 Autosomal genes associated with infertility

Gene	Implicated Disorder	Location	Mode of Inheritance	Reference
FSHB	FSH deficiency	11p13	Recessive	(Layman et al. 1997, Layman et al. 2002, Matthews et al. 1993, Phillip et al. 1998)
LHB	LH deficiency	19q13	Recessive	(Weiss et al. 1992)
PROP1	Combined pituitary hormone deficiency of GH, TSH, prolactin, and gonadotropins	5q35	Recessive	(Cogan et al. 1998, Fofanova et al. 1998, Wu et al. 1998)
HESX1	IHH caused by gonadotropin deficiency	3p21	Recessive, Dominant	(Dattani et al. 1998, Thomas et al. 2001)
LHX3	Combined pituitary hormone deficiency of TSH, prolactin, GH, FSH, and LH	9q34	Recessive	(Layman 2003)
LHR	Undermasculinization	2p21	Recessive	(Kremer et al. 1995, Layman 2003)
FSHR	FSH resistance	2p21-p16	Recessive	(Aittomaki et al. 1996, Beau et al. 1998, Tapanainen et al. 1997)
HSD17B3	17-Ketosteroid reductase deficiency	9q22	Recessive	(Geissler et al. 1994)
SRD5A2	5-α reductase type II deficiency	2p23	Recessive	(Andersson et al. 1991)
CYP21A2	Congenital adrenal hyperplasia (CAG)	6p21	Recessive	(Cabrera et al. 2001, Jaaskelainen et al. 2001)
CYP17	CAG, 17-OH deficiency	10q24	Recessive	(Layman 2003, Yanase et al. 1991)
STAR	Congenital lipoid hyperplasia	8p11	Recessive	(Layman 2003)
CYP19	Aromatase deficiency	15q21	Recessive	(Bulun 2000, Ito et al. 1993)
CYP11A1	Congenital lipoid hyperplasia	15q23-q24	Dominant	(Tajima et al. 2001)
HSD3B2	Type II 3-β hydoxysteroid dehydrogenase deficiency	1p13	Recessive	(Finnish-German APECED Consortium 1997, Layman 2003, Nagamine et al. 1997)
AIRE	Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED)	21q22	Recessive, Dominant	(Finnish-German APECED Consortium 1997, Nagamine et al. 1997)
NR5A1	Undermasculinization	9q33	Dominant	(Achermann et al. 1999)

Table 1.1 Autosomal genes associated with infertility

Gene	Implicated Disorder	Location	Mode of Inheritance	Reference
WT1	Frasier and Denys-Drash syndromes	11p13	Dominant	(Barbaux et al. 1997, Pelletier et al. 1991)
SOX9	Sex reversal, Campomelic dysplasia	17q24-q25	Dominant	(Foster et al. 1994)
AMH	Persistent müllerian duct syndrome	19p13	Recessive	(Imbeaud et al. 1996)
AMHR2	Persistent müllerian duct syndrome	12q13	Recessive	(Imbeaud et al. 1996)
DNAH5	Primary ciliary dyskinesia, Kartagener syndrome	5p15-p14	Recessive	(Olbrich et al. 2002)
DHH	Gonadal dysgenesis with minifascicular neuropathy	12q13	Recessive	(Umehara et al. 2000)
CFTR	Congenital bilateral absence of vas deferens, cystic fibrosis	7q31-q32	Recessive	(Ferlin et al. 2007, Georgiou et al. 2006)
SHBG	Androgen deficiency resulting in decreased spermatogenesis	17p13	Codominant	(Lazaros et al. 2008)
ESR1	Abnormal spermatogenesis and estrogen deficiency	6q25	Dominant	(Guarducci et al. 2006, Nuti and Krausz 2008, Yoshida et al. 2005)
ESR2	Abnormal spermatogenesis and estrogen deficiency	14q23	Dominant	(Nuti and Krausz 2008, Tuttelmann et al. 2007)
PTPN11	Noonan syndrome (one feature is un-descended testicles)	12q24	Dominant	(Tartaglia et al. 2001)
SF1	XY individuals with streak gonads	11q13	Recessive	(Parker 1998, Swain and Lovell-Badge 1999)
GATA4	Abnormal Testicular Development	8p23-p22	Dominant	(Swain et al. 1998, Tevosian et al. 2002)
WNT4	XY sex reversal	1p36-p35	Recessive	(Jordan et al. 2001, Vilain 2002)
DMRT1	Incomplete testis development in mice	9p24	Recessive	(Raymond et al. 2000, Zarkower 2001)
Dhh-PTch1	Impaired Leydig cell development in rats	9q22	Dominant	(Yao et al. 2002)
Fertlinβ	Lower rates of membrane fusion for fertilization to take place	8p11	Recessive	(Burkin et al. 1997)

1.3.4 Structural chromosome abnormalities

Structural chromosome abnormalities can influence reproductive success. The majority of men and about half of women with X-autosome translocations are infertile (Layman 2003). Breakpoints in genes involved in spermatogenesis and ovarian function can occur in X-autosome translocations. In addition, the X unaffected by an X-autosome translocation will be preferentially silenced in females through a process known as X-chromosome inactivation (XCI). XCI will be covered in more detail in later sections of this chapter. Chromosomes 15, 21, and 22 are the most common autosomes involved in X-autosome translocations because centromeric regions of these chromosomes are inclined to mis-pair with the X chromosome (Layman 2003). Y-autosome translocations can result in abnormal spermatogenesis due to disruption of genes involved in spermatogenesis. Autosome-autosome translocations can also lead to reduced fertility. In order for chromosomes to progress through meiosis they need to pair up to form synapses (Shah et al. 2003). Chromosomes that have undergone a balanced translocation pair up in a quadrivalent structure (Ferguson et al. 2008, Kirkpatrick et al. 2012, Shah et al. 2003). This process can take longer than pairing between normal chromosomes thereby delaying meiosis (Shah et al. 2003). In addition, translocated chromosomes are more likely to result in disjunction which results in gametes with unequal chromosome numbers (Shah et al. 2003). When translocated chromosomes pair during meiosis there are regions that are not paired (asynaptic) (Ferguson et al. 2008, Kirkpatrick et al. 2012). Asynaptic regions are more prone to fail meiosis resulting in the apoptosis of those germ cells (Shah et al. 2003). Robertsonian translocations (RTs) are 10 times more common in the male infertile population compared to newborns in the general population (Miller and Therman 2001). RTs are translocations between two acrocentric chromosomes with the retention on both the q (long) arms and loss of the p (short) arms. RTs attach to the sex vesicle during male meiosis (Miller

and Therman 2001). During male meiosis the X and Y chromosomes condense and interact with proteins to form a sex vesicle (Miller and Therman 2001). Inactivation of the sex vesicle could spread to the RT chromosomes and cause failure of meiosis (Ferguson et al. 2008, Kirkpatrick et al. 2012, Miller and Therman 2001).

1.3.5 Numerical chromosome abnormalities

Individuals with abnormal chromosome numbers have decreased fertility. Women with Turner syndrome have only one X chromosome (45, X). Ninety percent of individuals with Turner syndrome fail to undergo puberty and do not experience menstruation (Layman 2003). Individuals with Turner syndrome typically have streak ovaries and are only able to conceive via assisted reproductive technologies (ARTs) with a donor egg. It is thought that haploinsufficiency of multiple genes found on the X chromosome result in ovarian failure in these individuals (Layman 2003). Individuals who are 47, XXX undergo puberty normally and are fertile, but develop early menopause at around the age of 30 (Shah et al. 2003).

Men with Klinefelter syndrome have two X chromosomes and one Y chromosome (47, XXY). Individuals with this genotype are mainly sterile unless they are mosaic because genes on the extra X chromosome interfere with normal spermatogenesis (Shah et al. 2003). However, it is estimated that 25% of nonmosaic Klinefelter syndrome patients still have sperm in their ejaculate (O'Flynn O'Brien et al. 2010). The testicular environment in men with Klinefelter syndrome is altered and has been shown to result in increased errors in chromosome segregation (Shah et al. 2003). Therefore, even if these men are able to produce offspring naturally or though ARTs, there is a greater risk that their children will have some degree of aneuploidy (Shah et al. 2003).

Individuals with Down syndrome have three copies of chromosome 21 and have been shown to have reduced fertility. Women with Trisomy 21 range from being fertile to subfertile. However, men with this condition are mostly sterile. The effect of Trisomy 21 on male reproduction is unclear, but it is thought that in these men there is decreased proliferation of primordial germ cells and accelerated cell death (Shah et al. 2003).

1.4 Epigenetics

Epigenetics is the study of genetic traits in the absence of changes in DNA sequence. It involves chromosomal modifications that are heritable and can change chromosome functions without changing the DNA sequence. Genomic imprinting and X-chromosome inactivation are two major epigenetic phenomena. Imprinting will be discussed briefly while X-chromosome inactivation will be covered in more detail.

1.4.1 Genomic imprinting

Autosomal genes are normally expressed from both alleles concurrently. However, in the human genome, certain genes have preferential expression of only one parental allele through a mechanism known as imprinting (Manipalviratn et al. 2009). In genomic imprinting, one allele is expressed and the other is silenced based on the parent each allele came from (Manipalviratn et al. 2009). The epigenetic mechanisms of DNA methylation and histone modifications are utilized to achieve imprinting (Manipalviratn et al. 2009). DNA methylation involves the addition of a methyl group to cytosine residues found in cytosine guanine dinucleotides by DNA methyltransferases (DNMT) (Manipalviratn et al. 2009). A high frequency of cytosine-phosphate-guanine (CpG) dinucleotides are found in the promoter regions of genes (Manipalviratn et al. 2009). During gametogenesis DNMT3A and DNMT3B ensure that methylation patterns are laid down according to the gender of the individual

(Manipalviratn et al. 2009). Differentially methylated imprinted regions are regions where there is differing methylation status between male and female gametes (Manipalviratn et al. 2009). DNA methylation can prevent transcription by inhibiting binding of transcription factors and transcriptional machinery (Manipalviratn et al. 2009). DNA methylation can also activate other genes by adjusting chromatin configuration, enabling genes to interact with enhancers (Manipalviratn et al. 2009). Histone modifications such as methylation, acetylation, ubiquitylation, and phosphorylation are used in addition to DNA methylation to establish imprints. Alterations to histones can modify gene expression by changing the organization of chromatin structure and by altering interactions between regulatory factors and chromatin (Manipalviratn et al. 2009). There are over 80 imprinted genes in mammals and approximately 41 of those are known to be imprinted in humans (Morison et al. 2005). Most imprinted genes in humans are involved in regulating the growth and development of the embryo and placenta (Manipalviratn et al. 2009). The genetic conflict hypothesis suggests that imprinting evolved due to the discordance between the interests of the male and female genomes in the growth of their offspring (Moore and Haig 1991). It has been suggested that imprinted genes which are paternally expressed advance embryonic and placental growth while those that are maternally expressed inhibit embryonic and placental growth (Moore and Haig 1991).

1.4.2 X-chromosome inactivation

Diploid organisms can manage differences in copy numbers of single genes, but do not cope as well with large amounts of extra genetic material (Payer and Lee 2008). Aneuploidies in offspring usually result in abortion and those which survive usually have severe malformations and disabilities (Payer and Lee 2008). Chromosome aneuploidies present in live births normally involve the chromosomes with the fewest number of genes (Payer and Lee

2008). However, aneuploidies involving the X chromosome such as Turner syndrome (XO females) and Klinefelter syndrome (XXY males) show a much milder phenotype than aneuploidies involving autosomes (Payer and Lee 2008). As mentioned previously, the Y chromosome contains much less genetic material than the X chromosome. There are around 300 genes on the Y chromosome, but around 1500 on the X chromosome (Payer and Lee 2008). Therefore, there must be some process that enables the toleration of X aneuploidies and the extra X chromosome present in females compared to males. The mechanism that enables organisms to cope with these imbalances is known as X-chromosome inactivation (XCI). XCI results in one of the two X chromosomes in the somatic cells of females being transcriptionally silenced.

1.4.2.1 Mechanism

Studies of human X chromosome rearrangements have identified a region that is required for XCI called the X inactivation centre (XIC). The XIC is approximately 1 Mb and is found on the long arm of the X chromosome at Xq13 (Chang et al. 2006). Nine genes have been identified at the XIC with the most notable being the X-inactive specific transcript gene (*XIST*) (Chang et al. 2006). *XIST* produces a 17 kb transcript that is expressed from the inactive X chromosome, but not the active X chromosome (Brown et al. 1992). *XIST* does not contain any conserved open reading frames and its RNA is untranslated; indicating that it only functions as an RNA (Chang et al. 2006). *XIST* RNA has been shown to colocalize with the inactive X in female cells (Chang et al. 2006). Knock outs of *Xist* in female mouse embryonic cells inhibit XCI and *Xist* transgenes are able to induce inactivation of autosomes in mice (Chang et al. 2006). Before X-chromosome inactivation occurs, *XIST* is expressed from both X chromosomes at low levels (Panning et al. 1997, Sheardown et al. 1997). XCI occurs when *XIST* transcripts build up and are confined to the future inactive X (Xi) (Panning et al. 1997,

Sheardown et al. 1997). Stabilization of the transcripts at the Xi, but not the active X (Xa) is proposed to mediate this process (Panning et al. 1997, Sheardown et al. 1997). The promoter of *XIST* is methylated on the Xa and is therefore transcriptionally inactive (Hendrich et al. 1993). However, the promoter of *XIST* is unmethylated on the Xi indicating that it is transcriptionally active (Hendrich et al. 1993). *XIST* acts in *cis* to coat the X chromosome from which it was produced (Chang et al. 2006). It has been shown that female human preimplantation embryos start accumulating *XIST* RNA on one of the two X chromosomes at around the 8 cell stage (van den Berg et al. 2009). The *XIST* RNA continues to accumulate on one X chromosome during the morula and blastocyst stages (van den Berg et al. 2009). This correlates with the silencing of gene expression from the regions of the X coated with *XIST* (van den Berg et al. 2009). It is estimated that about 4-20 cells are present in the inner cell mass of the blastocyst when X inactivation occurs and that XCI begins prior to implantation of the embryo (van den Berg et al. 2009). It is not known when XCI occurs in the trophectoderm of the blastocyst, but there is no indication that it occurs separately from the ICM in humans (Peñaherrera et al. 2012). *XIST* is necessary for initiation of X-chromosome inactivation. However, once *XIST* expression from only one X chromosome has been established, a cascade of events occurs to ensure stability of the inactive X. These events mainly involve chromatin modifications and the order in which these processes occur has been determined in female mice embryonic cells.

1.4.2.2 Differences between the active and inactive X chromosome

The first process proposed to occur after *XIST* expression has been established from one X chromosome is the modification of histones. Histone tail modifications can change the electrostatic charge of histones, thereby altering their structural properties and interactions with DNA (Chang et al. 2006). Additionally, histone tail modifications can influence what

regulatory factors may be recruited to sites on the DNA with which they are associated (Chang et al. 2006). The active X is characterized by acetylation of H3 and H4 core residues of the nucleosome and methylation of H3 lysine 4 (Chang et al. 2006). Histone modifications associated with the inactive X include hypoacetylation of H3 and H4, di- and tri-methylation of H3 lysine 9, tri-methylation of H3 lysine 27, methylation of H4 lysine 20, and ubiquitylation of H2A lysine 119 (Chang et al. 2006). Histone modifications are not found uniformly on the X chromosomes but are mainly associated with gene promoters (Chang et al. 2006).

The next change that is observed is that regions of the inactive X replicate later than the rest of the genome (Willard and Latt 1976). The regions of the Xi that are not late replicating normally correspond to genes that do not undergo XCI such as the pseudoautosomal region (Chang et al. 2006). It is also suggested that histone modifications are involved in the differential timing of replication along the inactive X (Chadwick and Willard 2004).

Histones are octomers composed of two copies of each of the four histone core particles; H2A, H2B, H3, and H4. The H3 variant CENP-A replaces H3 in certain nucleosomes of the centromeric region of the Xi (Chang et al. 2006). The histone variants H3.3 and H2A.Z have been shown to be associated with active chromatin (Chang et al. 2006). The H2A variant, macroH2A is found throughout the Xi at inactivated regions (Chang et al. 2006). Dense regions of macroH2A are shown to co-localize with the Xi in female cells forming a macrochromatin body (MCB) (Chadwick and Willard 2001, Costanzi and Pehrson 1998). Antibodies specific for macroH2A immunoprecipitate with *XIST* RNA indicating that the two physically associate with one another (Gilbert et al. 2000).

DNA methylation is one of the last processes to occur in XCI. Methylation of CpGs in CpG islands tends to correlate with decreased gene expression (Chang et al. 2006). The addition of methyl groups to cytosines can alter the major groove of the DNA helix and thereby change its connection with proteins (Chang et al. 2006). In addition, DNA methylation of a gene can result in the recruitment of specific protein complexes such as those involved in histone modification. Hypermethylation of DNA is present at the 5' region of genes on the inactive X that undergo inactivation (Anderson and Brown 2002, Carrel et al. 1996, Cotton et al. 2009). DNA methylation plays less of a role in initiation of inactivation, but a greater role in maintenance of the inactivation state (Lock et al. 1987). Mouse cells which are deficient in *Dnmt1* and *Dnmt3/Dnmt3b (*involved in maintenance of methylation after replication and *de novo* methylation, respectively) display appropriate *Xist* expression and silencing of genes on the X chromosome (Panning and Jaenisch 1996, Sado et al. 2004). However, the reactivation of some X-linked genes occur in these cells after treatment with demethylating agents (Chang et al. 2006).

LINE-1 or long interspersed nuclear elements – 1 are thought to enable spreading of inactivation along the X chromosome. About 30% of the X chromosome is made of LINE-1 transposable elements; about twice as much as the average on other chromosomes (Chang et al. 2006). In addition, LINE-1 elements are more concentrated in regions of the X chromosome that undergo inactivation (Hansen 2003). It has been suggested that LINE-1s enable spreading of inactivation by XIST-mediated intrachromosomal pairing of LINE-1 repeats via looping (Hansen 2003). In the human genome, LINE-1 elements are predominantly silenced by hypermethylation of the 5' internal promoter (Chang et al. 2006). LINE-1 elements have been shown to be hypermethylated on both the inactive and active X (Hansen 2003). However, in cells of individuals with ICF syndrome who are deficient in

DNMT3B, LINE-1 elements are hypomethylated on the inactive X, but not on the active X and autosomes (Hansen 2003). This indicates that there are different allele-specific DNMTs that are responsible for methylation of LINE-1 elements on the Xi and Xa (Hansen 2003). X-chromosome inactivation and spreading is normal in ICF individuals, therefore, if LINE-1s are involved in spreading it may be in an unmethylated state (Hansen 2003). This evidence along with Lyon's hypothesis of LINE-1s being involved in spreading indicates that unmethylated LINE-1s may be used as way stations for spreading (Hansen 2003).

1.4.2.3 Maintenance

Once the inactive X has acquired the various features of inactivation, silencing is mainly stable. *XIST* is necessary for initiation of inactivation, but it appears as though it is not absolutely essential for maintenance of the silenced X chromosome (Payer and Lee 2008). Inactivation can be maintained after it has been established even if *XIST* is not present (Brown and Robinson 2000). However, in mice, after *Xist* deletions, macroH2A localization and the concentration of H3K27me3 vanish and some X-linked genes become reactivated (Payer and Lee 2008). Deletion of *Dnmt1* in mice results in the reactivation of the inactive X, signifying that it is needed for maintaining silencing on the Xi (Payer and Lee 2008). In addition, hypomethylation at the *Xist* promoter on the Xa leads to improper silencing of the Xa indicating the *Dnmt1* is also necessary for repression of *Xist* on the Xa (Payer and Lee 2008). Nuclear compartmentalization is thought to play a role in maintenance. The inactive X localizes to a region near the nucleolus in female cells and appears as a Barr body during the mid-to-late S phase of the cell cycle (Payer and Lee 2008). In *Xist* mutant mice cells, the inactive X does not localize to this region and chromatin repressive marks are lost along with the reactivation of some X-linked genes (Payer and Lee 2008). This suggests that the inactive state of the Xi is duplicated in this peri-nucleolar region during S phase in an *XIST*-dependant

manner (Payer and Lee 2008). During oogenesis, the inactive X is reactivated and this is correlated with the loss of *Xist* expression (McCarrey and Dilworth 1992). During spermatogenesis, *XIST* expression is observed and the single X chromosome is inactivated (Richler et al. 1992, Salido et al. 1992). Even though inactivation is very stable, there have been reports of inactivation occurring in testicular germ tumours and the over-expression of X-linked genes in breast and ovarian cancers, hence indicating reactivation (Chang et al. 2006).

1.4.2.4 Counting and choice

Diploid cells must have some method of measuring elements of the X chromosome and autosomes to assess the ratio of X chromosomes to autosomes in order to realize the need for X inactivation (Payer and Lee 2008). Multiple mechanisms have been suggested to be involved in this process. The "blocking factor" model suggests that there is a complex composed of X chromosome and autosomal factors which disrupt the balance between the X chromosomes by only binding to the X inactivation centre of the future active X (Nicodemi and Prisco 2007). This will inhibit *XIST* expression from the XIC of the active X and thereby prevent initiation of inactivation of that X (Payer and Lee 2008). In mice, deletions of genes *DXPas34*, *Tsix*, and *Xite* on the X chromosome result in abnormal numbers of inactive X chromosomes indicating that these are X chromosome components important in counting (Payer and Lee 2008). A second model, suggests that *XIST* expression is activated from one chromosome when a "competence factor" is expressed from more than one X chromosome in a diploid cell (Lee 2005). Another model suggests that X chromosome pairing is involved in counting and choice. Before XCI is initiated, the X inactivation centres of the two X chromosomes are shown to co-localize within the nucleus of mice embryonic cells (Payer and Lee 2008). The *Xite* and *Tsix* regions allow interaction between the X chromosomes and

enable information exchange to establish their future states (Xu et al. 2006). When an X chromosome improperly pairs with transgenes present on autosomes (preventing X-X pairing), the upregulation of *Xist* and XCI does not occur (Lee 2002). The molecular nature of these models is not yet known.

The choice of which X chromosome is to become inactivated is linked to the counting process, but how they are connected remains elusive. Ultimately, what determines which X is chosen to become the Xi is dependent on what regulates the expression of *XIST*. What regulates *XIST* expression in humans is not known. However in mice, expression of a gene at the 3' end of *Xist* called *Tsix*, produces an RNA that is antisense to *Xist* and hence opposes its expression (Brown and Robinson 2000). *Tsix* is expressed prior to inactivation and is thought to be an essential regulator of early *Xist* expression and the choice of which X is to be inactivated (Brown and Robinson 2000). Antisense transcripts are produced 3' to the human *XIST* gene, but they do not overlap the *XIST* gene (Chang et al. 2006). *Tsix* transcription is controlled by the gene products of *DXPas34* and *Xite* in mice (Payer and Lee 2008). *DXPas34* is bound by the insulator protein Ctcf in addition to another binding component and the transcriptional activator Yy1 (Payer and Lee 2008). In humans, CTCF and YY1 bind to the promoter of *XIST* and individuals with point mutations in the CTCF binding domain show skewed XCI with the mutant X being preferentially inactivated (Payer and Lee 2008).

1.4.2.5 Escape from inactivation

About 15% of genes on the X chromosome actually escape X-chromosome inactivation (Carrel and Willard 2005). Which genes are able to escape inactivation is not random along the X chromosome, but genes that escape are normally found in large clusters (Carrel and Willard 2005). Genes that escape inactivation include the majority of genes that

are located in the pseudoautosomal region of the X chromosome. These genes have Y homologues and therefore it is logical that they would not be subject to inactivation (Brown and Robinson 2000). Many genes that are concentrated on the end of the short arm of the X chromosome (Xp) escape XCI (Carrel and Willard 2005). It is thought that the long distance of this region from *XIST* may explain why it escapes inactivation. Furthermore, the mammalian Y and X chromosome are thought to have diverged from a pair of autosomes (Carrel and Willard 2005). Through rearrangements and repression of recombination, the X and Y have independently evolved (Carrel and Willard 2005). The Y chromosome has lost many of its genes and the loss of Y homologues is thought to drive the inactivation of the X-linked copy (Chang et al. 2006). Therefore, the genes on Xp may have lost their functional copy on the Y chromosome more recently in evolution and have not yet been completely inactivated (Chang et al. 2006). An additional 10% of genes on the X chromosome show variable levels of expression between females; with some genes being active on the Xi in some females but not in others (Chang et al. 2006). The fact that some genes escape inactivation contributes to a genetic dosage imbalance between males and females and indicates that dosage compensation is not needed for all X-linked genes with no Y homologue (Carrel and Willard 2005). It is proposed that this imbalance contributes to sex differences such as in disease susceptibility (Brown and Robinson 2000). The imperfect silencing of the X chromosome results in the phenotypes observed in individuals with X chromosome aneuplodies such as those with Turner and Klinefelter syndromes.

1.4.2.6 Causes of non-random X-chromosome inactivation

X-chromosome inactivation is normally a random process with each X having an equal likelihood of being inactivated. Normally, conservation of the inactive X chromosome through successive cell divisions after designation of inactivation will lead to a female who is

mosaic for groups of cells with either the maternal or paternal X chromosome active (Brown and Robinson 2000). Non-random or skewed inactivation occurs when there is preferential inactivation of a certain parental X chromosome. Mild skewing is when ≥75% of cells has the same parental X active and extreme skewing is when ≥90% of cells has the same parental X active (Robinson et al. 2005). There are several proposed causes of skewed inactivation. Primary non-random X inactivation refers to a bias in the choice of which X is to be inactivated at the time of inactivation (Newall et al. 2001). Secondary non-random inactivation is when events occur following X-chromosome inactivation to influence the ratio of cells with either the maternal or paternal X active (Newall et al. 2001). Examples of primary non-random inactivation include mutations in genes involved in the inactivation process, imprinting effects, and differences in alleles of the X controlling element (*Xce*) (Brown et al. 1997). Deletion of exons 1 through 5 of the *Xist* gene in mice leads to inactivation of the normal X chromosome (Marahrens et al. 1998). The lack of expression from *Xist* on the mutated X will prevent that X from being inactivated. There has been one report of a cytosine to guanine mutation in the *XIST* promoter at position -43 bp being present in two unrelated families with skewed X-chromosome inactivation (Plenge et al. 1997). Both families demonstrated the preferential inactivation of the X chromosome carrying the mutation (Plenge et al. 1997). However, in one of the families, not all the females with the transversion displayed non-random inactivation (Plenge et al. 1997). A cytosine to adenine mutation has been observed at the same position (-43 bp) of the *XIST* promoter in a female who lacked expression of *XIST* from a small ring X chromosome (Tomkins et al. 2002). Deletions in certain regions in *Tsix* in mice result in the mutated copy being preferentially inactivated (Lee and Lu 1999). In the extraembryonic tissue of mice, the paternal X chromosome is always inactivated (Chang et al. 2006). It is thought that an imprint on the paternal *Xist* allele may be

involved in this skewed inactivation (Brown and Robinson 2000). There have been reports of an imprint in the extraembryonic tissues affecting non-random inactivation in humans, but other reports have been conflicting (Chang et al. 2006). Allelic combinations of *Xce* present 3' to *Xist* in mice influences skewing (Simmler et al. 1993). However, an equivalent to *Xce* in humans is not known (Brown and Robinson 2000).

Selection can result in secondary non-random inactivation in which cells with certain X chromosomes active are selected for after X-chromosome inactivation has occurred. It is estimated that about 4-20 cells are present in the ICM of the blastocyst when X inactivation occurs (Brown and Robinson 2000). The ICM is the part of the blastocyst that will contribute to the fetus. From the few initial cells that are present during XCI to the billions of cells that will contribute to the body of a female there is the possibility for natural selection to occur and cause preferential survival of cells with a certain X activated (Brown and Robinson 2000). When chromosomal rearrangements involving the X chromosome are present, selection is normally used to make sure that there is the least amount of disruption in genetic dosage (Brown and Robinson 2000). In balanced X:autosome translocations, the normal X is preferentially inactive in cells (Belmont 1996). This occurs because spreading of the inactivation marks onto the translocated region of the autosome would result in monosomy for the autosome (Belmont 1996). Cells with autosomal monosomy would either die or have slow growth and would therefore be outgrown by cells expressing two copies of the autosome (Belmont 1996). In unbalanced X:autosome translocations, the translocated X is normally inactivated in cells (Belmont 1996). This is thought to occur to avoid the potentially severe phenotype that is observed in partial autosomal trisomy (Belmont 1996). These selection processes in cases of X-autosome translocations depends on the size and location of the translocated region (Brown and Robinson 2000). Translocations involving the distal regions

of the X chromosome normally show random inactivation (Brown and Robinson 2000). Decreased growth and viability of cells with X-linked mutations can lead to the preferential contribution of cells expressing the normal allele (Belmont 1996). Even if cells have only a slight selective advantage over other cells, skewing can still be observed over time if cells have a high turnover (i.e. blood cells) ((Belmont 1996). Diseases associated with skewed XCI and a normal phenotype in female carriers include Alpha-thalassemia/mental retardation syndrome (ATR-X), Barth syndrome (TAZ), Dyskeratosis congenita (DKC1), Dystonia-deafness-optic neuronopathy syndrome (DDP), hypohidrotic ectodermal dysplasia with immunodeficiency (NEMO), agammaglobulinemia (XLA), X-linked severe combined immunodeficiency syndrome (XLSCID), MECP2 duplication (MECP2), Wiskott-Aldrich syndrome (WASP), incontinentia pigmenti type 2, focal dermal hypoplasia, and X-linked dyskeratosis congenita (Belmont 1996, Orstavik 2009). In most of these cases, skewing has been observed in all blood cells, but in other instances it is only observed in particular blood cells (Belmont 1996). For example, for XLSCID, the skewing is only observed in certain immune cells such as B cells, T cells, and NK cells (Belmont 1996). In some cases, cells with mutated X chromosomes active are selected for. It is proposed that some mutations offer a selective advantage for cells even though they may be harmful to the individual overall. For example, a somatic X-linked mutation in the *PIG-A* gene which results in Paroxysmal nocturnal hemoglobinuria (PNH) prevents expression of a cell surface protein, which when missing, enables increased proliferation (Belmont 1996). Therefore, a blood cell precursor with this mutation has an advantage and most blood cells will only be produced from it, leading to monoclonality (Belmont 1996). Age can also contribute to secondary XCI. The frequency of extreme skewing (≥90%) increases from <3% in newborn to ~4% in women aged 30 to over 30% in women over 75 years of age (Brown and Robinson 2000). Skewing with

age is thought to occur due to small selective advantages of one X over the other or the depletion of the stem cell pool over time (Brown and Robinson 2000). Evidence to support the selective difference theory is that most monozygotic twins show skewing towards the same X chromosome as they get older (Christensen et al. 2000). Evidence to support stem cell depletion is that skewing tends to increase more considerably the older a female becomes (Brown and Robinson 2000).

In addition to primary and secondary non-random inactivation, skewed inactivation can also be the result of a small cell precursor pool size present during the time of inactivation (Brown and Robinson 2000). It has been suggested that monozygotic (MZ) twinning may result in skewed XCI by acting as a bottleneck to reduce the number of cells which contribute to each embryo (Belmont 1996). Studies have been conflicting on whether there is an increased rate of skewing in MZ twins compared to singleton pregnancies (Brown and Robinson 2000). Twinning would have to occur around the same time of inactivation to cause a decrease in the cell precursor pool (Brown and Robinson 2000). However, twinning in monochorionic MZ twins is proposed to occur several rounds of replication after XCI occurs and twinning in dichorionic MZ twins is proposed to occur before blastocyst development (Brown and Robinson 2000). Mosaic embryos can also contribute to a decrease in precursor pool size. For example, a zygote which was initially trisomic could lose the extra chromosome in some of its cells (Brown and Robinson 2000). This could lead to a smaller number of cells, mainly the diploid cells contributing to the ICM (Brown and Robinson 2000). The abnormal cells would mainly contribute to the trophectoderm resulting in confined placental mosaicisim (CPM) in which there is an abnormal chromosome make up observed in the placenta, but not the fetus. There have been suggestions that it is possible for only one diploid cell to contribute to the ICM at the time to inactivation (Brown and Robinson 2000).

1.4.2.7 Consequences and associations of non-random X-chromosome inactivation

Skewed X-chromosome inactivation towards the mutant allele can lead to the uncovering of X-linked recessive traits in individuals who are heterozygous for the mutation. Disorders associated with skewed XCI and an affected phenotype in females include Duchenne muscular dystrophy (DMD), Angioma serpiginosum, Incontinentia pigmenti, Microphthalmia with linear skin-defects syndrome (MIDAS), Otopalatodigital syndrome, Melnick-needles syndrome, Frontometaphyseal dysplasia, Orofaciodigital syndrome 1, and X-linked adrenoleukodystrophy (ALD) (Belmont 1996, Orstavik 2009). In addition, skewing in favour of the mutant X chromosome has been observed in several unrelated heterozygous females with haemophilia A (Bennett et al. 2008, Bicocchi et al. 2005, Miyawaki et al. 2010). It is proposed that the mutation resulting in haemophilia A is segregating with a mutation in *XIST* or mutations of other regulator sequences of *XIC* that would prevent inactivation of that X (Bicocchi et al. 2005). Skewed inactivation is thought to play a role in some cancers. Skewing resulting in cells predominant with an oncogene on the active X or cells predominant with a tumor suppressor gene on the inactive X can lead to cancer (Spatz et al. 2004). Non-random inactivation has been observed in patients with Wiscott-Aldrich syndrome who have a higher risk of leukaemia than the normal population (Spatz et al. 2004). Skewed inactivation has been observed in leukaemias, ovarian cancers, and breast cancer (Spatz et al. 2004). Age is thought to play a role in these processes. For instance, skewing increases with age and therefore if an oncogene is active on a particular X chromosome, the effects might not be observed until later in life if skewing goes towards the X chromosome with the activated oncogene (Spatz et al. 2004).

Skewed XCI has been associated with adverse pregnancy outcomes, developmental abnormalities, and intrauterine or neonatal deaths (Brown and Robinson 2000). It is reported

that the frequency of XCI ≥90% is greater in women with recurrent spontaneous abortions (RSA) than in women with normal pregnancies (Beever et al. 2003b). This association could be due to the preferential inactivation of a mutated X that would be lethal in male embryos who inherit it (Beever et al. 2003b). If this was the case, then RSAs should involve more male conceptus losses, however, this was not observed (Beever et al. 2003b). It has also been suggested that a decrease in ovarian follicles due to oocyte atresia or a restriction in precursor pool size during development of the embryo may be involved (Robinson et al. 2001). Any event that leads to a decrease in the number of cells contributing to the embryo can lead to a decrease in the oocyte pool number along with increased skewed XCI (Beever et al. 2003b). X-linked mutations resulting in skewed XCI can also result in increased oocyte atresia as observed in women with premature ovarian failure (Cunniff et al. 1991). Increased pregnancy losses involving trisomy has also been reported in women with ≥90% skewing (Beever et al. 2003b). The follicular reserve is thought to be important for aneuploidy risk and 50% of RSA cases are associated with a chromosome abnormality (Beever et al. 2003b, Robinson et al. 2001). Therefore, a small follicular reserve could lead to oocyte aneuploidy, fetal aneuploidy, and consequently RSA (Beever et al. 2003b). High XCI skewing in these cases could be due to poor early embryo growth which could also have resulted in a reduced follicular reserve (Beever et al. 2003b).

1.4.2.8 Methods to study X-chromosome inactivation

Reverse transcriptase PCR utilizing somatic-cell hybrids retaining either the Xi or Xa can be used to study which genes are subject to inactivation on the X chromosome (Brown et al. 1997). In addition, X-linked expression of genes can be determined by amplification of single-nucleotide polymorphisms of DNA and cDNA in heterozygous individuals (Carrel and Willard 2005). The two main methods used to measure skewed XCI include methylation

based assays and expression based assays. Gene expression assays require DNA and RNA. DNA is used to make sure that an individual is heterozygous for a polymorphism. RNA is reverse transcribed into cDNA and used to measure the level of expression of each allele (Brown and Robinson 2000). Expression methods however rely on the gene utilized being expressed in the tissue of interest (Kutsche and Brown 2000).

DNA methylation assays only require DNA and rely on differential methylation patterns between the active and inactive X. The 5' region of genes of the inactive X are hypermethylated compared to the active X, especially at areas rich in CpG dinucleotides (CpG islands) (Allen et al. 1992). Therefore, methylation sensitive enzymes such as *Hpa*II and *Hha*I with restriction sites in CpG islands can be used to digest only unmethylated regions on the active X (Allen et al. 1992). Amplified polymorphisms of an undigested and digested control can then be compared to determine skewing using densitometry of a silver stained polyacrylamide gel (Brown and Robinson 2000). XCI skewing can also be determined by automated fluorescence analysis using PCR primers labelled with a fluorescence dye followed by sizing of PCR products using capillary electrophoresis (CE). This technique will be covered in detail in the materials and methods section of Chapter 2.

1.5 Assisted reproductive technologies

Assisted reproductive technologies (ARTs) are referred to as all procedures that involve the handling of human oocytes, sperm, and/or embryos *in vitro* in order to achieve a pregnancy (World Health Organization 2002). Examples of ART include intrauterine insemination (IUI), *in vitro* fertilization (IVF), intracytoplasmic sperm injection (ICSI), gamete intrafallopian transfer, zygote intrafallopian transfer, tubal embryo transfer, gamete and embryo cryopreservation, oocyte and embryo donation, and surrogacy (World Health

Organization 2002). The process of superovulation and the three main ART procedures of IUI, IVF, and ICSI will be discussed.

1.5.1 Superovulation

In superovulation, hormones are used to stimulate the development and ovulation of ovarian follicles. Some hormones commonly used to achieve this task include clomiphene citrate, human chorionic gonadotrophin (hCG), human menopausal gonadotropins (hMGs), and gonadotropin releasing hormone (GnRH) (Carlson 2004, World Health Organization 2002). These hormones work to increase the serum levels of luteinizing hormone (LH) and follicle stimulating hormone (FSH), normally resulting in the development of multiple mature follicles (Carlson 2004). With the guide of ultrasound, an aspiration needle is inserted into the ripe follicle and the oocyte is sucked up (Carlson 1999). Retrieved oocytes can then be used for IVF and ICSI.

1.5.2 Intrauterine insemination

Intrauterine insemination is the process whereby thousands or millions of motile sperm are placed into the uterine cavity using a catheter (World Health Organization 2002). This procedure is used to enable the sperm to circumvent the cervical mucus (World Health Organization 2002). An ejaculate semen sample is washed to remove prostaglandins, bacteria, and immunocompetent cells (World Health Organization 2002). The sperm are centrifuged using a density gradient or allowed to swim up in a medium (World Health Organization 2002). It is important that insemination is done around the time of ovulation (World Health Organization 2002).

1.5.3 In vitro fertilization

In IVF, retrieved oocytes are incubated with spermatozoa in a fluid medium *in vitro* to be fertilized (Carlson 1999). Embryos at day 3 or day 5 are transferred using a catheter through the cervix into the uterine cavity (Carlson 1999). IVF is efficient for treating couples with unexplained fertility and female factor infertility, especially tubal disease (World Health Organization 2002). IVF is also used when IUI fails several times.

1.5.4 Intracytoplasmic sperm injection

Intracytoplasmic sperm injection involves the direct injection of a single spermatozoon using a microneedle into the cytoplasm of an oocyte through the zona pellucida (World Health Organization 2002). ICSI is mainly used to treat male factor infertility (Ma and Ho Yuen 2000, Ma and Yuen 2001). Dysfunctional sperm that would otherwise not be able to reach the egg or would be unable to penetrate it can be used to fertilize an egg. ICSI is generally used when IUI and IVF have failed multiple times.

1.5.5 Pregnancy outcomes

Since the advent of ARTs, there have been concerns about the safety of these procedures and how they might affect the health of children born by these methods. Superovulation is stimulating multiple follicles during one cycle, hence, it is possible that abnormal oocytes which would have undergone atresia are being induced to mature (Wramsby and Fredga 1987). Studies on the outcomes of IUI following superovulation have shown that there is a 1- to 2- fold increased risk for preterm birth as well as a 1- to 3- fold increased risk for low birth weight in singletons compared with pregnancies conceived naturally (Allen et al. 2006). However, there was no difference in the rates of congenital malformations between infants conceived by IUI following superovulation and infants conceived spontaneously (Allen

et al. 2006). The studies which produced this data controlled for maternal age and parity (Allen et al. 2006).

Many studies have looked at the obstetrical and perinatal outcomes of IVF and ICSI. Below are results from studies that controlled for maternal age and parity (Allen et al. 2006). Some studies also adjust for socio-demographic factors (Bonduelle et al. 2005). There are significantly increased rates of maternal gestational hypertension, gestational diabetes, placenta previa, and placenta abruption in singleton and twin IVF and ICSI pregnancies compared to natural conception pregnancies (Allen et al. 2006). IVF and ICSI singleton and twin pregnancies also have a 2-fold increased rate of labour induction and Caesarean delivery (Allen et al. 2006).

Singleton pregnancies following IVF or ICSI are associated with an elevated risk of neonatal death, preterm delivery (<37 weeks), low birth weight (<2500 g), very low birth weight (<1500 g), small for gestational age (<10^{th} percentile birth weight for gestational age), and neonatal intensive care (Allen et al. 2006). More multiple births are observed in IVF and ICSI pregnancies than in spontaneous pregnancies. This can be mainly attributed to the transfer of more than one embryo per treatment cycle during ART procedures (Allen et al. 2006). However, there is a higher rate of monozygotic twins in IVF pregnancies than in naturally conceived pregnancies (Allen et al. 2006). Morbidity and mortality increases with each additional fetus in a pregnancy (Allen et al. 2006). Studies show conflicting results when comparing preterm delivery, low birth weight, and small for gestational age between IVF and ICSI twin pregnancies compared to twin natural pregnancies (Allen et al. 2006).

It has been observed that singleton babies conceived by ICSI have a 1.6 fold risk of slower postnatal growth three years post partum (Allen et al. 2006). A multi-centre cohort

study showed that 5 year-old children conceived after IVF and ICSI were more prone to have had significant childhood illness, to have had a surgical operation, to need medical therapies, and to be admitted to hospitals (Bonduelle et al. 2005). After adjusting for maternal age, parity, and ethnicity, registry data revealed that there is a 2-fold increased risk for major congenital abnormalities in singleton and twin IVF and ICSI pregnancies compared to natural conceptions (Allen et al. 2006). Higher rates of chromosomal abnormalities in births and induced terminations as well as *de novo* chromosome abnormalities diagnosed prenatally are seen in ICSI conceived children than spontaneously conceived ones (Allen et al. 2006).

There has been evidence to show that ARTs may result in epigenetic alterations such as modifications in DNA methylation and genomic imprinting (Allen et al. 2006). The imprinting disorders, Beckwith-Wiedemann syndrome (BWS) and Angelman syndrome (AS) have been associated with ARTs. Prenatal overgrowth, abdominal wall defects, neonatal hypoglycaemia, hemihypertrophy, ear abnormalities, and macroglossia define BWS (Allen et al. 2006). Infants born with BWS are 18 times more likely to have been conceived through IVF than those without BWS (Allen et al. 2006). Nevertheless, the incidence of BWS in IVF conceived infants is very low at only about 1/4000 (Allen et al. 2006). AS is characterized by severe mental retardation, seizures, abnormal gait, frequent smiling and laughter, ataxia, hyper-reflexia, and hypotonia (Allen et al. 2006, Manipalviratn et al. 2009). Seven cases have been reported since 2009, in which a child with AS was conceived through IVF or ICSI (Manipalviratn et al. 2009). Five of these seven cases were due to an imprinting disorder (Manipalviratn et al. 2009). The small number of cases observed may be due to the fact that imprinting disorders resulting in AS are estimated to occur in only 1/12,000 births (Manipalviratn et al. 2009). However, the proportion of children with AS with an imprinting

defect as the etiological factor is higher than in the general population (Manipalviratn et al. 2009).

1.5.5.1 XCI and ARTs

It is it possible that an epigenetic mechanism in addition to imprinting is being affected by ARTs. X-chromosome inactivation is proposed to occur during the blastocyst stage and is therefore susceptible to aberrations during *in vitro* culturing in ARTs. One previous study investigated the XCI status in 22 female newborns conceived by IVF and 31 naturally conceived infants (King et al. 2010). They found no difference in the frequency of mild skewing (80-90%) (9.1% vs. 6.5%) or extreme skewing (>90%) (9.1% vs. 0%) when comparing IVF and control groups (King et al. 2010). However, they did find a significant difference in the mean level of skewing of between the IVF and NC groups (72.0% vs. 62.4%, P= 0.002) (King et al. 2010). Another study included 48 newborn females conceived by ICSI and 74 control samples of females aged 0-19 years (Robinson et al. 2005). No significant difference in the mean level of skewing (65.1%vs. 69.8%) or in the frequency of skewing ≥75% (18% vs. 35%) or ≥ 90% (4.6% vs. 10.8%) between the ICSI and control group was found (Robinson et al. 2005).

1.5.5.2 *FMR1* and ARTs

The association of *FMR1* premutation alleles with POF has been confirmed in many studies (Marozzi et al. 2000, Murray 2000, Sherman 2000, Sullivan et al. 2005, Wittenberger et al. 2007). In recent years, intermediate alleles (Bodega et al. 2006) as well as normal alleles (Bretherick et al. 2005) have also been associated with varying levels of POF. Little research however, has looked at how the size of *FMR1* alleles may be affecting women undergoing infertility treatments. One study looked at the effects of race on *FMR1* repeat counts in

infertile women and egg donors (Gleicher et al. 2010). Caucasians demonstrated higher rates of abnormal *FMR1* triplet repeat counts than African and Asian infertile populations (Gleicher et al. 2010). This suggests that the ability of *FMR1* repeat counts to denote risk for POF may vary between different ethnicities (Gleicher et al. 2010). The same group looked at the association of *FMR1* genotypes with IVF outcomes based on ethnicity (Gleicher et al. 2011). Genotypes were based on a normal range of repeats being between 26 and 34 (Gleicher et al. 2011). Individuals of all races with one allele below this normal range of repeats were shown to have poorer IVF pregnancy outcomes (Gleicher et al. 2011). No studies have investigated the transmission of *FMR1* alleles from women utilizing fertility treatments to their offspring produced by those procedures.

1.6 Rationale, hypotheses, and objectives

The widespread use of assisted reproductive technologies (ARTs) has led to questions about the short-term and long-term safety of these procedures. Pregnancies derived from *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI) have been associated with increased rates of chromosome abnormalities (Bonduelle et al. 2002, Wennerholm et al. 2000), congenital abnormalities (Hansen et al. 2002), low birth weight, pre-term births (Jackson et al. 2004, Kallen et al. 2002, Katalinic et al. 2004), multiple gestations (Schachter et al. 2001), and imprinting disorders (Manipalviratn et al. 2009). ART enables sub-fertile individuals to circumvent the natural selective pressures that are involved in human reproduction. Therefore, the risks of ART may be due to the underlying causes of sub-fertility in these individuals such as abnormalities in sperm or egg. IVF and ICSI involve unnatural induction of ovulation in women, *in vitro* handling of sperm and egg, as well as the growth of the embryo *in vitro*. Hence, any of these artificial processes could also be contributing to the increased rate of abnormalities in ART conceived pregnancies.

Abnormalities in imprinting, a type of epigenetic modification, have been associated with ARTs. Another epigenetic mechanism known as X-chromosome inactivation may also be altered as the process may be occurring during *in vitro* culturing in ARTs. XCI is a mechanism of dosage compensation that occurs to make sure that the majority of genes on the X chromosome are only expressed from one X chromosome in female cells. Skewing towards a certain X chromosome can lead to the uncovering of X-linked recessive traits in heterozygous women and has been associated with recurrent spontaneous abortion, cancer, and chromosome abnormalities (Beever et al., 2003; Brown & Robinson, 2000; Spatz et al., 2004). Therefore, higher rates of skewed XCI in the ART population would be of concern. The IVF and ICSI populations are subject to the risk factors of advanced maternal age, superovulation, *in vitro* handling of sperm and egg, and embryo growth in culture. These risks could cause epigenetic alterations, slow embryo growth, and cause ICM cell selection resulting in skewed XCI. In addition, ICSI is subject to the risk of male factor infertility and is a more invasive procedure in that the egg is pierced. Infertile males have higher risks of chromosome abnormalities such as aneuploidy in their sperm. This could result in slower growing embryos and/or the selection of certain cells to contribute to the ICM. Slower growing embryos due to chromosome abnormalities or the invasiveness of ICSI as well as ICM cell selection due to chromosome abnormalities can result in skewed XCI.

We hypothesize that ARTs can lead to significant effects on X chromosome skewing because XCI is established in the embryo during the time of *in vitro* culturing in ARTs. Therefore, we hypothesize that the frequency of skewed inactivation may be greater in ICSI and IVF conceived females compared to naturally conceived females. We also hypothesize that the frequency of skewed XCI may be higher in ICSI conceived pregnancies than in IVF

conceived pregnancies due to the more invasive nature of ICSI and its subjectivity to male factor infertility.

Greater numbers of CGG repeats have been associated with varying levels of premature ovarian failure in women. Therefore, it is possible that women utilizing ARTs are passing on *FMR1* alleles associated with reduced fertility to their offspring. Infertile males and females are observed to have greater numbers of chromosome abnormalities in their gametes compared to their fertile counterparts ((Dohle et al. 2002, Hristova et al. 2002, Schreurs et al. 2000). Meiotic repair mechanisms in subfertile individuals may not be as stringent and may result in meiotic replication slippage (looping out) or unequal meiotic crossovers leading to *FMR1* CGG repeat expansion. Some studies have indicated that repeat expansions can occur post-zyotically (Devys et al. 1992, Wohrle et al. 1993). Mosaicism has been observed in human preimplantation embryos due to mitotic errors during *in vitro* culturing (Munne et al. 1994). Therefore, ARTs may not only be passing on repeat ranges associated with reduced fertility in women, but could also be leading to the expansion of repeats into ranges that could cause Fragile X mental retardation (full mutation) and FXTAS (premutation) in offspring.

We hypothesize that the frequency of *FMR1* alleles with high CGG repeat counts and low numbers of AGG interspersions may be greater in females conceived by ICSI and IVF than in those conceived naturally due to the inheritance of alleles associated with reduced fertility from subfertile mothers, repeat expansion in subfertile parent's germlines, or repeat expansion during *in vitro* culturing.

The purpose of these studies was to determine the risks of abnormal X-chromosome inactivation and expanded *FMR1* allele mutations being present in females conceived by the

assisted reproductive technologies of intracytoplasmic sperm injection and *in vitro* fertilization compared to females conceived naturally.

Objective 1a: To compare the frequency of mild skewing (≥75%) and extreme skewing (≥90%) in ICSI, IVF and naturally conceived pregnancies.

Objective 1b: To compare the mean level of skewing in ICSI, IVF and naturally conceived pregnancies.

Objective 2a: To compare the number of CGG repeats in the *FMR1* alleles of female newborns conceived by ICSI, IVF, and by natural means.

Objective 2b: To compare the number of AGG interspersions in the *FMR1* alleles of female newborns conceived by ICSI, IVF, and by natural means.

Chapter 2 : Project 1- X-Chromosome Inactivation in Female Newborns Conceived by Assisted Reproductive Technologies

2.1 Introduction

Recent research on the safety of ARTs has been focused on the study of changes in gene expression without changes in DNA sequence, a phenomenon referred to as epigenetics. Abnormalities in imprinting, a type of epigenetic modification, have been associated with ARTs. Higher rates of the imprinting disorders; Beckwith Wiedemann syndrome and Angelman syndrome are observed in ART conceived infants compared to spontaneously conceived infants (Manipalviratn et al. 2009). It is proposed that another epigenetic mechanism known as X-chromosome inactivation (XCI) may be altered during embryo culture in ARTs. XCI is a mechanism that occurs early in embryo development to ensure the random silencing of one X chromosome in the somatic cells of females. Skewed XCI is when there is preferential inactivation of one of the parental X chromosomes in the majority of cells. X chromosome skewing can result from selection against a chromosome abnormality on one X chromosome, mutations of genes involved in X inactivation, a reduced number of precursor cells being present at the time of inactivation, or the selection of a small number of normal cells to contribute to the ICM of the blastocyst due to chromosomal mosaicism in the embryo (Brown and Robinson 2000). Skewed XCI can lead to the uncovering of X-linked recessive traits in heterozygous women and has been associated with recurrent spontaneous abortions, cancer, and chromosome abnormalities (Beever et al. 2003b, Brown and Robinson 2000, Spatz et al. 2004). In addition, XCI skewing has been shown to increase with age, with ≥90% skewing being present in 4.1% of females aged 0-19 years compared to 23.7% of females over the age of 60 years (Hatakeyama et al. 2004). The onset of X-linked recessive diseases could

be observed earlier in heterozygous women whom start with a high degree of skewing at birth. Therefore, higher rates of skewed XCI in the ART population would be of concern.

IVF and ICSI populations are subject to the risk factors of advanced maternal age, superovulation, *in vitro* handling of sperm and egg, and embryo growth in culture. These risks could cause epigenetic alterations, slow embryo growth, and/or cause cell selection resulting in skewed XCI. During superovulation, multiple ovarian follicles are generally stimulated to undergo ovulation. Oocytes that may have otherwise undergone atresia are recruited and used for IVF and ICSI (Wramsby and Fredga 1987). It is possible that these oocytes could have aberrations such as chromosomal and epigenetic abnormalities which could both result in skewed XCI. The majority of women utilizing fertility treatments are advanced in maternal age. Older women are more prone to having gametes with chromosome abnormalities such as aneuploidy (Hassold and Hunt 2001, Heffner 2004). Oocytes with an abnormal chromosome number could result in mosaic embryos with only a small number of normal cells contributing to the ICM. This could result in skewing as only a small number of cells will contribute to the fetus. Moreover, it is possible that mitotic errors could occur during *in vitro* culturing which could result in a mosaic embryo and skewed XCI (Munne et al. 1994). ICSI is also subject to the risk of male infertility and is a more invasive procedure. The invasiveness of ICSI may result in a slower growing embryo and a reduced precursor cell pool causing skewed XCI. Infertile males have higher risks of chromosome abnormalities such as aneuploidy in their sperm (Kirkpatrick et al. 2008). These abnormalities can be passed to their offspring via ICSI (Tang et al. 2010). This could cause cell selection of normal diploid cells contributing to the ICM and/or slower growing embryos. These could both result in a smaller precursor cell pool contributing to the fetus and skewed XCI.

In order to assess the risk of skewed XCI in females conceived by ARTs, we determined the frequency of mild skewing (≥75%) and extreme skewing (≥90%) as well as the mean level of skewing in populations of newborn females conceived by ICSI, IVF, and by natural means. We looked at the parent of origin of the skewed allele in an extremely skewed case to give us an indication of why skewing may be occurring. In addition, we determined the XCI status of different tissues at various sites of the placenta for this case in order to see if similar selective pressures were occurring in the extraembryonic tissues.

2.2 Materials and methods

2.2.1 Patient ascertainment

Patients that conceived through IVF and ICSI were recruited from several IVF centres across Canada, mainly at the UBC Centre for Reproductive Health, the Pacific Centre for Reproductive Medicine, the Genesis Fertility Centre, and the Calgary Regional Fertility Clinic. All viable female ART conceived newborns were analyzed regardless of pregnancy outcomes. Female twins were not selected for. Patients that had conceived naturally were recruited from hospitals across the lower mainland. Naturally conceived cases were excluded if pregnancy or congenital abnormalities were present. Informed consent was obtained for each patient prior to sample collection. A karyotype or comparative genomic hybridization (CGH) analysis of the chromosomes was available for all newborn cases studied. Placental tissue was available for the majority of cases. The parents of the newborn subjects in this study were not karyotyped.

2.2.2 DNA extraction from cord blood

Blood samples were collected in EDTA or sodium heparin tubes. The majority of genomic DNA was extracted from cord blood using the QIAamp DNA Mini Kit (QIAGEN).

However, some samples were extracted using the traditional salting out method from blood (Miller et al. 1988) followed by phenol-chloroform extraction (Bingle 2009) to remove the salts and further cleaning using the QIAamp DNA Mini Kit (QIAGEN).

2.2.3 Collection of placental tissues and extraction of DNA

For most cases, ten placental sites (chorionic villi and amnion) were available as well as samples of the umbilical cord and maternal decidua. At each site a small piece of amnion was carefully peeled away from the chorionic plate with tweezers and cut. Amnion was not always available as occasionally it was torn off during pregnancy. A small circular incision in the chorionic plate was made at each site and the plate layer was flipped up to allow the excision of only the chorionic villi. Precautions were taken to not cut too deeply as this would result in contamination with the maternal deciduas (endometrium). Figure 2.1 shows a portion of the human placenta. Tissues that were available for analysis are indicated in bold. Figure 2.2 represents a cross-section of a placental chorionic villi. Placental tissues were stored at -80°C in cryopreservation tubes until needed. Genomic DNA was extracted from placental tissues using the QIAamp DNA Mini Kit (QIAGEN).

Figure 2.1 Schematic diagram of a portion of the human placenta. Bolded words indicate extraembryonic tissues analyzed (adapted from Gude et al. 2004, Larsen 1998).

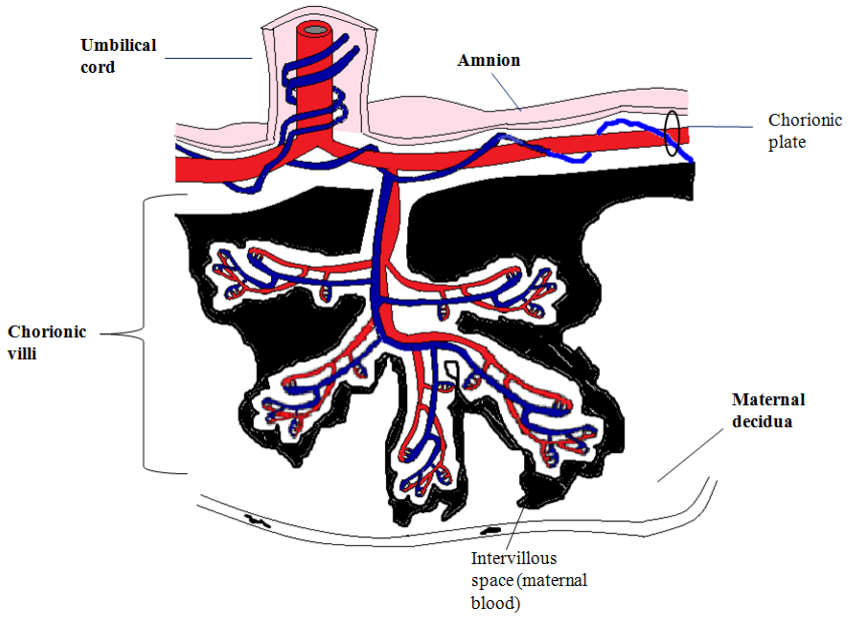
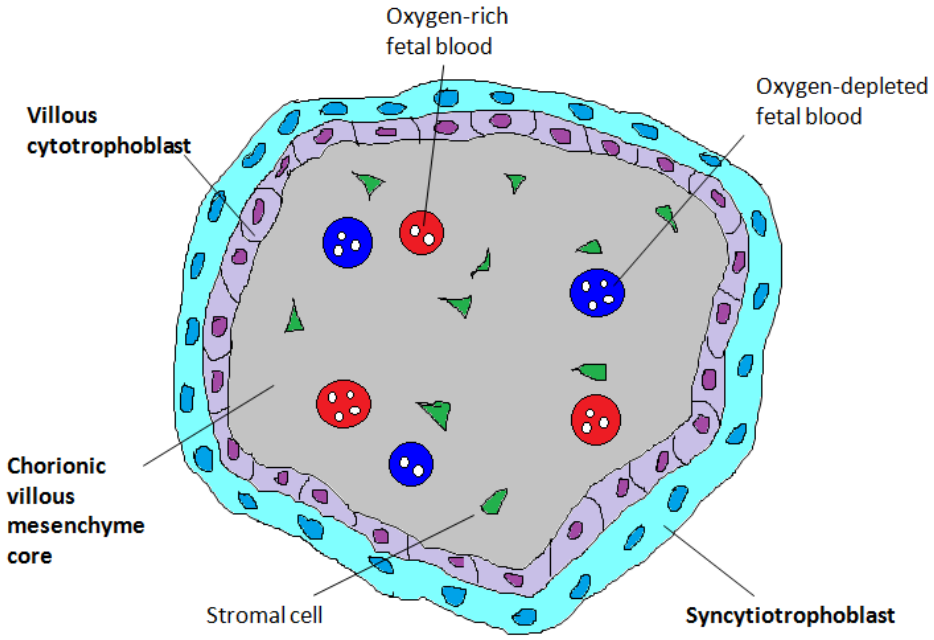


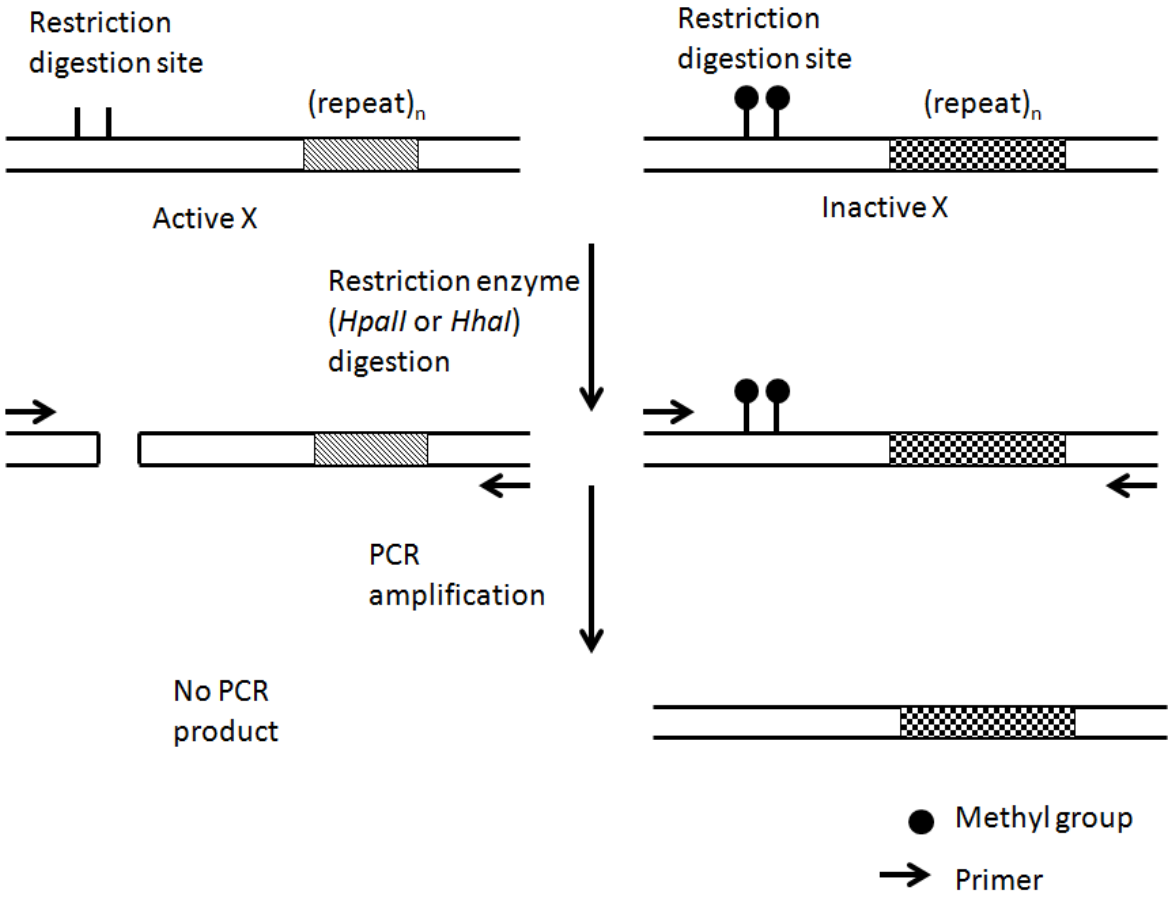
Figure 2.2 Cross section of a placental chorionic villi (adapted from Gude et al. 2004)



2.2.4 XCI assay

A DNA methylation-sensitive assay was used to determine the degree of XCI skewing as previously described, but with modifications (Beever et al. 2003b). Digestion of DNA with methylation-sensitive restriction enzymes was used to distinguish the inactive X chromosome from the active one (Beever et al. 2003b). Methylation sensitive enzymes such as *Hpa*II and *Hha*I will only cut restriction sites on the unmethylated, active X, leaving only the methylated, inactive X available for PCR amplification of the polymorphic repeat region (Figure 2.3).

Figure 2.3 Schematic representation of methylation-sensitive-enzyme based assay for determination of XCI skewing. *HpaII* and *HhaI* are methylation sensitive enzymes and will not cut at restriction sites that are methylated. Genes on the inactive X that have restriction sites methylated will not be cut and will therefore be available for PCR amplification. On the active X, the restriction sites are unmethylated and therefore the gene of interest will be unavailable for PCR amplification. PCR primers surround the differentially methylated restriction sites and the polymorphic repeat region of the gene.



2.2.4.1 Restriction enzyme digests

100 ng of genomic DNA was digested in 1x buffer 1 from New England Biolabs (NEB) (10 mM Bis-Tris-Propane-HCl, 10 mM $MgCl_2$, 1 mM Dithiothreitol, pH 7.0), 1 U of *RsaI*, and 2.5 U of *Hpa*II in a total volume of 20 µl for the *AR* and *FMR1* assays. 100 ng of genomic DNA was digested in 1x buffer 4 from NEB (1X NEBuffer 4, 20 mM Tris-acetate, 50 mM potassium acetate, 10 mM Magnesium Acetate; 1 mM Dithiothreitol, pH 7.9), 100 µg/ml Bovine Serum Albumin, 1 U of *RsaI*, and 2.5 U of *Hha*I in a total volume of 20 µl for the *DXS6673E* assay. Some samples were digested with similar reaction conditions with the inclusion of Invitrogen restriction enzymes instead of NEB restriction enzymes. *Rsa*I is not methylation sensitive and does not have restriction sites in amplified regions. It is thought to enable more efficient and complete digestion by making restriction sites more accessible for the methylation sensitive enzymes (Goto et al. 1997). For each sample an undigested control was similarly prepared with only 1U *Rsa*I. The samples were incubated at 37°C overnight and complete digestion was confirmed by PCR amplification of the 5`region of the *MIC2* gene located on the X chromosome.

2.2.4.2 Confirmation of complete digestions

MIC2 contains restriction sites for *Hpa*II and *Hha*I. This gene escapes XCI and is therefore unmethylated on both X chromosomes and should be completely digested by *Hpa*II and *HhaI* (Goodfellow et al. 1988). Two microlitres of digested and undigested samples were amplified with 1X Invitrogen PCR buffer (200 mM Tris-HCl (pH 8.4), 500 mM KCl), 400 nM of each primer, 2 mM $MgCl_2$, 333 µM of each dNTP, 4% dimethyl sulfoxide (DMSO), and 0.4 U of *Taq* (Invitrogen) in a total volume of 15 µl. PCR conditions were 95°C for 3 min (initial denaturation); 95°C for 1 min, 52°C for 1 min, and 72°C for 2 min, for 35 cycles with a final extension at 72°C for 7 min. Primer sequences were as follows: forward 5`-AGAGGTGCGT

CCGATTCTT-3` and reverse 5`-CGCCGCAGATGGACAATTT-3 ` (Anderson and Brown 2002). Five microlitres of 5X loading buffer was added to the PCR products and all of the sample was run on a 1% ethidium bromide or SYBR® Safe (Invitrogen) stained agarose gel for 45 minutes at 130 V. Fragments were visualized on a UV transilluminator (UVP, BioDoc-It system). The presence of a 400 bp band in the undigested sample but not in the digested sample indicated complete digestion. If digestion was incomplete, more restriction enzyme, buffer, BSA (if *Hha*I digestion) was added, the sample re-incubated overnight, and the *MIC2* PCR repeated to confirm complete digestion.

2.2.4.3 PCR amplification

Once complete digestion was confirmed, digested and undigested DNA was amplified by PCR using primers that flanked the differentially methylated restriction sites and a region of polymorphic repeats (Beever et al. 2003b) The androgen receptor (*AR*) PCR assay which relies on differential methylation of two *Hpa*II restriction sites near a polymorphic tri-nucleotide CAG repeat in the first exon of the X-linked AR gene was first used (Allen et al. 1992). If the *AR* locus was uninformative, skewing was determined at the fragile X mental retardation 1 (*FMR1*) (Hecimovic et al. 1997) or *DXS6673E* (Beever et al. 2003a) loci which both undergo XCI as well. *FMR1* has two *Hpa*II restriction sites near a CGG polymorphic repeat while *DXS6673E* has two *Hha*I restriction enzymes sites near a GA polymorphic repeat. PCR of *AR* was performed according to the modified protocol of Beever et al. (Beever et al. 2003b). Two microlitres of digested and undigested samples were amplified with 1X PCR buffer (Invitrogen), 400 nM of each primer, 2 mM $MgCl_2$, 333 μM of each dNTP, 4% DMSO, and 0.4 U of *Taq* (Invitrogen), in a total volume of 15 μl. PCR conditions were 95°C for 3 min (initial denaturation); 95°C for 1 min, 53°C for 1 min, and 72°C for 2 min, for 35 cycles with a final extension at 72°C for 7 min. Primer sequences were as follows: forward 5` -

GCTGTGAAG GTTGCTGTTCCTCAT-3` and reverse 5`-TCCAGAATCTGTTCC AGAGCGTGC-3` (Allen et al. 1992).

XCI was estimated at the *FMR1* locus according to the modified protocol of Hecimovic et al. (Hecimovic et al. 1997). Four to five-point-one microlitres of digested and undigested samples were amplified in 1X Expand long PCR buffer 1 (Roche), 10% DMSO, 350 μM dATP, 350 μM dCTP, 350 μM dTTP, 100 μM dGTP, 250 μM 7-deaza-GTP (Roche), 1 μM of each primer, and 1.25 U Expand enzyme mix (Roche), in a total volume of 10 μl. Cycling conditions were 94°C for 2 min (initial denaturation); 94°C for 10 s, 62°C for 30 s, and 68 °C for 2 min, for 10 cycles; 94°C for 15 s, 62 °C for 30 s, and 68°C for 2 min with an additional 20 s per cycle, for 30 cycles; and a final extension at 68°C for 7 min. Primer sequences were as follows: forward 5'-CTCAGCTCCGTTT CGGTTTCACTTCCG-3' and reverse 5'-AGCCCCGCACTTCCACCA CCAGCTCCTCC-3' (Fu et al. 1991).

PCR of the *DXS6673E* locus was performed according to a modified protocol of Beever et al. (Beever et al. 2003a). Two microlitres of digested and undigested samples were amplified with 1X PCR buffer (Invitrogen), 400 nM of each primer, 2 mM $MgCl_2$, 333 μM of dNTPs, 4% DMSO, and 0.4 U of *Taq* (Invitrogen) in a total volume of 15 μl. Cycling conditions were 95°C for 4 min (initial denaturation); 95°C for 1 min, 58°C for 1 min, and 72°C for 2 min, for 35 cycles, followed by a final extension at 72°C for 7 min. Primer sequences were as follows: forward 5'-ATGCTAAGG ACCATCCAGGA-3' and reverse 5'-GGAGTTTTCCTCCCTCAC CA -3' (Beever et al. 2003a).

2.2.4.4 Determining the degree of skewing

XCI skewing was estimated by automated fluorescence analysis. PCRs were performed with a forward primer labelled with the ABI Prism Dyes 6-FAM (*AR* forward

primer) or HEX (*FMR1* and *DXS6673E* forward primers). PCR products were submitted to the Robinson lab (CFRI) for capillary electrophoresis (CE). One microlitre of PCR product was mixed with 10 µl HiDi Formamide and 0.1 µl of ROX 500 size standard (Applied Biosystems). The mixture was denatured for 95°C for 5 minutes and chilled with icy water. The PCR products were then sized using CE on an ABI Prism 310 genetic analyzer. Fluorescence was detected by ABI Prism data collection software and analyzed using GeneScan Analysis software.

The peak areas of each allele were recorded for each digested and undigested sample pair. Stutter peaks (shadow bands), representing stutter during amplification of the repeat regions were included in the area for each allele. The peak areas of the digested samples were normalized in relation to undigested samples in order to determine the degree of skewing and to account for the often preferential amplification of the smaller allele (Beever et al. 2003b). Skewing was calculated using this previously published equation: 100*(d1/u1)/((d1/u1)+(d2/u2)) (Beever et al. 2003b). The more intense and less intense allele of the digested sample are represented by d1 and d2, respectively while the u1 and u2 represent the corresponding alleles for the undigested sample (Figure 2.4) (Beever et al. 2003b). This equation provides skewing values between 50-100% representing the % of skewing of the predominant allele (Beever et al. 2003b). If the *AR* alleles of a sample were within 3 bp of each other, shadow bands of the larger allele would overlap with the main band of the smaller allele (Beever et al. 2003b). Shadow bands account for ~30% of the main band, therefore the peak area of overlapping *AR* alleles were adjusted for this (Beever et al. 2003b). At the *DXS6673E* locus, multiple peaks were detected for each allele. The peak areas of five peaks were included in the area for each allele. Peaks were distinguished (from left to right on image) as sh3, sh2, sh1, m, and sh+ (sh= shadow band, m= main) (Figure 2.5). If the shadow

bands of the larger allele were overlapping with peaks of the smaller allele, sh3 was calculated to represent 9% of the amplification from sh2 to sh+, sh3 combined with sh2 was calculated to represent 34.6% of the amplification from sh1 to sh+, and sh3, sh2, sh1 combined was calculated to represent 127.7% of the amplification of m1 to sh+. Peak areas were adjusted accordingly (overlap values were based on data from 59 digested and undigested samples). Shadow bands were included in the area for each allele for the *FMR1* locus. If the shadow band of the larger allele overlapped with the main peak of the smaller allele, the sample was recorded as uninformative at *FMR1*.

Samples were classified as having <75% (random), ≥75% (mild), and ≥90% (extreme) skewing. These cut-off values were chosen as they were used in many human studies measuring skewing (Beever et al. 2003a, Gale et al. 1994, King et al. 2010, Lanasa et al. 1999, Plenge et al. 1997, Robinson et al. 2005). The entire XCI assay was repeated (i.e. new digestion and PCR amplification) and the values of the two tests was averaged if a sample was found to have ≥75% skewing.

Figure 2.4 Estimation of the degree of XCI skewing using the *AR* locus by automated fluorescence analysis. The peaks in blue represent *AR* PCR products amplified from the digested sample (D) while peaks in red represent *AR* PCR products amplified from the undigested sample (U). Samples **A** and **B** are both heterozygous females. The peak areas (include main peak and shadow peak) are shown for each allele (digested and undigested). The % of skewing was determined using the following calculation: 100*(d1/u1)/((d1/u1)+(d2/u2)) (Beever et al. 2003b). **A)** Example of random XCI skewing. **B)** Example of extreme skewing.

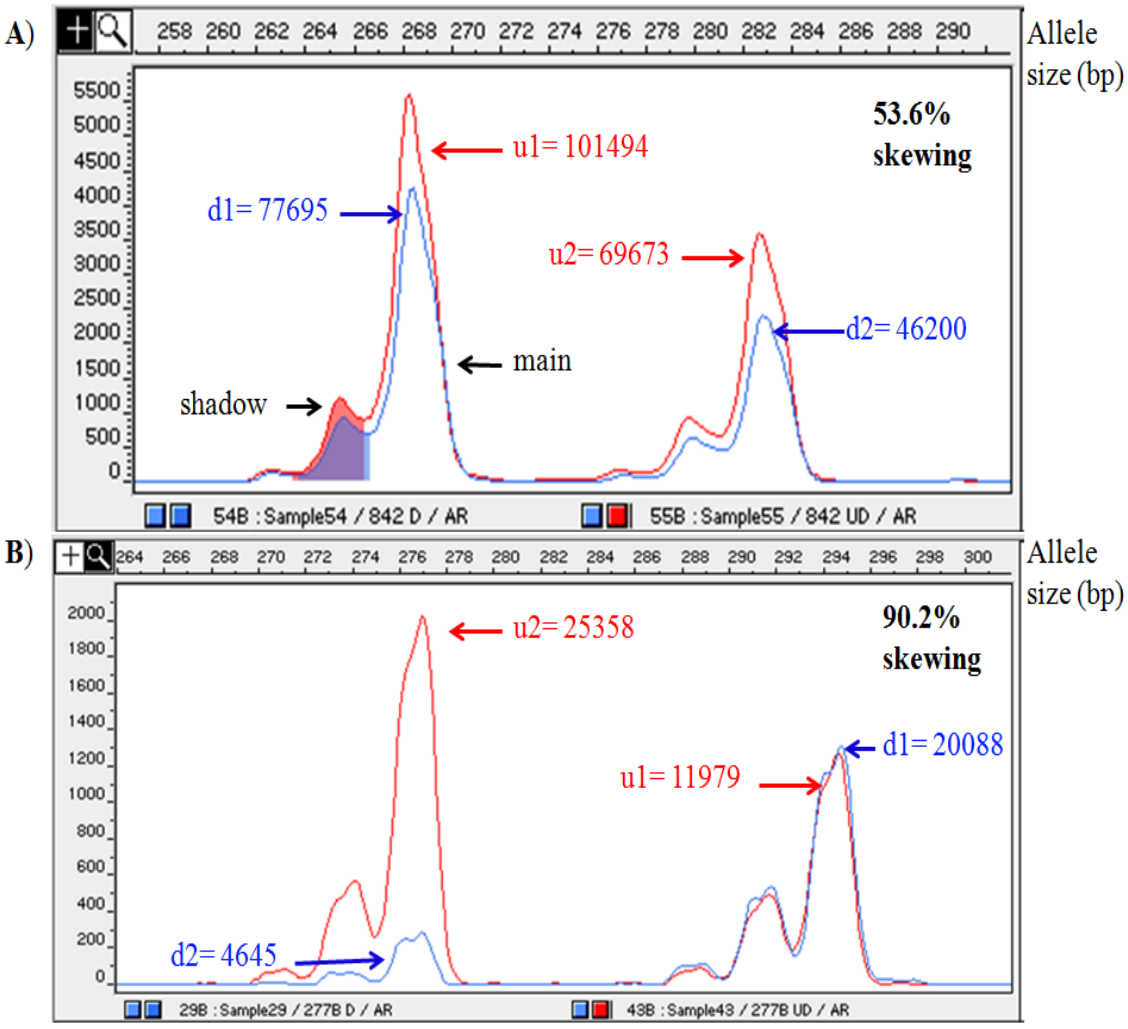
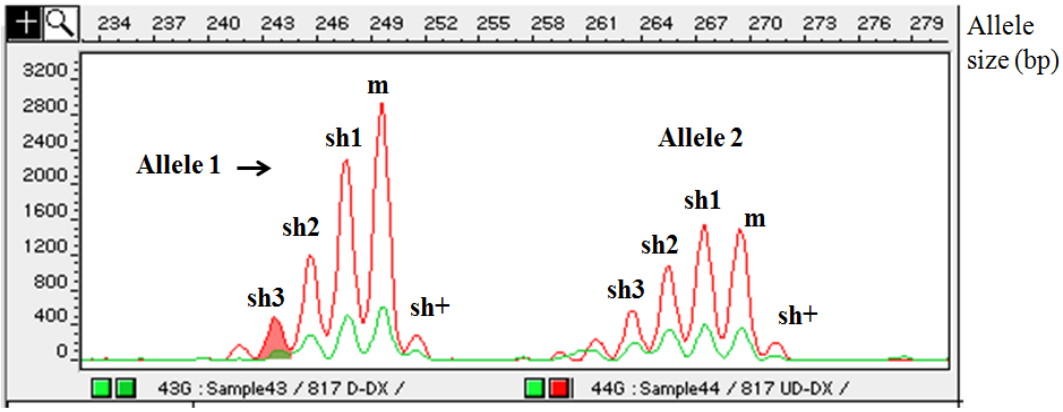


Figure 2.5 Estimation of the degree of XCI skewing using the *DXS6673E* locus by automated fluorescence analysis. The peaks in green represent *DXS6673E* PCR products amplified from the digested sample while peaks in red represent *DXS6673E* PCR products amplified from the undigested sample. The peak area of each allele included the main peak (m) and four shadow peaks (sh).



2.2.5 Statistical Analysis

A 2x3 Fisher's exact test was performed using VassarStats: Website for Statistical Computation (http://vassarstats.net/fisher2x3.html) to compare frequencies of mildly skewed (≥75%) and highly skewed (≥90%) XCI in the ICSI, IVF, and control populations (Lowry 2012b). A 3x5 Fisher's exact test was performed to compare the distribution of XCI skewing between the ICSI, IVF, and NC populations (Kirkman 1996). The Fisher's exact test can be used to determine whether two or more variables are associated (Lowry 2012a). This test is useful for smaller sample sizes, testing the null hypothesis (no association between the variables), and determining the probability of observing data due to chance (Lowry 2012a). The Kruskal-Wallis test was performed on GraphPad Prism 5.0 to compare the mean level of skewing between groups. The Kruskal-Wallis test compares the means of three or more unmatched groups (Lowry 2012a). This test is non-parametric and therefore compares values that are not distributed normally. A correlation test was performed using GraphPad Prism 5.0 to measure the reproducibility of the XCI assay. The Pearson correlation coefficient was used

to determine the association between independently replicated samples for the XCI assay. *P* values <0.05 were considered statistically significant.

2.3 Results

A total of 180 newborn female cord blood samples were tested for XCI status in this study. Subjects conceived by ICSI (n=70) included 17 individual females of a set of female twins, 2 individual females of a set of twins (gender of other twin unknown), and 3 female subjects of mixed gender twins. Karyotype abnormalities in the ICSI group included one case of 46,XX (75)/45,XY,-16 (2), one case of 46, XX, t(15;18), and one case of 46, XX, t(14:21). Subjects conceived by IVF (n=68) included 12 individual females of a set of female twins and 4 female subjects of mixed gender twins. Karyotype abnormalities in the IVF group included one case of 45,XX,-19(4)/46,XX (21). Subjects conceived naturally (n=42) included 2 individual females of a set of female twins and no karyotype abnormalities.

2.3.1 Distribution of XCI skewing in female newborns conceived by ICSI, IVF, and naturally

Informative results were obtained for 165 of the 180 cord blood samples analyzed. Uninformative samples were either homozygous for all three X-linked makers or had alleles that were too close together. A total of 15 samples were uninformative (8 ICSI, 4 IVF, 3 NC). Skewing was determined at the *AR* locus for 151 samples, the *FMR1* locus for 2 samples, and the *DXS6673E* locus for 12 samples. We found no significant difference between ICSI, IVF, and NC samples in the frequency of skewing ≥75% (8.1% vs. 6.3% vs. 12.8%) (p=0.486, Fisher's exact test), respectively. Nor was there a significant difference between the three populations in the frequency of skewing ≥ 90% (0% vs. 1.6% vs. 2.6%) (p=0.707, Fisher's exact test), respectively. Only two samples were found to have ≥ 90% skewing, one IVF and

one NC case. The distribution of skewing between the ICSI, IVF, and ICSI groups was also similar (p=0.191, Fisher's exact test) (Figure 2.6). Furthermore, there was no significant difference between ICSI, IVF, and NC samples in the mean level of skewing (64.7%±1.1% vs. 62.4%±1.0% vs. 63.8%±1.6%) (p=0.421, Kruskal-Wallis test). The skewing values of independently repeated samples showed good reproducibility (r=0.780, p<0.0001, Correlation test). The mean difference between independently replicated samples was 6.9%±1.1% with a range of 0.3% to 21.9% (n=28) (Figure 2.7).

Figure 2.6 Distribution of XCI skewing in female newborns conceived by ICSI, IVF, and naturally.

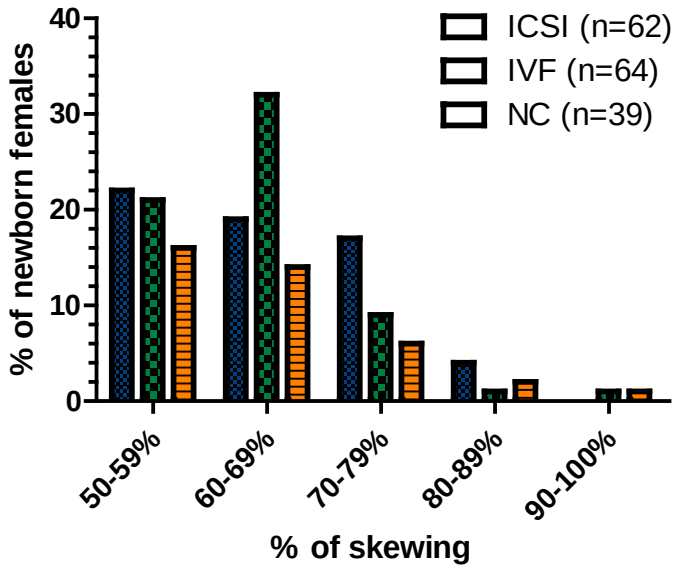
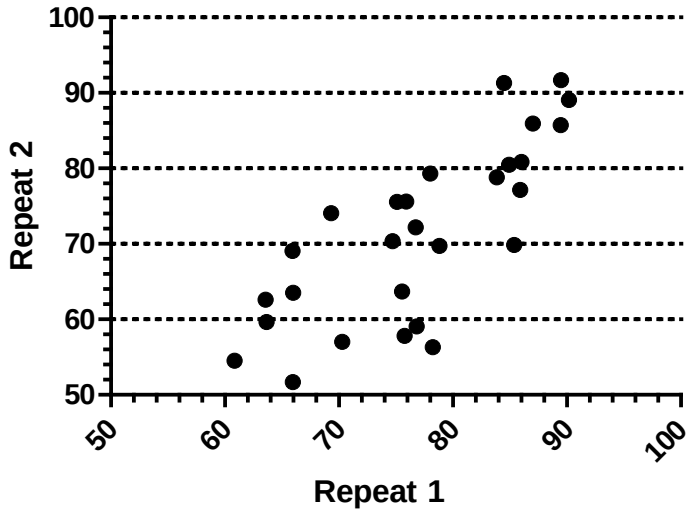


Figure 2.7 Reproducibility of automated fluorescence analysis. The XCI assay was replicated for 28 samples independently (r=0.780, p<0.0001, Correlation test).



2.3.2 XCI skewing pattern in the placental tissues and the parental origin of the skewed allele in the extremely skewed IVF case

Both of the extremely skewed cases, displaying 89.6% (IVF) and 90.6% (NC) skewing, had no karyotype abnormalities. The degree of skewing and the origin of the skewed allele for the IVF case of different placental tissue sites and cord blood is shown in Table 2.1. Chorionic villi for this case was available for all ten placental sites biopsied, however amnion was only available for six sites (Figure 2.8). The parental origin of the skewed allele was determined by sizing the *AR* alleles of the maternal decidua (uterine endometrium) of the placenta by automated fluorescence analysis. The decidua was contaminated with fetal villi, but which alleles were passed down to the infant could be determined by comparing the peak intensities of the alleles and by comparing the allele sizes of the decidua with the allele sizes of the cord blood (Figure 2.9). The maternal allele was preferentially inactivated in the cord blood, umbilical cord, 3/5 chorionic villi samples, and all of the four amnion samples that

showed mild or extreme skewing (Table 2.1). Placental analysis of the extremely skewed natural conception case was not performed.

Table 2.1 X chromosome skewing of placental tissues and cord blood of extremely skewed IVF case. The parental origin of the preferentially inactivated allele is listed for mild or extremely skewed tissues (bolded). S= site. Amnion for some placental sites was unavailable (N/A).

Tissue	% Skewing		Preferentially Inactivated Allele
Cord blood	**89.6%**		Maternal
Umbilical cord	**76.0%**		Maternal
	Villi	Amnion	
Placenta S1	60.0%	N/A	
S2	58.4%	58.4%	
S3	52.9%	52.9%	
S4	**87.4%**	**81.1%**	Maternal
S5	65.9%	**74.8%**	Maternal
S6	61.7%	N/A	
S7	**82.3%**	**92.6%**	Maternal
S8	**83.3%**	**74.9%**	Maternal
S9	**83.5%**	N/A	Paternal
S10	**93.0%**	N/A	Paternal

Figure 2.8 Location of the placental sites biopsied for the extremely skewed IVF case. The X marks the point of insertion of the umbilical cord. Chorionic villiwas available for all 10 sites while amnion was only available for sites 2-5, 7, and 8.

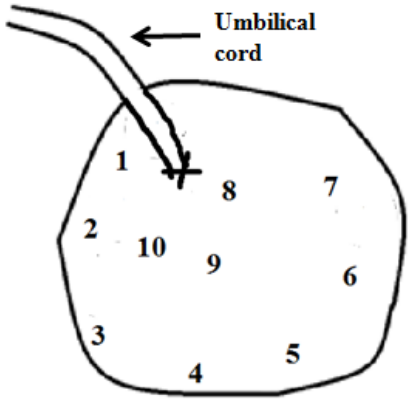
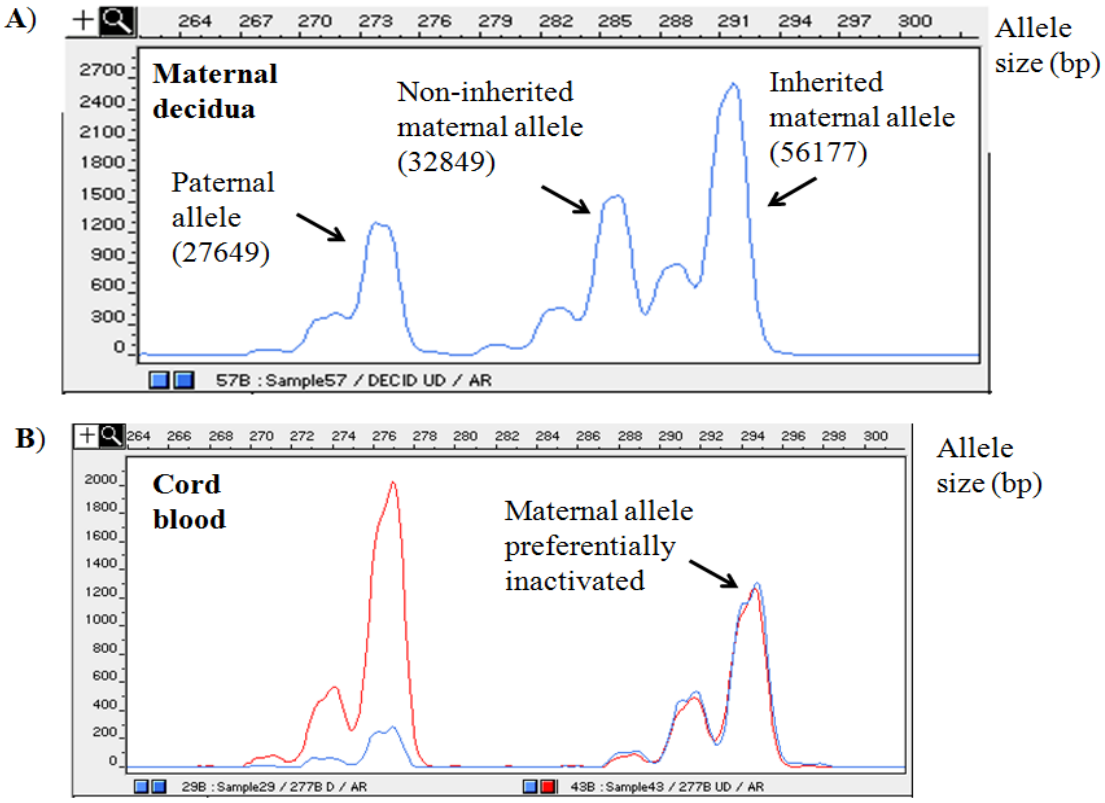


Figure 2.9 *Androgen receptor* allele sizing of placental maternal decidua and cord blood to determine parental origin of the skewed allele in the extremely skewed IVF case. A) *AR* allele sizing of maternal decidua. Three alleles are present due to contamination with placental fetal villi. The smallest allele was inherited by the infant as it corresponds to the smaller allele in the cord blood sample. The middle allele was only found in the decidua, indicating that it is the maternal allele that was not inherited. The largest allele is maternal and was inherited by the infant as the peak intensity of the allele is approximately double that of the other alleles, indicating that this allele was present in both the decidua and cord blood (area values of alleles indicated in brackets). Subsequently, it can also be determined that the smallest allele is paternal. B) XCI status of cord blood. Blue represents the digested sample and red the undigested sample. The maternal allele was preferentially inactivated as more digested product was amplified.



2.4 Discussion

We were able to determine the XCI status of 165 of the 180 cord blood samples analyzed. No significant differences were found between the informative ICSI (n=62), IVF (n=64) and NC (n=39) samples in the frequency of mild skewing (≥75%), extreme skewing (≥90%), or the mean level of skewing. Two extremely skewed cases were found, one in the IVF population and one in the NC population. Analysis of placental tissues of the extremely

skewed IVF case revealed that the maternal allele was preferentially inactivated in the cord blood. In this case it was shown that skewing status was similar between different tissues within the same site of a placenta, but that skewing was dissimilar between tissues of different sites.

Our findings indicate that assisted reproductive technologies are not associated with an increase in skewed X-chromosome inactivation. The occurrence of mild and extremely skewed XCI and the mean level of skewing was similar in the ICSI, IVF, and naturally conceived populations. This indicates that there is no significant reduction in the number of embryonic precursor cells present in the blastocysts conceived by IVF and ICSI compared to the blastocysts conceived by natural means nor in the blastocysts conceived by IVF compared to ICSI. In IVF and ICSI, it is likely that the best growing embryos are selected for transfer to the patient's uterus (Robinson et al. 2005). These embryos would likely have large numbers of cells contributing to the ICM of the blastocyst resulting in more random XCI.

Our data also suggests that X-chromosome inactivation is not altered during *in vitro* culturing. Some studies indicate that XCI is initiated in the morula and blastocyst stages before implantation (Puck et al. 1992, van den Berg et al. 2009). However, a recent study demonstrated that late blastocysts still demonstrated biallelic *FGD1* (undergoes XCI) expression indicating that chromosome-wide inactivation does occur until after the blastocyst stage (Okamoto et al. 2011). Therefore, it is possible that XCI is not occurring until after embryo implantation and hence XCI epigenetic programming would not be affected by *in vitro* culturing.

When chromosome mosaicism is present in the embryo, normal cells are generally selected to contribute to the ICM (Brown and Robinson 2000). This could result in a decreased precursor cell pool being present in the ICM at the time of inactivation and skewing

(Brown and Robinson 2000). The results of this study indicate that there is not a higher level of chromosome mosaicism in viable female blastocysts produced by artificial methods in contrast to those produced naturally or in blastocysts produced by ICSI in contrast to those produced by IVF as skewing is similar in all groups studied.

As previously suggested, it is possible that abnormalities in X-chromosome inactivation could be restricted to non-viable embryos (Robinson et al. 2005). If this were the case, we would not observe a greater number of XCI abnormalities in the newborns conceived by ARTs as all newborns studied resulted from a viable embryo.

One previous study investigated the XCI status in 22 female newborns conceived by IVF and 31 naturally conceived newborns (King et al. 2010). They found no difference in the frequency of mild skewing (80-90%) (9.1% vs. 6.5%) or extreme skewing (>90%) (9.1% vs. 0%) when comparing IVF and control groups (King et al. 2010). However, they did find a significant difference in the mean level of skewing of between the IVF and NC groups (72.0% vs. 62.4%, P= 0.002) (King et al. 2010). Another study included 48 newborn females conceived by ICSI and 74 control samples of females aged 0-19 years (Robinson et al. 2005). No significant difference in the mean level of skewing (65.1%vs. 69.8%) or in the frequency of skewing ≥75% (18% vs. 35%) or ≥ 90% (4.6% vs. 10.8%) between the ICSI and control groups was found (Robinson et al. 2005). A prior study evaluated the XCI patterns of 590 phenotypically unaffected newborns (Amos-Landgraf et al. 2006). They found that 4.9% had ≥80% skewing, 0.5% had ≥90% skewing, and 0.2% had ≥95% skewing (Amos-Landgraf et al. 2006). Our study adds to the results of these studies by evaluating aged matched infants conceived by IVF, ICSI, and naturally concurrently and by investigating a large group of samples.

The placental tissues of the extremely skewed IVF case were analyzed to determine XCI status and the parental origin of the skewed allele. In the cord blood, the maternal allele was preferentially inactivated indicating that there may be a chromosome abnormality on the maternal X chromosome and that the majority of cells that contributed to the ICM had the maternal X inactivated. It is also possible that there may be a mutation in the *XIST* gene or other genes involved in inactivation of the paternal X which is preventing inactivation of this X. Which parental allele was preferentially inactivated varied between placental sites (Table 2.1). Moreover, mild and extreme skewing was only observed in some amnion and chorionic villi placental site samples. It is has been observed that weaker selective pressures occur in the placenta that could result in skewed inactivation (Penaherrera et al. 2003). In the case of a paternally derived balanced (X;20) translocation, preferential inactivation of the non-translocated X was observed in the cord blood, umbilical cord, and amnion (Penaherrera et al. 2003). However, random inactivation was observed in the chorion and chorionic villi trophoblast and mesenchyme (Penaherrera et al. 2000). This indicates that certain placental tissues were able to cope with partial monosomy for autosome 20 (Penaherrera et al. 2000). In our extremely skewed IVF case, extreme skewing at all placental sites and tissues as well as skewing towards the same allele may not be observed because the placenta is better able to cope with the maternal X chromosome abnormality that would otherwise be detrimental to the fetus.

It is possible that variability of X chromosome skewing exists between the chorionic villi sites due to clonal populations of cells contributing to each placental site (Peñaherrera et al. 2012). Chorionic villi form through the initial budding of trophoblast, the invasion of the mesenchyme, and the development of fetal capillaries (Peñaherrera et al. 2012). Additional villi will branch off each initial mesenchymal villous forming a villous tree (Peñaherrera et al.

2012). Villous samples taken at different depths from the fetal to maternal side of the placental show similar skewing (Peñaherrera et al. 2012). This indicates that each villous tree derives only from cells of the initial villous bud (Peñaherrera et al. 2012). Therefore, skewing variability between placental sites in our study could be due to different populations of cells contributing to chorionic villi at each site. In addition, chorionic villi are composed of mesenchyme, cytotrophoblast, and syncytiotrophoblast. The villous mesenchyme is derived from the ICM of the blastocyst while the cytotrophoblasts and syncytiotrophoblasts develop from the trophectoderm. Therefore, the chorionic villi are made up of cells of different embryonic origin, some derived from the inner cell mass (maybe skewed) and some from the trophectoderm (maybe randomly skewed). However, this does not explain why extreme skewing is not observed in the umbilical cord or at all amnion sites since both of these are derived from the ICM of the blastocyst and hence should display similar skewing as the cord blood.

Although we did not find any association between skewed XCI and female newborns conceived by the ARTs of ICSI and IVF, our study is the largest to date that has looked at skewed XCI in relation to female newborns conceived by ICSI, IVF, and naturally. Previous studies have looked at limited numbers of only ICSI or IVF compared to naturally conceived infants. Our study is the first to look XCI in ICSI and IVF concurrently as well as assessing age-matched controls. In addition, we were able to decipher the parental origin of the skewed allele in an extremely skewed IVF case. In this sample, we observed skewing variability within the placenta which has been observed by others.

Chapter 3 : Project 2- *FMR1* CGG Repeat and AGG Interspersion Number in Female Newborns Conceived by Assisted Reproductive Technologies

3.1 Introduction

Fragile X syndrome (FXS), the most common cause of inherited mental retardation in males, results from the expansion of a trinucleotide CGG repeat located in the 5' UTR of the *Fragile X mental retardation 1 gene* (*FMR1*) to >200 repeats (full mutation) (Fu et al. 1991). Six to forty-five CGG repeats is considered normal, 45-54 is intermediate, and 55-200 is the premutation range (Nolin et al. 2003). Seventeen percent of male carriers in their 50s (Jacquemont et al. 2004) and 12.3% of women carriers (Coffey et al. 2008) of the premutation allele display symptoms of the neurodegenerative movement disorder known as fragile X-associated tremor/ataxia syndrome (FXTAS). Premutation alleles are also found in approximately 5% of all premature ovarian failure (POF) cases, 12% of familial POF cases, 3% of sporadic POF cases, but only in 0.4% of the general population (Murray 2000). In recent years, intermediate ranges of 41-58 repeats (Bodega et al. 2006) and normal ranges of 35-54 repeats (Bretherick et al. 2005) have been associated with POF. It has also been observed that repeats below and above the range of 26-34 repeats are associated with a decreased ovarian reserve (Gleicher et al. 2011). Alleles in the premutation range are prone to expansion to the full mutation range in subsequent generations through mitosis and meiosis due to the instability of large repeat tracts (Nolin et al. 2003).

Loss of AGG repeat interspersions within the CGG repeat tracts are thought to increase repeat instability (Dombrowski et al. 2002, Eichler et al. 1996). One to three AGG interruptions are generally found in normal and intermediate alleles while 0-1 are found in premutation alleles (Yrigollen et al. 2011). An instability threshold has been identified in which uninterrupted CGG tracts of greater than 34-38 repeats have been observed to result in

unstable transmission (Eichler et al. 1996). Loss of AGG interspersions can occur due to deletions or A to C transversions (Eichler et al. 1996).

Women achieving pregnancy through infertility treatments may display symptoms such as irregular ovulation that is indicative of a reduced primordial follicle pool. It is possible that some of these women may have undiagnosed POF and are passing on *FMR1* alleles associated with this syndrome of reduced fertility to their female offspring. It has been observed that infertile women demonstrate a mild shift towards *FMR1* alleles with higher CGG repeat counts (Gleicher et al. 2009). *FMR1* repeat expansion in the germlines of subfertile parents could also be occurring. Women utilizing infertility treatments are generally advanced in age. Advanced maternal age has been associated with an increased expansion from a premutation allele to a full mutation allele from mother to offspring (Ashley-Koch et al. 1998). Sperm aneuploidy is common in infertile men (Dohle et al. 2002, Hristova et al. 2002). Meiotic repair mechanisms in these men could be deficient which could result in meiotic replication slippage (looping out) or unequal meiotic crossovers leading to repeat expansion. Some studies have indicated that repeat expansions can occur post-zygotically due to mitotic errors (Devys et al. 1992, Wohrle et al. 1993). In ART, the early embryo is growing in culture media *in vitro*. Mosaicism has been observed in human preimplantation embryos due to mitotic errors during *in vitro* culturing (Munne et al. 1994). It is possible that in this unnatural environment, embryos are more prone to mitotic errors which could result in repeat expansion by mitotic replication slippage or unequal sister chromatid exchange in the post-zygotic stage. Therefore, there could be an increase risk of female offspring conceived through ARTs inheriting *FMR1* alleles with higher repeats and developing POF, FXTAS, and/or FXS. One study found that the intermediate *FMR1* alleles of POF patients lacked AGG interspersions (Bodega et al. 2006). Therefore, it is also possible that women with

undiagnosed and milder POF may also be passing on *FMR1* alleles with less AGG interspersions and hence increased instability to their children conceived by ARTs. In this study, we investigated the CGG repeat number and AGG interspersion number in the *FMR1* alleles of female newborns conceived by ICSI, IVF, and naturally.

3.2 Materials and methods

3.2.1 Patient ascertainment

The same cases were used as in Project 1 (Chapter 2).

3.2.2 DNA extraction from cord blood

The same methods were used as in Project 1 (Chapter 2).

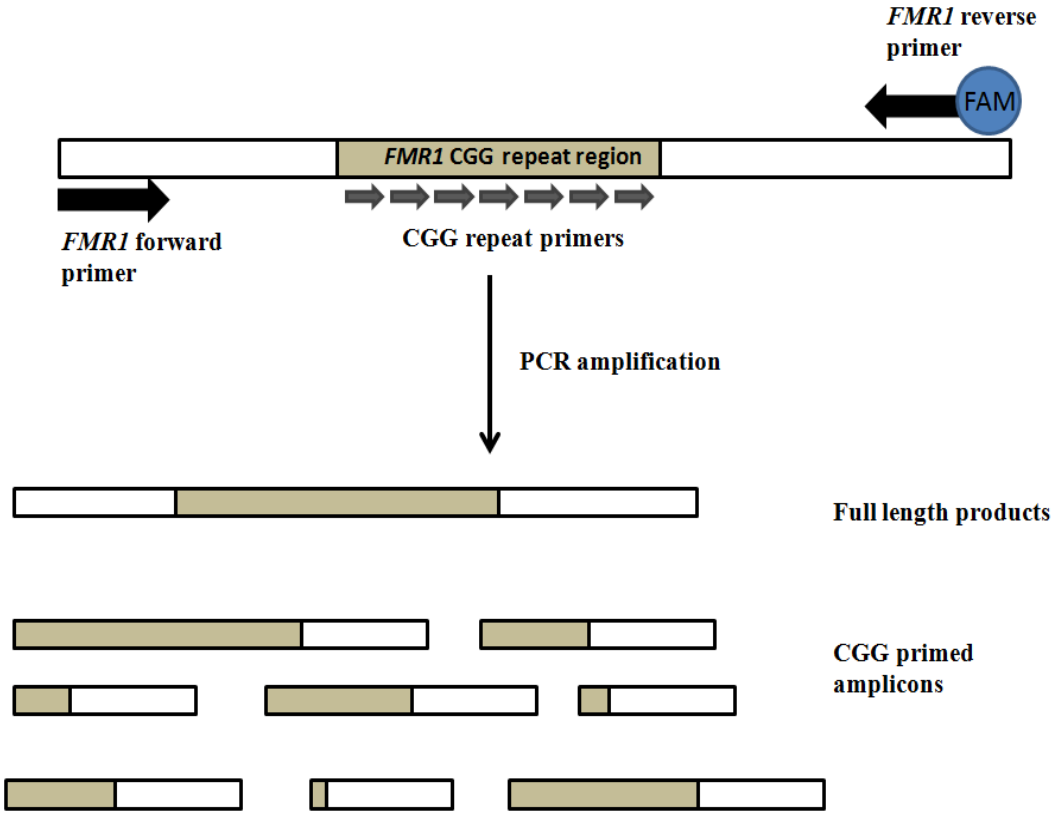
3.2.3 Determining *FMR1* CGG repeat length and AGG interspersion number

The *FMR1* repeat region was amplified by the novel AmplideX™ FMR1 PCR kit (Asuragen Inc, Item# 76008). Forty to one-hundred nanograms of genomic DNA (2μl of DNA at 20-50 ng/μl) was amplified in a master mix containing 11.45 μl of GC-rich AMP Buffer (Asuragen Inc.), 1.5 ul of gene-specific *FMR1* forward, reverse (FAM labelled) primers (Asuragen Inc.), 0.5 ul of *FMR1* CGG primer (Asuragen Inc.), 0.5 ul of diluent (Asuragen Inc.), and 0.05 ul of GC-rich polymerase mix (Asuragen Inc.). The gene-specific primer sequences were as follows: forward 5` -TCAGGCGCTCAGCTCCGTTTCGGTTTCA -3` and reverse 5`- FAM-AAGCGCCAT TGGA -GCCCCGCACTTCC -3` (Filipovic-Sadic et al. 2010). The CGG primer is composed of an unlabelled complementary five CGG repeat sequence. PCR conditions were 95°C for 5 min (initial denaturation); 97°C for 35 s, 62°C for 35 s, and 68 °C for 4 min for 10 cycles; 97°C for 35 s, 62 °C for 35 s, and 68°C for 4 min with an additional 20 s per cycle for 20 cycles; and a final extension at 72°C for 10 min. A control mix containing premutated and fully mutated *FMR1* alleles was included in each reaction

batch to ensure that premutated and fully mutated alleles were detectable. DNA of carriers of premutated and fully mutated alleles were purchased from the Coriell Institute (premutated: NA06894; fully mutated; NA07537). The amplified products were stored at -15 to -30°C in the dark until analysis by capillary electrophoresis (CE).

The PCR is designed with the conventional forward and reverse primers (gene-specific primers) and the addition of a third PCR primer (CGG repeat primer) that is only complementary to CGGs in the *FMR1* repeat region (Figure 3.1) (Chen et al. 2010). This three-primer PCR results in full length products that are generated from the gene-specific forward and FAM-labelled reverse primer combination (Chen et al. 2010). Additionally, a series of amplicons of different sizes will be produced from the combination of the FAM-labelled reverse primer and the CGG repeat primer which binds at different locations on the repeat region (Chen et al. 2010).

Figure 3.1 Three-primer repeat primed *FMR1* PCR. Full length products are produced from the forward and FAM- labelled reverse primer. A series of CGG primed amplicons are produced from the FAM-labelled reverse primer and CGG repeat primer bound at different locations on the repeat region.

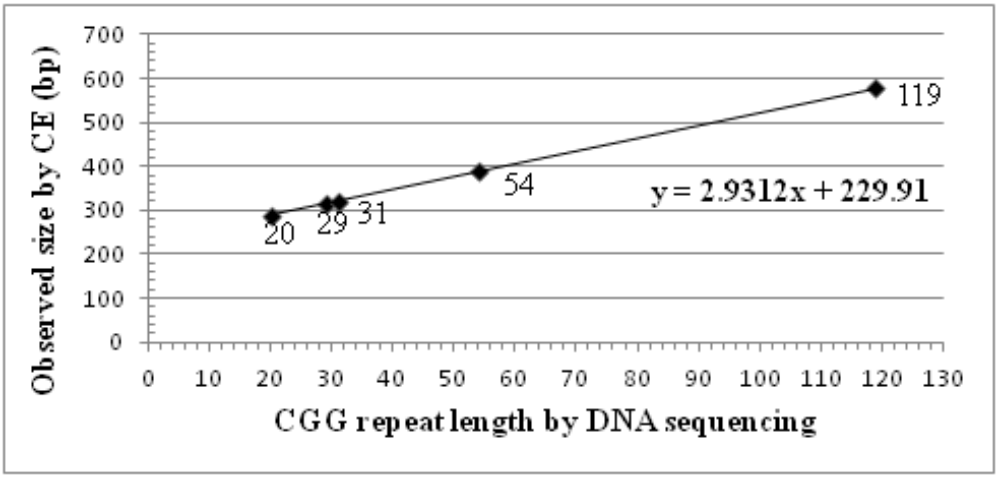


Full length PCR products and amplicons were evaluated on an ABI 3130xl Genetic Analyzer (Applied Biosystems) at the CMMT. Two microlitres of PCR product were mixed with 11 µl of Hi-Di Formamide (Applied Biosystems) and 2 µl of ROX1000 size ladder (Applied Biosystems). The mixture was heat-denatured for 95°C for 2 minutes followed by cooling on ice (protected from light) until transfer to the ABI machine. A 50 cm capillary was used for all injections with an applied voltage of 2.5 kV for 20s with a 40 minute run time at 15 kV on a Pop-7 gel polymer.

PCR products detected by CE were analyzed using GeneMapper 4.0 (Applied Biosystems). The size of the gene-specific full length products was determined by comparison

with the co-injected size standard. The number of CGG repeats for each allele was determined from the following calculation: #CGG= (Peak size- c_0)/ m_0. C_0 is the size correction factor and represents the DNA sequence surrounding the *FMR1* repeat region. M_0 is the mobility correction factor and accounts for the faster migration of the triplet region PCR products compared to the size standard. The size and mobility correction factors depend on the CE instrument, polymer type, capillary length, run conditions, and laboratory. C_0 and m_0 values were determined from a linear fit of expected CGG repeat length from DNA sequencing and base pair sizing by CE for the first five peaks of a pooled control of allele amplicons (provided by Asuragen Inc) (Figure 3.2). C_0 was determined as 229.91 bp from the y-intercept of the linear fit and m_0 was determined as 2.9312 from the slope.

Figure 3.2 Derivation of size and mobility correction factors. A linear fit of expected CGG repeat length from DNA sequencing and base pair sizing by CE for the first five peaks, 20, 29, 31,54, and 119 CGG repeats of a pooled control of allele amplicons. C_0 corresponds to the y-intercept (229.91) and m_0 corresponds to the slope (2.9312).

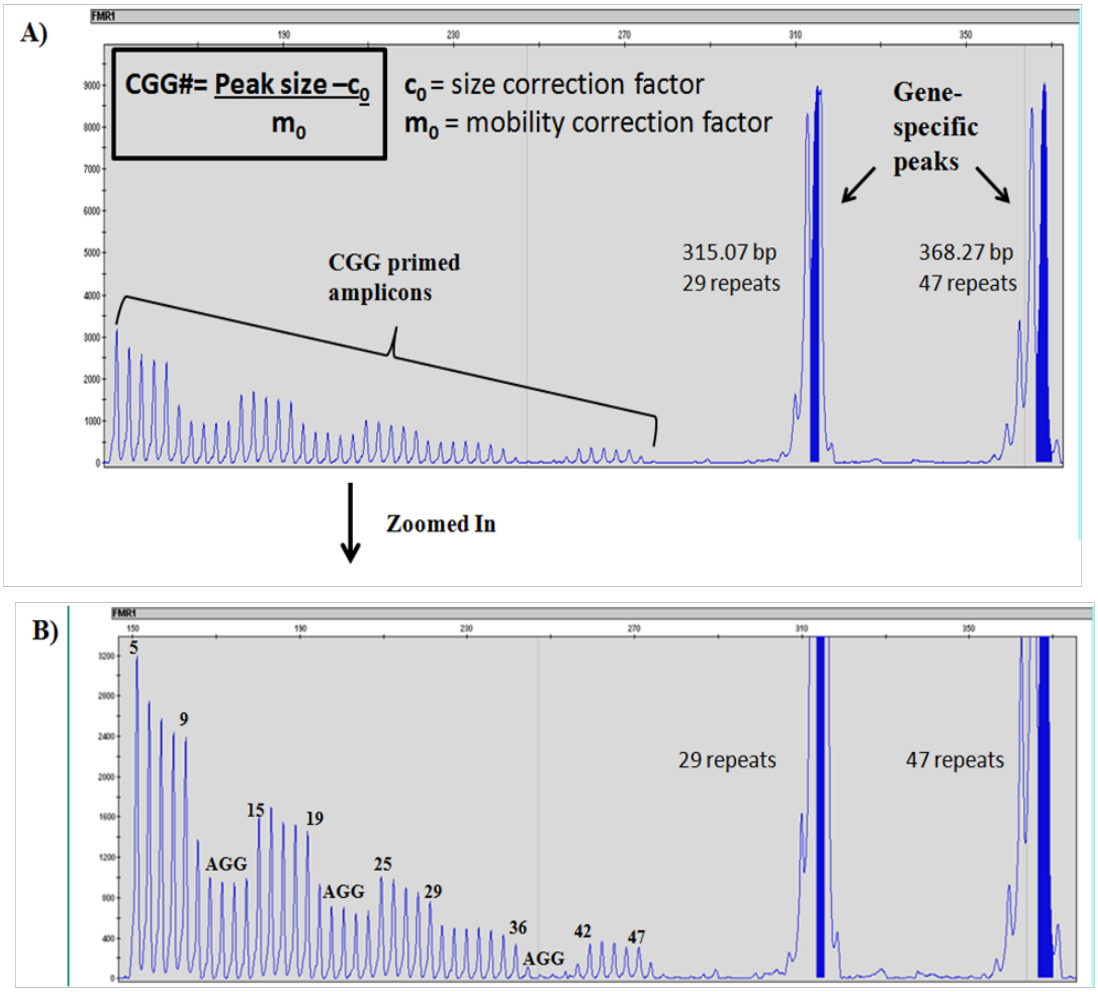


The peak size of each gene-specific full length product and the above equation was used to determine the *FMR1* CGG repeat number for each allele (Figure 3.3a). The highest point of the gene-specific peak was chosen to represent the size of each allele. Homozygous samples only showed one gene-specific peak and were recorded as having two alleles with the

same CGG repeat number. For homozygous samples, it was ensured that no PCR products were detected by CE in the premutation and full mutation range. Normal alleles were defined as <45 CGG repeats, intermediate alleles as 45-54 CGG repeats, premutation alleles as 55-200 CGG repeats, and full mutation alleles as >200 CGG repeats. *FMR1* PCR products could only accurately be sized up to 200 CGG repeats. Beyond 200 CGG repeats, the resolving threshold of the POP-7 polymer is exceeded. Nevertheless, these PCR products can be observed and categorized as >200 CGG repeat alleles.

As the repeat primer is composed of 5 complementary CGG repeats, the first product peak corresponds to amplicons with exactly 5 CGG repeats (Figure 3.3b) (Chen et al. 2010). The CGG repeat primer is specific for CGG repeats and will not bind to AGG sequences commonly found to interrupt the CGG repeat tract. Therefore, dips in the signal intensities correspond to the presence of an intervening AGG. The signal intensity will drop for the equivalent of 5 CGG repeats as each repeat unit on the repeat primer mismatches with the AGG sequence (Chen et al. 2010). Signal intensities that only drop down half way to baseline indicate that the AGG interspersion is only present on one allele. For the signal intensities to drop to baseline, an AGG must be present at the same position on both alleles (Chen et al. 2010). The total number of AGG interspersions (both alleles) was recorded for each sample as it was not always possible to determine the specific number of AGG interspersions on each allele.

Figure 3.3 Determining *FMR1* CGG repeat and AGG interspersion number by automated fluorescence analysis. A) Heterozygous female with one allele with 29 repeats (normal range) and the other allele with 47 repeats (intermediate range). The gene-specific peaks representing the full repeat lengths of each allele are seen on the right. The series of small peaks on the left represent the CGG primed amplicons. **B)** Close up of the CGG primed amplicons. The first amplicon peak (left) corresponds to amplicons with exactly 5 CGG repeats because the CGG repeat primer is comprised of 5 complementary CGG repeats. The first two AGG interspersions (from left to right) are only present on one allele as signal intensities only drop half way to baseline. The last AGG interspersion (farthest to right) is only found on the 47 repeat allele as the dip in signal intensity occurs after the 29 repeat amplicon (corresponds to 29 repeat allele). Signal intensities drop half way after the 29 repeat amplicon as only amplicons derived from the 47 repeat allele are contributing.



3.2.4 Statistical Analysis

A 2x3 Fisher's exact test was performed using VassarStats: Website for Statistical Computation (http://vassarstats.net/fisher2x3.html) to compare the frequencies of intermediate

alleles, intermediate carriers, premutation alleles, and premutation carriers (Lowry 2012b). A 3x6 Fisher's exact test was performed to compare the distribution of *FMR1* genotypes and the distribution of total AGG interspersion number between the ICSI, IVF, and NC populations (Kirkman 1996). The Kruskal-Wallis test was performed on GraphPad Prism 5.0 to compare the mean allele CGG repeat number, the biallelic mean, and the mean number of AGG interspersions. *P* values <0.05 were considered statistically significant.

3.3 Results

A total of 108 newborn female cord blood samples were analyzed in this study. Subjects conceived by ICSI (n=36) included 9 individual females of a set of female twins, 1 individual female of a set of twins (gender of other twin unknown), and 1 female subject of mixed gender twins. Karyotype abnormalities in the ICSI group included one case of 46,XX (75)/45,XY,-16 (2), one case of 46, XX, t(15;18), and one case of 46, XX, t(14:21). Subjects conceived by IVF (n=36) included 3 individual females of a set of female twins and 3 female subjects of mixed gender twins. Karyotype abnormalities in the IVF group included one case of 45,XX,-19(4)/46,XX (21). Subjects conceived naturally (n=36) included 2 individual females of a set of female twins and no karyotype abnormalities.

3.3.1 *FMR1* CGG repeat length

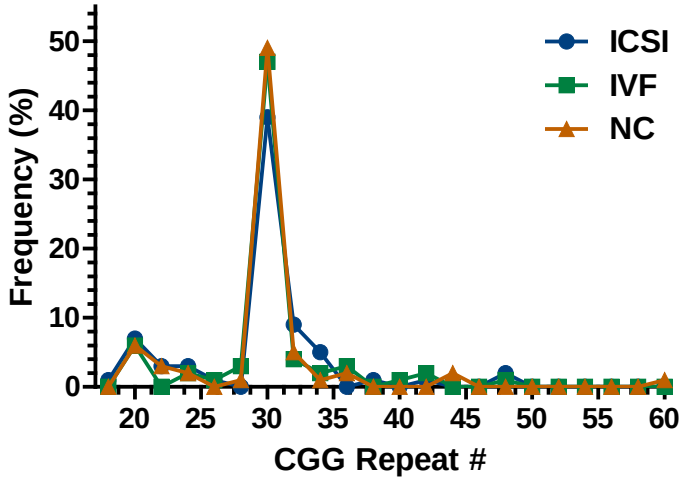
The *FMR1* allele frequencies for the ICSI, IVF, and NC populations are shown in Table 3.1. The majority of alleles were found with 29 and 30 CGG repeats (Figure 3.4). No significant differences were found between the ICSI, IVF, and NC groups in the frequency of alleles within the 29 to 30 repeat range (4.2% vs. 5.6% vs. 2.8%) (p=0.198, Fisher's exact test), respectively. No full mutation alleles were detected. There was one individual carrying a premutation allele (59 repeats) in the natural conception population. There were two

individuals carrying an intermediate allele in the ICSI population and one in the IVF population. No significant differences were found in the frequencies of intermediate alleles (2.8% vs. 1.4% vs. 0%) (p=0.775, Fisher's exact test), intermediate allele carriers (5.6% vs. 2.8%, vs. 0%) (p=0.771, Fisher's exact test), premutation alleles (0% vs. 0% vs. 1.4%) (p=1.0, Fisher's exact test), or premutation allele carriers (0% vs. 0% vs. 2.8%) (p=1.0, Fisher's exact test) in the ICSI, IVF, and NC groups, respectively. The mean allele CGG repeat number was similar between ICSI, IVF, and NC groups, respectively (29.2$\pm$0.6 vs. 29.6$\pm$0.6 vs. 29.3$\pm$0.7) (p=0.422, Kruskal-Wallis test).

Table 3.1 *FMR1* alleles in ICSI, IVF, and NC populations.

CGG Repeat #	ICSI	IVF	NC
18	1 (1.4%)	0 (0.0%)	0 (0.0%)
20	7 (9.7%)	6 (8.3%)	6 (8.3%)
21	1 (1.4%)	0 (0.0%)	1 (1.4%)
22	2 (2.8%)	0 (0.0%)	2 (2.8%)
23	2 (2.8%)	2 (2.8%)	2 (2.8%)
24	1 (1.4%)	0 (0.0%)	0 (0.0%)
26	1 (1.4%)	1 (1.4%)	0 (0.0%)
27	0 (0.0%)	3 (4.2%)	1 (1.4%)
29	11 (15.3%)	20 (27.8%)	25 (34.7%)
30	28 (38.9%)	27 (37.5%)	24 (33.3%)
31	5 (6.9%)	3 (4.2%)	2 (2.8%)
32	4 (5.6%)	1 (1.4%)	3 (4.2%)
33	4 (5.6%)	2 (2.8%)	1 (1.4%)
34	1 (1.4%)	0 (0.0%)	0 (0.0%)
35	0 (0.0%)	0 (0.0%)	1 (1.4%)
36	0 (0.0%)	3 (4.2%)	1 (1.4%)
37	1 (1.4%)	0 (0.0%)	0 (0.0%)
40	0 (0.0%)	1 (1.4%)	0 (0.0%)
41	1 (1.4%)	1 (1.4%)	0 (0.0%)
42	0 (0.0%)	1 (1.4%)	0 (0.0%)
43	0 (0.0%)	0 (0.0%)	1 (1.4%)
44	0 (0.0%)	0 (0.0%)	1 (1.4%)
47	1 (1.4%)	0 (0.0%)	0 (0.0%)
48	1 (1.4%)	1 (1.4%)	0 (0.0%)
59	0 (0.0%)	0 (0.0%)	1 (1.4%)
Total	72	72	72

Figure 3.4 Distribution of *FMR1* allele CGG repeat length in ICSI, IVF, and NC populations.



3.3.2 *FMR1* CGG repeat length biallelic mean.

To assess the average size of the *FMR1* alleles of an individual the CGG repeat lengths of the two alleles of each patient were averaged. There was no significant differences between the biallelic means of the ICSI, IVF, and NC groups, respectively (29.4±0.6 vs. 29.9±0.5 vs. 29.6±0.8) (p=0.231, Kruskal-Wallis test).

3.3.3 *FMR1* genotypes

It has been observed that repeats below and above the range of 26-34 repeats are associated with a decreased ovarian reserve (Gleicher et al. 2011). Therefore, Gleicher et al. determined *FMR1* genotypes based on whether or not a subject's alleles fell within this range (Gleicher et al. 2011). The distribution of *FMR1* genotypes was similar between the sample groups (Table 3.2) (p=0.690, Fisher's exact test).

Table 3.2 Distribution of *FMR1* genotypes classified by Gleicher et al. (Gleicher et al. 2011)**.** The genotypes are based on the normal range of *FMR1* CGG repeats being between 26-34 repeats. Norm= both alleles within range; het-norm/high= one allele above range; het-norm/low= one allele below range; hom-high/low= one allele above and the other below range; hom-low/low= both alleles below range; hom-high/high= both alleles above range.

	norm	het-norm/high	het-norm/low	hom-high/low	hom-low/low	hom-high/high	Total
ICSI	22	2	8	2	2	0	36
IVF	22	6	7	1	0	0	36
NC	24	2	6	1	2	1	36
Total	68	10	21	4	4	1	108

3.3.4 *FMR1* AGG interspersion number

The AGG interspersion pattern was not significantly different between ICSI, IVF, and NC populations (p=0.295, Fisher's exact test) (Table 3.3). The one ICSI sample that was found to have no AGG interspersions had *FMR1* alleles within the normal range (24 and 30 repeats). Furthermore, the average number of AGG repeats between ICSI, IVF, and NC groups was similar (3.3$\pm$0.2 vs. 3.4$\pm$0.2 vs. 3.4$\pm$0.1) (p=0.965, Kruskal-Wallis test).

Table 3.3 Distribution of total AGG interspersion number in ICSI, IVF, and NC populations.

	ICSI	IVF	NC
0	1 (2.8%)	0 (0.0%)	0 (0.0%)
1	0 (0.0%)	2 (5.6%)	0 (0.0%)
2	4 (11.1%)	3 (8.3%)	6 (16.7%)
3	12 (33.3%)	13 (36.1%)	11 (30.6%)
4	19 (52.8%)	14 (38.9%)	18 (50.0%)
5	0 (0.0%)	4 (11.1%)	1 (2.8%)
Total	36	36	36

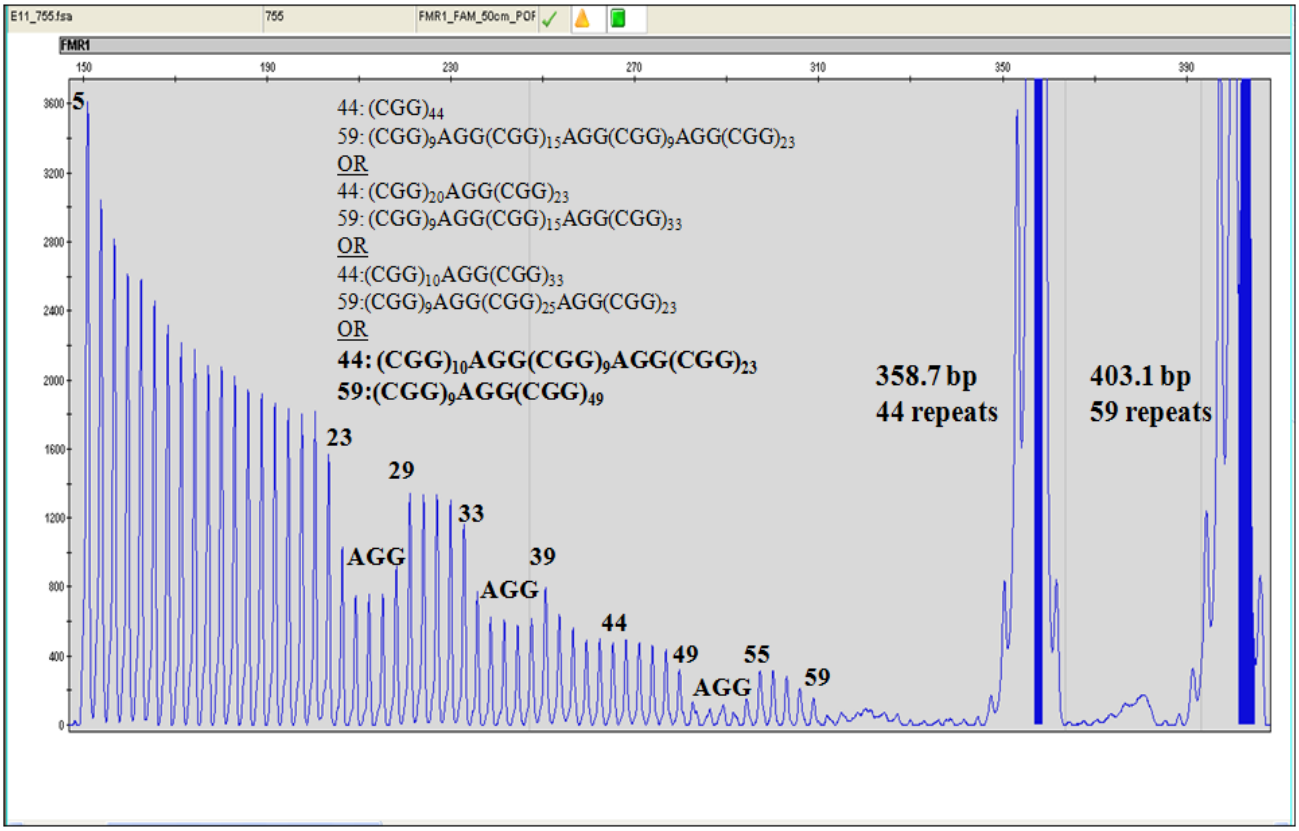
3.3.5 *FMR1* analysis and XCI skewing of NC premutation case

The one individual found to have a premutation allele was conceived naturally. This individual had *FMR1* alleles with 44 and 59 CGG repeats and a total of 3 AGG interspersions (Figure 3.5). It could not be determined exactly where each AGG interspersion is on each allele or how many are on each allele other than that there is at least one AGG interspersion on the premutation allele. However, the most likely location and number of AGG interspersions on each allele can be determined by comparing speculated haplotypes with known AGG haplotypes (Chen et al. 2010, Eichler et al. 1996). AGG interspersions are often spaced by 9 or 10 CGG repeats and clustered at the 5' end of the *FMR1* repeat region (Chen et al. 2010). Therefore, the locations of AGG interspersions are usually at the 10, 20 or 11, 21 positions relative to the starting 5'CGG repeat (Chen et al. 2010). Hence, the most likely sequence for the repeat region of the 44 repeat allele is $(CGG)_{10}AGG(CGG)_9AGG(CGG)_{23}$ and for the 59 repeat allele is $(CGG)_9AGG(CGG)_{49}$ (Figure 3.5). From the results of Project 1 (Chapter 2), this premutation case was found to have 53.8% (random) X chromosome skewing.

3.3.6 *FMR1* analysis of extremely skewed IVF case from Project 1

The 90% skewed IVF case from Project 1 had *FMR1* alleles within the normal range (30 and 31 repeats) and a total of 3 AGG interspersions. The extremely skewed natural conception case was not analyzed for *FMR1* repeat length and AGG interspersion number.

Figure 3.5 Determination of *FMR1* CGG repeat and AGG interspersion number in a naturally conceived female newborn with a premutation allele. This individual was heterozygous with alleles of 44 and 59 repeats. The first two AGG interspersions (left) correspond to dips in the signal half-way to baseline. These two AGG repeats could be on either allele. The AGG interspersion farthest to the right, corresponding to a drop in signal intensity to baseline, is on the 59 repeat allele as the signal intensity drop does not occur until after the amplicon corresponding to the 44 repeat allele. Four possible haplotypes for this sample were proposed. CGG-primed amplicons from left to right represent the CGG repeat primer binding closer to the reverse primer at the 3' end. Therefore, signal dips that are farther left correspond to AGG interspersions closer to the 3' end of the repeat region. From comparison with known haplotypes, the bolded repeat sequence configuration seen below is the most likely for this case.



3.4 Discussion

We were able to determine the CGG repeat number and total AGG interspersion number in 108 cord blood samples analyzed. No significant differences were found between the ICSI (n=36), IVF (n=36), and NC (n=36) populations in the frequency of premutation alleles, premutation allele carriers, intermediate alleles, or intermediate allele carriers. In addition, the mean allele CGG repeat number, biallelic mean, the distribution of *FMR1*

genotypes, the distribution of total AGG interspersion number, and the mean AGG interspersion number were similar between the groups. No full mutation alleles were observed. Only one premutation allele was found in a female newborn conceived by natural means. It was speculated that this premutation allele only contained a single AGG interspersion. Random X chromosome skewing was observed in the carrier of this allele. CGG repeat analysis of the extremely skewed IVF case from Project 1 (Chapter 2) revealed two normal repeat alleles.

Based on these preliminary results, it does not appear that females conceived by assisted reproductive technologies are at a higher risk for premature ovarian failure, fragile X-associated tremor/ataxia syndrome, or fragile X syndrome due to the inheritance of alleles with higher CGG repeats or greater instability (less AGG interspersions), or due to the expansion of CGG repeats within their parent's germlines or during *in vitro* embryo culturing.

The majority of alleles in all three groups were found with 29 and 30 CGG repeats which is comparable to what is observed in the general population (Figure 3.4) (Fu et al. 1991). It is proposed that the range of 29 to 30 repeats is indicative of life-long normal ovarian function and that divergence outside this range denotes a higher risk towards POF (Gleicher et al. 2010). As there were no differences between the groups in the frequencies of alleles within this range, it is not likely that the females conceived through ARTs are more at risk for POF. Premutation and intermediate alleles have been associated with varying degrees of POF. We did not observe any differences between the ICSI, IVF, and NC populations in the frequencies of alleles within these ranges, indicating that infants conceived by ART are not inheriting *FMR1* alleles that put them at a greater risk for POF. It has also been observed that repeats below and above the range of 26-34 repeats are associated with a decreased ovarian reserve (Gleicher et al. 2011). There were no differences between the three groups in the

distribution of genotypes based on this range, indicating that females conceived by ART are not at a greater risk for having a decreased ovarian reserve.

If increased expansions occur within the germlines of subfertile parents or during *in vitro* culturing of ART, then there should be higher rates of FXS, FXTAS and POF in children conceived by ARTs. However, this has yet to be observed in the literature either because it has not yet been investigated or the effect is not present. It is also possible that epigenetic factors are controlling *FMR1* gene expression rather than solely CGG repeat number. One study found that variability of *FMR1* expression is found in POF patients independent of CGG triplet repeat number (Schuettler et al. 2011). It has been suggested that there could be multiple transcriptional start sites (TSS) for *FMR1* due to expansions beyond a normal range (Schuettler et al. 2011). It is also proposed that C_pG methylation in the repeat region upstream of *FMR1* and compaction of the chromatin around *FMR1* influence the gene's transcription rate (Schuettler et al. 2011).

One study found that the distribution of *FMR1* alleles between infertile females of different ethnicities were distinctly different (Gleicher et al. 2010). Although Asian women experience poorer IVF outcomes than Caucasian patients, they did not have disproportionally more *FMR1* alleles with increased risk for POF (Gleicher et al. 2010, Gleicher et al. 2011). This shows that the ability of *FMR1* repeat length to denote POF risk may be limited to certain ethnic populations. The ethnic backgrounds of patients in our study were not available.

The distribution of total AGG interspersion number between the ICSI, IVF, and NC populations was similar. This implies that females conceived by ART do not have *FMR1* alleles with greater instability than females conceived normally. In the one ICSI sample that had no AGG interspersions on either allele, the repeat numbers of the alleles were both within the normal range (24 and 30 repeats). Uninterrupted CGG tracts of greater than 34-38 repeats

have been observed to result in unstable transmission from parent to offspring (Eichler et al. 1994). It is unlikely that the alleles for this case are unstable as the *FMR1* repeat regions are below this range for both alleles.

The proposed *FMR1* haplotype of the premutation natural conception case indicated that there is 2 AGG interspersions on the normal allele (44 repeats), but only 1 AGG interspersion on the premutation allele (59 repeats). Zero to one AGG repeats are generally found in premutation alleles (Yrigollen et al. 2011). Premutation alleles are very unstable and can easily expand to full mutation alleles (Chakravarti 1992). The premutation allele in this case only has 59 repeats which is on the lower end of the premutation range of 55-200 repeats. Therefore, this premutation allele is likely not as unstable as premutation alleles on the larger end of this range. Nevertheless, premutation alleles with as little as 59 repeats have been shown to expand to full mutation alleles within a single generation (Nolin et al. 2003). In one study which examined the transmission of intermediate and premutation alleles to their offspring, they observed a risk of 5.4% (2/37) of maternal alleles of repeat sizes 55-59 expanding to full mutations within a single generation (Nolin et al. 2003). However, those two 59-repeat alleles that expanded to full mutations in one generation did not have any AGG interruptions within the CGG repeat tract (Nolin et al. 2003). It is possible that the premutated allele could be unstable in later generations. As previously mentioned, uninterrupted CGG tracts of greater than 34-38 repeats can result in unstable transmission (Eichler et al. 1994). The 59 repeat premutation allele had a speculated sequence configuration of $(CGG)_9AGG(CGG)_{49}$. Hence, there is an uninterrupted CGG tract of 49 repeats which could be very unstable and expand in future generations. In one study, 30.9% (13/42) of maternal alleles with repeat size 55-59 were found to be unstable with 1 allele contraction and 12 expanding in the next generation (Nolin et al. 2003). The repeat size changes ranged from -1

to +63 (all still within premutation range) (Nolin et al. 2003). These mothers had no family history of fragile X (Nolin et al. 2003). The premutated allele in this naturally conceived female could become more unstable and lead to expansion in the next generation if an AGG interspersion deletion or an A to C transversion occurred during pre-meiotic mitotic divisions, meiosis, or post-zygotic mitotic divisions.

We were unable to determine the parental origin of the 59 repeat (premutation) *FMR1* allele in the naturally conceived newborn. It is unlikely that this female newborn will experience symptoms of POF or FXTAS as she had random (53.8%) X chromosome skewing, and hence there was not preferential activation of the premutated allele. In one study, three families carrying premutation alleles (63 to 163 repeats) were discordant for POF (Bodega et al. 2006). POF patients in these families always showed at least a twofold higher activation of the X chromosome with the intermediate/premutated allele than the corresponding healthy sister (Bodega et al. 2006). It was observed that the higher the repeat number the less X chromosome skewing was required to cause POF (Bodega et al. 2006). In one of the families, the POF patient only had 52% skewing towards the premutated allele, however the repeat size was 91 (Bodega et al. 2006). The corresponding healthy sister only had 8% skewing towards the premutated allele (Bodega et al. 2006). In another family, the POF patient had 94% skewing towards an intermediate allele of only 50 repeats while her healthy sister only had 53% skewing towards the same allele (Bodega et al. 2006). Some studies however, report no evidence for X inactivation effecting the manifestation of POF (Murray et al. 2000, Rodriguez-Revenga et al. 2009, Sullivan et al. 2005). Studies have also shown that skewing towards the premutation allele may result in different severities of FXTAS in women (Berry-Kravis et al. 2005, Hagerman et al. 2004).

The extremely skewed IVF case from Project 1 (Chapter 2) had both alleles in the normal range (30 and 31 repeats). This indicates that abnormal *FMR1* repeat length was likely not the cause of preferential X-chromosome inactivation.

Previous studies have investigated the *FMR1* repeat counts in the alleles of women undergoing fertility treatments. Some studies have shown that infertile women have *FMR1* alleles with slightly higher repeat numbers then women in the general population (Gleicher et al. 2009). Our study is the first to look at *FMR1* CGG repeat length and AGG interspersion number in the alleles of females conceived by ARTs to assess whether *FMR1* alleles associated with decreased fertility are being passed on and whether *FMR1* repeat expansion may be occurring in the germlines of subfertile parents or during embryo culturing. Although we did not find any significant differences between the females conceived by the ARTs of ICSI and IVF compared to those conceived naturally, our sample size is small and further study is warranted to ensure any lack of association.

Chapter 4 : Conclusions, Limitations, and Future Directions

4.1 Summary and conclusions

Since the birth of the first baby conceived by IVF in 1978, millions of babies have been born by assisted reproductive technologies. However, there have been concerns about the short and long term risks of ARTs to children conceived from these artificial methods. Pregnancies derived from *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI) have been associated with increased rates of chromosome abnormalities (Bonduelle et al. 2002, Wennerholm et al. 2000), congenital malformations (Hansen et al. 2002), low birth weight, pre-term births (Jackson et al. 2004, Kallen et al. 2002, Katalinic et al. 2004), multiple gestations (Schachter et al. 2001), and imprinting disorders (Manipalviratn et al. 2009). ART enables sub-fertile individuals to circumvent the natural selective pressures that are involved in human reproduction. Therefore, the risks of ARTs may be due to the underlying causes of sub-fertility in these individuals such as abnormalities in sperm or egg. IVF and ICSI involve unnatural induction of ovulation in women, *in vitro* handling of sperm and egg, as well as the growth of the embryo *in vitro*. Hence, any of these artificial processes could also be contributing to the increased rates of abnormalities in ART conceived pregnancies.

In Chapter 2 (Project 1), we investigated the occurrence of skewed X-chromosome inactivation in newborn females conceived by ICSI, IVF, and naturally. X-chromosome inactivation is the process by which one X chromosome in the somatic cells of females is transcriptionally silenced. Preferential inactivation of one X chromosome can result in the expression of X-linked recessive traits in heterozygous women. Skewed XCI can also be indicative of a small number of cells being present in the embryo at the time of inactivation and may suggest the presence of an X chromosome abnormality. We hypothesized that XCI may be altered in newborns conceived through ARTs as they are subject to *in vitro* culturing

conditions and a greater risk of chromosome abnormalities. We did not find that females born via the infertility treatments of ICSI and IVF were at a greater risk for having mildly skewed (≥75%) or extremely (≥90%) skewed X inactivation than females born by natural means. Nor did we find any significant differences in the distribution of skewing or mean level of skewing between the groups. These results indicate that XCI is either not affected by *in vitro* culturing conditions, occurs after ART treatments, or the effect is limited to non-viable embryos. Our results also suggest that viable blastocysts conceived through infertility treatments are not slower growing and do not have higher rates of chromosome mosaicism as compared to viable blastocysts conceived naturally. Our assessment of the placental tissues of an extremely skewed IVF case showed that preferential skewing was not present at all sites and tissues of the placenta. In addition, we observed that XCI status of chorion and amnion correlated well when they were from the same site. These findings are in agreement with other studies that have shown that weaker selective pressures (that would cause skewed XCI) occur in the placenta and that placental samples taken at the same site, but at different depths show similar skewing patterns (Penaherrera et al. 2000, Penaherrera et al. 2003, Peñaherrera et al. 2012) . Our results further support the findings of other studies that skewed XCI is not associated with ARTs (King et al. 2010, Robinson et al. 2005).

In Chapter 3 (Project 2), we assessed the *FMR1* CGG repeat and AGG interspersion number in newborn females conceived by ICSI, IVF, and by natural means. Higher ranges of triplet repeats in this gene have been associated with varying levels of POF, FXTAS, and FXS. The larger the repeat region and the less AGGs interspersed within the CGG repeat tract, the greater the susceptibility of the allele to expansion. We hypothesized that subfertile mothers could be passing on *FMR1* alleles associated with reduced fertility to their offspring or that repeat expansion may be occurring in the germlines of subfertile parents or during *in vitro*

culturing. Our findings did not support any of these hypotheses. We did not find any difference between the ICSI, IVF, and NC populations in the frequency of intermediate, premutation, and full mutation alleles. Nor did we find any differences in the mean allele CGG repeat number or biallelic mean. Differences in the distribution of *FMR1* genotypes and total AGG interspersion, as well as the mean AGG interspersion number were not observed between the groups. Our results suggest that females born through infertility treatments are not at a greater risk of having a decreased ovarian reserve or of developing FXTAS and FXS than babies conceived naturally. It is possible that epigenetic factors are controlling *FMR1* gene expression to cause toxic levels of *FMR1* mRNA in premutation carriers with POF or FXTAS. It is also possible that the ability of *FMR1* repeat length to denote POF risk may be limited to certain ethnic populations. However, the current sample size is too small to rule out any association between ARTs and *FMR1* CGG repeat number.

Despite the findings of some studies that ICSI and IVF are associated with increased rates of abnormalities in infants conceived by these methods, the rates of some of these abnormalities is quite low. The incidence of Beckwith-Wiedemann syndrome in IVF conceived infants is very rare at only about 1/4000 (Allen et al. 2006). Only seven cases have been reported as of 2009, in which a child with Angelman's syndrome was conceived through IVF or ICSI (Manipalviratn et al. 2009). In addition, some studies have failed to show an association between imprinting disorders (i.e. BWS and AS) and ARTs (Odom and Segars 2010). In summary, the two studies in this thesis indicate that female newborns conceived through ARTs are not at a greater risk of having skewed XCI or *FMR1* alleles with high triplet repeat numbers. These findings further support the claim that the risk of abnormalities in ARTs is relatively low.

4.2 Limitations

Although methylation based assays are the standard for determining XCI status, there are pitfalls to this method. Incomplete digestion with methylation sensitive enzymes could be occurring leading to the overrepresentation of an allele as being inactive. In addition, around 10% of the time, patients are homozygous for the androgen receptor gene causing us to determine the XCI status from other genes with differentially methylated restriction sites. Sometimes skewing values determined from different genes do not always correlate with each other (Beever 2002). However, the XCI status of mildly skewed and extremely skewed cases in our study were only determined at the *AR* locus. Another shortcoming of the XCI methylation based assay is that DNA methylation status can be altered. The three genes used in our study have differential methylation of two restriction sites near a polymorphic repeat. If only one of the restriction sites was improperly methylated it could result in false findings. It has also been shown that methylation status does not always correlate with gene expression. One study found that in neonates, the skewing values determined from DNA differed from those determined from RNA (El Kassar et al. 1998). Despite these limitations, the methylation XCI assay is still the gold standard when it comes to determining XCI status do to its feasibility and shown reliability. Assays to determine XCI status by examining SNPs near C_pG sites across the entire X chromosome are being developed and may be a more accurate way of determining XCI status in the future (Swierczek et al. 2012). Additionally, it is possible that there are tissue-specific differences in the degree of skewing. We determined XCI status from the leukocytes of cord blood. It has been shown that the degree of skewing in leukocytes does not always correlate with tissues from various body parts (Azofeifa et al. 1996, Gale et al. 1994). Nevertheless, it has also been shown that the methylation status of the majority of X-linked genes is similar between different tissues (Cotton et al. 2011).

Although the novel Asuragen *FRM1* PCR kit enables amplification of premutation and full mutation alleles there are limitations to the information this assay can provide. PCR analysis enables accurate sizing of *FMR1* alleles, but does not provide information on *FMR1* methylation status (Sherman et al. 2005). Methylation status of *FMR1* plays a role in the expression of the gene and toxic levels of *FMR1* mRNA is what is thought to contribute to the formation of FXTAS and POF in premutation carriers. In addition, *FMR1* CGG repeat length does not always correlate with *FMR1* expression (Schuettler et al. 2011). Therefore, although we were able to accurately determine the CGG repeat number of the *FMR1* alleles of the subjects in our study, we were not able to determine *FMR1* mRNA and FMRP levels which would have given us a more complete picture of what is going on. Another shortcoming of this assay is that in full mutation alleles the exact CGG repeat number cannot be determined due to the threshold of the capillary electrophoresis gel polymer. Hence, full mutation alleles can only be categorized as having >200 repeats. Additionally, the exact number and position of AGG interspersions within the *FMR1* repeat tract normally cannot be determined for each allele. This information can only be speculated by comparing with well known *FMR1* haplotypes.

In Project 2 (Chapter 3), if we are really trying to examine if subfertile mothers are passing on high repeat *FMR1* alleles to their children or if repeat expansions are occurring in the germlines of subfertile parents or during *in vitro* culturing then we need to do *FMR1* testing on the parent's blood. If we observe *FMR1* alleles with higher CGG repeat counts in females conceived by ARTs compared to spontaneously, then we can only speculate what might be the cause without the parent's blood. If we had access to parental blood we could see if there is higher occurrence of high repeat *FMR1* alleles in mother's utilizing fertility treatments compared to those whom conceived naturally. In addition, we could see if the

FMR1 repeat length changed from parent to child to decipher whether expansion may be occurring within germlines or during *in vitro* culturing. Currently, we only have access to the maternal deciduas of the placenta which can only give us information about the mother's *FMR1* alleles. In addition, the decidua is normally contaminated with fetal villi making it difficult to determine which allele was passed on by the mother.

4.3 Future directions

More samples need to be analyzed to rule out any associations between ARTs and skewed XCI or abnormal *FMR1* triplet repeat counts. This is especially true for Project 2 as only 36 samples were analyzed in each population group and this is the first study that has looked at the *FMR1* repeat length in newborn females conceived by ICSI and IVF. The inclusion of intrauterine insemination (IUI) samples in both projects would aid in isolating the effect of *in vitro* culturing conditions. In IUI, sperm in injected into the uterine cavity of the female recipient, and hence fertilization and growth of the early embryo occurs *in vivo*.

For Project 1, further analysis into the cause of extreme skewing in the two cases we found should be performed. The XCI status in the placental tissues of the naturally conceived extremely skewed case needs to be determined. In addition, common X chromosome abnormalities that cause extreme skewing can also be explored in our two extremely skewed cases. This would be difficult however, as certain X chromosome abnormalities have been shown to cause skewing in some individuals, but not others. Ideally, it would be useful to investigate the XCI status of infants conceived from embryos that underwent PGD (preimplantation genetic diagnosis). The main method of PGD is to remove one or two blastomeres from the day 3 embryo (6 to 8 cell stage) for genetic diagnosis before implantation. Therefore, it would be interesting to see if X chromosome skewing would be

affected by this as there could potentially be a smaller precursor cell pool present at the time of inactivation. However, getting approval for this study would be very difficult as parents likely do not want further testing after the invasive procedure of PGD. A less used, alternative PGD method is to remove one or two cells from the trophectoderm of the blastocyst for genetic diagnosis before implantation. This may not have an effect on the X chromosome skewing status of the fetus as the trophectoderm does not contribute to the fetal tissues.

For Project 2, the premutated NC case should be further explored. Although it is unlikely that we will ever have access to the parental blood of this case, we have access to the maternal decidua of the placenta of this newborn. We should analyze the *FMR1* alleles in the decidua to see if we can decipher whether or not the premutated allele was passed on from the mother and whether expansion in the germline or during *in vitro* culturing may have occurred. As mentioned above, *FMR1* CGG repeat number may not always accurately portray the level of *FMR1* expression. We do not have RNA for the cord blood for the majority of our cases so we would not be able to determine levels of *FMR1* mRNA. However, one study used X chromosome skewing values at the *AR* and *FMR1* loci to determine a genotype repeat size that may be a better indicator of the relative activity of each of the alleles (Bretherick et al. 2005).

If our hypotheses for Project 2 are correct, then high repeat *FMR1* alleles could also be present in newborn males conceived by ARTs. Premutation *FMR1* alleles are associated with POF as well as FXTAS. Therefore, if subfertile mothers are passing on *FMR1* alleles associated with reduced ovarian reserve to their daughters they could be passing on *FMR1* alleles that put their sons at a higher risk of developing FXTAS. In addition, if *FMR1* repeat expansions are occurring in the germlines of mothers utilizing fertility treatments or during *in vitro* culturing, then it is possible that males born through ARTs would have higher repeat

FMR1 alleles that put them at a greater risk of developing FXTAS and FXS. In the future, we should investigate the *FMR1* CGG repeat and AGG interspersion number in the alleles of male newborns conceived by ARTs.

Overall, the findings of our two studies add to the knowledge about the safety and risks associated with assisted reproductive technologies. Although we did not find that females conceived by ICSI and IVF at a greater risk for having abnormal X chromosome skewing or unstable *FMR1* alleles than females conceived spontaneously, the number of samples analyzed is too small to rule out any associations. Further research is warranted as there are few studies which have looked at the XCI status in infants conceived by ARTs and we are the first to investigate the *FMR1* allele repeat size in babies conceived artificially.

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