X-CHROMOSOME INACTIVATION PATTERNS IN HUMAN EXTRAEMBRYONIC AND FETAL TISSUES

by

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Abstract

X-chromosome inactivation (XCI) compensates for dosage of X-linked genes in female mammals. XCI is generally random and clonally inherited in embryonic tissues, XCI patterns in the placenta appear to be distinct and vary among different mammalian species. The aim of this thesis was to further our understanding of XCI patterns in human placentae with three main goals:

1) To determine if XCI shows a parental bias in human placenta as it does in mouse and other mammals, XCI patterns were studied in amnion, chorion, trophoblast and mesenchyme of 14 normal female term placentae, and 11 first trimester placentae using methylation based assays (*AR* and *FMR-1*). XCI patterns were heterogeneous within most term placentae, in both direction and degree of skewing, indicating that there is no clear parental bias to XCI in term placentae. However, a trend towards the preferential inactivation of the paternally derived X was observed in trophoblast of first trimester placentae. Investigation of a placenta with a paternally derived X/autosome translocation also supports the idea that preferential inactivation of the paternal X is probably not a requirement for normal development in humans as it is in mice.

2) To determine whether all or most X-linked genes are hypomethylated in human extraembryonic tissues, XCI was studied in 20 CVS samples, using methylation-based assays for 7 X-linked genes (*MAOA, ARAF, AR, XIST, DXS6673E, GRIA3E* and *FMR-1*). Incomplete methylation was observed for all genes except *XIST*. The low methylation suggests either gene derepression, or that an alternative mechanism of silencing is involved in gene regulation in placenta.

3) To use XCI studies to understand the characteristics of cell dynamics in cases of trisomy preferentially confined to the placenta, I studied XCI in embryonic and extraembryonic tissues from 25 such cases. Extremely skewed XCI was found in diploid fetal but not placental tissues of most of these individuals. This may result from the selective elimination of trisomic cells from the embryonic precursor cell pool subsequent to XCI. A significant correlation between high levels of skewing and abnormal fetal outcome was evident, suggesting that when high levels of trisomy are found in the placenta, there is an increased probability of "hidden" trisomy mosaicism in the fetus.

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List of Abbreviations

AF	amniotic fluid
BrdU	bromodeoxyuridine
CPM	confined placental mosaicism
CVS	chorionic villi sampling
DMSO	dimethylsulfoxide
dpc	days post coitum
dy	days
EDTA	ethylendiamine-tetraacetic acid
ES cells	embryonic stem cells
FITC	fluorescein isothiocyanate
HBSS	Hanks balanced salt solution
hCG	human chorionic gonadotropin
ICM	inner cell mass
ICSI	intracytoplasmic sperm injection
IUD	intrauterine death
IUGR	intrauterine growth restriction
LB	live born
LINES	long interspersed nuclear elements
LMP	last menstrual period
mat	maternal
mo	months
NCBI	National Center for Biotechnology Information
ND	neonatal death
pat	paternal
PBS	phosphate buffer saline
PK	proteinase K
SAB	spontaneous abortions
SDS	sodium dodecyl sulphate
SPSS	Statistical Package for the Social Sciences

TA	therapeutic abortions
UPD	uniparental disomy
wks	weeks
Xa	active X
Xa^P	active paternal X
Xa^M	active maternal X
XCI	X-chromosome inactivation
Xi	inactive X
XIC	X-chromosome inactivation center
Xi^P	inactive paternal X
Xi^M	inactive maternal X
X^M	maternal X
X^P	paternal X

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Dedication

A mi adorada abuela, inspiracion de tantos suenos idos,

A mi madre querida, el pilar de toda mi vida

A mis hijos Martin Alejandro y Nicole Gabrielle

quienes me enseñaron el significado de amor absoluto

y se han convertido en mis alas, mi sonrisa,

mis suenos...

Chapter 1: Introduction

1.1 Opening remarks

The extraordinary complexity of the processes of embryonic development and placentation in humans has fascinated researchers for decades. In an attempt to unravel the details of the story and given the obvious ethical limitations of the studies in humans, many researchers have resorted to other species such as the mouse. In the process it has been discovered that while there are many features that we share as fellow mammals, there are many also that make us unique as a species. In the past decade, with the development of *in vitro* fertilization techniques, researchers have been able to follow and document the early stages of embryonic development; however, what happens inside the womb remains largely unknown. This knowledge becomes particularly relevant for the understanding of pathological conditions in the embryo and its supporting placenta, such as the presence of trisomy mosaicism. Throughout the present study I will try to demonstrate the importance of the use and understanding of epigenetic factors such as XCI as indirect tools to gain an insight into some aspects of human in-utero growth and development. The relevance of studies as the present goes beyond the prenatal period, considering that in many cases, postnatal outcome is largely based on events occurring during embryonic and fetal development.

1.2 X-chromosome inactivation (XCI)

1.2.1 XCI as a mechanism for dosage compensation in mammals.

In 1961 Mary Lyon first hypothesized that in mammalian females one of the two X chromosomes undergoes inactivation, to compensate for the dosage imbalance that would

result from having two copies of the X chromosome relative to the single copy present in males (Lyon 1961). By this time, Barr and Bertram had shown the presence of a heteropyknotic "sex chromatin", later called Barr body, in the nucleus of female cells and Ohno had proposed that this structure was indeed an X-chromosome (Barr and Bertram 1949; Ohno et al. 1959). Based on her observation of the pattern of expression of X-linked coat color genes in female mice, on the known viability of XO female mice and on Barr's and Ohno's observations, Lyon proposed a mechanism by which one of the X-chromosomes at random was genetically inactivated in female mammals and could be visualized cytogenetically as the Barr body, usually at the periphery of the cell nucleus (Barr and Bertram 1949; Ohno et al. 1959; Lyon 1961). Soon after, with the use of biochemical markers such as enzyme variants of Glucose-6-phosphate dehydrogenase (*G6PD*), Davidson and colleagues showed evidence of this process in humans (Davidson et al. 1963).

The need for a mechanism of dosage compensation of sex linked genes is not exclusive to mammals. In *Drosophila melanogaster*, dosage compensation is ensured through hypertranscription of the single X-chromosome present in the males. In the nematode *C. elegans,* both X chromosomes are down regulated in the XX hermaphrodite (summarized in Heard et al. 1997; Franke and Baker 2000; Meyer 2000). The human X and Y chromosomes, like those of other animals, are believed to be derived from a pair of autosomes that have undergone differentiation throughout evolution constituting the chromosomal basis of sex determination (Graves 1996; Lahn and Page 1999). It has been hypothesized that during mammalian evolution and the acquisition of sex-determining function, the ancestral Y chromosome underwent a series of inversions that suppressed recombination in specific regions (Lahn and Page 1999). This lack of recombination is thought to have led to high levels of differentiation in these regions. In fact, it is believed that the Y chromosome has

gradually lost many of its genes, resulting in a dosage imbalance between males and females, which was ultimately compensated for by the up-regulation of homologous genes on the X-chromosome in males. The restoration of optimal expression in females was then likely achieved by X inactivation. (Adler et al. 1997; Jegalian and Page 1998). Also, it is evident that the X-chromosome not only retained most of its original genes but also acquired some additional autosomal genes throughout evolution (Spencer et al. 1991; Graves 1996; Graves et al. 1998; Jegalian and Page 1998). Various regions of the X and Y chromosome retained homology, in particular those that undergo obligatory recombination in male meiosis or pseudoautosomal regions (PARS). There are two limited regions of sequence identity between X and Y that allow pairing and recombination during meiosis: PAR1, which is located in distal Xp and Yp and PAR2, located in distal Xq and Yq (Graves et al. 1998).

1.2.2 The XCI process

The remarkable biological process of XCI can be better understood in terms of three different stages:

Marking: The process of marking involves issues of choice of the X to be inactivated (this process is mostly at random) and counting, as it is clear that only one X remains active per each diploid set of chromosomes, implicating limited autosomal factors in an initial activating mark (Riggs 1990). It is believed that during random XCI, counting and choice precede or occur at the onset of initiation of XCI. Studies in chimaeric mice showed that marking or commitment to inactivation occurs prior to differentiation at or shortly after 3.5 dpc, while XCI was not observed in the inner cell mass until at least 5.5 dpc (Gardner and Lyon 1971). Studies of marking in humans rely on indirect evidence provided by the distribution of XCI skewing. The term XCI skewing refers to the non-random pattern of XCI

in which one of the X chromosomes is inactivated more often than the other in a given tissue or individual. In other words, it represents any deviation from a 1:1 ratio of maternal to paternal inactive X chromosomes in each individual or in a specific tissue. Using data on XCI skewing, it is possible to estimate the number of embryonic precursor cells that gave rise to the individual or the specific tissue. Based on this method, the number of embryonic precursors at the time of commitment to XCI has been estimated as 14-16 cells (Fialkow 1973; Tonon et al. 1998). Other studies using maximum likelihood analysis (Puck et al. 1992) and simulations of XCI patterns compared to actual observed distributions of skewing in blood and buccal mucosa of normal women (Monteiro et al. 1998) have estimated the haematopoietic and embryonic precursor cell pool (number of cells in the ICM) at the time of marking as ~10-20 cells and 4 - ≤16 cells respectively. Earlier studies based on protein isoforms of *G6PD* had estimated that XCI occurs when approximately 20 embryonic precursor cells are present (Fialkow 1973).

Major players at the marking stage are the X-chromosome inactivation centre (XIC), a 1Mb region that contains several elements thought to have a role in XCI and where marking is believed to occur (Willard 1996). There are several loci in this region with known important functions in the process: one is the X inactive specific transcript (*XIST* gene), necessary in *cis* for inactivation to occur (Brown et al. 1991). This gene is expressed at low levels from both X chromosomes in the early stages of human embryonic development but later on is expressed exclusively from the inactive X (Brown et al. 1991; Daniels et al. 1997). A second gene in the region generates a transcript antisense to *Xist* prior to the onsent of XCI and is called *Tsix*. Deletions of this gene in mice, in which it is thought to be a critical regulator of early *Xist* expression, lead to the inactivation of the deleted X, suggesting that

this is the site for the activating mark to occur (Lee et al. 1999; Lee and Lu 1999). Studies on the recently identified human *TSIX* have shown, however, that the human gene lacks the key regulatory elements necessary for an imprinted function such as that in mice and that the antisense *TSIX* transcripts are unable to repress *XIST*. Unlike in the mouse, *TSIX* was not found to be maternally imprinted in placental tissues and it was found to be transcribed in both embryonic and extraembryonic tissues throughout embryogenesis (Migeon et al. 2002). A third locus of interest is *DXPas34*. This is a CpG rich region that lies 3' of *XIST* and is hypermethylated on the active X chromosome in somatic cells. This locus reportedly contains the main initiation site for *TSIX* transcription (Courtier et al. 1995; Lee et al. 1999). Finally, there is the X-chromosome controlling element locus (Xce), which has been described in mice, and whose existence is still controversial in humans (Brown and Robinson 2000). Xce affects the choice of the X to be inactivated or to remain active. X chromosomes with strong Xce alleles are likely to remain active (Avner and Heard 2001).

Two genetically distinct autosomal mutations (X-inactivation autosomal factors 1 and 2 or *Xiaf1* and *Xiaf2*), have been identified through a phenotype-driven genetic screen involving chemical mutagenesis in the mouse. Seemingly, these two mutations have dominant effects on X-chromosome inactivation choice early in embryogenesis. As a result, primary XCI patterns are significantly different from those predicted by their Xce genotypes (Percec et al. 2002). Additionally, it has been reported that imprinted XCI in mouse that leads to preferential inactivation of the paternally derived X in extraembryonic tissues, depends in part on the polycomb group (Pc-G) protein *Eed*. Loss of this gene leads to reactivation of the inactive X chromosome in extraembryonic tissues but apparently has no effect in somatic cells (Wang et al. 2001).

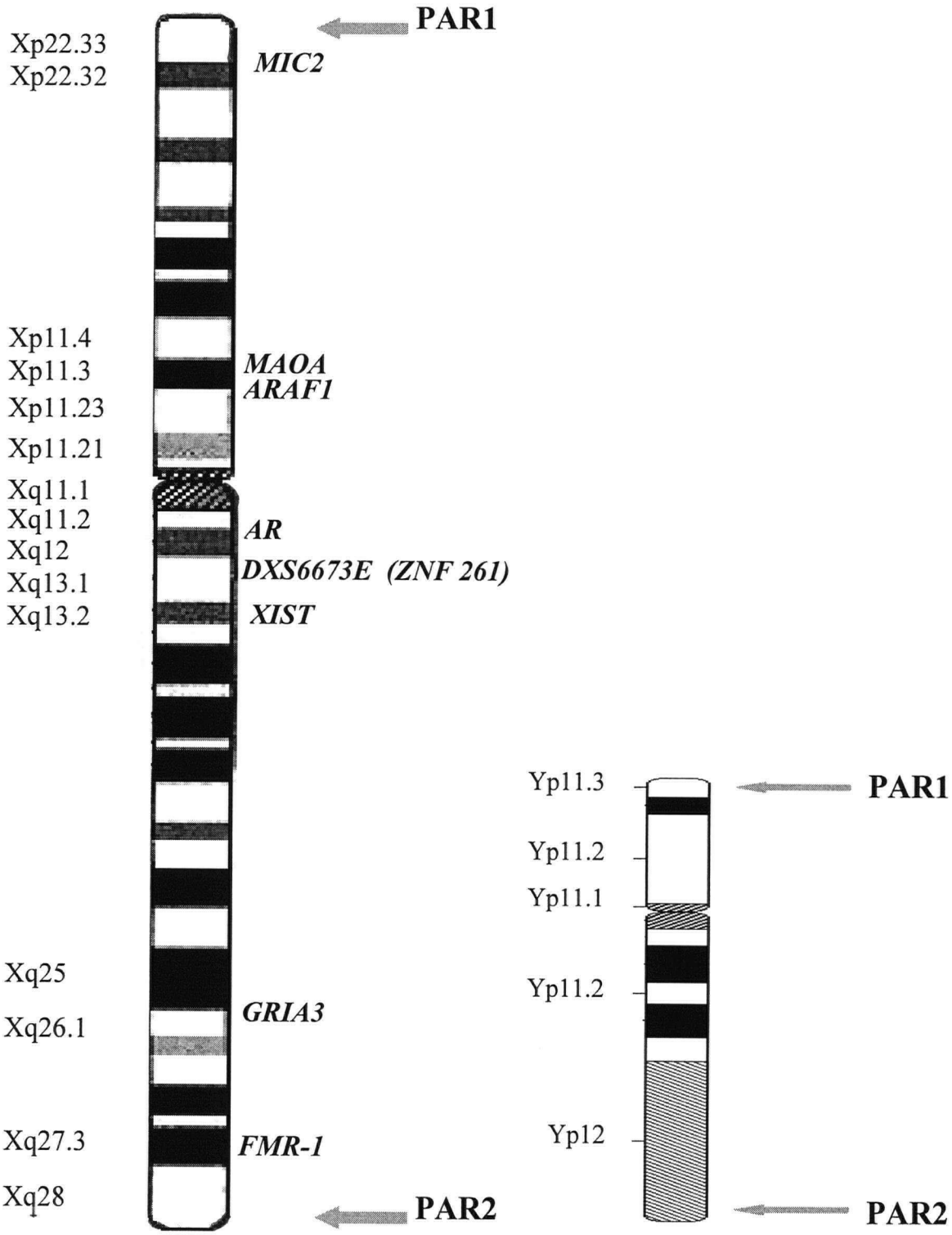
Inactivation: This process involves initiation and subsequent spreading of XCI. *XIST* plays a major role in this stage. This gene has been found to be both necessary and sufficient for inactivation (Penny et al. 1996; Marahrens et al. 1997; Wutz and Jaenisch 2000). The inactivation process has proven to be developmentally regulated, as *Xist* expression was thought until recently to only be capable of inducing inactivation in embryonic cells and not in somatic cells (Wutz and Jaenisch 2000). However, studies in fibrosarcoma cell lines expressing ectopic human *XIST* have shown that *XIST* RNA can induce *de novo* chromosomal inactivation in post-differentiation cell lines (Hall et al. 2002). From studies in mouse ES cells, it seems clear that ongoing *Xist* expression alone can induce inactivation in such cells; however, this process is reversible, unless cells have differentiated. Upon differentiation, additional features of XCI such as late replication and histone deacetylation are included in the process, rendering it irreversible (Wutz and Jaenisch 2000). Once *XIST* is stably expressed, it works as a large functional RNA that co-localizes and coats what will be the inactive X chromosome. XCI then spreads along the X chromosome with the help of "way stations" that allow the boosting of the inactivation signal. The nature of these elements is still unknown (Gartler and Riggs 1983; White et al. 1998), however it has been suggested that interspersed repetitive elements of the LINE type, which are found in higher numbers on the X chromosome, act as booster elements to promote the spread of *Xist* RNA. The contact of this functional RNA with the LINE elements causes them to be sensed as repeated elements and this activates the cell machinery that is involved in repeat-induced gene silencing (Lyon 1998).

XCI does not affect all X-linked genes equally and there are numerous genes that escape inactivation. These genes are either clustered in specific regions or interspersed between genes that are subject to XCI. The largest survey of expression of X-linked genes to

date, using somatic cell hybrids, has shown that at least 15% of genes on the human inactive X escape inactivation (Carrel et al. 1999). A more recent study of this kind using cDNA arrays has identified additional genes that are also expressed from the inactive X-chromosome (Sudbrak et al. 2001). Genes that escape XCI generally display features similar to their homologues in the active X, such as early replication, unmethylated CpG islands and histone H4 hyperacetylation (reviewed in Heard et al. 1997). Several genes that escape inactivation in humans have Y homologues and many are clustered in the XpPAR (PAR1) and XqPAR (PAR2) regions (see Figure 1.1). Other genes that escape XCI have no reported Y homologues or have non-functional Y homologues and the biological significance of an increased dosage of these genes in females is unknown (Disteche 1995). The number of genes that escape XCI in mice is considerably smaller and in fact there are various genes that escape XCI in humans but not in mice (e.g the *ZFX* gene), a fact that possibly explains the better prognosis of murine XO fetuses (Disteche 1997). The explanations for these differences between mice and humans are still speculative.

In addition to genes that are subject to XCI and those that escape this process, there is a third category of genes that show variable expression from the Xi. These genes are therefore expressed in some females and not expressed in others (Anderson and Brown 1999; Carrel et al. 1999).

Figure1.1 Ideogram of the human X and Y-chromosomes, indicating the pseudoautosomal regions and the position of the genes studied in this thesis (modified from NCBI http://www.ncbi.nlm.nih.gov)



Maintenance: It is likely that *XIST* expression induces changes upon the inactive X-chromosome that are ultimately responsible for the stable maintenance of the inactive state. There are multiple factors that appear to contribute in an additive manner to the extremely stable maintenance of XCI, namely chromatin condensation and peripheral location, late replication, histone hypoacetylation, Macrohistone H2A1 localization and hypermethylation at the 5' ends of genes (reviewed in Brown 2001). Once established, XCI can be maintained even in the absence of *XIST* expression (Brown and Willard 1994). Furthermore, the disruption of any given factor that contributes to the maintenance of the inactive state does not cause reactivation *per se* but contributes to an increased frequency of reactivation (Brown 2001).

The order of events leading to a stable inactive state is still unclear and some studies have found differences between this process during mouse ES cell differentiation as compared to *in vivo* mouse embryos (Constanzi et al. 2000). Timing differences for these factors between embryonic and extraembryonic tissues have been suggested (Brown and Robinson 2000). Regardless of the sequence of events, somatic inactivation in humans is very stable and clonally heritable. The redundancy of the different epigenetic modifications, mentioned above, seems to ensure the stability of the inactivation process (Avner and Heard 2001). At the gene level, however, inactivation is less stably maintained and more sensitive to loss of methylation as demonstrated by studies in somatic cell hybrids (Gartler and Goldman 1994) and studies in extraembryonic tissues of humans (Migeon et al. 1986).

In marsupials, overall instability of the inactive state is characteristic, as loci on the inactive X are readily reactivated in culture (Migeon et al. 1989). The instability of XCI differs between species and between X-linked genes (Cooper 1993). There is also evidence for tissue-specific inactivation of different loci on the paternal X (Graves 1996). In

eutherians, including mice and humans, the inactive state seems relatively more stable although there are several exceptions to this rule as the reported reactivation of the certain genes in aging mice (e.g. ornithine transcarbamylase (*Otc*) locus) (Wareham et al. 1987; Brown and Rastan 1988) and the reactivation of X-linked genes through the use of demethylating agents (Hansen et al. 1996) and hybridization of human cells (chorionic villi) with mouse cells (Migeon et al. 1986).

Features of the Inactive X

Late replication: There are several features that characterize the inactive X such as the fact that it replicates asynchronously relative to its homologue that remains active. This characteristic is one of the first to be detected on the inactive X and is shared by both eutherians and metatherians (man, mice and marsupials) (reviewed in Gartler and Riggs 1983). Studies of replication timing are often done using Bromodeoxyuridine (BrdU) (Verma and Babu 1995) which is a thymidine analog that can be incorporated into DNA during replication. Labelling the DNA with BrdU during the final hours of culture allows the visualization of the late replication pattern. BrdU substituted DNA can be detected through either fluorescent staining or Giemsa methods. More efficient methods of detection use antibodies for BrdU which specifically bind the chromosomal DNA that has been substituted with the base analog. The antibodies are usually coupled with a fluorochrome such as FITC that allows detection by fluorescence microscopy (Verma and Babu 1995). Studies of a series of X-linked genes that are subject to inactivation in humans, have found that in all cases the active X alleles of the genes replicated earlier than the alleles on the inactive X. Additionally, when replication patterns were analyzed for genes that escape XCI, both alleles were found to replicate synchronously (reviewed in Heard et al. 1997). These results suggest

that replication timing of different X-linked genes reflects their specific activity status regardless of the status of the X chromosome where they are located.

Chromatin conformation: Another feature of the inactive X is its chromatin conformation. The inactive X chromosome is characterized by tightly packed DNA that forms a structure called the Barr body, which is usually located towards the periphery of the cell's nucleus (Barr and Carr 1961).

Histone deacetylation: Modifications in the acetylation status of lysine residues in the core histones (protein components of the nucleosome involved in DNA packaging) are also associated with XCI in mammals. In mice and humans, the inactive X chromosome (Xi) is depleted of the acetylated isoforms of histones H2A, H3 and H4, suggesting a role of histone hypoacetylation in heterochromatinization. The finding of hyperacetylated histones in the X pseudoautosomal regions, where many genes escape XCI further supports this idea (reviewed in Heard et al. 1997).

Cytosine methylation: The addition of a methyl group to cytosine residues at carbon atom 5 (5-methylcytosine) within the context of a CpG dinucleotide has been associated with stable maintenance of the inactive state, so it is considered an important component of the XCI process. Perhaps, one of its most interesting characteristics is the fact that methylation patterns can be inherited from one cell to its daughter cells as a consequence of the properties of DNA methyltransferases that are capable of methylating hemi-methylated DNA (methylated on one strand only) (reviewed in Heard et al. 1997). While there are no clear differences in terms of global methylation patterns between the inactive and the active X

(Bernardino et al. 1996), individual CpG islands associated with 5' end of genes are heavily methylated on the inactive X and unmethylated on the active X of somatic tissues (Tribioli et al. 1992; Carrel and Willard 1996). However, detailed methylation studies on CpG islands at various murine and human X-linked genes (Pfeifer et al. 1990a; Pfeifer et al. 1990b; Hornstra and Yang 1994) have shown that several unmethylated CpG's are interspersed with methylated CpG's on the inactive X chromosome. Specifically, human *PGK1* reportedly has 60 out of 61 CpG's methylated in 800-bp of the promoter region in the inactive X-chromosome (Pfeifer et al. 1990b). The mouse *Pgk1* promoter, on the other hand, has only 1 CpG consistently methylated (Tommasi et al. 1993), indicating that there is no clear conservation of 5' region methylation patterns within and across species. It seems possible that for some X-linked genes, global promoter methylation patterns rather than CpG site-specific methylation correlates with the activity status, although there is still no certainty that all the relevant CpG sites have been looked at (Heard et al. 1997).

The methylation status of some X-linked genes is less clear in extraembryonic tissues of both mouse and human. For example, the *Hprt* locus is relatively unmethylated in murine extraembryonic tissues (Kratzer et al. 1983). Also, Migeon and colleagues showed a tendency of cultured human chorionic villi cells to reactivate X-linked genes. This has been linked to the hypomethylation of CpG islands in the promoter regions of these genes (Migeon et al. 1986).

As mentioned above, the primary role of methylation in XCI is likely the stabilization of the inactive state in the Xi, at least for some of the genes that have been studied in mammals. This view is supported by the relative instability of inactive X-linked genes in both marsupials and human extraembryonic tissues both of which have been associated with hypomethylation of CpG islands in X-linked genes (Migeon et al. 1986;

Cooper 1993). On the other hand, DNA methylation is not exclusive to the inactive X, as several methylated sites have been described on the active X-chromosome which are mostly located on non-transcribed sequences and the 3' ends or bodies of X-linked genes (Yen et al. 1984; reviewed in Heard et al. 1997). These methylation patterns are conserved and are common to various genes, suggesting a role of this type of methylation on XCI (Heard et al. 1997). The mechanism by which CpG island methylation represses the expression of X-linked genes is still being investigated. The prevention of binding of transcription factors, changes in accessibility of the chromatin and stabilization of the DNA preventing transcription have all been suggested to explain methylation associated gene silencing (Heard et al. 1997).

XCI in development

Studies in mice and humans have shown that during the early stages of female embryogenesis in placental mammals, both X chromosomes are active, whether they are derived from the sperm or the oocyte. In mice, late replication of one of the two X's is not seen until the blastocyst stage, at around 3.5-4.5 days post coitum (dpc) and some genes demonstrate biallelic expression from the 2-cell to the blastocyst stage (Singer-Sam et al. 1992; Latham and Rambhatla 1995). Takagi and Abe (1990) have suggested that this increased dosage of gene product is tolerated early in development because only a few genes are being transcribed at this stage in gestation. Later in development, a greater number of genes are active and therefore, inactivation overcomes the detrimental dosage effect (Takagi and Abe 1990). The process of XCI takes place in a developmentally-regulated manner associated with tissue differentiation. In mouse embryonic development, XCI occurs first in the trophoectoderm, followed by the primary endoderm (both precursors of extraembryonic

tissues), and subsequently in the epiblast, which gives rise to the embryo proper (Monk and Harper 1979). It is known to occur as early as 3.5-4.5 dpc (blastocyst stage) in murine extraembryonic tissues, which are the first cell lineages to differentiate and are characterized by non-random XCI (Takagi and Sasaki 1975). On the other hand, in the cells that give rise to the embryo proper XCI occurs later, at approximately 5.5-6.5 dpc (around the time of implantation) and the process is usually random (Monk and Harper 1979). Although biochemical studies have suggested that XCI is complete by the onset of gastrulation (6.5 dpc) (Monk and Harper 1979), studies of different embryonic tissues in transgenic mice have shown that the onset of XCI occurs at different times in different tissues. Using mouse embryos, these studies showed that XCI starts in the epiblast at day 5.5 dpc and is complete in all embryonic tissues by day 11.5 (Tan et al. 1993). Tissues such as the gut, cranial mesoderm, heart and notochord are reportedly the last to undergo XCI. However, the reliability of these results is in question given that a lacZ transgenic system was used to assess gene expression and that recent studies have demonstrated that the lacZ sequences interfere with expression of transgene constructs (Cohen-Tannoudji et al. 2000).

In the mouse female germline, the inactive X is reactivated at the onset of meiosis, at around 12.5-13.5 dpc. The reactivated X-chromosome remains active in the oocytes throughout ovulation and the fertilization process, until inactivation occurs during preimplantation development (reviewed in Heard et al. 1997). In the male germline, the opposite situation is seen, as the single active X is inactivated before the onset of meiosis. The X's chromatin is late replicating, highly condensed and pairs with the Y chromosome. The Y chromosome is also inactivated forming the sex vesicle. The reason for the inactivation of both X and Y in spermatogenesis is unknown although it may be related to the prevention of abnormal recombination between this chromosome pair that does not share

many homologous regions. It has been reported that the X and Y-chromosomes are reactivated post-meiotically (reviewed in Heard et al. 1997).

It is important to remember that most of what we know about that process and timing of XCI in development comes from studies of mouse and very little has been proven in humans. The exact timing of XCI is therefore still unknown in humans, although based on the studies in mice, it is thought to occur in the late blastocyst stage (Gartler and Riggs 1983).

1.2.3 Non random XCI

From its early description in 1961 (Lyon 1961), the process of XCI was believed to be random in terms of the choice of an X to be inactivated. Thus, female mammals are mosaics for XCI, with cell populations in which either the maternal X or the paternal X are inactive. This pattern of inactivation is stably maintained through subsequent cell divisions. In some instances it is possible to visualize the mosaic pattern of expression of certain genes, although, the effects of other factors such as migration and cell mixing could contribute to mask it. However, there are several known mechanisms that lead to non-random XCI resulting in the prevention of the symptoms of X-linked diseases in females and the normal phenotype of carriers of balanced X/autosome translocations amongst other outcomes. These mechanisms are: Primary non-random XCI, stochastic processes, reduced precursor cell population and secondary skewed XCI (selection), which will be described individually in the following section.

Primary non-random XCI

Primary skewed XCI refers to the preferential inactivation of a given X-chromosome at the time of XCI. Three main phenomena can be classified within this category: Genetic variants (which include *Xist* and *Tsix* mutations and the effect of different

alleles of the Xce locus on XCI), autosomal influences and imprinting effects (Brown and Robinson 2000).

Xist and Tsix mutations: a few induced deletions of sections of genes like *Xist* and *Tsix* have been described in mouse that result in non-random XCI (as summarized in Brown and Robinson 2000). A C to G transversion has also been reported in 2 families with skewed XCI, although its presence is not always correlated with high levels of skewing (Plenge et al. 1997; Tomkins et al. 2002). Given that *XIST*'s transcription is fundamental to initiate XCI, mutations that compromise its availability, stability and localization could potentially lead to skewed patterns of XCI. Similarly, given *Tsix*'s role as a regulator of *Xist* expression, mutations compromising this gene could also have an impact on choice of the X to be inactivated.

Alleles of the Xce locus (skewing in mice): this locus that is believed to lie 3' to *Xist*, affects the choice of X to be inactivated or to remain active in female mice. In heterozygotes for different Xce alleles, those X chromosomes that carry a strong Xce allele remain active over an X chromosome that carries a weak Xce allele (Cattanach 1975) through a mechanism that remains unexplained. This causes a 20-30% distortion from random XCI. The various alleles are correlated with different expression levels of *Xist* and the methylation of a repetitive element, within the CpG island of the *Tsix* locus (*DXPas34*) (Avner et al. 1998; Lee et al. 1999). The ability of the Xce locus to influence imprinted XCI in extraembryonic tissues in mouse is controversial (Rastan and Cattanach 1983; West and Cattanach 1985).

Autosomal influences: At least two distinct autosomal mutations designated as autosomal factors 1 (*Xiaf1*) and 2 (*Xiaf2*) that exert dominant effects on X-chromosome choice in early embryonic development have also been identified in mice through a phenotype-driven genetic screen involving chemical mutagenesis. The role of these *trans*-acting autosomal factors in the XCI pathway is not yet clear, although they seem to specifically affect an early step in the XCI pathway, perhaps interacting with some *cis*-acting elements in the Xic (Percec et al. 2002).

Imprinting effects: In marsupials, exclusive paternal X-chromosome inactivation is characteristic of most embryonic and extraembryonic tissues (Cooper 1993). This observation is largely based on studies of replication timing in hybrid progeny with morphologically distinct X chromosomes or studies of the expressed isoforms of protein variants of X-linked genes such as *G6PD* and *PGK* (Cooper et al. 1971; Richardson et al. 1971; Sharman 1971). XCI in marsupials appears to be unstable as some loci are reactivated in culture. XCI is also incomplete, as different loci on the inactive paternal X are inactive in different tissues (reviewed in Graves 1996). Marsupials share with eutherians several characteristics of the inactive X such as late replication, chromatin condensation and histone acetylation, but there is not an apparent role of DNA methylation in marsupial XCI and attempts to find a marsupial *Xist* homologue have failed so far. This raises doubts and questions over the requirement of *Xist* expression for XCI, at least in marsupials, as well as over the possible role of this gene on imprinted XCI (see below) (reviewed in Graves 1996).

Preferential inactivation of the paternally derived X is also a characteristic of extraembryonic tissues of mice and bovines (Takagi and Sasaki 1975; West et al. 1977; Xue et al. 2002). Preferential inactivation of the paternal X has been demonstrated for the extra-embryonic tissues (trophoectoderm and primitive ectoderm of the yolk sac) of the mouse and

rat, while random XCI was observed in the mouse embryo. Specifically, cytological analysis of replication timing of embryos heterozygous for the Cattanach translocation and studies based on X chromosome dimorphisms have shown that 80-90% of late-replicating X chromosomes are paternal in origin (Takagi and Sasaki 1975; Wake et al. 1976).

Studies in mice have suggested that the basis for this imprint relies on the resistance of the maternal X to be inactivated and a predisposition of the paternal X to inactivation. It has been suggested that the epigenetic modifications that lead to the parent-of-origin effects in imprinted XCI likely involve changes in the X chromosome as a whole and possibly occur during oogenesis and spermatogenesis. Alternatively, the basis for the imprint could lie on the *Xist* gene itself, given its major role in initiation of XCI (Heard et al. 1997). The latter alternative seems likely given that the expression profile of the *Xist* gene in mouse early embryonic development is indeed imprinted. In fact, Kay and colleagues have demonstrated that the *Xist* transcripts that can be detected prior to XCI are only of paternal origin. Moreover, subsequent random XCI in the embryonic tissues of the mouse implies that the erasure of the imprint, is preceded by random expression of *Xist* from either the paternal or maternal allele (Kay et al. 1993; Kay et al. 1994). Studies in mice carrying *Xist* deletions have corroborated this gene's role in imprinted XCI as mice with deletions on the maternal allele were shown to develop normally, while murine embryos carrying a deletion on the paternal allele died soon after implantation, largely due to poor development of extraembryonic tissues. These findings suggest that only the paternal *Xist* allele is able to initiate XCI in the mouse placenta (Marahrens et al. 1997). Methylation has been suggested as the most likely mechanism or signal for this imprint on *Xist,* based on the notion that methylation is a major candidate for the imprinting signal in genomic imprinting in general. Studies in both somatic and extraembryonic tissues have shown that full methylation of *Xist*

on the active X-chromosome is associated with no transcription of this gene and an active status for the X chromosome (Norris et al. 1994). Furthermore, studies of *Xist* expression in female and male germline show that *Xist* expression is related to *Xist* demethylation and XCI of a given X homologue (Zuccotti and Monk 1995).

As to what predisposes the mouse paternally derived X to have imprinted XCI there are several theories. There was speculation of potential leakiness of the inactive state that the paternal X could carry over from spermatogenesis to the early embryo predisposing the paternal X to preferential inactivation in the early stages of differentiation (Monk and McLaren 1981). If this is true, it seems that this mechanism is reversible given that XO mice with only a paternally derived X (X^P), that is X^PO, survive and do well in development, (although they are slightly growth restricted), suggesting that the single X^P is active in extraembryonic tissues (Papaioannou and West 1981; Tada et al. 1993). These findings argue in favor of a theory that states that imprinted XCI is inabsolute in mice and that a number of cells escape imprinting in extraembryonic tissues. In this regard, Huynh and Lee have proposed a model in which the paternal X is transmitted to the zygote in a pre-inactivated state, therefore it is assumed that the male gamete initiates and establishes the imprint, while the zygote only maintains this state in the extraembryonic lineages and erases this imprint in the embryonic tissues, leading to random XCI. Furthermore, they suggest that imperfect zygotic maintenance of the imprint is responsible for the inabsolute nature of the imprint in extraembryonic tissues in mice (Huynh and Lee 2001). Additionally, experiments with cloned mouse embryos have shown that epigenetic marks can be removed and reestablished on either the paternally or maternally derived X during cloning (Eggan et al. 2000).

Whether preferential paternal XCI occurs in developmentally comparable tissues in human placenta has remained unclear, despite several studies investigating this subject (see Table 1.1). This is largely due to the fact that such studies have been affected by methodological limitations and biases.

Given that XCI is clonally maintained in successive generations of cells (Lyon 1961; Davidson et al. 1963) it is assumed that the cells in the term placenta are representative of the earlier cells existing at the onset of XCI in extraembryonic tissues. Based on these assumptions, several studies in humans have been performed using either polymorphic X-linked enzymes such as *G6PD* or assays based on differential methylation of X-linked genes, on the active and inactive X (Ropers et al. 1978; Migeon and Do 1979; Migeon et al. 1985; Harrison and Warburton 1986; Harrison 1989; Mohandas et al. 1989). Some of these studies found evidence for preferential inactivation of the paternally derived X chromosome (X_i^P) in human placentae (Ropers et al. 1978; Harrison and Warburton 1986). Others, on the contrary, found random XCI in human extra-embryonic tissues (Migeon and Do 1979; Migeon et al. 1985; Mohandas et al. 1989)(see Table 1.1 for details on these studies).

A more recent study by (Goto et al. 1997) based on the analysis of the methylation status of *Hpa II* sites in the Androgen Receptor (*AR*) gene, showed preferential methylation of the paternally derived allele in 2 samples of cultured trophoblast. In contrast, no evidence of preferential paternal allele methylation for *AR* was found in the mesodermal cells, and both paternal and maternal alleles were found to be hypomethylated in various samples of this tissue. An additional study by Looijenga et al (1999), using a similar approach, found that methylation of the *AR* gene in full term placenta was specific for trophoblast cells and that it was not biased in terms of the parental origin of the X. Thus, some placentae were shown to have the paternal X preferentially inactive and some other

placentae were shown to have the maternal X preferentially inactive. However, some other placentae had a heterogeneous pattern of XCI when two samples (located side by side) from each placenta were studied. This pattern is characterized by the presence of some sites with the maternal X preferentially inactive and some sites with the paternal X preferentially inactive within the same placenta. Detailed study of two additional placentae showed a very heterogeneous pattern of XCI (Looijenga et al. 1999). A more recent XCI study in human trophoblast of early pregnancy used a methylation based assay and a polymorphism in the Phosphoglycerate kinase (*PGK)* gene (Uehara et al. 2000). This study concluded that there was a degree of non-randomness due to predominant paternal XCI in about half of the samples of human trophoblast that they examined. The other half showed heterogeneous XCI patterns. They suggested that skewing of XCI exists in the human trophoblast but that the degree of skewing due to X_i^P is likely restricted (Uehara et al. 2000)(Table 1.1).

Table 1.1 X-chromosome inactivation studies in human extraembryonic tissues.

Extraembryonic tissue[a]	# of informative cases	# of samples per case[b]	gestational age	type of assay[c]	study results[d] (% cases)	reference
Studies that found preferential inactivation of the paternally derived X						
placenta	22	1	full term	G6PD isoforms	preferential expression mat allele (59%)	**(Ropers et al. 1978)**
amnion(d,c) chorion (d,c) chorionic villi (c)	42	1	full term	G6PD isoforms	preferential expression mat allele amnion Xa^M(50-64%) chorion Xa^M(40-50%) chorionic villi Xa^M (58%) random XCI in some cases	**(Harrison and Warburton 1986)**
soft villous cytotrophoblast (e,c)	13	1 large	full term	G6PD isoforms	preferential Xi^P in cytotrophoblast (54%) exclusive Xi^P (38%) random (8%) random XCI in stromal cells	**(Harrison 1989)**
trophoblast (d,c,z) mesoderm (d,c,z)	2 2	1 1	CVS 10-12 wks	methylation test *AR* gene	preferential XCI of the paternally derived X (100%) hypomethylation in CVS mesoderm in 50% of experiments random XCI in 50% of experiments	**(Goto et al. 1997)**

Extraembryonic tissue[a]	# of informative cases	# of samples per case[b]	gestational age	type of assay[c]	study results[d] (% cases)	reference
Studies that found random XCI						
amnion chorionic villi chorion**	12	1	full term	G6PD isoforms	no results for amnion chorion contaminated random expression of G6PD isozymes in 33% cases (villi) preferential expression on maternally derived allele in 50% of cases **(villi)	**(Migeon and Do 1978)** **
chorion chorionic villi	9	1	TA 7-13 wks post LMP	G6PD isoforms	random XCI in extraembryonic tissues random XCI in embryonic tissues	**(Migeon and Do 1979)**
chorion (c) chorionic villi (c) chorionic villi	5 1	...	SAB 11-16 wks term	G6PD electrophoretic phenotype (ssc)	incomplete dosage compensation of G6PD only in chorionic villi random XCI in human chorionic villi	**(Migeon et al. 1985)**
chorionic villi (d,c)	4	2	first trimester CVS	G6PD electrophoretic phenotype (ssc) methylation test *HPRT* assay	random XCI in human chorionic villi	**(Mohandas et al. 1989)**

Extraembryonic tissue[a]	# of informative cases	# of samples per case[b]	gestational age	type of assay[c]	study results[d] (% cases)	reference
chorionic parenchyma (d)	79 pairs	1	term IUD (~4 cases) preterm (~9 cases)	methylation test for DXS255 locus using M27β probe	random (symmetric) XCI in chorionic mesoderm (81%) cord (90%) amnion (86%)	**(Bamforth 1996)**
trophoblast (d,m) Stroma (d,m)	9 females 2 females	1* 9*	full term	methylation test *AR* assay	absence of methylation in stroma (100%) random XCI in umbilical cord heterogenous XCI in trophoblast (60%)	**(Looijenga et al. 1999)**
anchoring villi (trophoblast) (d,z) branch villi (trophoblast) (d,z)	6 6	~5^ ~6+	TA (7-8wks)	methylation test *PGK* assay	different levels of skewed XCI in human trophoblast restricted degree of skewing due to preferential inactivation paternally derived Xi (40% of samples) random XCI (47% of samples)	**(Uehara et al. 2000)**

[a] c= cultured; d=direct preparations; m=tissues isolated by microdissection; e= enzymatic separation of tissues z= enzymatic plus mechanical separation of tissues.

[b] *=4 adjacent samples from each site (~0.5cm^3); ^= 30 different anchoring villi were studied in all 6 cases; +=38 different branch villi were analyzed in 6 cases.

[c] ssc= single cell clones

[d] the term "preferential" refers to any deviation from the expected 1:1 ratio of inactivation for the two X-chromosomes or expression of the two alleles of an X-linked gene. However, in some of the studies cited in this table, this term is not clearly defined.

**= maternal contamination seen or suspected in the samples tested.

Stochastic processes

Using data on the distribution of skewing amongst newborn populations (any deviation from the 1:1 ratio in the inactivation of the two X-chromosomes) and also using computer simulations, it has been estimated that about 4-20 embryonic precursor cells are present at the time of XCI (Puck et al. 1992; Monteiro et al. 1998). If it is assumed that the choice of the X chromosome to be inactivated is indeed random, the relatively small number of cells that contribute to the embryo might lead to skewing of inactivation purely due to chance. This is illustrated in studies of control populations where the mean contribution of each X chromosome to the pool of inactive X's is approximately 50%, but at the extremes of the distribution lie individuals with substantial deviations from the 1:1 ratio (reviewed in Belmont 1996). As mentioned earlier in this chapter, several studies have looked at the distribution of skewing in newborn and adult women. Most of these studies were carried out using peripheral blood, although there are a few studies including other tissues (Sharp et al. 2000). Based on the data obtained from studies in blood, skin and skeletal muscle and under the assumption of a purely stochastic process, the variance of the distribution of skewing in these tissues suggests that about 10-25 cells constitute the common precursor cell pool (number of embryoblasts) at the time of XCI (Fialkow 1973)

Reduced precursor cell population

Studies of XCI in humans have demonstrated the presence of different levels of skewing in different tissues within the same individual (Gale et al. 1994; Sharp et al. 2000). It is possible that the number of precursor cells present at the time of commitment to XCI (marking of X to be inactivated) is an important determinant of the distribution of skewing. Given the very limited number of both embryonic and extraembryonic precursor cells present

in the human embryo at the onset of XCI, it is clear that any process that causes further reduction of these already limited cell pools is likely to have an impact on the level of skewing. In other words, there is a greater probability of non-random XCI occurring in a tissue or tissues that have undergone a reduction in the number of precursor cells (Fialkow 1973). To illustrate this, Beever (2002), explains that assuming a 50% chance for inactivation of each X, 100% skewed XCI is expected for 6% of embryos if commitment to inactivation occurs at the 5-cell stage of embryonic development. On the other hand, extremely skewed XCI (100%) is seen in only 0.2% of embryos when commitment to XCI occurs at the 10-cell stage (Beever 2002). In this way, a process analogous to genetic drift causes deviations of random XCI due to random sampling of embryonic cells. Two events that may be related to a reduction in the embryonic precursor pool size are: twinning and mosaicism preferentially confined to the placenta.

It has been reported that there is an increased frequency of skewed XCI in monozygotic (MZ) twins compared to singletons (Goodship et al. 1996). This association was suggested to be due to a reduction of the embryonic precursor pool of cells that give rise to each twin, as a result of embryo splitting. For XCI skewing to be predominant in this type of twins, the splitting would have to occur around the time of initiation of XCI (Brown and Robinson 2000). However, splitting in monozygotic-monochorionic twins is believed to occur several rounds of replication after the commitment to XCI, therefore, is very unlikely that skewed XCI is associated with the twinning event. Similarly, in monozygotic-dichorionic twins, the split is estimated to occur before the blastocyst stage and therefore, well before the commitment to XCI. Moreover, some studies have not found significant differences in XCI patterns between MZ twins and singleton births (Monteiro et al. 1998).

A developmental event that seems to better illustrate the reduction of the precursor cell pool as a cause for XCI skewing is the presence of trisomy mosaicism. Specifically, when the trisomic cell line is preferentially confined to the placenta and the trisomy is of meiotic origin. A trisomic rescue event leads in many cases to the development of a fetus that is predominantly diploid and that shows high levels of skewing in most diploid fetal tissues (Lau et al. 1997; Peñaherrera et al. 2000). It has been hypothesized that most of these diploid tissues might be derived from a single diploid embryonic precursor cell present at the time of commitment for XCI, with the remaining trisomic cells being negatively selected from contributing to the embryo (Lau et al. 1997; Peñaherrera et al. 2000; Robinson et al. 2000).

Secondary skewed XCI (Selection)

Secondary skewed XCI refers to the situation in which random XCI occurs, but it is followed by cell selection processes that result in deviations from the expected 1:1 ratio of paternal to maternal inactive X chromosomes. Cell selection is a very significant cause of non-random XCI, therefore, it is not surprising that this area of research has received much attention in the past few decades. In trying to understand the impact of cell selection in XCI skewing it is important to consider that most tissues/organs in the adult are the result of countless rounds of cell division, from the starting point of only a few embryonic precursor cells at the time of XCI, which in humans is believed to occur in the late blastocyst stage (Gartler and Riggs 1983). Given the large number of cell divisions talking place in human development, even small biases in cell viability or proliferative capacity can lead to skewing of inactivation (Brown 2001). Indeed, the numerous cell divisions are likely to increase the chance of skewing by genetic drift.

There are several factors associated with increased cell selection that can lead to non-random XCI some of which would confer a growth disadvantage to the cells, while others are likely to confer them a growth advantage. Amongst those that commonly confer a growth disadvantage to the cell are:

Chromosome rearrangements involving the X: a chromosome rearrangement that involves the X chromosome, whether or not a disease locus is disrupted, could favor the survival of cells with either the normal or the rearranged X active, depending on the situation. Usually these rearrangements include either balanced or unbalanced X/autosome (X/A) translocations, interstitial or terminal deletions, isochromosomes, ring chromosomes and insertions affecting the X (Mattei et al. 1982; Rastan 1983; Schmidt and Du Sart 1992; Pegoraro et al. 1997). The presence of a large chromosome rearrangement generally leads to the selection of the products that cause the least disruption of the gene dosage or minimal genetic imbalance. The rearranged X is therefore, generally inactivated in cases of deletions and unbalanced X/A translocations, while the normal X is usually inactivated in cases of balanced X/A translocations. The efficiency of this selection depends on a number of factors that will be discussed in Chapter 5.

Mutations of X-linked genes: in cases of X-linked gene mutations that confer a growth disadvantage to the cells, the selection process can either affect the cells in the whole individual or could be restricted to certain tissues. Therefore, non-random XCI could be either constitutional or confined to a specific tissue or tissues. Some examples in this category are the immunohematological disorders caused by X-linked recessive gene defects that lead to abnormalities of different hematopoietic cell lineages, such as X linked agammaglobulinemia (XLA), and Wiskott-Aldrich syndrome amongst many others (Van den Veyver 2001). It is believed that XCI skewing in these female carriers, most likely protects

them from the disease phenotype through selection against the cells in which the X carrying the mutation remains active.

Although most of the X-linked mutations that have been studied convey a growth disadvantage to the cell that ranges from mild to severe, there are cases in which the mutation conveys a growth advantage to the cell. In these cases, skewed XCI can occur due to selection in favour of cells with the mutant allele on the active X chromosome. While in some cases this could be advantageous, in some other cases, female heterozygotes for a mutant allele that have become hemizygous due to skewing could manifest the clinical symptoms of X-linked diseases normally only seen in males (i.e. adrenoleukodystrophy) (Migeon et al. 1981).

Monoclonality: Skewing due to a growth advantage of a particular cell or cells occurs when all cells of a given tissue are derived from a single cell. This type of growth is seen in leukemias, solid tumors, myeloproliferative disorders and myelodysplastic syndromes (Hotta 1997). For these reasons, XCI studies have become a major means to study monoclonality in human cancer.

Age related increase in skewing: Several studies in humans have shown that the percentage of individuals in the population with highly skewed XCI increases with age. Using a cut-off of 90% to designate extreme skewing, several studies have demonstrated that non-random XCI increases from approximately 3% in neonates, to 4-7% in young females (~30 year-old), ~30% in 75 year-old women or older (Fey et al. 1994; Busque et al. 1996; Gale et al. 1997; Sharp et al. 2000). This type of skewing can be explained by small selective differences between the two X chromosomes, that allow preferential growth of one type of cell over

another allowing for increase in skewing over time with cell turnover. Studies in elderly monozygotic twin pairs as well as long-term studies in hybrid cats support this explanation (Abkowitz et al. 1998; Christensen et al. 2000). In female Safari cats, XCI skewing develops with aging due to hemizygous expansion of hematopoietic stem cells that bear a specific X chromosome haplotype. This haplotype is predominant over another as it is favored by selection (Abkowitz et al. 1998). In humans, a positive correlation in the direction of skewing has been observed between aging monozygotic twin pairs. It has been suggested that non-pathologic polymorphisms could cause small differences in the ability of cells to proliferate and therefore promote biased hematopoietic cell expansions. Thus, it seems likely that X-linked genetic factors influence human hematopoietic stem cell kinetics (Tonon et al. 1998; Christensen et al. 2000). Alternative explanations for increased skewing with age include: a) The hematopoietic stem cell pool decreases over time as a result of cell senescence (cell depletion), therefore blood cell production is supported by a decreased number of progenitors and b) Clonal expansion of abnormal hematopoietic stem cells as seen in neoplasia, can lead to XCI skewing. However, myelodysplasia is a rare occurence in humans (reviewed in Tonon et al. 1998; Christensen et al. 2000).

1.3 The Human Placenta

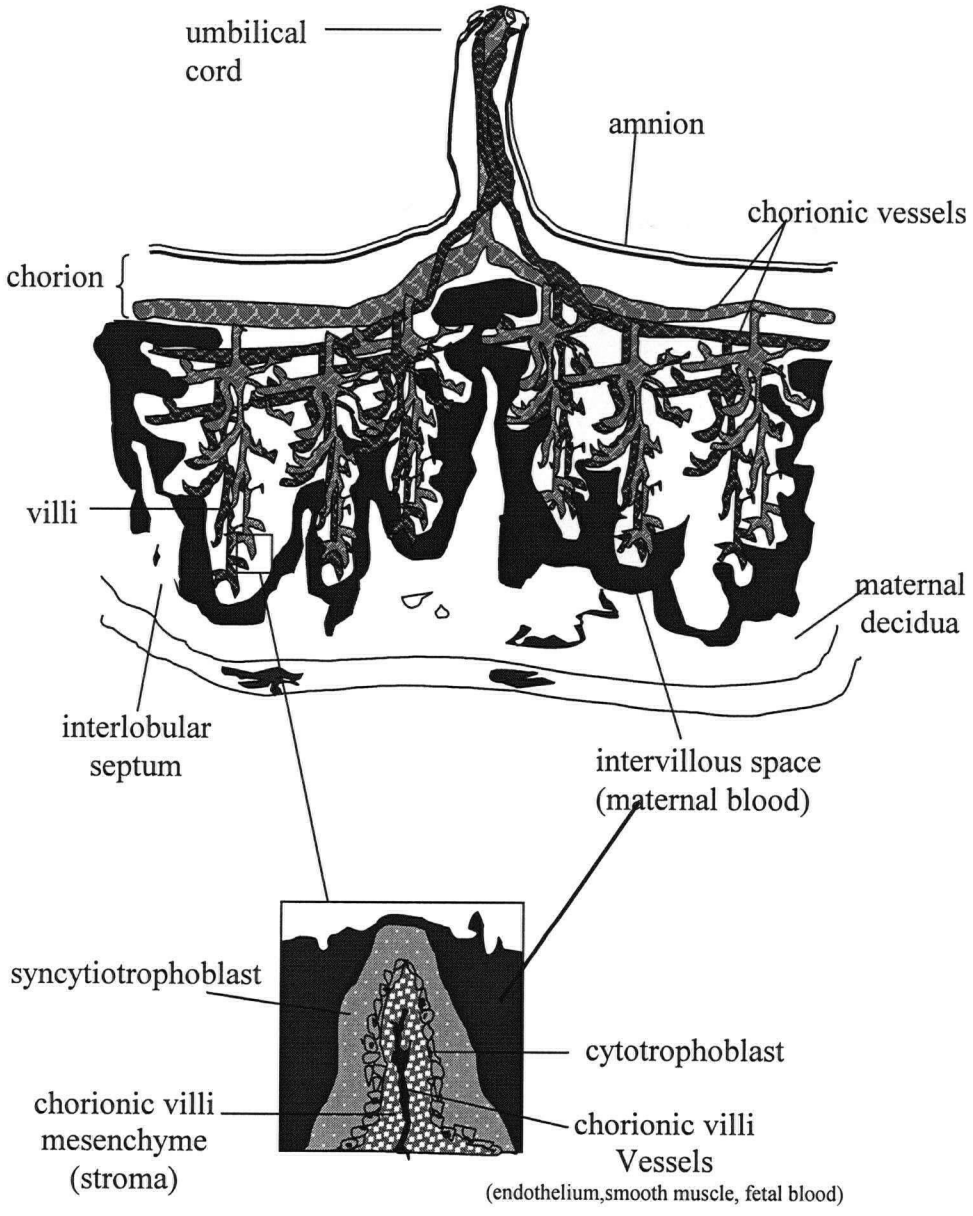
1.3.1 Basic Morphology and Physiology

In spite of its crucial role in embryonic development, the human placenta is one of the least studied human organs. This structure represents an interface between the maternal and fetal circulations that long was thought to have a passive role in human gestation.

Interesting new research in the field of placental pathology and physiology has demonstrated, however, that the human placenta plays a major role in the regulation of embryonic and fetal growth *in-utero* and that the influence of the placental status and placental support goes well beyond the prenatal period of human development.

The human placenta, is a discoid structure that weighs approximately 500 g at term. Viewed from the fetal side, it is possible to distinguish the insertion of the umbilical cord either at the center or usually off-center in the placental disc. A sagital section of this organ allows the visualization of the different placental components, which will be described below (see Figure 1.2).

Figure 1.2 Schematic diagram of the human placenta indicating the different extraembryonic tissues studied. Modified from (Lewis and Perrin 1999) and (Larsen 1993).



Placental membranes

The placental membranes include the reflected or free membranes and the portion of the membranes still attached to the placental disc on the fetal side of the placenta. In both cases the two components are amnion and chorion. Maternal decidua is found in contact with the outermost surface of the chorion (Lewis and Perrin 1999). The placental membranes form the embryonic/fetal sac that completely surrounds the embryo/fetus and contains the amniotic fluid (AF).

Amnion: This is a thin membrane (can be peeled from the chorion) that forms the amniotic sac. It has two main layers: the amniotic ectoderm which is believed to be a derivative of the epiblast in the inner cell mass (ICM) and the amniotic mesoderm or mesenchyme, whose origin is still unclear but is believed to be a derivative of both the hypoblast in the ICM (extraembryonic mesoderm) and the embryonic mesoderm of the primitive streak (Bianchi et al. 1993; Whittle et al. 2000; Robinson et al. 2002)

Chorion: This is a thick membrane whose outermost layer is often fused to the maternal decidua at term. The chorion arises developmentally as a derivative of the ICM's hypoblast. The hypoblastic layer of the bilaminar embryonic disc gives rise to the extraembryonic mesoderm that eventually lines the blastocyst cavity spreading around the cytotrophoblast surface and forming the chorion (Lewis and Perrin 1999).

Amniotic Fluid: This is the fluid that surrounds the embryo throughout gestation. Its volume varies with gestation between 200-1000 ml. Early production of amniotic fluid is believed to be transmembranous, from the amnion, fetal lung secretions and across the fetal skin. As

pregnancy advances, AF is largely secreted by the lungs, but after 16 weeks gestation, fetal urine seems to be the major contributor. The volume is regulated by intramembranous absorption towards the fetal vessels and across the chorionic plate as well as by fetal swallowing (Lewis and Perrin 1999).

Maternal decidua: This term refers to the stromal layer of the endometrium (the lining of the uterus) in which cells have undergone a series of physiological and morphological changes in preparation for the establishment and maintenance of pregnancy. The portion of the endometrium that underlies the implanted embryo is known as decidua basalis and constitutes the maternal portion of the placenta (Larsen 1993; Lewis and Perrin 1999).

Placental parenchyma: This structure is defined to include the placental chorionic villi, organized in 15-25 lobes or cotyledons visible from the maternal side of the structure. Placental chorionic villi form the villous tree or placentome, which is the name given to the elaborate network of progressively smaller branches in which stem villi are divided as they extend from the chorionic plate (chorion on the placental disc) (Larsen 1993). Human placentation is characterized by the fact that maternal blood directly bathes the placental villi (haemochorial placenta). Chorionic villi have two major components (see Figure 1.2), the trophoblast (differentiated in cytotrophoblast and syncytiotrophoblast) and the mesenchyme. The mesenchyme in turn has several components such as chorionic villi stroma, endothelium and smooth muscle from the chorionic villi vessels.

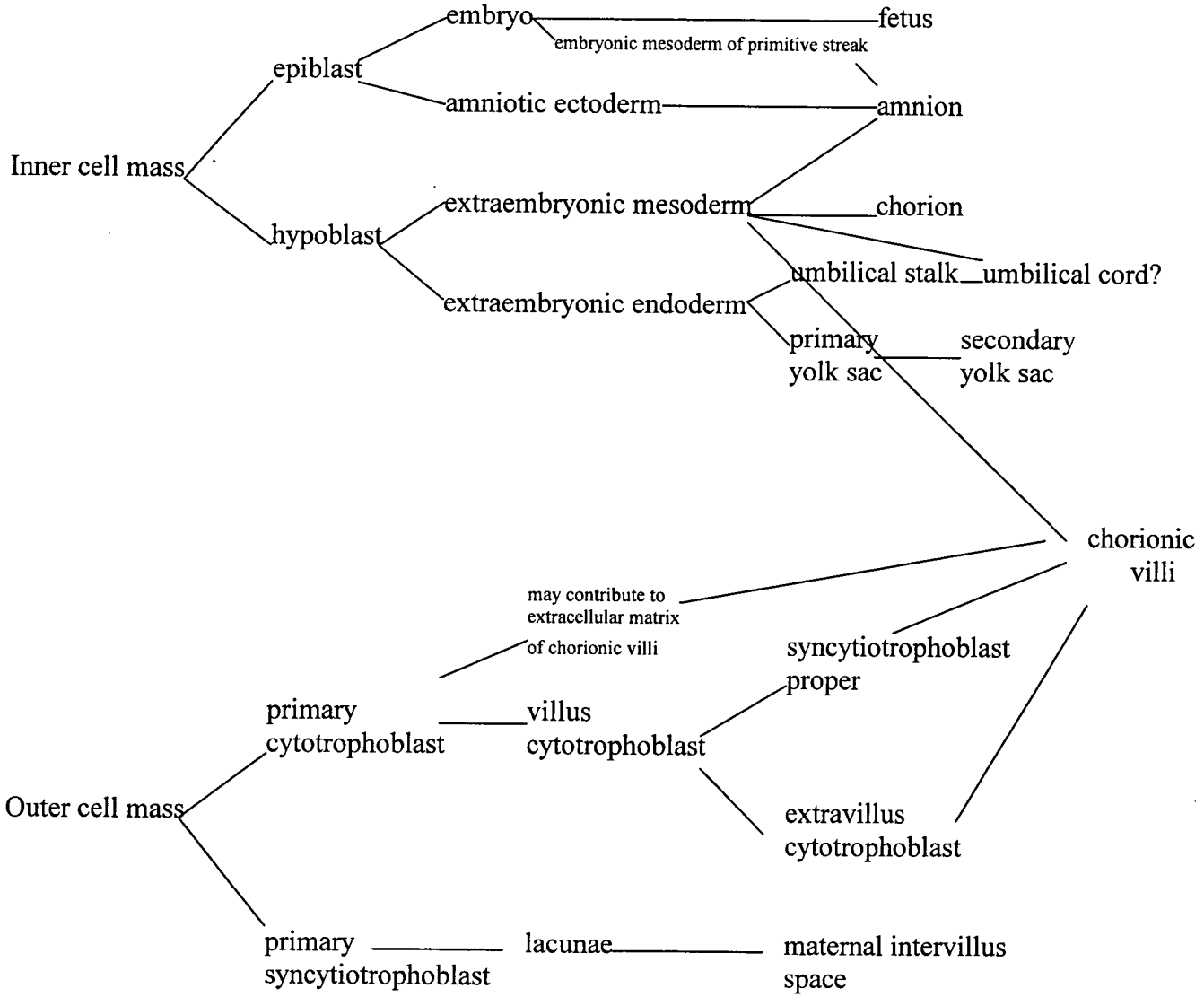
Trophoblast: The trophoblast forms a thin single layered epithelium that constitutes the outer wall of the blastocyst at around day 5 of embryonic development (Larsen 1993). Derived

from the cells of the outer cell mass of the morula, it is the first tissue to differentiate in the developing embryo. This layer eventually differentiates into three distinct types of tissue: a) syncytiotrophoblast: This is the outer layer of proliferating cells that coalesce post-mitotically to form a syncytium (mass of cytoplasm containing multiple nuclei). This tissue is highly invasive in nature and therefore is responsible for the blastocyst implantation via the invasion of the maternal decidua. It is also active in protein and steroid synthesis such as human chorionic gonadotropin (hCG). b) cytotrophoblast: This tissue corresponds to the inner layer of the trophoblast that forms the wall of the blastocyst. It remains mitotically active, keeping the cell membranes intact. Cytotrophoblast and syncytiotrophoblast are both components of the chorionic villi, and finally, c) extravillous cytotrophoblast: This is a highly invasive type of trophoblast that forms columns that anchor the placenta to the uterus. It also participates in the invasion and transformation of the maternal uterine vessels that ensures a constant supply of maternal blood to the fetus (Larsen 1993; Moore 1993). This type of trophoblast expresses a non-classical major histocompatibility antigen HLA-G, which is trophoblast specific and prevents destruction by the immune system (reviewed in Lewis and Perrin 1999).

Mesenchyme: This is a connective tissue derived from the hypoblastic layer of the ICM of the blastocyst that gives rise to the extraembryonic mesoderm. This tissue appears during the formation of the secondary chorionic villi, forming the core of the secondary stem villi where the chorionic villi vessels develop later in development (tertiary stem villi). Its components, aside from the fetal blood vessels (endothelium and smooth muscle in some cases) are fibroblasts and Hofbauer cells (special phagocytic cells that express MHC class II determinants in the 1st trimester)(Lewis and Perrin 1999). The stroma is the connective tissue

where the placental vessels that connect with vessels forming in the embryo proper develop, establishing a true utero-placental circulation. This tissue constitutes the core of the secondary and tertiary chorionic villi. A diagram showing the developmental origin of the different tissues is shown in Figure 1.3.

Figure 1.3 Developmental origin of embryonic and extraembryonic tissues and structures



Modified from: (Lewis and Perrin 1999)

1.3.2 Trisomy mosaicism

It has been estimated that at least 10-30% of fertilized human eggs are aneuploid (mostly trisomic and monosomic) (reviewed in Hassold and Hunt 2001). Studies of aneuploidy in human gametes have shown that human chromosome segregation during meiosis is very error prone, leading to 2-5% aneuploidy in sperm and 18-25% aneuploidy in human oocytes (Martin et al. 1991; see review in Hassold and Hunt 2001). Abnormal chromosome numbers in humans have important clinical consequences, such as spontaneous abortions (~35% of which are aneuploid), mental retardation and developmental abnormalities. In all, Hassold and colleagues have estimated that at least 5% of all human recognized pregnancies are aneuploid (Hassold and Hunt 2001), with 4% being trisomic (Hassold and Jacobs 1984). Triploidy, monosomy X and trisomy 16 comprise about 23% of the total chromosome abnormalities seen in spontaneous abortions (Hassold 1986). In live births, only trisomies 13,18 and 21, as well as monosomy X are seen in non-mosaic state. Thus trisomies for most other chromosomes are seen in mosaic state either generalized to fetus and placenta or confined to the placenta (Robinson et al. 1999).

Chromosomal mosaicism, is the presence of two or more cell lines with different chromosome complements within one individual. Most commonly it refers to the presence of a trisomic cell line mosaic with a diploid cell line. Trisomy mosaicism can be either generalized (found in placenta and embryo) or confined (usually to the placental tissues but occasionally to the fetal tissues). Generalized mosaicism usually involves chromosomes 13,18,20,21 and the sex chromosomes in live births. True fetal mosaicism occurs in about 0.1-0.3% of amniocentesis (Wilson et al. 1989) and its outcome varies according to the chromosome involved and the origin and level of the trisomy.

Confined placental mosaicism (CPM) was first described in term placentae of infants born with unexplained intrauterine growth restriction (IUGR)(Kalousek and Dill 1983). It refers to the discrepancy between the chromosomal constitution of the chorionic tissue and the embryonic/fetal tissues and is seen in about 1-2% of viable pregnancies ascertained by chorionic villus sampling (CVS) (Mikkelsen 1985; Ledbetter et al. 1992; Teshima et al. 1992). CVS is a procedure used for prenatal diagnosis at 10-12 weeks gestation in which tissues used for analysis are withdrawn, under ultrasonographic guidance, from the villous area of the chorion through either transcervical or transabdominal procedures (Thompson et al. 1991). Some cases that are positive for mosaic aneuploidy on CVS, are followed-up using amniocentesis. The latter is a procedure done at ~ 16wks gestation by which a sample of amniotic fluid is removed transabdominally from the amniotic sac under the guidance of ultrasonography (Thompson et al. 1991).

A study in term placentae from normal pregnancies where multiple sites were examined has shown CPM in 5% of cases (Artan et al. 1995). It is however, almost impossible to completely rule out the possibility of fetal mosaicism affecting one or more tissues, upon diagnosis of CPM. "Occult" trisomy mosaicism confined to specific tissues in the fetus can go undetected given that most researchers never test every fetal tissue (Benn 1998). For this reason, the term trisomy mosaicism preferentially confined to the placenta will be used throughout this manuscript in lieu of CPM.

Molecular studies of trisomy in fetuses and live borns have shown that maternal meiotic non-disjunction due to abnormal recombination plays a major role in its etiology (reviewed in Hassold and Hunt 2001). Mosaicism involving a trisomic and a diploid cell line could arise either through the loss of a chromosome in a trisomic conceptus or through the gain of a chromosome in a normal diplod conceptus. The former involves a process known as

trisomic zygote "rescue" and is seen in cases where the trisomy is of meiotic origin, while for the latter, the trisomy is of somatic origin and the non-disjunction event occurs in early embryonic development. The resulting mosaic pattern in the conceptus depends on several factors, such as the cell lineages that are affected by the mutational effect, the time of the event itself, and the processes of cell selection based on the viability of the cell with the mutation. In general, a non-mosaic diploid fetus with trisomy confined to both of the chorionic villi components of the placenta (trophoblast and mesenchyme) implies that the zygote was originally trisomic and the loss of a chromosome occurred in an embryonic precursor cell during development. The presence of mosaicism for a trisomy that is present in a specific region of the placenta or is confined to a specific cell lineage of the placenta implies that a somatic or mitotic error occurred in a placental precursor cell of a normal diploid conceptus (Kalousek and Vekemans 2000). It is important to point out that at present it is not known how trisomic cells are eliminated from contribution to the fetus proper. In the search for additional means to help us understand these processes in human placenta and eventually predict the outcome of the pregnancy, XCI offers as a promising tool that needs to be explored.

Three types of CPM have been described based on cytogenetic findings regarding the presence or absence of the aneuploidy in different placental cell lineages (Kalousek 1993): Type I CPM, in which the aneuploidy is confined to the trophoblast. This is the most common type and has been described for trisomy 3. It is usually associated with a normal outcome, however up to 22% of pregnancies of this type are reported to result in spontaneous abortions, IUGR and/or perinatal morbidity (Johnson et al. 1990). Type II CPM, in which the aneuploidy is confined to the chorionic villous stroma (mesenchyme). This type has been shown in both normal pregnancies as well as cases of fetal IUGR and intrauterine fetal death

(IUD) (Kalousek et al. 1987; Kalousek et al. 1991). Type III CPM, in which the aneuploid cell line is found in both trophoblast and mesenchyme, with outcome varying from IUD or severe IUGR to completely normal (Kalousek 1993). Types I and II are commonly the result of a somatic gain of a chromosome in a normal diploid conceptus (somatic origin of the trisomy), while type III is often associated with a meiotic origin of the trisomy (Kalousek 1993; Robinson et al. 1997). To assess the type of CPM for a given case, trophoblast is analyzed through direct preparations or short-term chorionic villi cultures while the mesenchyme is analyzed using long-term cultures of villous mesenchyme.

In one third of the cases, trisomic zygote rescue leads to uniparental disomy (UPD), a condition were both copies of a chromosome have originated from a single parent (Kalousek et al. 1993; Ledbetter and Engel 1995). Uniparental disomy for certain chromosomes is associated with clinical abnormalities in humans. These could be the result of homozygosity for recessive deleterious genes or genomic imprinting (the differential expression of genes based on their parent of origin)(Ledbetter and Engel 1995).

1.4 Statement of purpose

My overall goal was to investigate the mechanism of XCI in human extraembryonic tissues during development. This knowledge is important if we are to determine the extent to which mouse development can be extrapolated to humans and if we want to shed some light into the mechanism of dosage compensation across different groups of mammals from an evolutionary point of view. Several specific hypotheses were addressed:

I hypothesized that paternally imprinted XCI, which is characteristic of extraembryonic tissues of mouse and marsupials is also a feature of human placenta but possibly confined to the trophoblast. To study this I analyzed XCI patterns in four different

extraembryonic tissues (amnion, chorion, trophoblast and mesenchyme) of a series of term and first trimester placentae. Additionally, I examined XCI patterns in cord blood and placenta of a phenotypically normal female carrier of a balancedX/autosome translocation of paternal origin.

Secondly, because promoter hypomethylation in human extraembryonic tissues has been suggested for X-linked genes such as *AR* and *G6PD* it is possible that this feature is common to other X-linked genes. Therefore, I hypothesized that there would be hypomethylation of the promoter regions of all or most X-linked genes in extraembryonic tissues in humans. To explore this, the methylation status of the promoter regions of 7 X-linked genes (*MAOA, ARAF, AR, XIST, DXS6673E, GRIA3E* and *FMR-1*) was analyzed in normal female CVS samples.

Finally, given that increased levels of extreme skewed XCI have been found in diploid fetal tissues of cases of trisomy mosaicism preferentially confined to the placenta, I hypothesized that these are due to a reduction in the embryonic cell pool that contributes to the embryo. This reduction may be the consequence of selective elimination of trisomic cells from the embryonic precursor cell pool subsequent to XCI. Additionally, when high levels of trisomy are found in the placenta, there is an increased probability of "occult" trisomy mosaicism in the fetus. I hypothesized that the prevalence of trisomy in some fetal tissues is correlated with poor fetal outcome and increased levels of skewed XCI. This would allow one to explore the possibility of using XCI assays as a tool to aid in the prediction of embryonic/fetal/live born outcome in mosaic trisomy cases. To investigate these possibilities, I studied XCI in embryonic and extraembryonic tissues of mosaic trisomy cases in which the trisomy is confined predominantly to the placenta and correlated these findings with data on presence of trisomy, conventional cytogenetics and clinical outcome.

Chapter 2: Materials and methods

2.1 Subjects and Sample collection

2.1.1 *Term and first trimester placentae (placental sampling method)*

A total of 22 placentae (14 female /8 male) from full term deliveries and 35 first trimester placentae (11 female/ 24 male or uninformative females). Amongst the first trimester placentae, 23 cases of therapeutic (TA) and 12 cases of spontaneous abortions (SAB) were included in this study. Fresh term placentae were obtained from either healthy voluntary donors with prior informed consent (see Appendix I) or from anonymous healthy deliveries at BC Women's Hospital, Vancouver, BC (some were controls for a study on IUGR, collected by Dr. Valerie Desilets in collaboration with Dr. Dagmar Kalousek). Specimens from anonymous TA's (<12wks gestation) were obtained from BC Women's Hospital and specimens from anonymous SAB's (8-13wks gestation) were obtained from the department of embryo-fetal-pathology at BC Women's Hospital, as part of a study by Paul Yong on the effect of trisomy in the growth of human trophoblast.

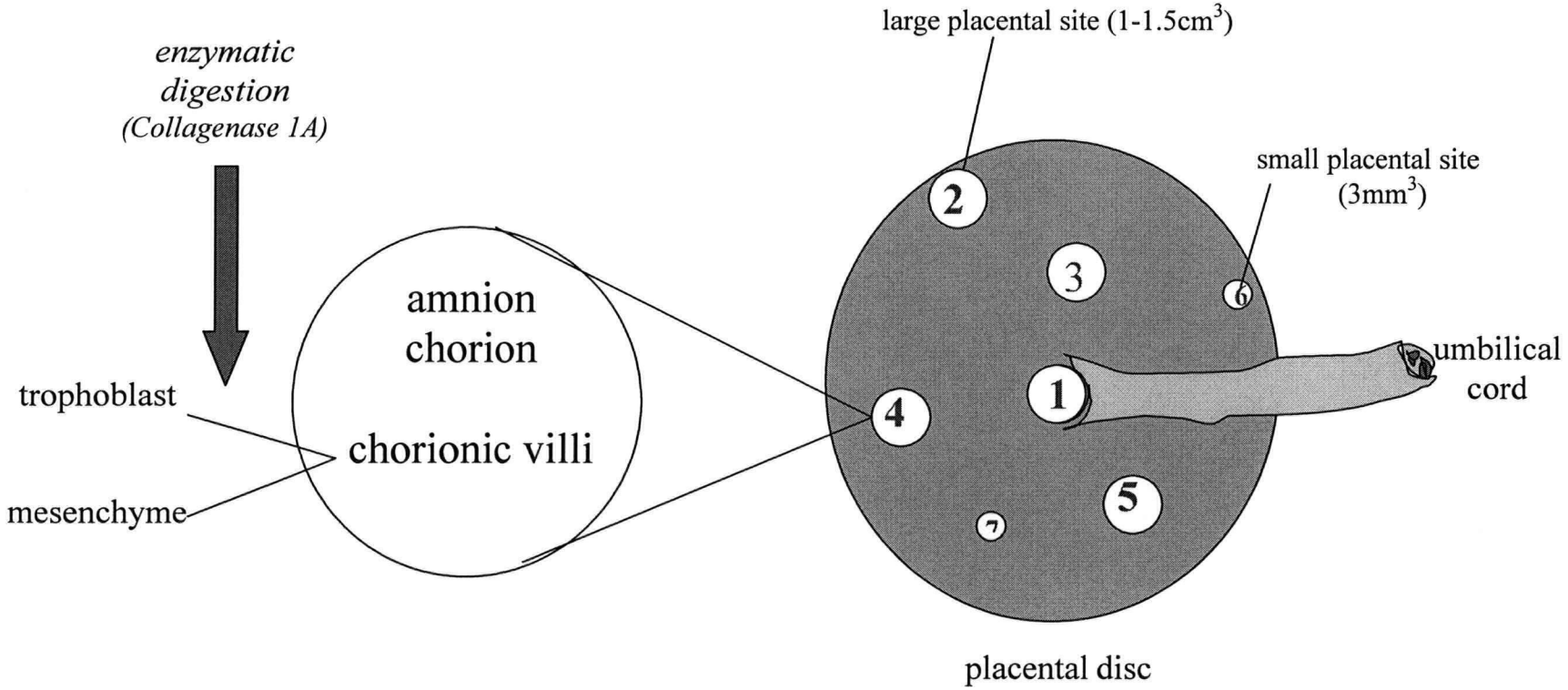
Placental sampling: Samples of approximately 1-1.5 cm^3 from 5 sites within the term placentae were collected from the fetal side of the placenta to avoid contamination with the maternal decidua. At least one of the sites was located near the origin of the umbilical cord and another towards the periphery of the placental disc. In a subset of placentae, 2 additional sites were sampled from the fetal side of the placental disc, limiting the size of the sample to $3mm^3$, to allow for comparisons between two different sample sizes. Additionally, 2 more samples were obtained from the maternal side of the placental disc to allow for comparisons regarding sample location. The approximate location of each of the sites was measured and

recorded in a schematic map for each placenta (as shown in Figure 2.1) (see Appendix II) (based on Henderson et al. 1996). Details on placental weight, size, shape and number of vessels in the umbilical cord were also recorded (see Appendix III). Chorionic plate, amnion and chorionic villi were subsequently isolated from each of the samples, cleaned under the dissection microscope and washed with physiological saline solution. A sample of the umbilical cord was also obtained. Samples of cord, chorion and amnion were directly used for DNA extraction, whereas the chorionic villi samples were subjected to a digestion with collagenase IA (Sigma 350U/mg) in order to separate the chorionic villi mesenchyme (mostly stromal core) from the trophoblast (modified from Simoni et al. 1983). Briefly, ~50mg of chorionic villi were incubated for 5-20 minutes at room temperature in 1ml of collagenase I A solution (25 mg collagenase SIGMA (350units/mg) in 20 ml of Hanks Balanced Salt Solution (HBSS) with Ca and Mg). After incubation, samples were vortexed briefly. If the supernatant became cloudy, 2ml of HBSS were added to each sample and the digest was left to settle for 2 minutes, after which the supernatant (containing the trophoblastic cells) was carefully collected and cells were pelleted. The remaining villi, stripped from the trophoblastic layer were then washed in saline, finely minced and washed in saline again. DNA was then separately extracted from each of these two components of the chorionic villi.

Maternal blood, which is normally coating the placenta, was collected for DNA extraction in 7 ml vacutainer tubes with EDTA. Possible contamination of the maternal blood with amniotic fluid and/or fetal blood was tested for using several autosomal markers. Additionally, maternal decidua, when available was also sampled from the maternal side of the placenta and carefully separated from the chorionic villi under the dissection microscope. Whenever possible, fetal blood was also collected from the umbilical cord for DNA

extraction. In the first trimester placentae, the smaller amount of tissue available and the loss of structural morphology, made it impossible to obtain more than 1 sample of each of the placental tissues and the maternal decidua. The samples were processed in the same way as for the term placentae.

Figure 2.1 Schematic representation of the placental sampling method.



2.1.2 *X/autosome translocation case*

In Chapter 4, I discuss a case of a female carrier of a balanced X/autosome translocation of paternal origin. This female was conceived through intracytoplasmic sperm injection (ICSI), followed by in vitro fertilization (IVF) from a normal mother and a father carrier of the translocation. Details on clinical and cytogenetic findings are given in the corresponding chapter.

2.1.3 *CVS samples (cultures) and replication studies of the X-chromosomes*

This study discussed in Chapter 5, involved the use of 20 leftover anonymous samples of CVS cultures diagnosed as normal females and obtained from the Cytogenetics Laboratory of BC Children's and Women's Hospitals (CVS1-20). Four additional cases (CVS-JF 1 and 4) were used for BrdU-FISH studies to assess replication timing of the X chromosomes.

2.1.4 *Mosaic trisomy cases*

A total of 25 cases of mosaic trisomy referred to the laboratories of Dr. D.K. Kalousek and Dr. W.P. Robinson (as part of a study of placental mosaicism) from various centers were included in the study described in Chapter 6. Only cases of meiotic origin of the trisomy for which fetal and parental bloods were obtained, were included in this study, with prior informed consent. In some of the cases, placental tissues were also available for study. The inclusion of cases of meiotic origin is based on our previous findings of a significant number of cases of skewed XCI in diploid fetal tissues of mosaic trisomy cases of meiotic origin, while no similar effect was true for cases of somatic origin (Lau et al. 1997). The majority of cases were sampled by Irene Barrett (Dr. D.K. Kalousek lab.), who also did the conventional cytogenetics and FISH studies on them. Fetal tissues were also identified and

washed in saline prior to DNA extraction. Duplicate tissue samples were processed in our laboratory by myself and laboratory technicians, in a similar way to that described for the term placentae.

2.2 Tissue cultures and harvest

CVS cultures were subcultured using supplemented AmnioMAX-C100 medium (GIBCO) with added 0.22% Fungizone-Amphotericin B (250μg/ml) (GIBCO) in a CO_2 incubator, at 37 °C for 3-4 days until they were almost confluent. Subsequently the cells were harvested and pelleted for DNA extraction. For harvesting, the old culture media was discarded and bleached, and the cultures were washed with PBS to eliminate any residual media. Then, each culture was incubated with 2ml of trypsin in Citrate Saline 0.25% at 37°C for approximately 5 minutes until the cells detached from the dish. At this point, 1 ml of medium was added to each culture to stop the enzymatic activity of the trypsin. Cells were completely loosened by gentle flushing with media and subsequently collected in 15 ml falcon tubes.

Immunostaining of small samples of CVS subcultures was done using Cytokeratin 7 (DAKKO). This is a known marker of trophoblastic cells in chorionic villi, not expressed in mesenchymal cells (Blaschitz et al. 2000). Cytokeratin 7 immunostaining was used to assess the purity of the CVS samples (which are expected to be predominantly chorionic villi mesenchyme). This procedure was done in collaboration with Paul Yong.

2.3 BrdU assay for the presence of an inactive X: replication study (CVS- JF)

2.3.1 *BrdU incubation and harvest*

In a subset of CVS samples (see CVS-JF 1-4), in addition to the extraction of DNA for XCI studies, subcultures were used for replication studies using bromodeoxyuridine (BrdU) and fluorescence in situ hybridization (FISH) with anti-BrdU. The BrdU-FISH component of the study, was done by Jane Gair in collaboration with Dr. Peter Lansdorp laboratory. In brief, semi-confluent CVS cultures were incubated with 0.1 ml of BrdU (1000X) per each 10 ml of culture (Boehringer-Mannheim). Incubation was done for 6 hours after which 0.2 ml of Karyo-Max Colcemid solution (10μg/ml) (GIBCO) were added per each 10 ml of culture for 1-2 hours before harvesting. Finally, cells were harvested using the trypsinization procedure described above and each cell pellet was incubated in 8 ml of hypotonic solution of 0.075M KCl (Stem Cell Research) in a 37°C water bath for 30 minutes. The preparation was spun and the supernatant removed, after which, the cell pellet was fixed with 3:1 methanol:acetic acid (Fisher) and used to drop several slides for the FISH preparations.

2.3.2 *BrdU FISH*

Slides prepared as described above were treated with 1X PBS pH 7-7.5 for 10-15 minutes. They were subsequently fixed with 4% formaldehyde in PBS (for 2 min) to preserve cell morphology and washed 3 times for 5 minutes in 1X PBS. Later, the slides were treated with a solution of pepsin in acid water (1mg/ml) at 37°C for 10 minutes and washed twice with PBS (2 minutes each time). At this point, the slides were subject to a second fix in 4% paraformaldehyde (2 min) followed by 3 more washes in PBS (5 min each). Then the slides were dehydrated using a short series of ethanol washes (70%, 90% and 100%) and finally air dried in preparation for denaturation and staining.

Slides were denatured with pre-warmed 70% formamide in 4X SSC (2 min) in a 37°C water bath and later subject to a second series of ethanol washes (70%, 90% and 100%) (2 min each). A blocking solution (1% BSA, 0.1% Tween 20 in 1X PBS) was used to incubate the slides for 20 min. The slides were then covered with 50 µl of a 1:25 solution of anti BrdU FITC: blocking solution and then placed in a humid incubation chamber for 20 min at room temperature in the dark. Slides were kept wet and then washed 3 times (5 min each) with 1X PBS. A fixation step with 4% formamide (10 min), followed by a series of ethanol washes (70%, 90%, 100) preceded the final step that involves counterstaining with DAPI. The slides were visualized under a fluorescence microscope.

2.4 DNA extraction protocol

2.4.1 *DNA extraction from fresh blood*

In most of the cases of normal term placentae studied in Chapter 3, maternal blood and cord blood were collected from the placenta. In most of the mosaic trisomy cases, discussed in Chapter 6, maternal blood and patient's blood were obtained after birth (peripheral blood). A salting out method was used to extract DNA from fresh blood (modified from Miller et al. 1988). Depending on the sample 0.5 to 7 ml of fresh blood were drawn from the patient (peripheral blood). Alternatively blood was collected from the vessel used to transport the placenta or from the segment of the umbilical cord attached to the placenta after delivery. Blood was collected in vacutainer tubes with EDTA and then transferred to 50 ml conical tubes. Ice-cold erythrocyte (EC) lysis buffer (155 mM NH_4Cl; 10 mM $KHCO_3$; 0.1 mM Na_2EDTA pH 7.4) was added to the blood bringing the total volume to 40ml. The samples were then lysed at 4°C until the mixture turned translucent or was no longer opaque (signalling that most red blood cells were completely lysed). The tubes were

then centrifugated at 1500rpm (Jouan CR412 bench-top centrifuge) at 4°C for 15 minutes and the supernatant was decanted and bleached. The lysis was repeated if the pellet had still some blood in it (red pigment visible). The remaining leukocyte pellet was then resuspended in SE buffer pH 8.0 (75mM NaCl; 25mM Na_2EDTA). The amount of SE buffer varied according to the size of the pellet from 1-5 ml. Proteinase K (PK)(20mg/ml) and 10% sodium dodecyl sulfate (SDS) were added to the buffer in amounts equivalent to 1/200 of the volume and 1/20 volume respectively and the lysate was incubated overnight at 37°C. Following a 5 minute incubation at 55°C, 6M NaCl was added to the lysate in an amount equivalent to 1/3 of its volume, the mixture was vortexed vigorously for 30 seconds and centrifugated for 15 minutes at 3000rpm at room temperature. The clear supernatant was transferred to a clean 50 ml conical tube. Two volumes of ice-cold 95% ethanol were then added to precipitate the DNA. A sealed glass pasteur pipette was used to fish out the DNA which was then washed in 70% ethanol and dissolved in TE buffer (10mM Tris/HCl pH 8; 1mM EDTA). DNA concentration was estimated be either spectrophotometry or by direct visualization of the genomic DNA using a 1% agarose gel stained with ethidium bromide.

2.4.2 *DNA extraction from frozen blood*

DNA was extracted from frozen blood using a modification of the procedure described above using phenol-chloroform for the extraction (as described in Sambrook et al. 1989). Frozen blood was thawed and lysed in EC lysis buffer as described above for fresh blood. Every step of the lysis procedure thereafter using SE buffer, PK and SDS was identical to the one described for the salting out method. Once the pellet had been completely lysed overnight, however, 1 volume of DNA grade phenol pH7.8 was added to the lysate and the mixture was vortexed for 30 seconds and centrigugated at 5000rpm for 5 minutes at 4°C. The top layer (aqueous layer) was the carefully transferred to a clean conical tube and 1

volume of a 50:50 mixture of phenol-chloroform was added to the sample. The mixture was vortexed for 30 seconds and centrifuged again at 5000 rpm at 4°C for 5 minutes. The top layer was then transferred to a new tube in which 1 volume of chloroform was added to the mixture. This was followed by centrifugation as described above and transfer of the aqueous layer to a clean tube in which 1/10 of volume of 3M sodium acetate (pH 6) was added to the sample, along with 2 volumes of ice-cold 95% ethanol to precipitate the DNA. DNA was collected and dissolved as described for the fresh blood. Occasionally, no precipitate was visible, therefore the sample was left at -20°C overnight. The DNA was then pelleted by centrifugation at 5000 rpm at 4°C for 35 minutes. The pellet was washed in 70% ethanol, air-dried and dissolved in TE buffer as described above.

2.4.3 *DNA extraction from tissue sections and tissue cultures*

DNA was extracted from tissue sections or pellets obtained from enzymatic separation or tissue culture harvests using a salting out method (Miller et al. 1988). Up to 50 mg of tissue were finely minced using razor blades and transferred to 1.5ml eppendorfs. The sample was then washed 2-6 times (depending on level of contamination with maternal blood) with PBS until the supernatant and tissue sections were clean. Tissue lysis buffer (200-500µl) (0.01M Tris-HCl pH 7.6, 0.01M EDTA pH 8.0, 0.1M NaCl, 2% SDS, 0.039 M DTT and 20µg/ml of proteinase K) was then added to the tissue or cell pellet along with additional 10-30µl of proteinase K (20mg/ml) and the lysate was incubated for 1-3 days in a 37°C water bath. When the lysate had a homogenous appearance (no visible pieces of tissue), 1/3 of the volume of 6M NaCl was added to the sample and it was vigorously vortexed for 30 seconds. The mixture was then centrifuged at 13000rpm in a Biofuge 13 microcentrifuge at 4°C for 5 minutes. The clear supernatant was transferred to a clean 1.5 eppendorf tube in

which 2 volumes of ice cold 95% ethanol were added to precipitate the DNA. Genomic DNA was either fished out, or precipitated overnight and pelleted by microcentrifugation as described for the blood extraction, washed in 70% ethanol and later dissolved and stored in TE buffer.

2.5 XCI assays

XCI was tested in all of the samples using methylation-sensitive assays to estimate the degree of skewing. The basis for these assays is the existence of methylated deoxycytosine residues at the CpG islands (usually at the 5' ends) of various X-linked genes on the inactive X chromosome (Yen et al. 1984; Tribioli et al. 1992). Certain restriction enzymes such as *HpaII* and *HhaI* are methylation sensitive and therefore can only cut their corresponding restriction sites when the site is not methylated. Given that we expect that X-linked genes that are subject to XCI will have one allele that is methylated (on the inactive X) and one allele that is not methylated (on the active X), some of these genes can be used to study XCI patterns. Suitable genes should have restriction sites for methylation sensitive enzymes such as *Hpa II* and *HhaI* and also a polymorphic marker (e.g. microsatellite) near the restriction site, allowing the distinction between paternal and maternal alelles.

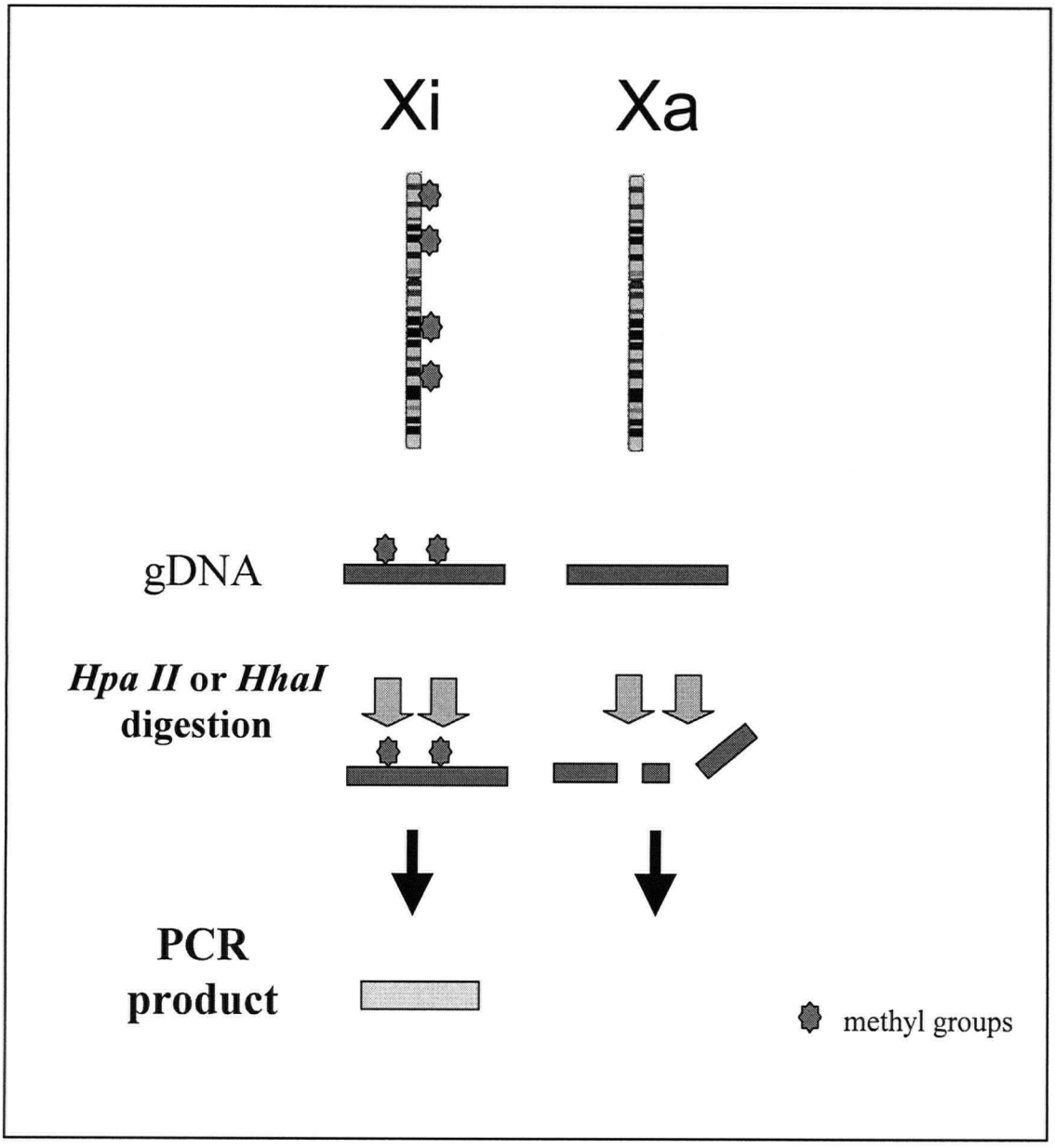
In a methylation-based assay, genomic DNA is digested with a methylation sensitive enzyme and then the region of the gene of interest that encompasses the restriction sites and the polymorphic marker is amplified by PCR. A PCR product is expected only from the allele that was not cut by the restriction enzyme (inactive allele), while the allele that was cut by the restriction enzyme would yield no PCR product (see Figure 2.2).

Three different genes that meet the criteria delineated above have been used in this study for the methylation based assays: Androgen Receptor (*AR*), Fragile X Mental Retardation (*FMR1*) and *ZNF261* (Figure 1.1).

2.5.1 *Restriction enzyme digests*

Three hundred nanograms of each genomic DNA sample were co-digested with 10U of *RsaI* and 10 U of *Hpa II* or *Hha I* in 1X buffer 1 (10mM Bis Tris Propane-HCl; 10mM $MgCl_2$; 1mM dithiothreitol at pH7) or buffer 4 (20mM Tris Acetate; 10mM magnesium acetate; 50mM potassium acetate; 1mM dithiothreitol at pH 7.9 with added bovine serum albumin 100µg/ml) respectively (all enzymes and buffers from New England Biolabs-NEB), in a total volume of 20 µl. The secondary cutter *RsaI* was used to facilitate co-digestion by the methylation sensitive enzymes *Hpa II* or *Hha I* and to increase the accuracy and reproducibility of our results (Beever 2002). An internal control, referred to as the "undigested sample" was prepared for each sample by digesting 300 ng of the same DNA sample with 10U of *RsaI* and 1X NEB buffer only. DNA samples from male individuals or fathers of the probands were also assayed as digestion controls. All the samples were incubated overnight in a 37°C water bath.

Figure 2.2 Schematic representation of the methylation-sensitive-enzyme based assay for assessment of XCI status. Methylation sensitive enzymes *HpaII* or *HhaI* are not able to cut restriction sites that are methylated. Methylated genomic DNA from the inactive (Xi) that is left intact is then amplified by PCR. Unmethylated restriction sites from the active X (Xa) will be cut by the restriction enzymes, thus, yielding no PCR product by PCR. In somatic tissues, the promoter regions of several X-linked genes on the inactive X are methylated, while equivalent regions on the active X are unmethylated.



2.5.2 *Test for completeness of digestion: the MIC 2 gene*

Following *Hpa II* or *HhaI* treatment, samples were checked for completeness of digestion through PCR amplification of a segment in exon 1 of the pseudoautosomal *MIC2* gene (Xp22.32), which is unmethylated on both X chromosomes as it escapes XCI (Goodfellow et al. 1988). As the amplified segment has several *HpaII* and *HhaI* restriction sites, no PCR product is expected from completely digested samples whereas undigested samples allow the amplification of a 400 bp product. This assay was performed as previously published by (Anderson and Brown 2002). PCR conditions were as follows: the reaction mix was prepared with 1X Rose Taq buffer (20mM Tris HCl pH 8; 10mM KCl, 0.1% Triton X 100; 50 μg/ml nuclease free BSA; 2mM $MgCl_2$), 4% DMSO, 125μM dNTP, 400nM of each primer and 0.2U of Rose Taq Polymerase in a total reaction volume of 15μl. One microlitre digested DNA and undigested (control) DNA was added to the reaction mix and amplified under the following PCR conditions: initial denaturation at 95°C for 3 minutes, denaturation at 95°C for 1 minute, annealing at 52°C for 1 minute and extension at 72°C for 2 minutes, for 35 cycles, with a final extension at 72°C for 7 minutes. The undigested sample was used as an internal control to determine a baseline level of amplification of one allele relative to the other. Primer sequences for the *MIC2* gene were as follows: *MIC2*-A 5'-AGAGGTGCGTCCGATTCTT-3' and *MIC2*-B 5'-CGCCGCAGATGGACAATTT-3' (as described in Anderson and Brown 2002). One microlitre of agarose loading buffer (blue juice) (50% glycerol; 0.1% xylene cyanol; 0.1% bromophenol blue; 25mM EDTA and 50mM Tris pH4) was added to each PCR reaction and all 15 μl of PCR product were run and visualized on 1% agarose gels stained with ethidium bromide. Incomplete digestion was evidenced by the presence of a band at the 400bp level, in which case, 5 U of *HpaII* were added to the sample and incubated at 37°C overnight, followed by PCR amplification and

visualization of products as described above. This assay was shown to detect at least 4.5% of incompletely digested sample in our laboratory (Beever 2002).

2.5.3 *AR assay*

The methylation status of the Androgen Receptor gene, located in Xq11.2-q12 (Figure 1.1) has been shown to correlate with XCI status in humans (Allen et al. 1992). In this assay, the methylation pattern of two *HpaII* restriction sites adjacent to a polymorphic CAG repeat in the 1st exon of the gene was evaluated. This was done according to a protocol described previously (Allen et al. 1992). *AR* is a highly informative marker with heterozygosity at this locus estimated to be 90%. PCR conditions were as follows: the reaction mix was prepared using 1X Rose Taq buffer, 200μM dNTP, 500nM of each primer and 0.2U of Rose Taq Polymerase in a total reaction volume of 15μl. A total of 1.5 μl of DNA from each sample were added to the reaction mix and amplified using the following PCR conditions: initial denaturation at 95°C for 5 minutes, denaturation at 95°C for 45 seconds, annealing at 60°C for 30 seconds and extension at 72°C for 30 seconds, for 25 cycles, with a final extension at 72°C for 7 minutes. Primer sequences were as follows: F primer 5'-GCTGTGAAGGTTGCTGTTCCTCAT-3' and R primer 5'-TCCAGAATCTGTTCCAGAGCGTGC-3'. The size of the PCR product is ~ 280bp.

2.5.4 *FMR1 assay*

The methylation status of the fragile X mental retardation-1 gene (*FMR1*), located on Xq27.3 (Figure 1.1) has also been shown to correlate with XCI status in humans (Carrel and Willard 1996). The *FMR1* assay evaluates the methylation pattern of two *HpaII* restriction sites near a polymorphic CGG repeat located in the 5' UTR of the gene and was

performed according to the method described by (Carrel and Willard 1996; Hecimovic et al. 1997). This is a moderately informative marker with a heterozygosity value of 63% (Fu et al. 1991). PCR conditions were as follows: the reaction mix was prepared using the 1X PCR buffer #1 (Expand Long Template PCR System kit from ROCHE), 1% DMSO, 116μM dATP, 116 μM dCTP, 116 μM dTTP, 100μM dGTP, 250 μM 7-deaza GTP, 1μM of each primer and 0.35 U of Taq enzyme mix (Expand Long PCR kit-ROCHE) in a total reaction volume of 10μl. Only 1 μl of DNA for each sample was added to each reaction tube and amplified using the following PCR conditions: Initial denaturation at 97°C for 4 minutes, denaturation at 97°C for 30 seconds, annealing at 65°C for 45 seconds with an additional 20 seconds per cycle, an extension at 68°C for 4 minutes, for 25 cycles with no final extension. Primer sequences were as follows: F primer 5'-GCTCAGCTCCGTTTCGGTTTCACTTCCGGT-3' and R primer 5'-AGCCCCGCACTTCCACCACCAGCTCCTCCA-3'. The product size is ~300 bp.

2.5.5 *ZNF261(DXS6673E) assay*

The methylation status of the *ZNF261 (DXS6673E)* gene, mapped to Xq13.1 has been recently shown to correlate with XCI status in humans (Beever et al. 2003). For the DXS6673E assay, the methylation status of two *Hha I* sites in the vicinity of a polymorphic $(GA)_6$ CATA $(GA)_{20}$ repeat in exon 1A of the gene was evaluated (Van der Maarel et al. 1996). This is highly informative marker with a heterozygosity value of over 90% (Beever et al. 2003). PCR conditions were as follows: the reaction mix was prepared with 1X Rose Buffer with no $MgCl_2$, 200μM dNTP, 0.5mM $MgCl_2$, 1μM of each primer and 0.1 U of Rose Taq in a total reaction volume of 25 μl. Two microlitres of sample were added per reaction and PCR was done under the following conditions: initial denaturation at 95°C for 4 minutes,

denaturation at 95°C for 1 minute, annealing at 54°C for 1 minute and extension at 72°C for 2 minutes, for 35 cycles with a final extension at 72°C for 7 minutes. Primer sequences were as follows: *DXS663E*-A 5'-ATGCTAAGGACCATCCAGGA-3' (Beever et al. 2003) and *DXS6673E*-B 5'-GGAGTTTTCCTCCCTCACCA-3' (same as primer DXS6673E-2 in Carrel and Willard 1999).

2.5.6 *Visualization of PCR products of AR, FMR1 and DXS6673E XCI assays*

Separation of PCR products for all 3 markers and quantification of band intensities was performed using two different methods due to the acquisition of new equipment in the lab in recent years. A) Silver-stained polyacrylamide gels evaluated by densitometry (NIH image software- http://rsb.info.nih.gov/nih-image)
B) Automated fluorescent fragment analysis using an ABI Prism 310 Genetic Analyzer (GeneScan Analysis Software version 3.1.2).

For densitometry, 3-8 µl of PCR product (both undigested (control) and digested samples) was mixed with an equal volume of urea loading buffer (4.2%urea, 0.1% bromophenol blue, 5mM EDTA and 0.1% xylene cyanol) and the mixture was denatured at 95°C for 5 minutes, immediately chilled on ice, and then loaded on to a 5% polyacrylamide gel (50% urea). The product was separated by electrophoresis at 55W for 60 min, after which the gel was blotted onto blotting paper, transferred to a 10% methanol/10% acetic acid solution for 15 minutes, washed in 10% ethanol for 10 minutes and soaked in a 0.5% Nitric Acid solution for 30 seconds to remove background. Following two washes with water, the gel was stained with 0.2% silver nitrate for 20 minutes, rinsed once with water and developed with a solution of 0.28M Na_2CO_3 and 0.0185 % formaldehyde. As bands appear, the gel was fixed with a 10% acetic acid solution for a few minutes and then washed briefly with water.

Finally the gel was dried on blotting paper at 80°C for 35 minutes using a gel dryer. Each gel was subsequently scanned using Apple Color OneScanner Dispatcher Software and later analyzed by densitometry using NIH image software. This program converts allele bands into peaks and allows the calculation of peak areas that represent band intensities. The average intensity reached on either side of the peaks was used to set a baseline or background level for each of the samples (Figure 2.3). In this manner, the areas under the peaks corresponding to both undigested and digested samples (run side by side on the gel) were calculated and used to estimate XCI skewing as detailed below. This method showed good reproducibility in our laboratory ($r=0.8$, $p<0.0001$-correlation test).

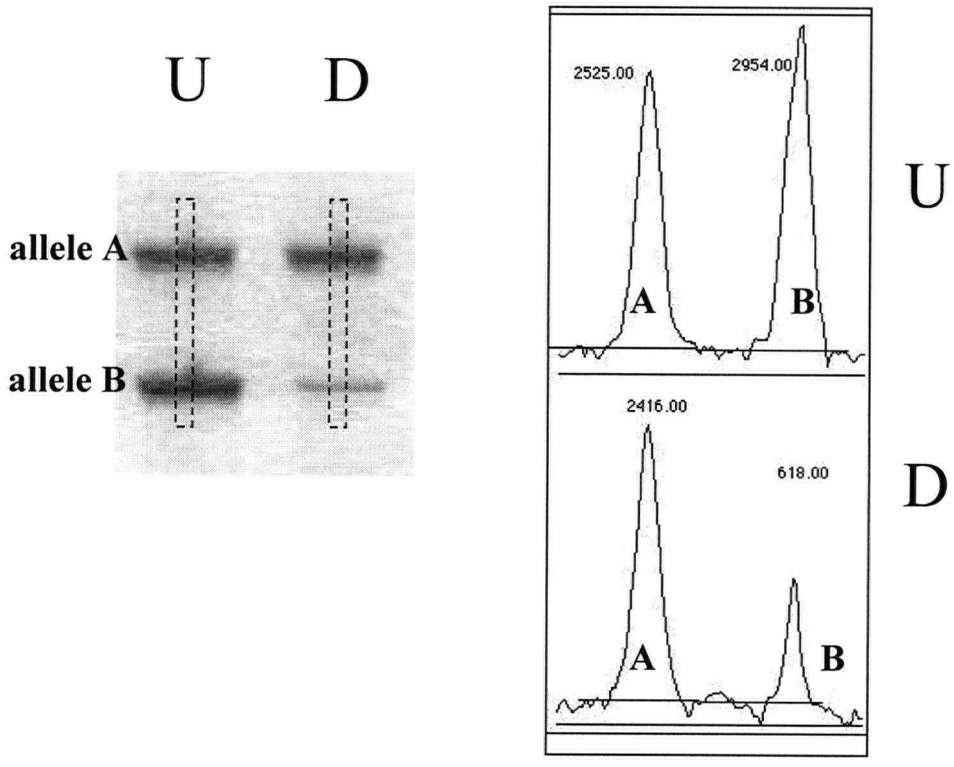
The automated fluorescent analysis required that 1 of each pair of primers were labelled with either fluorescent HEX dye (ABI Prism) (markers *AR* and *DXS6673E* or 6-FAM fluorescent dye (ABI Prism) (*FMR1* marker). PCR for each of the 3 markers was performed as described above. One microlitre of PCR product was mixed with 10.8 µl of deionized formamide and 0.2 µl of ROX 500 size standard (ABI) and denatured at 95°C for 5 minutes, immediately chilled on icy water and finally run by capillary electrophoresis using an ABI Prism 310 Genetic Analyzer. Using both the Data Collection software and the GeneScan Analysis software, the alleles for each marker are displayed as peaks and their areas are automatically calculated. Peak areas are then used to estimate the degree of skewing (Figure 2.4). The reproducibility of this method was higher than the one for densitometry ($r=0.9$, $p<0001$).

2.5.7 *Calculation of the degree of skewing*

The data obtained from both the densitometry of silver stained gels and the automated fluorescent analysis was used to calculate the degree of XCI skewing using the following formula: $(D_1/U_1)/(D_1/U_1+D_2/U_2)$ where D_1 represents the peak area of the digested

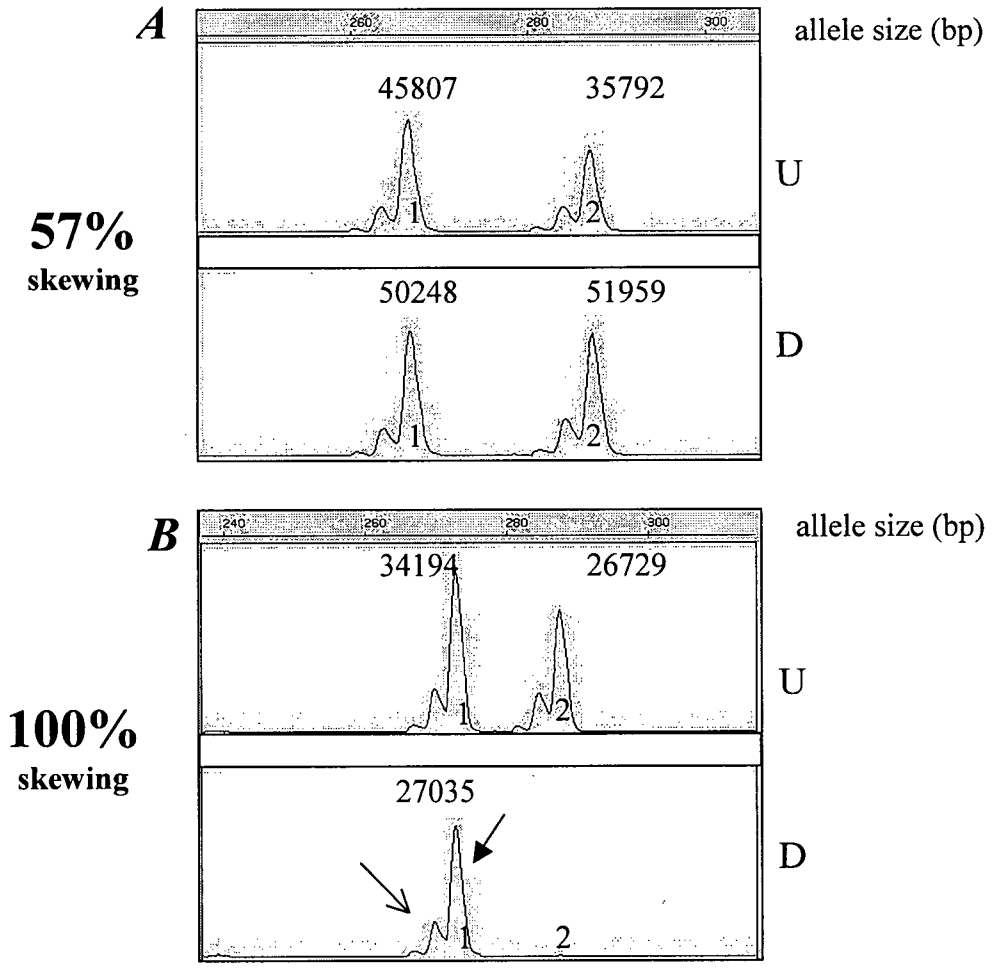
sample of the more intense allele, D_2 represents the peak area of the digested sample of the less intense allele, and U_1 and U_2 represent the peak areas of the corresponding undigested samples. In this manner, the degree of skewing was estimated relative to the most intense allele (the preferentially inactive allele) and therefore, values are given within the range of 50-100% skewing. However, when comparing tissues from the same individual, X inactivation is measured relative to the larger allele, giving a value of 0-100% skewing. The undigested sample, as explained before, was used in every case as an internal control allowing the normalization of measurements and overcoming the bias posed by possible differential amplification of certain alleles of a given marker (see Figures 2.3 and 2.4).

Figure 2.3 Estimation of the degree of XCI skewing by densitometry. Silver stained polyacrylamide gels were scanned and analyzed with NIH image software. Undigested (U) and digested (D) samples were run side by side. A section of each lane (alleles A and B) is selected from the image (dotted rectangles) and used for the densitometry analysis in which the program calculates the density of each band and shows it as peak on a plot. The background line or baseline is added manually allowing the program to calculate the areas under each of the peaks (numbers above the peaks). These data is used to calculate skewing using the formula described in section 2.5.7.



skewing = 82.1%
preferential inactivation
of allele A

Figure 2.4 Estimation of the degree of XCI skewing by automated fluorescent analysis ABI-310. In sections *A* and *B* the upper rectangle shows the PCR products amplified from the undigested sample (U) and in the lower rectangle, the PCR products of the digested sample (D). Alleles 1 and 2 are shown both heterozygous females. The area under each peak, including the main bands (filled arrows) and the shadow band (light arrows) in each case is indicated above each of the peaks. Also indicated is the % skewing in each case as calculated by the formula given in section 2.5.7. Sample ***A*** shows random XCI while sample ***B*** shows extreme XCI skewing.



2.6 Assays to assess the methylation status of other X-linked genes in CVS

In order to further assess the methylation status of several X-linked genes on CVS, four additional genes, some with no available DNA polymorphisms, were used to study the CVS samples: *ARAF1, MAOA, GRIA3 and XIST* (Figure 1.1). These assays were performed using methylation sensitive enzymes *Hpa II* (for the first 3 genes) and *Hha I* (for *XIST*).

2.6.1 ARAF1 assay

This gene maps to Xp11.4-p11.2 and is subject to XCI (Anderson and Brown 1999). In this assay, the methylation status of 8 *HpaII* sites in the promoter region of the gene (Anderson and Brown 2002) was tested using the same *Hpa II* digests and control samples used for the polymorphic gene assays (*AR* and *FMR1)*. PCR conditions were as follows: the reaction mix consisted of 1X Gibco Taq buffer, 1mM $MgCl_2$, 200μM dNTP, 800nM each primer and 0.625 U of Gibco Taq in a total volume of 25μl. Two microlitres of sample were added per reaction and PCR was done under the following conditions: initial denaturation at 95°C for 5 minutes, denaturation at 95°C for 1 minute, annealing at 58°C for 1 minute and extension at 72°C for 2 minutes, for 32 cycles with a final extension at 72°C for 7 minutes. Primer sequences were as follows: ARAF-m1 5'-TGCCAAAGCCCTAAGGTCA-3' and ARAF-m4 5'-CGCTGTCGACGATGGTCT -3'. Ten microlitres of the PCR product mixed with 1 μl of agarose loading buffer were run on a 1% agarose gel, loading the undigested and digested samples side by side. Product size (509 bp) was confirmed using a φ X174 DNA-Hae III ladder.

2.6.2 MAOA assay

The monoamine oxidase A (*MAOA*) gene maps to Xp11.3-Xp11.4 and is also subject to XCI. In this assay the methylation status of 6 *HpaII* sites located upstream of the first exon of the gene in the vicinity of a polymorphic GT dinucleotide/VNTR region (located 1kb 3'of 1st.exon) (Hendriks et al. 1992) was investigated using *Hpa II*-only digests. The secondary cutter *Rsa I* was not used as there were restriction sites for this enzyme within the amplified region. PCR conditions were as follows: the reaction mix contained 1X buffer #3 (Expand Long PCR kit –Roche), 500 nM dNTPs, 400nM each primer, 250μM $MgCl_2$ and 1.75 U of Taq polymerase mix (Expand long PCR-Roche). Two microlitres of digest were added to a final reaction volume of 25 μl and PCR was done under the following conditions: initial denaturation at 94°C for 2 minutes, denaturation at 94°C for 30 seconds, annealing at 53°C for 30 seconds and extension at 68°C for 80 seconds, for 35 cycles with a final extension at 68°C for 7 minutes. Primer sequences were as follows: *MAOA* -3.6 5'-ACATTCTAAACCTAATAACTC-3' and *MAOA-3.3* 5'-CAATAAATGTCCTACACCTT-3'. The resulting PCR products (1.2-1.4 kb) were then cut with *SacI* to reduce the size of the DNA fragments that encompass the polymorphic region. Digestion was done using 1X buffer 1 (NEB), 1% BSA, 1 U of *SacI* and 9 μl of PCR product, for a total volume of 20 μl that was incubated at 37°C overnight. Six to eight microlitres of the digested PCR product were mixed with urea loading buffer, denatured at 95 °C and loaded into a 5% polyacrylamide gel, where they were electrophoresed for 1 h at 55W. The undigested and digested (*HpaII*-only) samples were run side by side and the gel was silver-stained.

2.6.3 *GRIA3 assay*

This glutamate receptor gene (*GRIA3)* maps to Xq25-q26 and is subject to XCI. In this assay, the methylation status of 3 *HpaII* sites in the 5' UTR promoter region of the gene (Gecz et al. 1999) was tested using the same *Hpa II* digests and control samples used for the polymorphic gene assays (*AR* and *FMR1)*. PCR conditions were as follows: the reaction mix consisted of 1X Taq buffer (Gibco), 1.5mM $MgCl_2$, 200 μM dNTPs, 600 nM primer mix, and 0.625 U of Gibco Taq, in a total reaction volume of 25 μl. Two microlitres of sample were added per reaction and PCR was done under the following conditions: initial denaturation at 94°C for 5 minutes, denaturation at 94°C for 1 minute, annealing at 58°C for 1 minute and extension at 72°C for 2 minutes, for 35 cycles with no final extension. Primer sequences were as follows: GLI-1 5'-ATCCACGTTAGGAGTGGTGT-3' and GLI-2 5'-AAGCGATTTCTGTCCCTATG-3' (Betty Lai (Brown Lab.), personal comunication). Ten microlitres of the PCR product mixed with 1 μl of agarose loading buffer were run on a 1% agarose gel, loading the undigested and digested samples side by side. Product size (~410bp) was estimated using a φ X174 DNA-Hae III ladder.

2.6.4 *XIST (AT2;29r)* assay

The X inactive specific transcript gene (*XIST)* maps to Xq13.2 and is expressed from the inactive X. *XIST* is methylated on the active X and therefore can also be used for methylation-based assays. In this assay, the methylation status of one *Hha I* site located in the promoter region/exon 1 of the gene, was tested using the same *HhaI* digests used for the *DXS6673E* assay. PCR conditions were as follows: the reaction mix consisted of 1X Gibco Taq buffer, 1.5 mM $MgCl_2$, 200μM dNTPs, 1μM primer mix and 0.625 U of Gibco Taq Polymerase in a total reaction volume of 25 μl. Two microlitres of sample were added per

reaction and PCR was done under the following conditions: initial denaturation at 94°C for 5 minutes, denaturation at 94°C for 1 minute, annealing at 54°C for 1 minute and extension at 72°C for 2 minutes, for 30 cycles with no final extension. Primer sequences were as follows: *XIST*-AT2 5'-TATGCTCTCTCCGCCCTCA-3' and *XIST-29r* 5'-AATCAGCAGGTATCCGATACC-3' (Brown et al. 1992; Tinker and Brown 1998). Ten microlitres of the PCR product mixed with 1 µl of agarose loading buffer were run on a 1% agarose gel, loading the undigested and digested samples side by side. Product size (~550bp) was estimated using a φ X174 DNA-Hae III ladder.

2.6.5 *XIST 3':5'* assay

The *XIST* gene maps to Xq13.2 and is expressed from the inactive X. This assay was used only to test the presence of amplifiable DNA in both the undigested and digested sample from the methylation based assays (Brown et al. 1992). This allows distinction between the finding of no PCR product in the reaction due to hypomethylation and therefore complete digestion with *HpaII*, versus instances in which there is no PCR product due to absent or non-amplifiable DNA. Given that these primers amplify a segment within exon 6 of the *XIST* gene which contains no *Hpa II*, *HhaI* or *RsaI* restriction sites, both undigested and digested samples are expected to yield a PCR product. PCR conditions were as follows: the reaction mix consisted of 1X Gibco Taq buffer, 1.5 mM $MgCl_2$, 200µM dNTPs, 1µM primer mix and 0.625 U of Gibco Taq Polymerase in a total reaction volume of 25 µl. Two microlitres of sample were added per reaction and PCR was done under the following conditions: initial denaturation at 94°C for 5 minutes, denaturation at 94°C for 1 minute, annealing at 54°C for 1 minute and extension at 72°C for 2 minutes, for 30 cycles with no final extension. Primer sequences were as follows: *XIST*-3'

GAAGTCTCAAGGCTTGAGTTAGAAG and *XIST*- 5'-TTGGGTCCTCTATCCATCTAGGTAG. Ten microlitres of the PCR product mixed with 1 µl of agarose loading buffer were run on a 1% agarose gel, loading the undigested and digested samples side by side. Product size (~185bp) was estimated using a φ X174 DNA-Hae III ladder.

2.7 Statistical Analysis

The data in the present study was analyzed using the following tests:

Descriptive statistics: Summary statistics (i.e mean or average and sums) were calculated using Microsoft Excel 2000 and/or Statistical Package for the Social Sciences (SPSS)10.0.

Fisher's exact Test and Chi Square test: These two tests determine the existence of an association between two or more variables. The Fisher's Test, can be applied when small sample sizes are involved; however both tests allow to test the null hypothesis (H_0= no association between the variables) and to determine the probability of observing the obtained data due to chance. As the Fisher's test calculates exact probabilities and is directional, it was used as the preferred method when possible. The chi-square test was used when the Fisher's test could not be applied (mostly situations in which the large sample did not allow the complex calculation required for a Fisher's test). Both tests were performed using VassarStats: Website for Statistical Computation-Lowry R, 1999. (http://faculty.vassar.edu/lowry/VassarStats.html).

Kolmogorov-Smirnov One-Sample Test: This test assesses the degree to which an observed distribution of categorical frequences differs from an expected pattern (on the null hypothesis). It is similar to the Chi-square goodness of fit test. The Kolmogorov-Smirnov test was performed in this thesis using SPSS 10.0.

Exact Binomial probability: It is used for calculation of exact binomial probabilities in a standard "k out of n" situation in which "k" is the number of times a binomial outcome is observed or is estipulated to occur, "p" is the probability that the event will occur on any particular occasion, "q" is the probability that the event will not occur on any particular occasion and "n" is the number of occasions or opportunities for the event to occur. This test was performed using VassarStats: Website for Statistical Computation-Lowry R, 1999. (http://faculty.vassar.edu/lowry/VassarStats.html)

Correlations: A correlation test is a measure of the relationship that exists between two paired variables. The Pearson correlation coefficient was used throughout this study and this test was performed using VassarStats: Website for Statistical Computation-Lowry R, 1999. (http://faculty.vassar.edu/lowry/VassarStats.html).

Mann-Whitney test: The Mann-Whitney test is a non-parametric test that estimates the differences between the means of two independent samples. As this test does not make assumptions of the distribution of the population it is used to compare values that are not distributed normally. This test combines the two samples, ranking the measures from highest to lowest. In this case the null hypothesis maintains that if both samples are drawn from the same population, the sum of ranks of each sample should be roughly the same. This test was performed using SPSS 10.0

Chapter 3: X-chromosome inactivation patterns in human term and first trimester placentae.

3.1 Introduction

X chromosome inactivation (XCI) is one of the most fascinating and well-studied epigenetic processes and yet much remains to be discovered about the timing and mechanism of XCI during early human development. XCI is generally random, and it is stably maintained through successive cell divisions (Lyon 1961). There are, however, a number of examples of non-random XCI in which the choice of X chromosome to remain active at the onset of XCI is affected (summarized in Chapter 1, section 1.2.3).

In the early 1970's, reports of preferential inactivation of the paternally derived X for various species of marsupials provided one of the first evidences of genomic imprinting in mammals (Cooper 1971). Preferential paternal XCI was subsequently described in eutherian embryonic tissues (mouse and rat) (for review see Gartler et al. 1992). A number of studies in human extraembryonic tissues have followed, often showing conflicting results (summarized in Chapter 1 and Table 1.1). For example, some showed evidence of imprinted XCI in human extraembryonic tissues and others did not.

Previous studies of XCI in human placenta have been confounded by one or more of the following factors (see Table 1.1): 1) Samples limited to one stage of development: In many studies only a small number of cases were studied, limited to either term placentae, CVS or first trimester placental samples. It is possible that imprinted XCI in placenta

depends on the developmental stage of the embryo as has been implicated for other imprinted genes such as H19 (Jinno et al. 1995; Adam et al. 1996), highlighting the need for a more comprehensive study that includes several samples from different developmental stages. 2) The use of a single sample or site from each of the studied cases as representative of the placenta as a whole: A study by Henderson and colleagues on the distribution of mosaicism in human placentae showed a patchiness in the distribution of aneuploid cells on the placental disc, suggesting that the sampling of multiple sites from each placenta would give a more accurate idea of the placenta as a whole (Henderson et al. 1996). 3) Limited informativeness of markers: Most of the early studies reported in the literature have used rare protein isoforms, usually restricted to specific human groups (e.g. isoforms of human *G6PD*). 4) Treating the placenta as one tissue type: Most previous studies have either used a "placental sample" for which the tissue composition was not indicated or samples that included only the components of the chorionic villi (Table 1.1). Although in some studies the components of the chorionic villi (trophoblast and mesenchyme) were sampled and analyzed separately, in other studies they were analyzed together (villi). 5) The use of cultured cells for XCI assays: In several studies, assays were performed using cultured cells, which introduces a possible culture bias in the results due to preferential growth of certain cells. Because XCI is clonal, results can change in culture if there are selective advantages for the growth of certain cells. 6) The potential for differential expression of certain protein markers: Possible differences in the level of expression (translational bias) or detection of different protein isoforms could bias XCI studies based on them. Additionally, some protein isoforms could be favoured by selection over others in nature. 7) Maternal contamination: Contamination of placental samples could occur with either maternal blood that surrounds the placenta or maternal decidua, which is in intimate contact with the chorionic villi in the maternal side of the

placental disc. As the mother shares at least one allele with the case or patient, the presence of maternal contamination in any given fetal sample will render the calculation of skewing for that sample, inaccurate.

The goal of the present study was to overcome methodological limitations and biases of previous studies in order to achieve a clear understanding of the various aspects of the XCI process in human placenta. Thus, this study was designed to 1) assess XCI patterns in placentae from different gestational ages (first trimester and term placentae). 2) reduce the sampling bias by examining multiple sites from each placenta; 3) increase informativeness of the cases studied by the use of at least two X-linked markers (*AR* and *FMR1* assays); 4) use placental samples in which each of the different extraembryonic tissue components have been carefully isolated either by dissection or enzymatic separation; 5) use direct preparations (DNA extractions from tissue sections) to assess XCI status in order to avoid possible culture biases. 6) avoid the possibility of maternal contamination biasing the results through the exclusion of samples showing evidence of maternal contamination when tested with either the X-linked markers or the genotyping of a number of autosomal markers.

The knowledge of the characteristics of the XCI process in human extraembryonic tissues in the context of imprinted XCI seen in other mammals is likely to shed some clues into the evolution of the XCI process itself. Furthermore, it is important to understand the similarities and differences between humans and mice since the mouse is frequently used as a model organism in genetic studies. Additionally, the inclusion of various placental tissues in this study might allow the establishment of developmental correlations between tissues. In general, a better knowledge of the process of XCI in humans, which may differ from other

species, may lead to a greater understanding of the potential impact of X-linked gene expression in extraembryonic tissue growth and development. Finally, a clear understanding of the XCI inactivation patterns in normal placentae, would allow us to establish comparisons with similar studies in pathological conditions that may involve impaired or abnormal placental or fetal development.

3.2 Results

A total of 22 term placentae were analyzed in this study. Fourteen were from female newborns and all were informative for the *AR* assay. The remaining 8 were derived from male newborns and therefore were used as digestion controls (see Chapter 2.1). In order to study samples from an earlier developmental stage, eight females informative for either the *AR* or *FMR1* assays were identified from a total of 23 first trimester therapeutic terminations (TA) (≤12 weeks gestation). Three additional informative females were identified in a sample of 12 spontaneous abortions (SAB) (8-13 weeks gestation). The remainder of the cases in both groups were either males (20 cases) or females either uninformative (2 cases) for either XCI assay used in this study or with an XO constitution (2 cases). The total number of samples tested for each group of placentae is shown in Table 3.1.

As described in chapter 2, two visualization methods were used in this thesis; silver stained polyacrylamide gels or assesment of the samples with the 310ABI genetic analyzer. All the assay results on term placentae were visualized on silver stained polyacrylamide gels. Some of the TA's and all the SAB's were analyzed using the 310ABI genetic analyzer, which was purchased a few years into the study and allows a faster, more efficient and reproducible analysis of the results. A study done in our laboratory has shown

no statistically significant differences between the two methods ($p=0.107$ Wilcoxon signed-rank test, two-tailed t-test) and highly correlated estimates of XCI skewing ($r=0.986, p<0.0001$) (Beever 2002). The same study showed a positive but poor correlation ($r=0.673$, $p<0.0001$) between the *AR* and *FMR1* assays used to study XCI, yet the two assays did not significantly differ ($p=0.453$, Wilcoxon signed-rank test, two-tailed t-test). For these reasons all samples are considered together, regardless of the XCI assay used to study them and the visualization method used to determine skewing.

The term "preferential" inactivation that is used throughout this chapter implies any skewing above 50% unless a different cut-off value is indicated. Any deviation from the 1:1 or 50:50 ratio was included in this study to allow comparisons with previous studies that used this criterion. However samples were further classified into two groups for the XCI analysis using a cut-off of 75% skewing: those with <75% skewing and those with ≥75% skewing. This cut-off value was chosen based on several considerations. This value (≥75% skewing) has been used in other studies (Gale et al. 1994) probably related to the fact that the lowest value of skewing found in a study of extraembryonic tissues in mice, where preferential XCI of the paternally derived X was reported, was 79% (Takagi and Sasaki 1975). For all samples showing more than 70% skewing, digestion and amplification were repeated and the reported values in such cases are the average of two separate readings (I thought this would allow a more accurate classification of samples that show low levels of skewing versus those that show moderate to extreme levels of skewing). In order to measure "extreme levels of skewing" a higher cut-off value of ≥90% skewing was also used, based on the data from several studies in humans that use this cut-off value to measure "extreme XCI skewing" (Plenge et al. 1997; Lanasa et al. 1999; Sangha et al. 1999; Beever 2002).

Table 3. 1

Number of skewed sites at different cut-off values of skewing. The parent of origin of the inactive X for each tissue type is given for all those placentae that were informative for parent of origin. Note that the values in column 1 for total samples tested include some samples that showed 50% skewing (no detectable bias).

tissue type (number of placentae tested)[a]	cut-off values	>50% skewing		≥75% skewing		≥90% skewing	
	Total samples tested[a]	total samples[a] >50% skw.	informative samples only[b] $Xi^P : Xi^M$	total samples[a] ≥75% skw.	informative samples only[b] $Xi^P : Xi^M$	total samples[a] ≥90% skw.	informative samples only[b] $Xi^P : Xi^M$
A) term placentae (n=14)	n = 322^	n = 297	52 : 76	n = 72	13 : 23	n =24	4 : 9
amnion	74	64	7 : 23 **	11	0 : 7***	0	....
chorion	78	69	12 : 18	4	0 : 3	0	....
trophoblast	82	79	19 : 13	37	8 : 7	19	3 : 6
mesenchyme	88	85	14 : 22	20	5 : 6	5	1 : 3
B) pre-term placentae[c] (n=11) TA+SAB	n = 33^	n = 30	14 : 11	n = 8	2 : 1	n = 6	0 : 1
amnion	3	2	2 : 0	0	....	0	....
chorion	5	5	1 : 3	2	0 : 1[d]	2[d]	0 : 1[d]
membranes	3	3	2 : 1[d]	0	....	0	....
trophoblast	11	10	7[d] : 1[e] *	4	2[d] : 0	2	....
mesenchyme	11	10	2[d] : 6[d]	2	....	2	....

[a] includes placentae that are informative and non-informative for parent of origin of the inactive X.

[b] includes only placentae informative for the parent of origin of the inactive X (Xi^P= paternal X preferentially inactive and Xi^M= maternal X preferentially inactive).

[c] there were no obvious differences between the two kinds of first trimester placentae analyzed in this study (TA's and SAB's), therefore the data for both of them has been combined.

[d] hypomethylation is likely in some samples, therefore the result may not accurately reflect inactivation status.

[e] the only sample skewed towards the preferential inactivation of the maternally derived X in trophoblast at the ≥50% cut-off was a SAB.

* $p<0.035$ (1-tail) ** $p < 0.005$(2-tail) ***$p=0.15$ (2-tail) Compared to the expected 1:1 ratio through exact binomial probability test.

^ these totals include samples that showed 50% skewing.

3.2.1 *Effect of sample size and sample location in the distribution of skewing in placentae.*

In an effort to assess the effect of sample size in XCI skewing (non-random XCI) and given the possibility of a clonal "patchy" growth of placental tissues, 1-2 sites of approximately $3mm^3$ (small sites) were taken from the fetal side of term placentae in 7 cases (see Chapter 2.1). The level of skewing observed in both small sites ($3mm^3$) and large sites ($1\text{-}1.5\ cm^3$) is shown in Table 3.2, where a cut-off of $\geq 75\%$ skewing was used to divide the samples in two groups. While there is a trend towards more XCI skewing in the small sites as compared to the large sites, this difference was not significant for any of the four placental tissues considered in this study (amnion, chorion, trophoblast and mesenchyme) (Table 3.2).

To test the possibility of site location within the placenta affecting the level of skewing, one site was collected at the base of the umbilical cord (usually located in a central position), 1 or 2 sites at the periphery of the placental disc, and 3-5 sites in intermediate locations. Additionally two sites were obtained from the maternal side of the placenta. The data shows no bias in skewing due to site location (see Table 3.3). The lack of effect of both sample size and sample location on the level of skewing allowed all the sampled sites in each placenta (small and large) to be considered together for the rest of the data analysis.

Table 3.2. Effect of sample size in the distribution of XCI skewing in term placentae. Number of samples in each extraembryonic tissue per size category that showed skewing above and below a cut-off of 75% skewing.

sample size	small		large		all		total	P value[a]
tissue	<75%	≥75%	<75%	≥75%	<75%	≥75%		
amnion	9	1	54	10	63	11	74	n.s.
chorion	11	1	63	3	74	4	78	n.s.
trophoblast	5	7	40	30	45	37	82	n.s
mesenchyme	9	3	59	17	68	20	88	n.s
	34	12	216	60	250	72	322	

[a] Fisher's exact test (one tail): no significant differences were found for the degree of skewing vs sample size at the ≥75% cut-off value, in the pair-wise comparisons for any tissue (e.g. small vs large in amnion)

Table 3.3. Effect of sample location within the placental disc in the distribution of XCI skewing in term placentae. Number of skewed sites of each extraembryonic tissue per location category that showed skewing above and below the cut-off value of 75% skewing.

	cord		middle		periphery		maternal side		total	P value[a]
	<75%	≥75%	<75%	≥75%	<75%	≥75%	<75%	≥75%		
amnion	13	0	37	9	13	2	0	0	74	n.s
chorion	11	0	47	4	16	0	0	0	78	n.s
tropho	8	3	29	19	6	9	2	6	82	n.s
mesenchyme	12	1	43	8	13	3	5	3	88	n.s
	44	4	156	40	48	14	7	9	322	

[a] Fisher's exact test (two tails): no significant differences were found for the ≥75% level of skewing in any of the pair-wise comparisons for any tissue (e.g. cord vs middle, cord vs periphery, middle vs periphery)

3.2.2 *XCI skewing in term and first trimester placentae*

One of the objectives of this study was to determine the prevalence of moderate to extreme skewing in term and first trimester human placentae. All female term placentae (14 cases) analyzed in this study showed at least 1 site in one tissue with levels of skewing of ≥ 75% (moderate to extreme skewing). Ten of those 14 placentae showed one or more sites in one tissue with ≥90% skewing (extreme XCI skewing). Skewing levels of ≥75% were found in 5 of 11 first trimester placentae, while extreme skewing was only seen in 3 of 11 cases. This data shows significant differences between first trimester and term placentae when moderate to extreme skewing is considered ($p=0.002$) and borderline significance between the two groups ($p=0.04$) when only extreme skewing is considered.

3.2.3 *Degree and direction (parent of origin of the inactive X) of skewing in human extraembryonic tissues*

To test the possibility of imprinted XCI in placental tissues in humans, as seen in mice and marsupials, the parent of origin of the inactive X had to be established. Thus samples of maternal blood or maternal decidua had to be available for study and the mother had to share only one of the alleles present in the placenta. Only 6 of 14 term placentae, 5 of 8 TAs and all 3 SABs met these criteria and therefore were informative for parent of origin of the inactive X. Table 3.1 summarizes the results for these placentae based on the XCI assays using (*AR* and *FMR1*). The degree of XCI skewing and parent of origin of the inactive X were analyzed in each of the 4 different placental tissues studied (amnion, chorion, trophoblast and mesenchyme) with the following results:

Amnion

Every term placenta analysed in this study showed some degree of skewing at one or several sites of this tissue. If any deviation from the 1:1 ratio (or 50% skewing) is considered (as has been done by several previous studies), 8 out of 14 term placentae showed heterogeneous patterns of XCI, in which either the paternal X or the maternally derived X was found to be inactivated in different sites within the same placenta. In 5 of 14 cases skewing occurred towards the same allele and one case showed only 1 site with >50% skewing. When any deviation from a 1:1 ratio is considered, a significant majority of amnion samples (23/30) from 6 informative term placentae showed preferential inactivation of the maternal X ($p=0.0052$; two tailed Exact binomial probability) (Table 3.1). When a higher cut-off value of ≥75% skewing was used, a total of 11 of 74 (15%) of amnion samples from 5 of 14 term placentae showed this level of skewing (Tables 3.1 and 3.4). Using the ≥75% cut-off value, all three cases where multiple sites were skewed within the same placenta showed inactivation of the same allele within the placenta and in all 7 informative samples, the maternal X was preferentially inactivated ($p=0.015$ two tail; Exact binomial probability)(see Table 3.1). First trimester placentae were excluded from this analysis due to the small number of cases for which amnion was available.

Chorion

The chorion was rarely extremely skewed for XCI. However, when all deviations from a 1:1 ratio are considered in the chorion samples, all but one term placenta (13/14) showed sites with some level of skewing and in all of these placentae a heterogeneous pattern of XCI was seen. Skewing in these cases also showed a slight trend towards the inactivation of the maternally derived allele (18/30 samples) (see Table 3.1). Only 4 of 78 (5%) samples

in three different placentae showed a degree of skewing above the ≥75% cut-off value (Tables 3.1 and 3.4). Using the ≥75% cut-off value, all three informative samples also showed preferential inactivation of the maternally derived X (Table 3.1). First trimester placentae were excluded from this analysis due to the small number of cases for which chorion was available.

Mesenchyme

If any deviation from a 1:1 ratio is considered, all term placentae showed some degree of XCI skewing in the mesenchyme. All but one term placenta showed a heterogeneous pattern of XCI in this tissue, although there was a trend towards the inactivation of the maternally derived allele (22/36 samples) (see Table 3.1). The mesenchyme showed ≥ 75% skewing in 10 of 14 placentae, in a total of 20 of 88 (23%) of samples (Table 3.4). At this cut-off level, skewing in the same direction was seen in 3 of 5 placentae with multiple mesenchymal sites available for study. In the other two placentae, XCI skewing was heterogeneous and no overall bias in terms of the parental origin of the inactive X was evident. Only 5 samples were found to be skewed when a higher cut-off value (≥90%) was used, and 3 of 4 informative samples show inactivation of the maternally derived allele. At this cut-off level skewing was heterogeneous in 1 of two placentae where multiple sites were available. The sampling of only one placental site in first trimester placentae precluded a similar analysis to that of the term placentae in these cases. However, considering all the first trimester placentae together and any deviation from a 1:1 ratio of skewing, the majority of mesenchymal samples (6/8) were skewed towards the inactivation of the maternally derived X although this bias is not significant (see Table 3.1). Only two

samples showed a higher degree of skewing (≥90%), although none of these were informative for parent of origin of the inactive X (Table 3.1)

Trophoblast

When any deviation from a 1:1 ratio is considered, 10 of 13 term placentae showed XCI skewing and in all cases, a heterogeneous pattern of XCI was observed. In 3 term placentae inactivation occurred towards the same allele. No data for this tissue is available for PX1. A total of 19 of 32 samples with any detectable level of skewing showed a trend towards preferential inactivation of the paternally derived allele (Table 3.1). The trophoblast showed levels ≥ 75% skewing, in at least one site from all 13 of 13 placentae analysed for this tissue. Six of ten placentae that showed ≥ 75% skewing at multiple sites were skewed in the same direction. At the ≥ 75% cut-off value, no parental bias to skewing was evident from the data on informative term placentae. A similar result was true at a higher cut-off value of ≥90% in which 5 of 7 placentae showed skewing in the same direction (Table 3.1). The sampling of only one placental site in first trimester placentae precluded a similar analysis in these cases. However, considering all the first trimester placentae together, a borderline significant majority of trophoblast samples (7/8) were skewed towards the preferential inactivation of the paternally derived X ($p=0.035$ one tail; Exact binomial probability) (see Table 3.1).

Given that all the term placentae for which trophoblast samples were available showed sites with levels of skewing ≥75%, it is of interest to determine if the trophoblast showed significant differences in terms of degree of skewing relative to the other 3 tissues studied. A comparison of the number of samples that showed <75% skewing and ≥75%

skewing in different extraembryonic tissues of term placentae shows significantly more skewing in trophoblast (37/82 samples) compared to each of the other 3 tissues ($p \leq 0.002$) (Table 3.4).

Table 3.4. Number of samples showing less or equal/more than 75% skewing in different extraembryonic tissues of normal term pregnancies. There is significantly more skewing in trophoblast than in the rest of tissues examined.

Tissue	number of placenta	degree of skewing [a,b]		comparison with Trophoblast[c]
		>50%--< 75%	≥ 75%	
amnion	14	63	11	P<0.0001
chorion	14	74	4	p<0.0001
mesenchyme	14	68	20	p=0.002
trophoblast	13	45	37	---

[a] The values included in this table are based on the percentages of skewing of the preferentially inactive allele (using the *AR* assay).

[b] The number of samples analyzed for each tissue is not the same due to the elimination of some samples because of either contamination or DNA degradation.

[c] The Chi square test (http://vassun.vassar.edu) was used for these comparisons.

XCI is mostly heterogeneous in term placentae

Heterogeneity of XCI skewing could be examined in all placentae even when parent of origin of the inactive X was not available by assesing whether the larger or the smaller allele were preferentially inactivated in every sample. Using the ≥75% cut-off, most of the term placentae (8 cases) showed heterogeneous patterns of XCI; that is, the presence of skewing in two sites of the same placenta, in opposite directions, as previously explained. Heterogeneous XCI could occur both within one tissue type or between different placental tissues in a given placenta. For example Figure 3.1 shows the distribution of skewing at different sites in all 4 different tissues in one placenta (PX23). There were however, a few placentae (4 cases) in which skewing tended toward the same allele (unidirectional X inactivation). For example, Figure 3.2 shows the distribution of skewing in placenta PX5, in which the direction of skewing was similar in different tissues and within a given tissue. In the remaining term placentae (2 cases), only one site was found to be skewed at the ≥75% level. Ten of 14 term placentae showed one or more sites with extreme skewing (≥90%). Three of those 10 placentae showed a heterogeneous pattern of XCI, while 6 of 10 showed extreme skewing towards the same allele and 1 of 10 showed extreme skewing in only one site. Therefore, although the XCI pattern in term placentae is for the most part heterogeneous, there are some placentae where skewing occurs towards the same allele (unidirectional skewing). This is illustrated graphically in Figure 3.3 (a,b,c and d).

First trimester placentae

Previous studies have shown that genomic imprinting for certain genes such as H19 depends on the developmental stage of the embryo (Jinno et al. 1995; Adam et al. 1996).

This suggests that there is a possibility for developmental differences in a potentially imprinted XCI. In an attempt to address this possibility, XCI was also examined in first trimester placental samples. All the first trimester placentae (TA and SAB) were considered together due to the small sample size in each of the two studied groups. Preferential inactivation of the maternal X was seen in at least one sample from each tissue studied except amnion for which only two of three samples were informative. It is noteworthy that neither moderate nor extreme skewing were very common. The only sample showing greater than 90% skewing was one sample of chorion, which showed preferential maternal XCI. Thus, there is certainly not a strong imprinting bias in first trimester placentae. Nonetheless, 7 of 8 samples of trophoblast that showed any level of skewing (including 2/2 samples of trophoblast with ≥75% skewing), showed preferential inactivation of the paternally derived X ($p=0.035$ one tailed; Exact binomial probability) (Table 3.1).

Figure 3.1. Distribution of skewing in term placenta PX23
(an example of a placenta where skewing values are very heterogeneous)

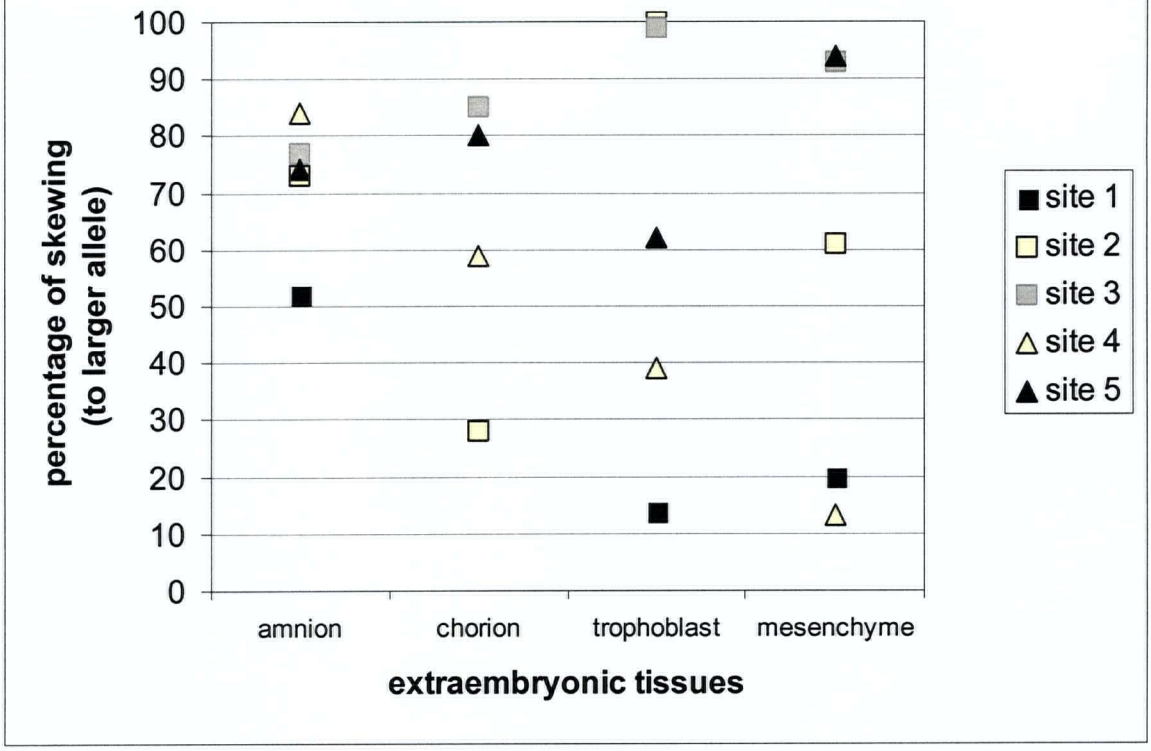


Figure 3.2 Distribution of skewing in term placenta PX5
(an example of a placenta for which skewing was relatively homogeneous)

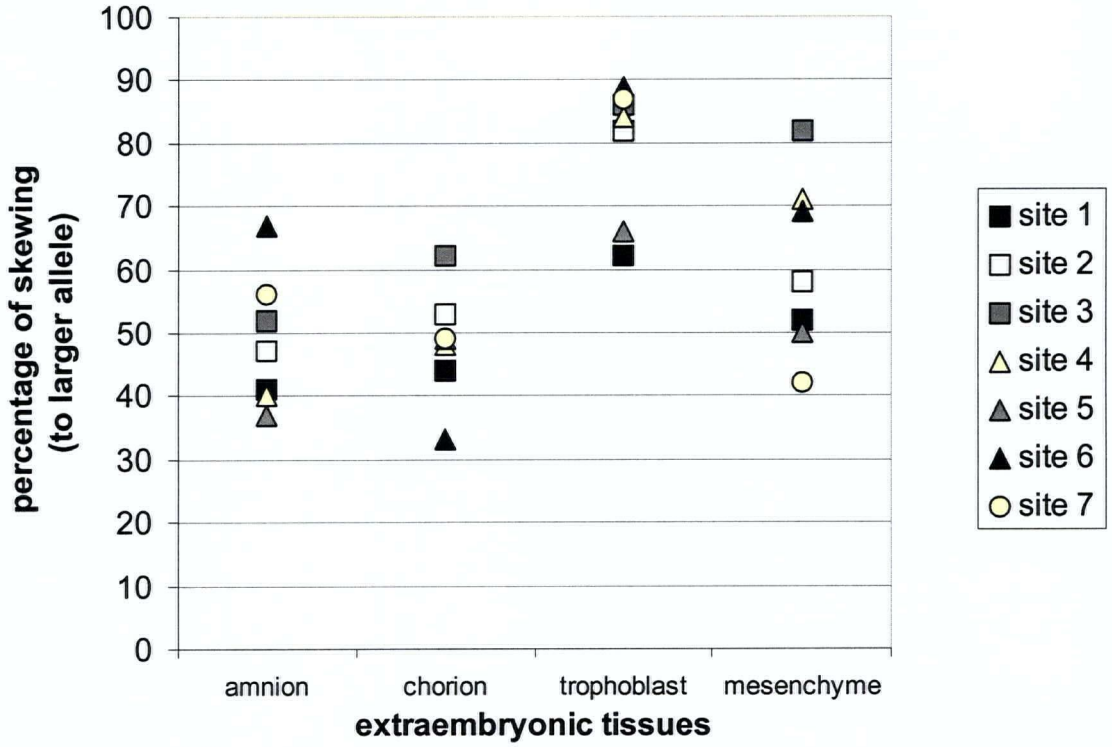


Figure 3.3 Distribution of skewing in extraembryonic tissues from term placentae (N=14).

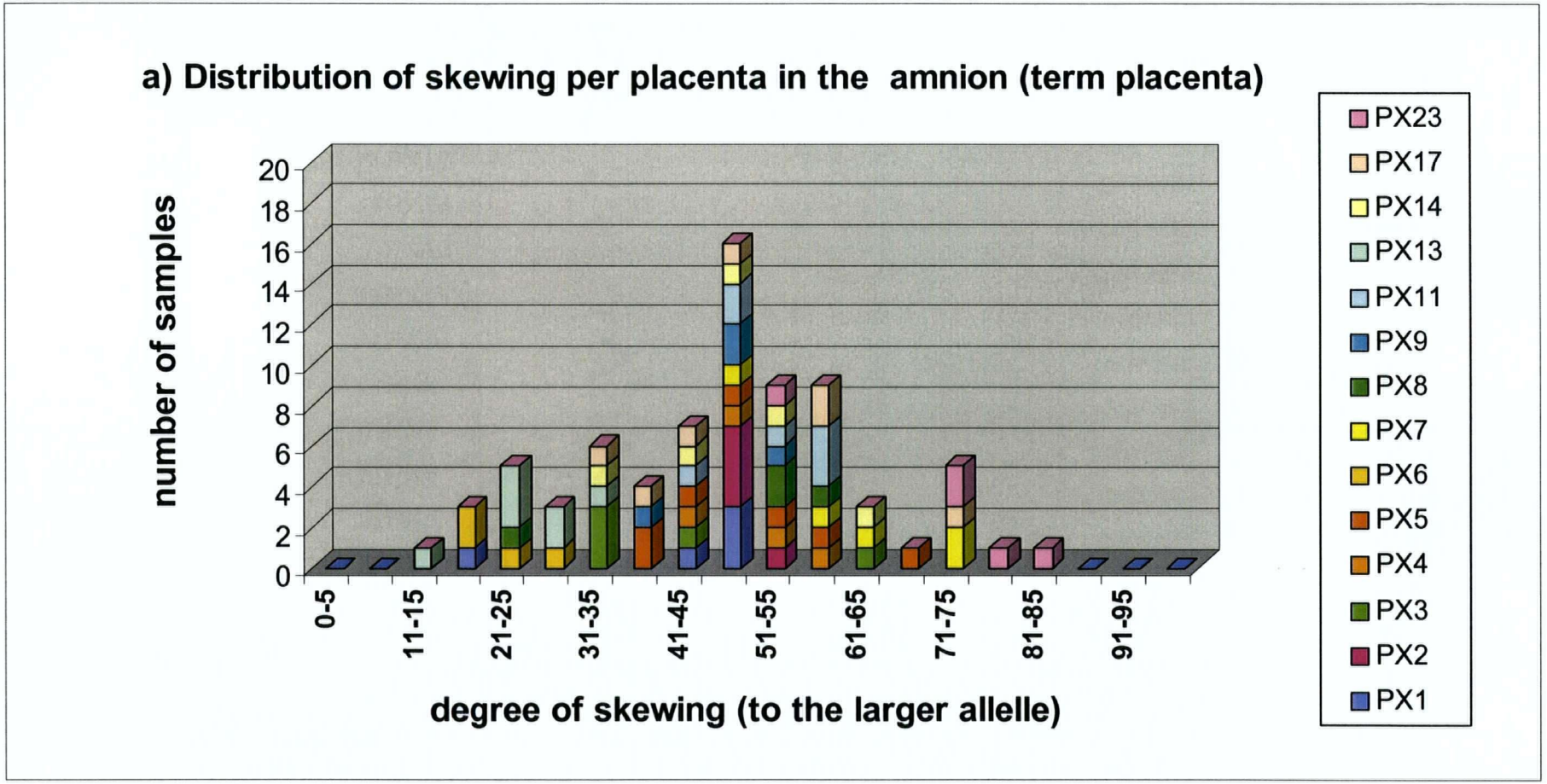


Figure 3.3 Distribution of skewing in extraembryonic tissues from term placentae (N=14).

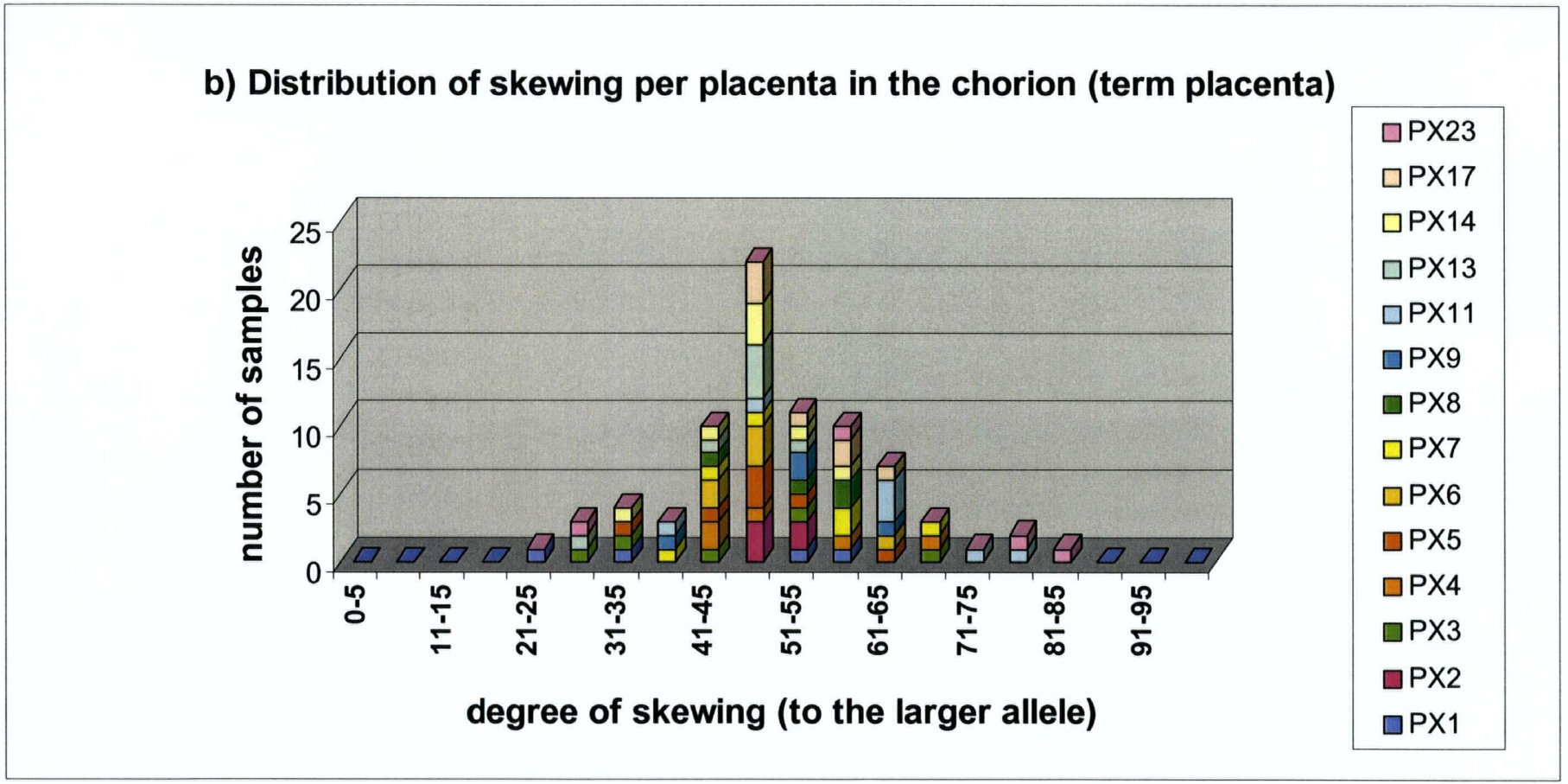


Figure 3.3 Distribution of skewing in extraembryonic tissues from term placentae (N=13).

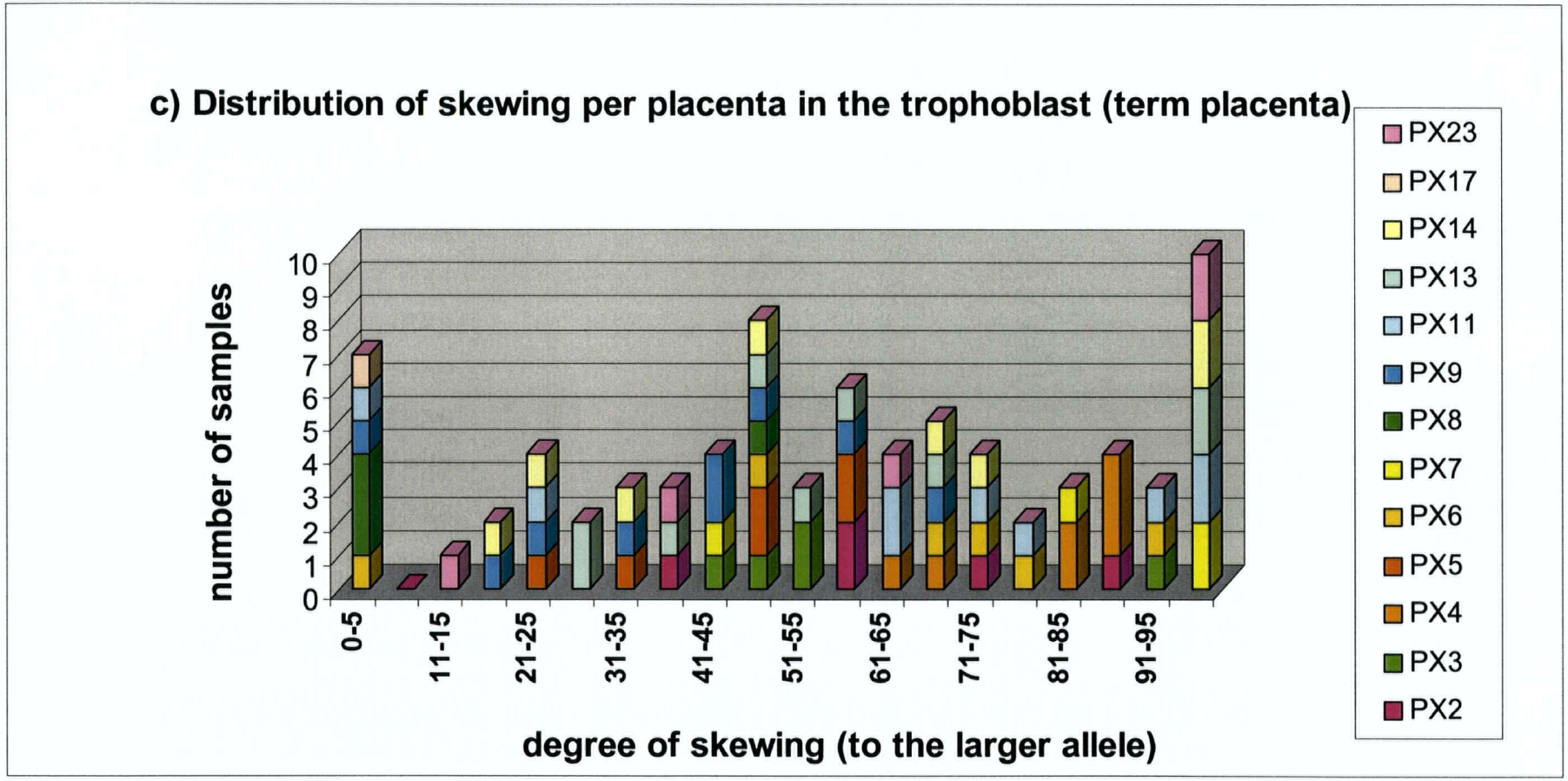
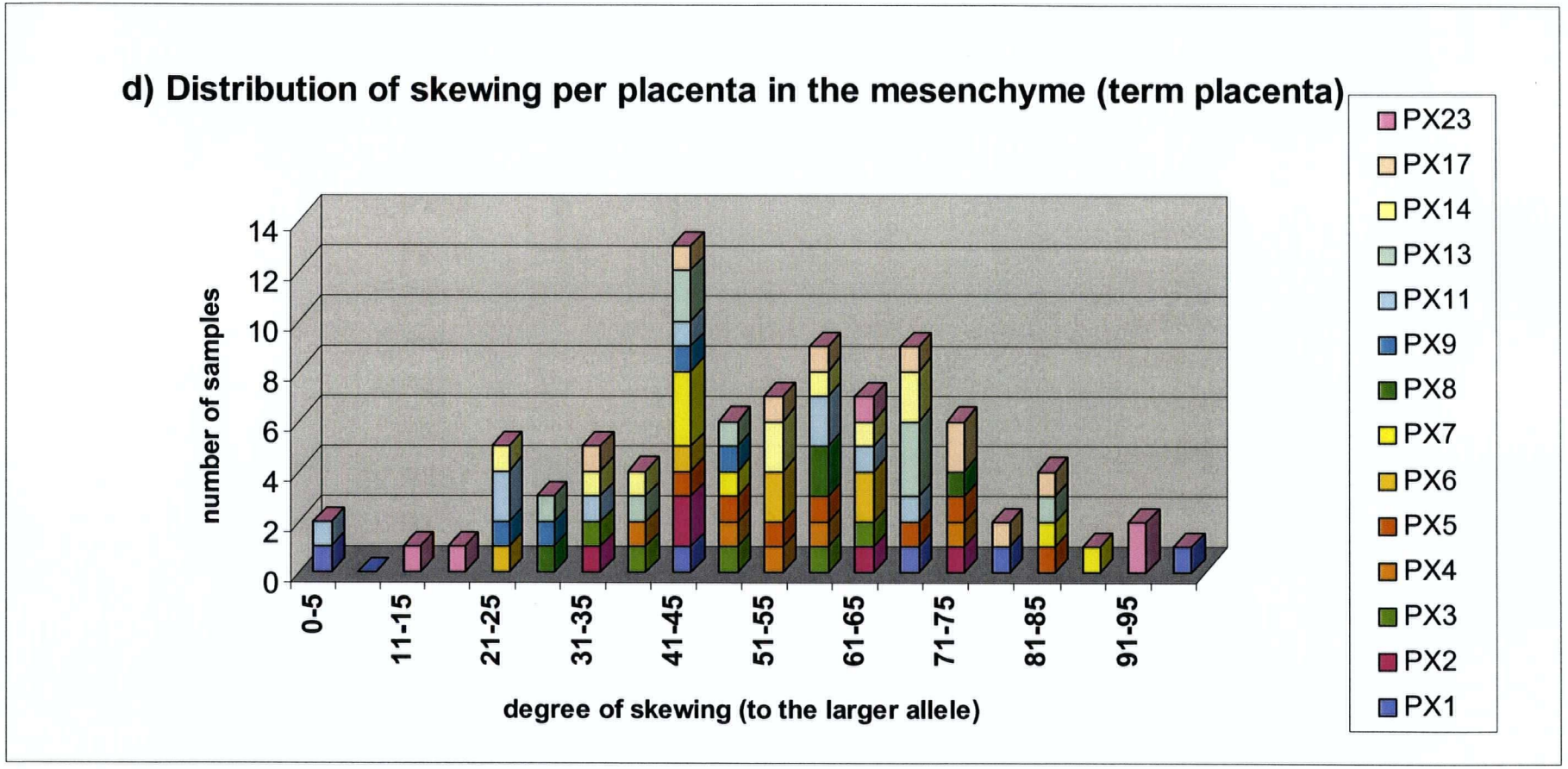


Figure 3.3 Distribution of skewing in extraembryonic tissues from term placentae (N=14).



3.2.4 *Distribution of skewing for different tissues in term placentae*

The parental origin of the inactive X could be determined in 6 of the14 female term placentae. However, as there is seemingly no parent of origin bias to skewing, the distribution of skewing in all term placentae was examined considering only the percentage of skewing for the larger allele (the upper allele on the gel) for 5-9 sites in each placenta. The data, shown in Figure 3.3 graphically demonstrates a seemingly normal distribution of the level of skewed XCI for amnion and chorion (Figure 3.3 a and b). The distributions of skewing for the mesenchyme and trophoblast do not appear normal, with a larger variance, a more ragged distribution and an increased frequency of extreme skewing (≥90% skewing) in the trophoblast (Figure 3.3 c and d). The Kolmogorov-Smirnov test for normality was applied and the results showed that the data from chorion does not follow a normal distribution ($p<0.01$). For the amnion, mesenchyme and trophoblast, on the other hand, normality was not rejected (see Table 3.5 and Figure 3.4 a,b,c and d). A detailed analysis of the contribution of each individual placenta to the overall distribution of skewing in different tissues shows that these results are not biased by the data derived from only a few placentae. They instead reflect the tendency seen across most of the placental samples (Figure 3.3 a,b,c and d).

3.2.5 *Correlation of skewing within a placenta (term placentae)*

If XCI or the designation of the X chromosome to be inactivated occurs prior to tissue differentiation in a small pool of cells we may expect a correlation in XCI skewing between different tissues. Such an effect is often observed when XCI is examined in different

fetal tissues (Gale et al. 1994; Azofeifa et al. 1996; Sharp et al. 2000) leading to the conclusion that the various tissues originated from a limited precursor cell pool. To investigate the possibility of a correlation of skewing between different tissues obtained from a specific site within a given placenta, pair-wise correlations between tissues were tested. For this analysis, unmatched samples of either of the four placental tissues were eliminated from the Pearson-correlation calculations (this refers to samples that were available for 1 tissue but were not available for some other tissue at a specific placental location or site). Detailed results are shown on Table 3.6.

A slightly positive but non-significant correlation was seen between amnion and each one of the other 3 extraembryonic tissues studied (Table 3.6). Similarly there was not a significant correlation between chorion and mesenchyme, nor chorion and trophoblast (Table 3.6). In fact, the correlation between chorion and trophoblast was negative. On the other hand, a significant correlation ($p<0.0001$) of the degree of skewing was found between the mesenchyme and the trophoblast when pairs of corresponding sites were analyzed, although a positive but non-significant correlation was found between these two tissues when non-corresponding sites were paired together (Table 3.6).

Figure 3.4 **Graphic representation of the distribution of skewing of 4 extraembryonic tissues (amnion, chorion, mesenchyme and trophoblast).** The X-axis in each figure represents the degree of skewing and the Y axis represents the number of samples. Each figure has an outline of the normal distribution for comparison.

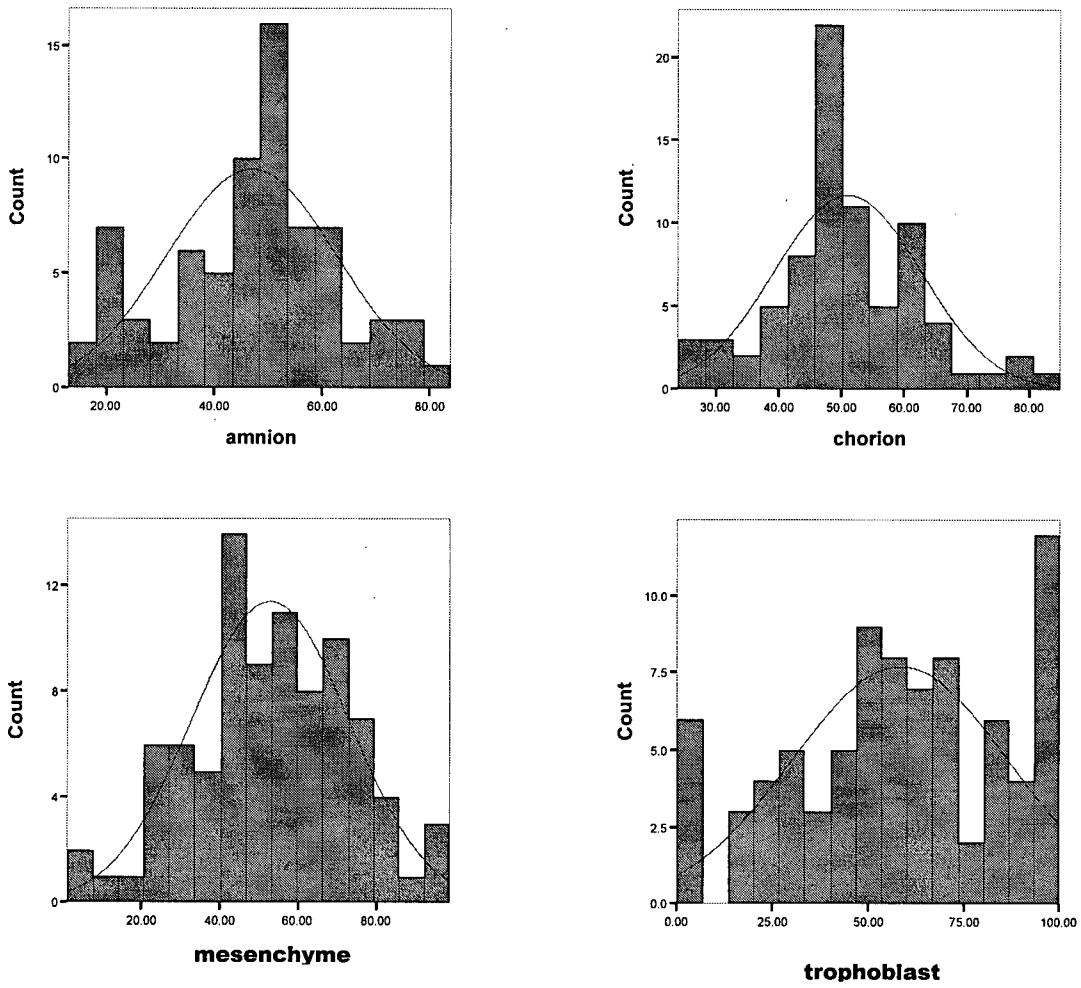


Table 3.5. Kolmogorov-Smirnov test for normality in each of the 4 extraembryonic tissues from term placentae.

tissue	Kolmogorov-Smirnov		
	statistic	df	significance
amnion	0.091	67	0.200
chorion	0.138	67	0.003*
trophoblast	0.072	67	0.200
mesenchyme	0.059	67	0.200

Table 3.6. Correlations of XCI skewing between different tissues within one placental site[a] . This table includes data from all 14 female term placentae.

tissue	**Amnion**	**Chorion**	**Trophoblast**
Amnion	....	....	....
Chorion	r= 0.151, p= 0.2, n= 72	....	....
Trophoblast	r= 0.118, p= 0.3, n= 68	r= -0.11, p= 0.3, n= 73	....
Mesenchyme	r= 0.136, p= 0.2, n= 74	r= 0.07, p= 0.5, n= 78	r= 0.625, p<0.0001, n=82

[a] Pearson correlation (p 2-tailed)
r= correlation coefficient
n= total number of samples tested
The correlation between pairs of non-corresponding sites between mesenchyme and trophoblast is (r= 0.151, p=0.1745, n=82)

3.3 Discussion

Although preferential inactivation of the paternally derived X is seen in marsupials and extraembryonic tissues of mice, the present study does not show the same effect in similar tissues in humans.

Preferential paternal XCI in human placenta

The results reported in this thesis show no tendency towards the preferential inactivation of a paternally derived allele in human term placentae. In fact, there was a tendency towards preferential maternal XCI in all tissues analyzed with the exception of the trophoblast and this difference was statistically significant in amnion. This is in contrast to previous findings in murine extraembryonic tissues and in several tissues in marsupials (Cooper et al. 1971; Richardson et al. 1971; Takagi and Sasaki 1975; Frels 1980) as well as several studies in humans that showed imprinted XCI in the extraembryonic tissues (Ropers et al. 1978; Harrison and Warburton 1986; Harrison 1989; Goto et al. 1997).

Although the number of analyzed samples is small, the results from the 1st trimester placentae follow similar patterns as those observed in term placentae, with the exception of the trophoblast. Remarkably, when all deviations from a 1:1 ratio are considered in first-trimester placentae, the trophoblast shows a tendency towards the inactivation of the paternally derived X chromosome with the exception of one placenta (SAB) that showed preferential XCI of the maternally derived X. While this tendency is only of borderline statistical significance, these findings leave open the possibility that there are developmentally related differences in terms of XCI bias. It is possible that in early gestation the trophoblast has a tendency towards the inactivation of the paternally derived X that is subsequently replaced by random XCI in the developing placenta. This could be related to

the fact that the trophoblast is the first tissue to differentiate in embryonic development of mice and humans. Changes in imprinting status dependent on stage of development have been reported for H19 (Jinno et al. 1995; Adam et al. 1996). Additionally, a tendency towards imprinted XCI in early placenta would explain conflicting results when samples from different gestational ages are compared. For example, a study of XCI skewing on CVS samples (10-12 wks gestation) by Goto and colleagues showed preferential inactivation of the paternally derived X in microdissected sections of trophoblast (Goto et al. 1997), while a recent study by Looijenga and colleagues (Looijenga et al. 1999), using term placental samples (~38-42wks gestation) found a predominantly heterogeneous pattern of XCI in the placental trophoblast.

In contrast to the results of Looijenga and of this thesis for term placentae, a study by Harrison on fresh trophoblast cells from term placentae showed almost exclusive expression of the maternal G6PD allele in all of 13 samples studied (Harrison 1989). To explain the discrepancies between these findings, it could be argued that gene methylation is not necessarily correlated with gene expression in every case, or perhaps that a predominantly maternal expression is a gene specific effect that affects G6PD in particular. Additionally, maternal contamination is a possibility, although this is a factor that was clearly controlled for in Harrison's study.

It is possible that the XCI imprint has been conserved in mice and continues to affect XCI in extraembryonic tissues throughout gestation, while in humans, the imprint is evident very early in gestation but as embryonic development progresses, relaxation of the imprint leads to random XCI in extraembryonic tissues. A possible mechanism for this transition may involve reactivation followed by de novo inactivation in tissues such as the

trophoblast. Evidence for reactivation (unstable inactivation) of the inactive X chromosome was found in a study by Migeon and colleagues where hybrids derived from a clone of chorionic villi cells (heterozygous for G6PD A) and mouse A9 cells, showed that loci such as *G6PD*, *HPRT* and *PK* were expressed on both human X chromosomes indicating that X-chromosome inactivation is completely reversible in cells of trophoblastic origin (Migeon et al. 1986). It is also possible that not all cells in this tissue undergo XCI at once, so that cells undergoing inactivation at earlier stages of development are affected by the imprint, whereas cells undergoing XCI at later stages of development undergo random XCI.

Whether XCI in human extraembryonic tissues is random in early gestation as well as at later stages of embryonic development, or shows a transition from imprinted XCI at earlier stages in development to random XCI at later stages of embryonic development, it is clear that the situation in humans is not entirely similar to that in the mouse extraembryonic tissues. The presence of several placental sites with preferential maternal XCI in a number of the samples of all the extraembryonic tissues analyzed in this study argues against preferential paternal XCI as a requirement for normal development in humans. The viability of human conceptuses with either XO or supernumerary X chromosomes, regardless of the parental origin of the missing or additional X chromosomes (Harvey et al. 1990; Hassold et al. 1990; Leal et al. 1994) represents strong evidence against stringent non-random XCI. In contrast, in mice with only maternally derived X's (e.g. X^M X^M), biparental embryos with supernumerary maternally derived X's (e.g. $X^M X^M Y$ or X^M $X^M X^P$) and monosomic $X^P O$ animals are all associated with impaired cell differentiation and reduced viability possibly due to the failure of dosage compensation (by inability to inactivate the extra X^M's or due to the inactivation of the only X^P in the monosomic cases) (reviewed in Huynh and Lee 2001). It can be argued however, that those X aneuploidies that survive in

humans are perhaps "imprinting escapees", that represent a minority of these types of conceptuses, given that 99% of monosomy X cases and 21% of other sex chromosome aneuploidies are spontaneously aborted (Thompson et al. 1991). However, the similar frequencies of maternally and paternally derived sex chromosome aneuploidies between spontaneous abortions and live births (Hassold et al. 1996) argue against this explanation, since outcome should be influenced by the parental origin of the X if there were a parental bias to XCI. Additionally, a recent study by Migeon and colleagues has shown that the human *TSIX* lacks the regulatory elements needed for the imprinting function of *Tsix* in mice. They concluded that human *TSIX* antisense transcripts are unable to repress *XIST* and both genes are transcribed from the inactive X (Migeon et al. 2002). The lack of maternal imprint in this gene in human placental tissues provides an additional argument against the possibility of imprinted XCI in human extraembryonic tissues.

Association of the degree of XCI skewing with sample size or sample position within the human placenta.

Regardless of the direction of skewing, it is clear that moderate to extreme XCI skewing is not a characteristic of all placentae. Although most placental sites showed some evidence of deviation from a 1:1 ratio, only a minority of term placental sites and even fewer first trimester placental sites showed evidence of ≥75% or ≥90% skewing. Potential differences in the percentage of skewing due to sample size were analyzed but showed no clear effect on XCI skewing. It could be argued that the size selected for the small samples (sites) was not "small" enough to detect these differences, however, there was such heterogeneity of XCI skewing from site to site when the larger samples (sites) were analyzed, that these differences are likely less important. No association of a particular location within

the placental disc with certain levels of skewing was evident. However, there are clear differences from site to site within most placentae in both the percentage and direction of skewing, making it very difficult to consider a single placental site as an accurate representation of the placenta as a whole.

Site-to-site heterogeneity in human placentae

A unique feature of this study is that several placental samples (sites) were obtained and analyzed from each individual placenta, which gives us a more representative sample of the placenta as a whole. Sample bias has been a major problem in many of the previous studies as explained in the introduction to this chapter. When extraembryonic tissues are analyzed separately, it seems clear that if any deviation from a 1:1 ratio is considered, most term placentae show a heterogeneous pattern of XCI in amnion, chorion, mesenchyme and trophoblast. When a more stringent cut-off of $\geq$ 75% skewing is applied however, the chorion shows very few sites with this level of skewing to allow statistical analysis. The mesenchyme continues to show a mostly heterogeneous pattern of XCI, while the amnion and trophoblast show a tendency towards skewing in the same direction. However, amnion tended to be skewed towards the maternal allele and trophoblast towards the paternal allele in this data set. Finally, when an even higher cut-off value ($\geq$ 90%) is applied to the data, only tissues such as the trophoblast and the mesenchyme show samples with this level of extreme skewing and XCI skewing is usually, but not always, seen following the same direction within a placenta.

The number of samples of amnion that showed skewed XCI ($\geq$ 75%) is largely due to the contribution of only two placentae PX6 and PX13, in which this tissue showed higher levels of skewing in the same direction. In both of these placentae, there were also

extreme levels of skewing in cord and/or cord blood. It has been reported that a sample of amnion at term is comprised of ~70% of amniotic ectoderm (Whittle et al. 2000), a derivative of the epiblast in the inner cell mass of the embryo. Given that the epiblast will give rise to the embryo proper, the finding of a correlation of XCI skewing between amnion samples and fetal cord or cord blood from the same placenta is expected.

This study's findings of a predominantly heterogeneous pattern of XCI in the trophoblast correlate with those of Looijenga et al (1999), who reported that the trophoblast shows a "coarser" XCI pattern, with differences between samples and sites within a single full-term placenta. The authors did not state the cut-off value that was used to assess skewing; however, it was assumed that they considered any deviation from a 1:1 ratio in this analysis as indicative of "preferential" inactivation of a given X chromosome. This group also suggested that this pattern might be explained by clonal expansion of a limited number of trophoblastic progenitor cells with either an inactive maternal X or an inactive paternal X, but they cautioned that this idea had to be proven by the analysis of a large number of samples taken from an informative placenta (Looijenga et al. 1999). In this study, I have overcome this problem by analyzing a series of placental sites and have obtained similar results to those previously reported by Looijenga and colleagues.

Distribution of skewing in term placentae

The analysis of the distribution of skewing in different placental tissues demonstrates that amnion has an approximately normal distribution (Figure 3.4 a). This distribution suggests that XCI is mostly random in this tissue, with deviations from a 50:50 ratio due simply to random sampling effects. The distribution in the chorion is not normal

(see Figure3.4 b). This tissue exhibits a considerably narrower distribution of skewing which may indicate greater mixing of cells. The distribution of XCI skewing in mesenchyme is normal, with a broader distribution of values relative to amnion and chorion. This may reflect lower numbers of precursor cells or less admixing between cell populations during the development of the placenta relative to tissues such as the chorion. As the mesenchyme encompasses a series of individual components such as villi stroma (stromal core), endothelium and smooth muscle (from the small chorionic villi vessels), results on the mesenchyme reflect the situation in this array of tissues, rather than that of each individual component. A more detailed analysis involving the isolation of each of these tissues would be necessary in order to draw any conclusions regarding XCI in the various components of the mesenchyme.

The trophoblast showed a higher tendency towards extreme skewing compared to the rest of tissues considered in this study and an overall normal distribution of values for skewing (Figure 3.3 c)(Table 3.5). These findings correlate with those of other studies which reported evidence of skewed XCI confined to the chorionic villi trophoblast (Harrison 1989; Goto et al. 1997; Looijenga et al. 1999). A detailed analysis of the contribution of each individual placenta to the overall distribution of skewing shows that the extremely skewed category (>90%skewing) in the trophoblast includes samples from at least 10 different placentae (Figure 3.3 c). This indicates that increased skewing in the trophoblast is not an effect of sample bias (ie. the small number of placentae analyzed). The trophoblast undergoes differentiation very early in development, and it is possible that XCI occurs earlier in the trophoblast compared to other extraembryonic tissues, at a time when there are fewer progenitor cells present. This might lead to increased levels of extreme skewing in this tissue. In fact, studies in mice have demonstrated that trophoectoderm formation occurs at

approximately 3 days postcoitum, and that XCI occurs at this point in development, whereas it occurs at 5-6 days postcoitum in embryonic cells (Takagi and Sasaki 1975; Tan et al. 1993). However, retention of clonal coherence and therefore a decreased cell migration and intermingling capacity in the trophoblast could also explain the increased levels of XCI skewing in the trophoblast. In fact, although not specific for the trophoblast, it has been reported that different tissues in the pre-gastrulation embryo in mouse show marked differences in clonal cohesion, clone dispersal, cell intermingling and cell migration (Gardner and Cockroft 1998).

The timing of XCI in human placenta

The analysis of the correlation of the degree of skewing for different extraembryonic tissues within one placental site (Table 3.6), showed no significant correlation of skewing between the amnion and any of the other tissues included in this study. This suggests that XCI in the amnion, occurs independently from other extraembryonic tissues. As already discussed, the evidence of a large embryonic component (originating from the epiblast) making up most of the term amnion supports this conclusion and explains the correlation found between skewing in amnion and the umbilical cord and cord blood. The chorionic plate (chorion) also showed no correlation between the degree of skewing seen in this tissue and any other tissue studied. These findings suggest that XCI occurs independently in chorion and, as very little skewing was observed, perhaps it occurs at a time when a large progenitor cell pool is available for inactivation.

It is possible that a high correlation between different tissues within one placental site reflects sample contamination, particularly when the two tissues are in intimate contact with each other. A significant correlation of skewing between the trophoblast and

mesenchyme was found in this study when pairs of corresponding sites (two tissues from the same site in a given placenta) were analyzed. In contrast, a positive but non-significant correlation was found when pairs of non-corresponding sites (trophoblast and stroma from a different site within a given placenta) were analyzed (Table 3.6). This is very suggestive of unavoidable contamination during the enzymatic process of separation of these two tissues. In spite of these findings, the distributions of skewing in the two tissues were not identical and the trophoblast showed higher levels of extreme skewing relative to the other tissues (including the mesenchyme) (see Table 3.4). This might imply that a smaller precursor cell pool gives rise to the trophoblast at the onset of XCI, which seems to correlate with the fact that trophoblast is the first tissue to differentiate in the developing human and mouse embryo. The greater site-to-site variation seen in this tissue also suggests lower levels of cell mixing. Thus, cells near one another are more likely clonally related than cells in distant locations (as discussed earlier).

Although it is difficult to interpret these findings, the results seem to suggest that XCI is mostly a random process in amnion, chorion and mesenchyme. The situation in the trophoblast is less clear and suggests a tendency towards preferential paternal XCI at earlier stages of embryonic development followed by a possible relaxation of the imprint at later stages of development. Whether these findings are correlated to the timing of the XCI process in the embryonic progenitors of these tissues, the progenitor cell pool size, the degree of cell migration leading to cell intermingling that characterizes the growth of each of these tissues, a combination of these factors or some other factor, still remains unclear.

Insights into the evolution of XCI

The occurrence of preferential paternal XCI (Xi^P) in marsupials has usually been considered a "primitive" condition from which random XCI evolved in eutherians, once they diverged from marsupials (Cooper 1993). The idea has been that imprinted XCI was ancestral and that random XCI evolved to confer upon the somatic tissues of the embryo the benefits of "quasi-heterozygosity". This hypothesis is supported by the presence of Xi^P in some eutherians and in marsupials, and the fact that in eutherians this imprint is erased in those cells that give rise to the embryo proper (Cooper 1993). However, if one considers that marsupial and eutherian mammals have been evolving for a similar period of time, it is difficult to label one strategy as more "primitive" than the other. It has been argued that the incomplete and unstable XCI seen in marsupials might serve a purpose in the dosage regulation of certain X-linked genes that are important for marsupial sexual differentiation, while the complete and stable inactivation seen in eutherians is perhaps a recent evolutionary acquisition due to their independence from this particular pathway of sexual differentiation (Graves 1996). In this context, the idea that genomic imprinting evolved to limit the effects of growth factors in either the embryo or extraembryonic tissues due to differential parental investment seems applicable to eutherian mammals but less relevant in marsupials which have highly underdeveloped extraembryonic tissues (Graves 1996). Therefore, many agree that the data points towards an ancestral XCI mechanism that was likely unstable, incomplete and paternal (Graves 1996). Recent studies in mice have shown a "relaxed" imprinting in extraembryonic tissues, with as many as 30% of cells escaping imprinting. It has then been proposed that the relaxed imprinting of extraembryonic tissues of mice represents a transitional stage in an evolutionary pathway that goes from an absolutely imprinted

mechanism in marsupials to a random mechanism as reported by some groups including this study for human placentae (Huynh and Lee 2001).

Recently, however, an alternative hypothesis has been proposed in which XCI is thought to begin as a randomly expressed gene cluster, on the undifferentiated proto-X chromosome in an ancestral mammal. Eventually (under the influence of a controlling element) the X acquired a parent-specific imprint in rodents and marsupials, with the purpose of perhaps minimizing maternal-fetal incompatibilities (Ohlsson et al. 2001). As evidence for this hypothesis, some underlying elements of randomness have been found in imprinted XCI in eutherians, such as random XCI in parthenogenetic ($X^M X^M$) mouse embryos and breakdown of imprinted XCI in studies of *Xist* expression of embryos produced from crosses between different strains of mice bearing different *Xce* alleles (as reviewed by Ohlsson et al. 2001). However, the lack of evidence for underlying random XCI in marsupials, in which X–linked genes are either only maternally expressed or biallelically expressed in certain tissues, seems to favour the hypothesis that imprinted XCI is the ancestral state (Ohlsson et al. 2001). Although this issue has not yet been resolved, it is clear from the results of this thesis and other studies in humans that imprinted XCI is not a requirement for normal long term development of human extraembryonic tissues. It is possible, however, that imprinting effects are confined to the chorionic villi trophoblast in the first trimester of human gestation with subsequent relaxation of the imprint as pregnancy progresses. As much as this finding could be used to argue in favour of imprinted XCI as the ancestral state, one can also argue that the findings in rodents and marsupials represent a evolutionary feature uniquely developed by these two groups, presumably for their own adaptative purposes and therefore, not shared with humans.

The analysis of a larger number of informative samples from first trimester placentae should help clarify the trend observed in this study for the placental trophoblast in early gestation. Additionally, expression studies targeting the same genes that were investigated in this thesis using methylation based assays, might shed some light into the mechanics of the XCI process itself. It is clear that while a good correlation of methylation with expression has been reported in previous studies for genes such as *AR* and *FMR1*, this issue has not been investigated for human placentae. For obvious ethical reasons, it is impossible to investigate XCI at its onset in the human late blastocyst stage or during early gastrulation, as has been done for the mouse and other mammals. Therefore, we will have to find alternative ways to unravel the features of the process through studies of early human IVF embryos (as has been done for the studies of *XIST/TSIX* expression) and studies of extraembryonic tissues obtained either through prenatal sampling or at birth.

Chapter 4: X-chromosome inactivation (XCI) patterns in placental tissues of a paternally derived bal t(X;20) case

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4.1 Introduction

Females carrying a balanced X-autosome translocation generally exhibit non-random X-chromosome inactivation (XCI) that is attributed to selective growth of cells that have inactivated the normal X. This prevents gene dosage imbalance, which could potentially result from inactivation of autosomal genes translocated to the X or from functional disomy of genes that normally undergo XCI (Disteche et al. 1979; Mattei et al. 1982; Schmidt and Du Sart 1992). It has been estimated that approximately 77% of cases with a balanced X-autosome translocation exhibit complete non-random inactivation of the normal X chromosome, based on both studies of replication timing as well as molecular evaluation of XCI status (Schmidt and Du Sart 1992). These cases have translocation breakpoints distributed all along the X chromosome and the majority of carriers are either phenotypically normal or show gonadal dysgenesis, reproductive problems and/or single gene disorders. A minority (<9%) show multiple congenital abnormalities and/or mental retardation (Schmidt and Du Sart 1992).

In ~23% of X-autosome translocation carriers, the derivative X is inactivated in variable proportions of cells (Schmidt and Du Sart 1992; Wolff et al. 1998) and interestingly in these cases, the breakpoints tend to be located in distal Xp and Xq (Schmidt and Du Sart

1992). The outcomes in these cases are variable, but carriers often (>80% of cases) show mental retardation and/or multiple congenital abnormalities or single gene disorders (Schmidt and Du Sart 1992). Presumably in such cases there is sufficient abnormal gene expression resulting from either spreading of inactivation into the autosomal segment or failure to inactivate part of the X to result in an abnormal phenotype, yet insufficient alterations to influence cell viability and proliferation. Even if cell viability is compromised, a late acting gene or a gene product with tissue specific function might also explain this apparent dichotomy, particularly if gene impairment due to the random XCI does not affect development per se but rather is responsible for specific cell death later on in life.

Non-random inactivation is also seen in the extraembryonic tissues of rodents, as discussed in the previous chapter, in which the paternal X chromosome is preferentially subject to XCI (Takagi and Sasaki 1975). The previous chapter has tried to address the situation in human extraembryonic tissues and presents strong evidence that XCI is mostly random in human placental tissues. These findings corroborate those of some previous studies (Migeon and Do 1979; Looijenga et al. 1999) although due to sample limitations it is still difficult to completely extrapolate these results to the earliest stages of development. The presence of random XCI in human extraembryonic tissues is also supported by the absence of imprinted *XIST* expression in early human pre-implantation embryos (Daniels et al. 1997; Ray et al. 1997) and the viability of individuals with supernumerary X chromosomes of maternal origin (i.e. $X^P X^M X^M$ or $Y X^M X^M$) (Harvey et al. 1990; Hassold et al. 1990; Leal et al. 1994), not seen in mice (reviewed inHuynh and Lee 2001).

Preferential inactivation of the paternally derived X during early placentation is a requisite for normal embryonic development in mice (Goto and Takagi 1999). If preferential inactivation of the paternally derived X chromosome were also true for human

extraembryonic tissues, then a paternally derived X-autosome translocation would result in unbalanced gene expression in extraembryonic tissues through preferential activation of the non-translocated maternal X. While males with X-autosome translocations are often phenotypically normal, they are almost invariably sterile as a consequence of meiotic imbalance during spermatogenesis (Madan 1983) that leads to either azoospermia or oligospermia. Thus, there are few reported cases of males that reproduced and in no case was the aberration transmitted to the offspring (Buckton et al. 1971; Lichtman et al. 1978).

We recently obtained the placenta from a pregnancy in which a balanced X-autosome translocation involving chromosomes X and 20 was transmitted from father to daughter with the use of intracytoplasmic sperm injection (ICSI) (Ma et al. 2002)(Ma *et al.*, 2002 in preparation). In brief, a couple was referred to ICSI after being ascertained due to primary infertility in the 39 year old male-partner, who presented with severe oligozoospermia. Chromosome analysis was performed on peripheral blood lymphocytes of the couple, and the husband's brother and mother using GTG banding. While the female-partner showed a normal chromosome complement, the male partner, his brother and his mother showed a balanced whole arm translocation involving chromosomes X and 20. The breakpoints appeared to be at or near the centromere in both chromosomes and the translocation led to the fusion of the two long arms and the two short arms. The karyotype of the male-partner was described as follows: 46, XY, t(X;20) (p10; p10). The couple decided to undergo *in vitro* fertilization (IVF) combined with ICSI and an appropriately grown, healthy female was delivered at term. Chromosome analysis of a cord blood sample of this baby girl confirmed a balanced karyotype, 46, XX, t (X;20)(p10;p10)*pat* that had been previously seen at amniocentesis (Figure 4.1). Postnatal follow-up has also been normal.

Samples were taken for XCI studies from cord blood, umbilical cord and 4 different placental tissues (amnion, chorion, trophoblast and mesenchyme) collected from 5 different, non-adjacent sites, within the placenta of the baby girl carrier of the translocation, using the protocol described above for normal term placentae. Maternal samples of both peripheral blood and the maternal blood from the placenta were also included in the study to allow us to determine the parental origin of the inactive X. A blood sample from the paternal grandmother (translocation carrier) was also available for analysis (Figure 4.2).

Figure 4.1 Karyotype of the baby girl carrier of the X/A translocation and partial karyotypes of this girl, her father and paternal grandmother showing both the normal and the derivative chromosomes X and 20 (reproduced with permission from Dr. Sai Ma). Long arrows and arrowheads indicate the translocated chromosomes in the child, her father and paternal grandmother. The corresponding normal homologues are also shown along with ideograms of the normal chromosomes X and 20.

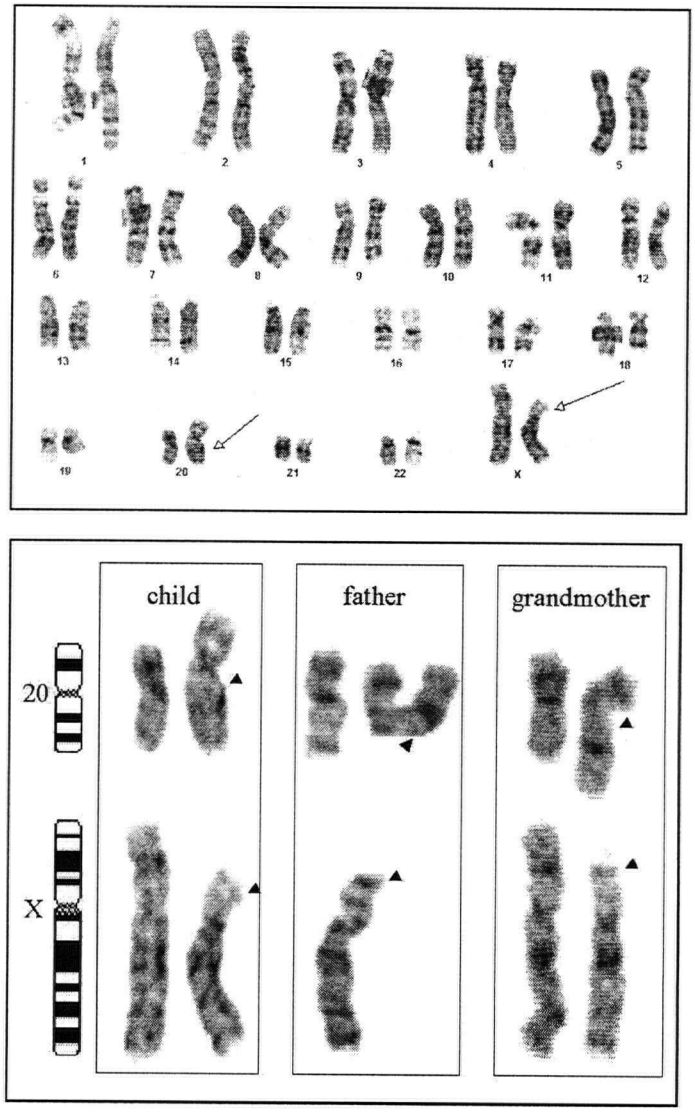
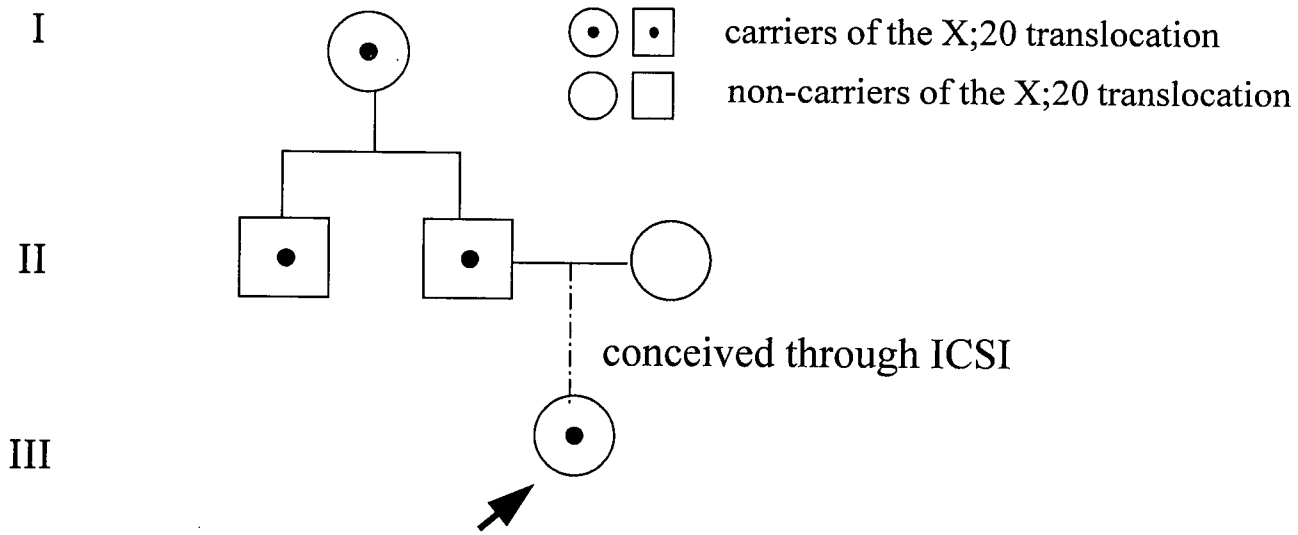


Figure 4.2 Pedigree of the X/A translocation case. (modified with permission from Dr. Sai Ma).



Arrow indicates young female carrier of the translocation (conceived through ICSI)

This case thus provided a unique opportunity to examine the process of X-chromosome inactivation during placental development. This chapter includes the report on the analysis of XCI patterns in placental tissues and cord blood from this female, her non-carrier mother and her paternal grandmother who also carries the translocation.

4.2 Results

XCI was assayed in every DNA sample using methylation-based tests for three different X-linked markers: Androgen Receptor (*AR*), Fragile X Mental Retardation (*FMR1*) and *DXS6673E*.

This case was informative for all three markers used in the present study. The data for XCI in placental tissues, umbilical cord, cord blood, maternal blood and grand-maternal blood is presented in Table 4.1. Extreme XCI skewing with preferential inactivation of the maternal X (X_i^M) (the non-translocated X) was evident in all sites of the amnion, cord and cord blood using both *AR* and *FMR1* markers. Preferential inactivation of the maternally derived X, was also observed in samples of the extra embryonic tissues; however, multiple sites in the chorion, trophoblast and mesenchyme showed either random XCI or only a moderate level of skewing (Table 4.1 and Figures 4.3 and 4.4).

While there was very high reproducibility for XCI values obtained using two different sets of digests of the same sample assayed with the same polymorphic marker (correlation coeficient r=0.97 for *AR* and r=0.94 for *FMR1*), a lack of correspondence between XCI values obtained from the three different markers was evident.

Interestingly, each of two separate maternal blood samples also showed extremely skewed XCI. The mother showed preferential inactivation of the same *AR* allele that was preferentially inactivated in her daughter, but a different *FMR1* allele from that

transmitted to her daughter. The mother was uninformative for marker *DXS6673E* (Table 4.1). The paternal grandmother who was also a carrier of the translocation was uninformative for both *FMR1* and *DSX6673E*. Assessment of skewing of inactivation with the *AR* assay showed highly skewed XCI with preferential inactivation of the normal X chromosome (represented by allele b) as opposed to the translocated one (represented by allele c), which she shares with her grandchild (Table 4.1 and Figures 4.3, 4.4 and 4.5).

Table 4.1. Percentage of XCI in different placental tissues, umbilical cord, cord blood and maternal blood of a female carrier of a paternally derived X/autosome translocation.

AR (*Hpa II* digests)[a]								
tissue (genotype)[b]	AMNION (ac)	CHORION (ac)	TROPHOBLAST (ac)	MESENCHYME (ac)	CORD (ac)	CORD BLOOD (ac)	MATERNAL BLOOD (ad)	pat GM BLOOD (bc)
site 1	94 mat	100 mat	88 mat	61* mat	95 mat	96 mat	97 allele a	91 allele b
site 2	95 mat	59 mat	88 mat	70 mat				
site 3	95 mat	81 mat	87 mat	86 mat				
site 4	95 mat	91 mat	78 mat	61 mat				
site 5	96 mat	91 mat	61 mat	52 mat				
FMR1 (*Hpa II* digests)[a]								
Tissue (genotype)[b]	AMNION (ab)	CHORION (ab)	TROPHOBLAST (ab)	MESENCHYME (ab)	CORD (ab)	CORD BLOOD (ab)	MATERNAL BLOOD (ac)	pat GM BLOOD (bb)
site 1	100 mat	100* mat	NA	98* mat	100mat	99 mat	100 allele c	homozygous
site 2	100 mat	100 mat	NA	100* mat				
site 3	93 mat	100* mat	NA	100* mat				
site 4	100 mat	100* mat	NA	100* mat				
site 5	100 mat	100* mat	61* mat	100* mat				
DXS6673E (*HhaI* digest)[a, c]								
tissue (genotype)[b]	AMNION (ab)	CHORION (ab)	TROPHOBLAST (ab)	MESENCHYME (ab)	CORD (ab)	CORD BLOOD (ab)	MATERNAL BLOOD (aa)	pat GM BLOOD (bb)
site 1	84 mat	83 mat	NA	100* mat	90 mat	86 mat	homozygous	homozygous
site 2	71* mat	63* mat	78* mat	90* mat				
site 3	NA	81* mat	NA	100* mat				
site 4	77 mat	77 mat	NA	87* mat				
site 5	90 mat	79 mat	NA	75* mat				

[a]: percentages shown are, for most of the samples, the average of two independent assays carried out using two different digestions. *AR* and *FMR1* were tested using the same two digests.

[b]: based on this genotype preferentially inactivated alleles are designated as maternal (mat) or paternal (pat) in origin. Preferentially inactive maternal alleles are designated using the corresponding letters of the alphabet.

[c] : XCI values calculated for this marker may be inaccurate due to allele overlap at the level of stutter bands.

*: the PCR product yield in the digested sample was very low compared to the internal control (sample not digested with either *Hpa II* or *Hha I*), therefore the value estimated for skewing is not reliable. The low PCR product yield could be evidence for gene hypomethylation.

NA: no amplification of the digested sample in these cases. GM: grandmother

Figure 4.3 Results from the automatic fluorescent analysis (ABI Prism) of extraembryonic tissues, cord blood and maternal blood of a pat bal t(X:20) case using the *AR* assay. The plot shows methylation- based analysis results for the following samples: amnion (*a*), trophoblast (*b*), mesenchyme (*c*), umbilical cord (*d*), maternal blood (*e*) and cord blood (*f*). Note the extreme levels of skewing evident in samples a,d,e, and f. Also some degree of hypomethylation in samples b and c. The *AR* alleles for both the patient (ac) and her mother (ad) are indicated. The undigested sample is shown with a red curve(short arrows), the digested sample with a green curve (long arrows). The percent skewing calculated based on the ABI data is indicated in every case. The maternal allele (mat) is preferentially inactivated in every tissue of this case. Allele **a** is preferentially inactivated patient's mother (*d*).

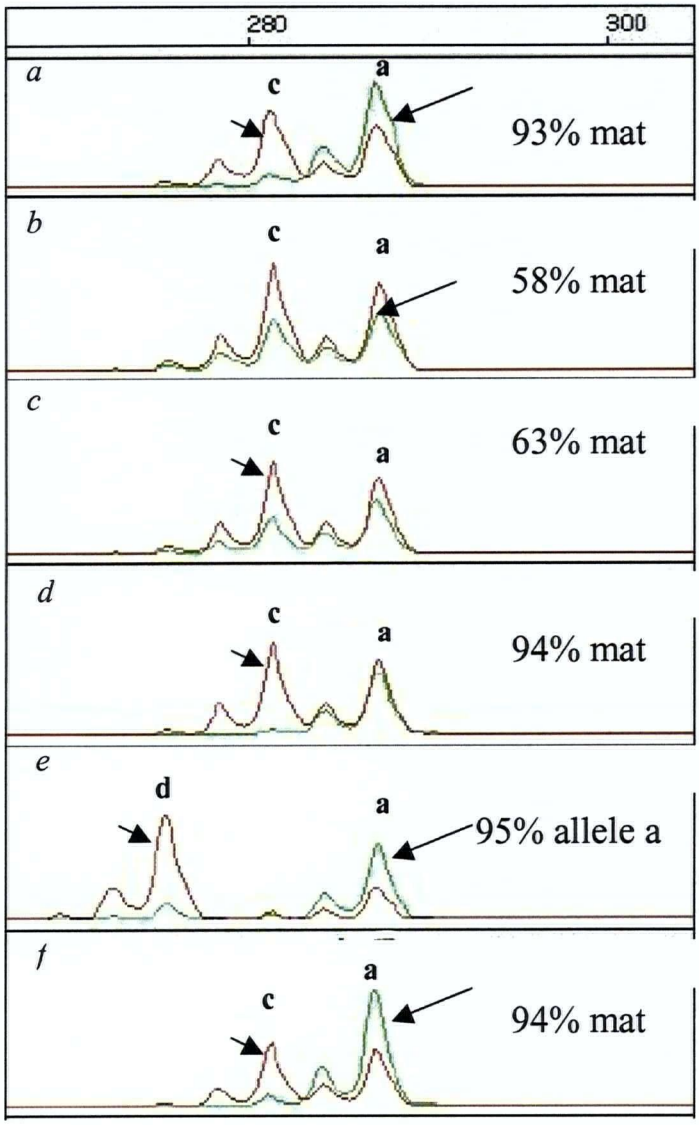


Figure 4.4 Results from the automatic fluorescent analysis (ABI Prism) of extraembryonic tissues, cord blood, umbilical cord and maternal blood of a pat bal t(X:20) case using the *FMR1* assay. The plot shows methylation- based analysis results for the following samples: amnion (*a*), chorion (*b*), trophoblast (*c*), umbilical cord (*d*), cord blood (*e*) and maternal blood (*f*). Note the extreme levels of skewing evident in samples a,d,e, and f. Also high degree of hypomethylation in samples b and no PCR product from the digested sample in c. The *FMR1* alleles for both the patient (ab) and her mother (ac) are indicated. The undigested sample is shown with a red curve (short arrows), the digested sample with a blue curve (long arrows). The percent skewing calculated based on the ABI data is indicated in every case. Samples marked with * show skewing values that are not reliable due to low product yield from the digested sample, possibly indicative of hypomethylation. The maternal allele (mat) is preferentially inactivated in every tissue of the patient. Allele **c** is preferentially inactivated in the patient's mother (*f*).

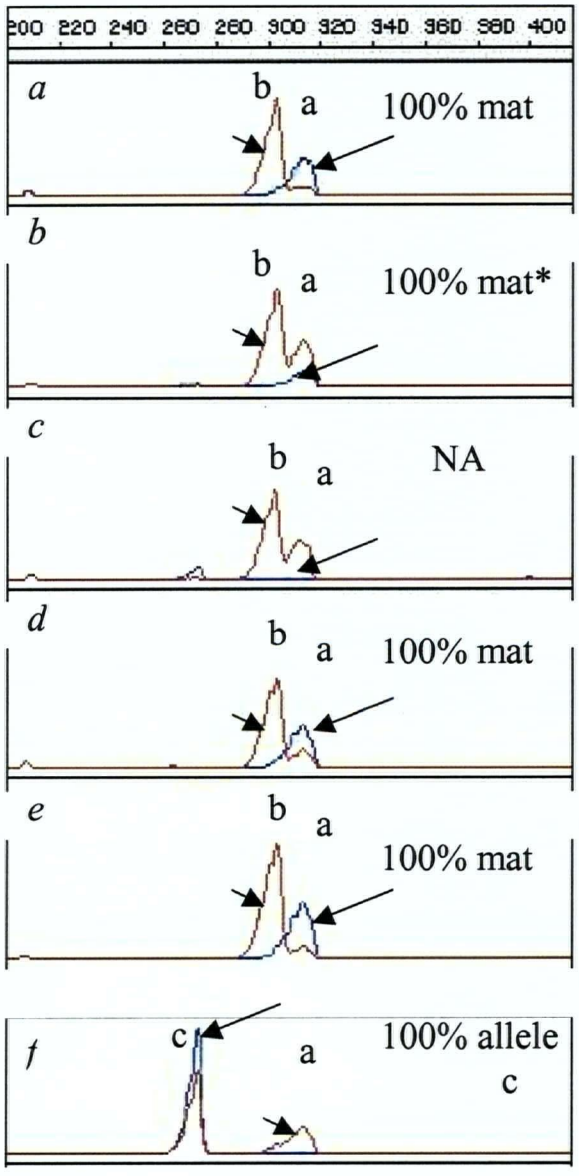
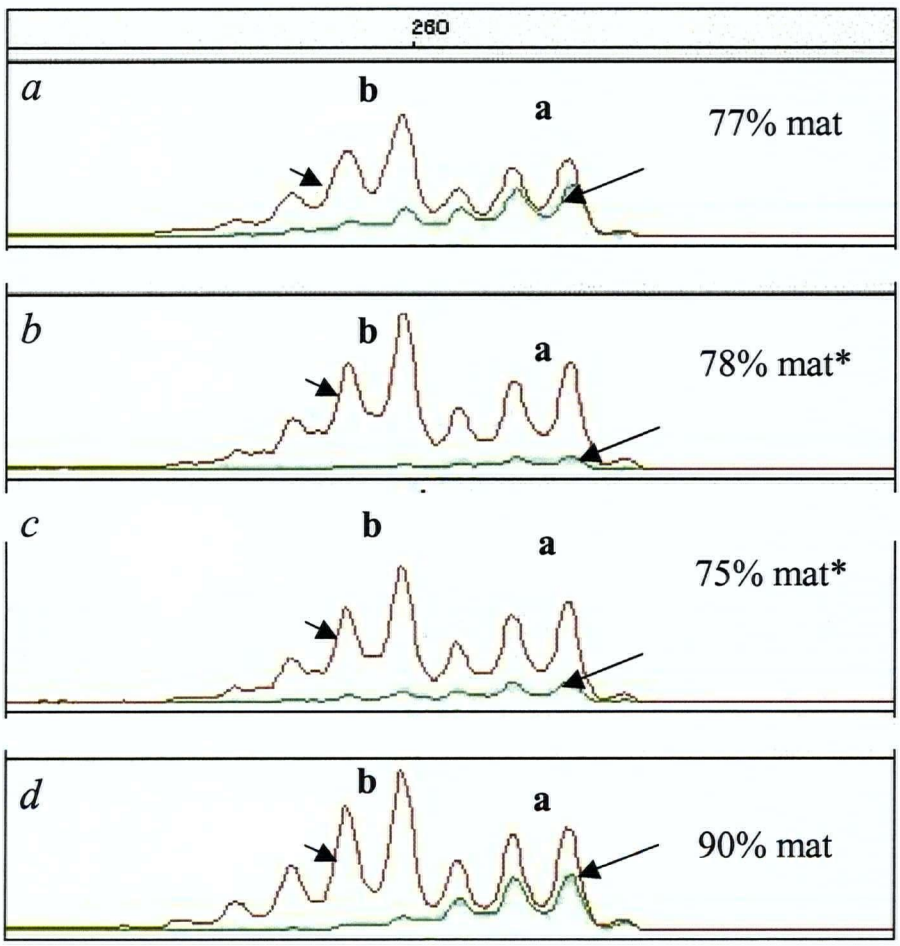


Figure 4.5 Results from the automatic fluorescent analysis (ABI Prism) of extraembryonic tissues, umbilical cord of a pat bal t(X:20) case using the *DXS6673E* assay. The plot shows methylation- based analysis results for the following samples: amnion (*a*), trophoblast (*b*), mesenchyme (*c*), umbilical cord (*d*) Note the extreme levels of skewing evident in samples *a* and *d*. Also high degree of hypomethylation in samples *b* and *c*. The *DXS6673E* alleles for the patient are labelled (ab). The undigested sample is shown with a red curve (short arrows), the digested sample with a green curve (long arrows). The percent skewing calculated based on the ABI data is indicated in every case. Samples marked with * show skewing values that are not reliable due to low product yield from the digested sample, possibly indicative of hypomethylation. The maternal allele (mat) is preferentially inactivated in every tissue of the patient.



4.3 Discussion

The cord blood of this female with a paternally derived X;20 translocation, showed preferential inactivation of the normal X chromosome, as is seen for the majority of maternally inherited balanced X-autosome translocations (Table 4.1) (Hellkuhl et al. 1982; Schmidt and Du Sart 1992; Kalz-Fuller et al. 1999). While there are reports of discrepancies between XCI patterns for different embryonic tissues within the same patient (Hellkuhl et al. 1982), umbilical cord and amnion in this study's case showed a clear bias towards the inactivation of the non-translocated X chromosome. As DNA extracted from amnion most likely represents cells predominantly derived from early embryonic ectoderm (see Figure 1.3) (Robinson et al. 2002), this seems to suggest that this non-random XCI pattern may be found for most embryonic tissues. Most likely non-random XCI is a consequence of an early cell selection process, which favours the growth of cells that have inactivated the normal X and thus have a balanced dosage of expressed genes. A correlation between XCI skewing in amnion, cord blood and umbilical cord was also evident in the study of normal term placentae, detailed in the previous chapter.

It is worth noting that in this case the mother of the female carrier of the X/autosome translocation showed extremely skewed XCI. Although this finding could be due to chance, inheritance of skewed XCI has been reported to occur in rare situations (Pegoraro et al. 1997; Plenge et al. 1997; Orstavik et al. 1999; Tanner et al. 1999). While *XIST* mutations have been found in some of these cases (Plenge et al. 1997), the other studies have excluded *XIST* or the XIC region as the cause of non-random XCI (Pegoraro et al. 1997; Orstavik et al. 1999; Tanner et al. 1999) and the Pegoraro study has found that a deletion in the Factor VIII gene (*F8C*) located at Xq28, is linked to XCI skewing in their pedigree. An unrecognized X-linked disorder affecting both mother and daughter could explain skewing

by affecting proliferation of specific cell populations after random XCI (Migeon and Haisley-Royster 1998). The fact that mother and daughter do not share the same alleles in the preferentially inactive X for the *FMR1* gene could be explained by meiotic recombination. However, no X-linked disease has been reported in this family. Also, given that 7% of controls evaluated in our laboratory show extreme skewing, chance likely plays an important role, even in families where more than one female is observed with skewed XCI. Thus, the presence of the X-autosome translocation is the most likely cause of skewing in the child and it is very likely that the skewing in the mother was coincidental.

The analysis of skewing of XCI in extraembryonic tissues was confounded by the lack of correlation between XCI values obtained from the 3 different markers that were used in this study. The various methylation-based assays to test for XCI were used under the assumption that the methylation status of a gene correlates with its expression status and/or accurately reflects inactivation status (Allen et al. 1992; Carrel and Willard 1996; El-Kassar et al. 1998). However, hypomethylation of these genes in the chorionic villi may confound interpretation of these results (Peñaherrera et al. 2001). A separate study of cultured CVS samples that will be described in Chapter 5, demonstrates that in many of the cases, *AR* was methylated normally, as we would expect for somatic tissues, whereas genes such as *FMR1, DXS6673E, ARAF1* and *MAOA* are usually hypomethylated in cultured chorionic villus stroma (a component of the mesenchyme). Additionally, there was no evidence of *AR* hypomethylation in control placentae (Chapter 3). However, it is important to note that these results were obtained after this chapter's case was studied.

Based on these findings it is possible that the lack of correlation for XCI values for these three markers in the present case is mostly due the fact that *FMR1* and *DXS6673E* are likely hypomethylated in chorionic villi mesenchyme and the trophoblast, and perhaps

even the placental chorion, and thus the methylation status does not correlate with XCI status for these loci in placental tissues.

Additional evidence of hypomethylation comes from our finding of poor yield of amplification relative to that of the internal control, in the digested samples of the tissues mentioned above, when tested for *FMR1* or *DXS6673E* (see * values in Table 4.1 and Figures 4.4 and 4.5). These differences in product yield lead to inaccurate estimation of skewing, in which some samples could appear as extremely skewed (100%) when they are not. Similar results were found for samples of chorionic villi trophoblast and mesenchyme of control term placentae and a few similar samples of first trimester placentae assayed for XCI using the *FMR1* markers (as will be detailed in Chapter 5). Meanwhile, only limited evidence of hypomethylation or extremely skewed XCI was found when the same samples were assayed using the *AR* markers. Furthermore, our findings of gene hypomethylation for *FMR1* and *DXS6673E* in the present study are also consistent with the hypomethylation generally considered to be associated with extraembryonic tissues in humans (Migeon et al. 1985; Luo et al. 1993; Goto et al. 1997; Looijenga et al. 1999; Bird 2002).

Thus, only the results obtained with the *AR* assay were used to interpret the inactivation status in the placental tissues. Unlike the situation in the embryonic tissues, highly skewed XCI patterns were not a characteristic of all extraembryonic tissues in the placenta, in fact, although most sites showed some bias towards the inactivation of the non-translocated X, some of the sites showed completely random XCI (Table 4.1). The breakpoints were not located in distal Xp or Xq, as has been found in the majority of cases of balanced X-autosome translocations that lack skewing of inactivation. This interesting finding has several important implications. First, it seems likely that the selective forces acting against cells that inactivate the translocated X are either weaker or absent in placental

tissues relative to their fetal counterparts. The fact that no site was found showing preferential inactivation of the translocated X would argue in favour of a weaker selective force rather than complete absence of cell selection in these tissues. How some placental tissues seem to tolerate a functional partial disomy of the X chromosome and partial monosomy of an autosome is not yet clear, particularly given that when these findings are true for embryonic tissues, they are often associated with mental retardation and malformations (Hellkuhl et al. 1982; Schmidt and Du Sart 1992). However, studies of confined placental mosaicism (CPM) have shown that trisomies and monosomies for various chromosomes can be tolerated in placenta and that mosaicism can remain undetected because its consequences are not always severe (Kalousek 1994; Kalousek and Vekemans 1996).

Second, these findings may shed some light on the XCI inactivation process itself, in regards to extra-embryonic tissues in humans. For the past three decades there has been controversy around the evidence for imprinted XCI in human extra-embryonic tissues. Several studies have claimed that preferential XCI of the paternally derived X (X_i^P) as seen in embryonic and extra-embryonic tissues of marsupials (Cooper et al. 1971; Richardson et al. 1971) and extra-embryonic tissues of mice (Takagi and Sasaki 1975), is also a characteristic of human extra-embryonic tissues (Ropers et al. 1978; Harrison and Warburton 1986; Harrison 1989; Goto et al. 1997). Others have argued against these findings, showing evidence of random XCI, with no parental bias effect in humans (Migeon and Do 1979; Migeon et al. 1985; Mohandas et al. 1989; Peñaherrera et al. 1998; Looijenga et al. 1999). Given that in the present case, the paternally derived X is involved in the translocation, and that none of the placental samples analyzed in this study had this X chromosome preferentially inactivated, it seems unlikely that X_i^P is a requirement for normal development in humans as it is for mice and marsupials (Goto and Takagi 1999). Furthermore, the absence

of placental sites showing X_i^P, argues against a long-term parental bias to XCI in human extra-embryonic tissues. It may still be argued however, that very strong selective pressures favouring the growth of cells inactivating the maternal X, may have overcome an initial bias favouring the inactivation of the paternal X. Additionally, it is not clear whether the XCI status on term placentae represents the situation in early placental stages.

This case represented a unique opportunity to gain some insight in the XCI process in human extraembryonic tissues. It would be interesting to verify its findings in extraembryonic tissues of additional cases of balanced X-autosome translocation carriers including cases of both paternally and maternally derived translocations in which the somatic tissues show the expected preferential inactivation of the normal X homologue; however these cases are relatively rare.

Chapter 5: XCI patterns in CVS samples

5.1 Introduction

Incomplete dosage compensation in human chorionic villi (extraembryonic tissue), possibly linked to hypomethylation of the promoter regions of genes in the inactive X chromosome, has been reported (Migeon et al. 1985; Monk et al. 1987; Goto et al. 1997). Whether this instability of inactivation is related to inhibition of *de novo* methylation and/or specific demethylation is not yet clear.

In 1985, Migeon and colleagues showed that dosage compensation for *G6PD* in human cultured chorionic villus cells is incomplete and that the two alleles for this gene were hypomethylated (Migeon et al. 1985). A subsequent study of human-mouse cell hybrids derived from human cloned chorionic villus cells and mouse A9 cells showed reversal of XCI and reactivation of human genes such as *HPRT, G6PD* and *PGK1* in such hybrids, suggesting that the inactive state is less stable in human chorionic villi relative to somatic tissues (Migeon et al. 1986). Hypomethylation in human extraembryonic mesoderm was also shown by Monk (1987) (Monk et al. 1987). Goto and colleagues found that in at least 50% of their experiments, DNA extracted from mesodermal cells from two human chorionic villi samples did not amplify after digestion with methylation sensitive restriction enzymes. They concluded that the chorionic villi mesoderm was largely unmethylated at the *Hpa II* sites tested in the *AR* assay. They suggested that only a low proportion of the alleles in mesodermal cells are methylated, regardless of their parental origin (Goto et al. 1997). In 1999, Looijenga and colleagues used microdissection to separate the trophoblastic and mesodermal (stroma) components of the human chorionic villi in two placentae. They found that the stromal cells lacked methylation of the *Hpa II* sites tested with the *AR* gene assay

(Looijenga et al. 1999). These results were based on the analysis of 9 samples (size=~0.5cm^3) from each of two term placentae. Due to the small number of samples studied in the two studies mentioned above and the fact that only *AR* was tested in both cases it was important to investigate the possibility of hypomethylation in human placental stroma using a larger number of placentae and several X-linked genes.

My initial study of normal first trimester and term placentae (reported in Chapter 3) showed some evidence of gene hypomethylation in a few of the samples tested (see [d] in Table 3.1). The lowest levels of methylation were seen in samples analyzed using the *FMR-1* assay. Similarly the study of XCI in a placenta from a balanced (X/autosome) translocation case (described in Chapter 4), showed evidence of gene hypomethylation in chorion, trophoblast and mesenchyme when samples were assayed using the *FMR1* and *DXS6673E* loci (see Table 4.1). The studies in this chapter were undertaken to establish whether gene hypomethylation in the chorionic villus stroma was a common characteristic in human placentae. Given that the enzymatic separation procedure used with the normal placental samples does not allow isolation of pure chorionic villus stroma, we decided to use cultured chorionic villous samples (CVS) for this study. It is known that the trophoblastic cell component of the chorionic villi, as well as the endothelium and smooth muscle components of the chorionic villi mesenchyme do not grow well in culture, therefore CVS cultures are largely composed of stromal cells. The methylation status of seven X linked genes was tested in these samples and several controls to explore the possibility of inter-gene variability in gene methylation. This was done using the methylation sensitive enzyme-based assays described in Chapter 2. An additional marker, (XIST 3':5') was used to verify the presence of amplifiable DNA in these samples. This marker is able to amplify DNA present in both the undigested and digested samples, because the segment that is amplified, lacks the restriction

sites for either of the two methylation sensitive enzymes (*Hpa II* or *HhaI*) or the secondary cutter (*RsaI*) used in this thesis. This is an accurate means of confirming the presence of amplifiable DNA and ruling-out the possibility of PCR artifact, particularly in those samples that exhibit no PCR amplification using other markers.

To further assess the possibility of gene-specific hypomethylation in placenta, several samples of chorion, trophoblast and mesenchyme from normal term placentae were tested using the *FMR1* assay (samples of maternal blood, cord blood and maternal decidua were used as controls, as XCI has been well established in tissues of embryonic origin). Both placental samples and controls had been previously tested using the *AR* gene assay and had shown no clear evidence of hypomethylation when visualized in silver-stained polyacrylamide gels.

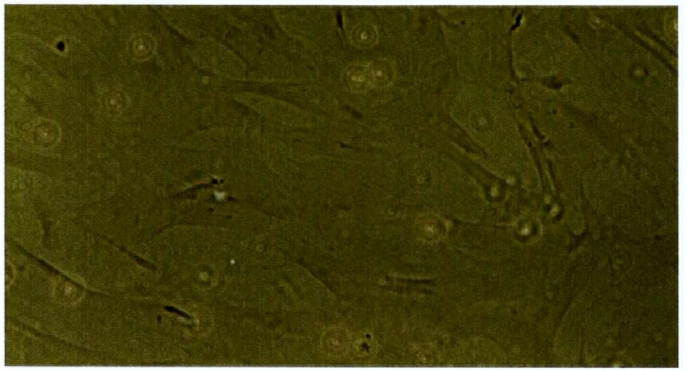
Finally, a study on replication timing of the X chromosome based on the use of BrdU-FISH was also performed using 4 of the CVS samples used for the methylation studies. The BrdU-FISH component of this study was performed by Jane Gair. The purpose of the study was to test for the presence of a late replicating (presumably inactive) X chromosome in the CVS cultures, given that gene hypomethylation in extraembryonic tissues does not necessarily preclude the persistence of an inactive X chromosome.

5.2 Results

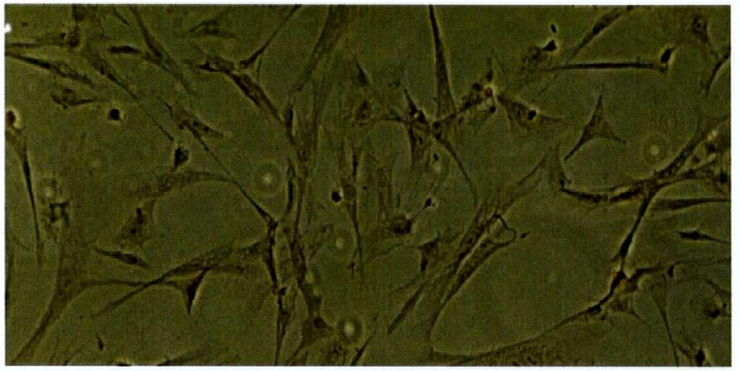
A total of 20 CVS cases with normal female karyotype were used in this study. Several control samples (mostly maternal blood or decidua, fetal blood, amnion and umbilical cord) were also tested alongside the CVS samples. The immunohistochemistry using Cytokeratin 7 antibodies (see Chapter 2) which are specific for the trophoblastic component of the chorionic villi, showed no evidence of trophoblastic contamination in the CVS cultures (see Figure 5.1). Seven X-linked genes were tested for their methylation status

in these samples and controls (*AR, FMR1, DXS6673E, MAOA, GRIA3, XIST AT2:29r* and *ARAF*) and the primers *XIST 3':5'* were used to confirm the presence of amplifiable DNA in the digests.

Figure 5.1 Immunocytochemistry analysis with Cytokeratin 7 antibodies of a CVS sample and a control culture of trophoblastic origin. The analysis demonstrates that the majority of the cells that are found in long-term CVS cultures are of mesenchymal (stromal) origin as very few of the cells are labelled during the procedure (Slides prepared by Paul Yong and Maria Peñaherrera, photography by Paul Yong).



CVS



trophoblast

Throughout this study, the term hypomethylation is used to refer to the observation of little or no PCR product from the digested sample, relative to the amount of product observed from the undigested sample after PCR. In theory, if PCR amplification is within the linear range, we expect the total PCR product from the digested sample to be approximately half that of the undigested sample. This is assuming that the *HpaII* site(s) on the active allele of the gene is always unmethylated, while that/those on the inactive allele is always methylated. The percentage amplification of the digested sample relative to the undigested sample was estimated for a subset of the control and placental samples used in this thesis that were studied using the A*R* and *FMR1* assays and quantified by ABI (see Chapter 2). The Mann-Whitney test was used to assess the difference between the means of the two types of samples (placental samples versus control (embryonic) samples) for each of these two loci.

Significant differences between the means for placental versus embryonic tissues were found for both *AR* and *FMR1* ($p<0.001$). Specifically, both *AR* and *FMR1* demonstrated lower levels of amplification of the digested samples in placental tissues (mean=23% for *AR* and mean=10% for *FMR1*) as compared to the control tissues (mean=71% for *AR* and mean= 61%for *FMR1*) (see Figures 5.2 and 5.3). Based on these findings, the results of the methylation tests were analyzed considering 3 levels of amplification for the digested sample: a) normal amplification, as expected for genes that do not escape XCI in somatic tissues. This was defined in this study as >30% amplification of the digested sample relative to the undigested sample (as quantified by ABI output for *AR*, *FMR1* and *DXS6673E,* by densitometry on polyacrylamide gels for *MAOA* or estimated by eye on agarose gels for *ARAF, XIST* AT2:29r and *GRIA3 (*see Figures 5.4, 5.5 and 5.6)); b) poor amplification, with PCR product levels of the digested sample ranging from >0% to ≤30% of that of the

undigested sample or resulting in a faint band on agarose gels; and c)no amplification with absence of PCR product (0%) from the digested sample on the ABI (no band present on agarose or polyacrylamide gels). The results are summarized in Table 5.1.

Table 5.1 Results of the methylation based assays of several X-linked genes in CVS and control samples.

marker	cytogenetic location	sample type	N	normal amplification [a]	poor amplification [b]	no amplification [c]	Fisher's (1 tail) p value [e]
MAOA	Xp11.4-p11.3	CVS	14	0	6	8	5.1×10^{-7}
		controls [d]	10	10	0	0	
ARAF	Xp11.4-p11.2	CVS	15	0	2	13	0.001
		controls [d]	3	3	0	0	
AR	Xq11.2-q12	CVS	18	7	5	6	0.0002
		controls [d]	25	23	2	0	
DXS6673E	Xq13.1	CVS	8	0	0	8	0.00016
		controls [d]	7	7	0	0	
XIST AT2:29r	Xq13.2	CVS	11	9	0	2	0.67 (n.s.)
		controls [d]	7	6	0	1	
GRIA3	Xq25-q26	CVS	16	0	14	2	0.00005
		controls [d]	5	5	0	0	
FMR1	Xq27.3	CVS	17	0	3	14	3.2×10^{-8}
		controls [d]	15	14	1	0	

[a] normal amplification is defined as digested sample showing >30% of the amplification of the undigested sample quantified by ABI output for *AR* and *FMR1* and judged by eye from agarose gels (for *MAOA, ARAF, DXS6673E, XIST* AT2:29r *and GRIA3*).

[b] poor amplification is defined as digested sample showing <30% but more that 0% of the amplification of the undigested sample quantified by ABI output for *AR* and *FMR1* and judged by eye from agarose gels (for *MAOA, ARAF, DXS6673E, XIST* AT2:29r *and GRIA3*).

[c] no amplification of PCR product from the undigested sample. However, presence of amplifiable DNA was confirmed using primer *XIST3':5'*.

[d] control samples used in this study included: umbilical cord, cord blood, maternal peripheral blood and maternal decidua.

[e] Fishers exact test between samples that showed normal amplification and those that showed some level of hypomethylation (poor or no amplification) in this study. n.s.=not significant.

Figure 5.2 Percentage amplification of the undigested sample versus the digested sample in placental samples and controls (*FMR1* assay). Mean values indicated for each group.

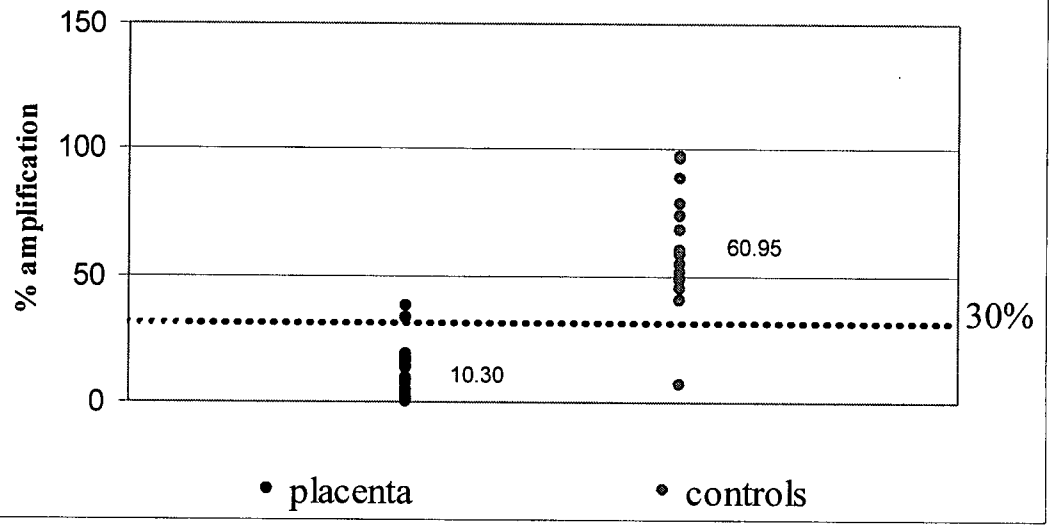


Figure 5.3 Percentage amplification of the undigested sample versus the digested sample in placental samples and controls (*AR* assay). Mean values indicated for each group.

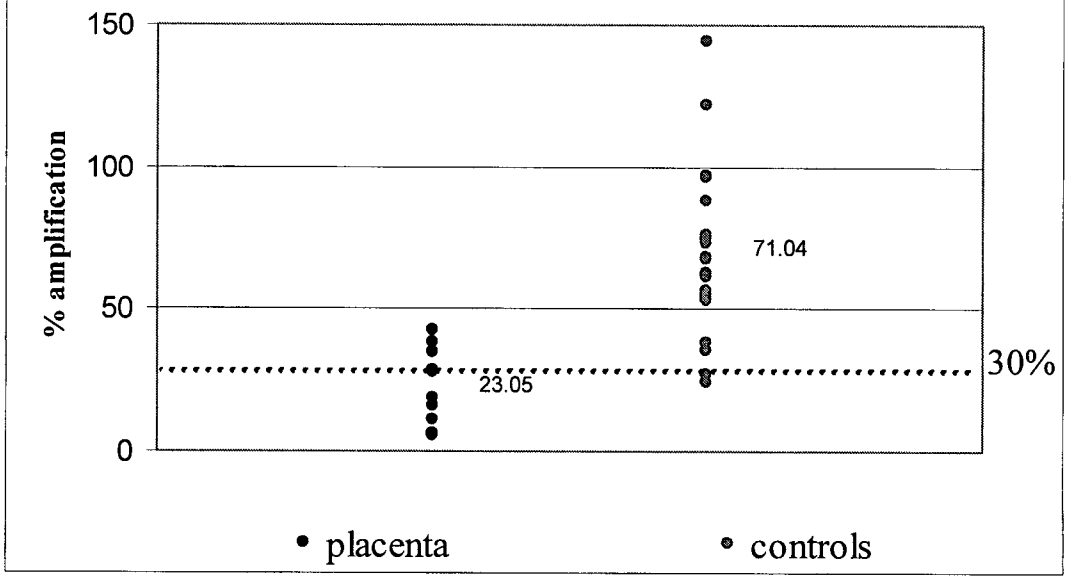


Figure 5.4 Silver-stained polyacrylamide gel showing the results for the methylation-based assay using *MAOA*. Undigested (U) and digested (D) samples are run side by side. All samples shown in this gel had almost complete absence of the PCR product of the digested sample (hypomethylation), with the exception of CVS 2 and CVS 9 where a faint band can be seen in the digested sample relative to that of the undigested sample.

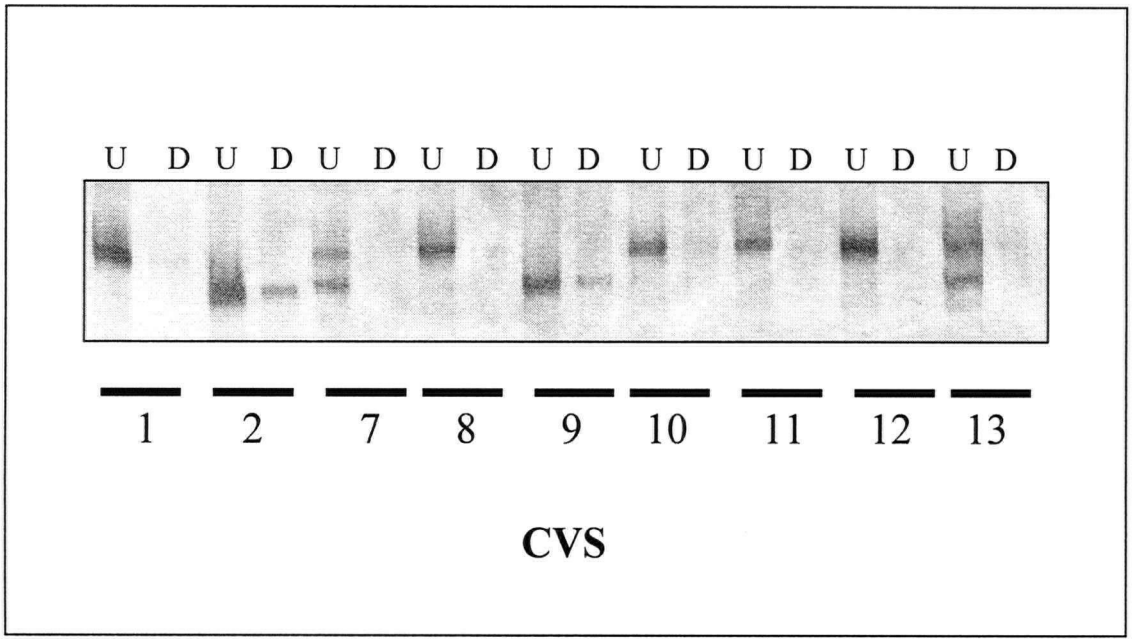


Figure 5.5 Agarose gel stained with ethidium-bromide showing the results for the methylation-based assay using *ARAF*. Undigested (U) and digested (D) samples are run side by side. All CVS samples shown in this gel had almost complete absence of the PCR product of the digested sample (hypomethylation), with the exception of CVS 11 where a faint band can be seen in the digested sample relative to that of the undigested sample. None of the control samples showed hypomethylation (c1 and c4= cord blood, c2=maternal blood, c3=peripheral blood) *L*= φX174 Hae RF DNA ladder).

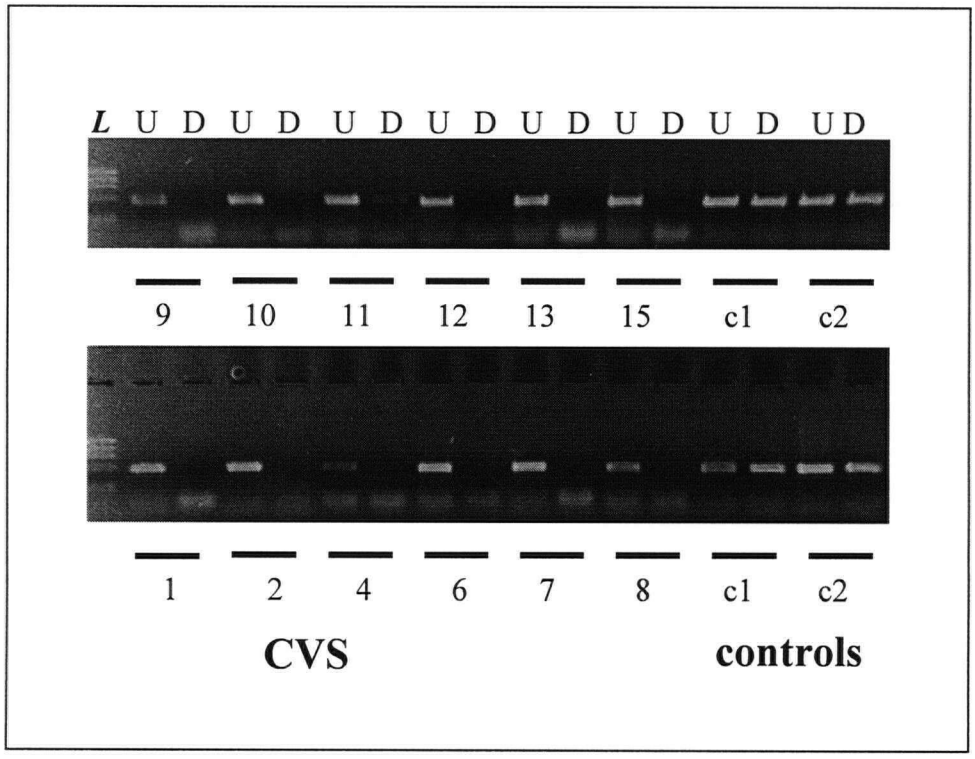
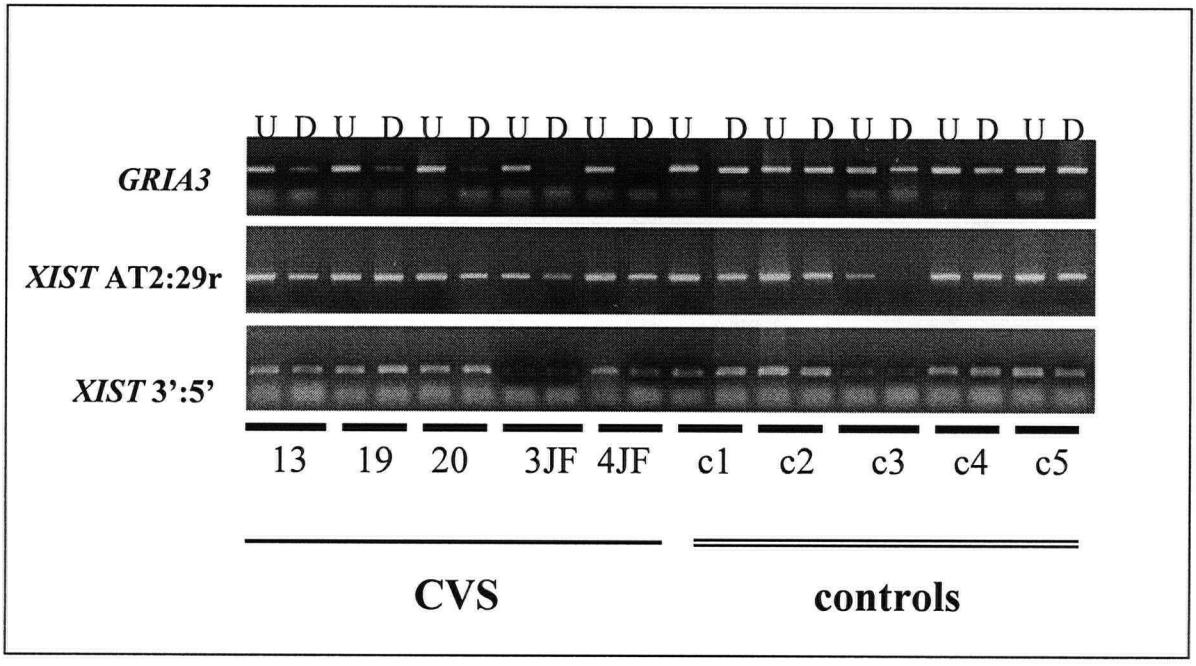


Figure 5.6 Agarose gel stained with ethidium-bromide showing the results for the methylation-based assay using *GRIA3, XIST AT2:29r, XIST 3':5'*. Undigested (U) and digested (D) samples are run side by side. All CVS samples tested with *GRIA3* show a faint band in the digested sample relative to that of the undigested sample (hypomethylation). None of the control samples showed hypomethylation (c1 and c2=maternal blood, c3=decidua , c4 and c5= cord blood). All CVS samples tested with *XIST AT2:29r* showed normal amplification of the digested sample. All but one of the controls (c3) showed a similar result. Using *XIST 3':5'*, all samples were shown to have amplifiable DNA after digestion.



In summary, some degree of hypomethylation of *HpaII* and/or *HhaI* sites was evident in six of seven X-linked genes tested in this study (*MAOA, ARAF, AR, DXS6673E, GRIA3, FMR1*). While genes such as *MAOA, ARAF, DXS6673E, GRIA3* and *FMR1* showed hypomethylation in every CVS sample tested and none of the control cases, *AR* showed this effect only in a subset of CVS samples and *XIST* was clearly methylated in the great majority of CVS samples tested.

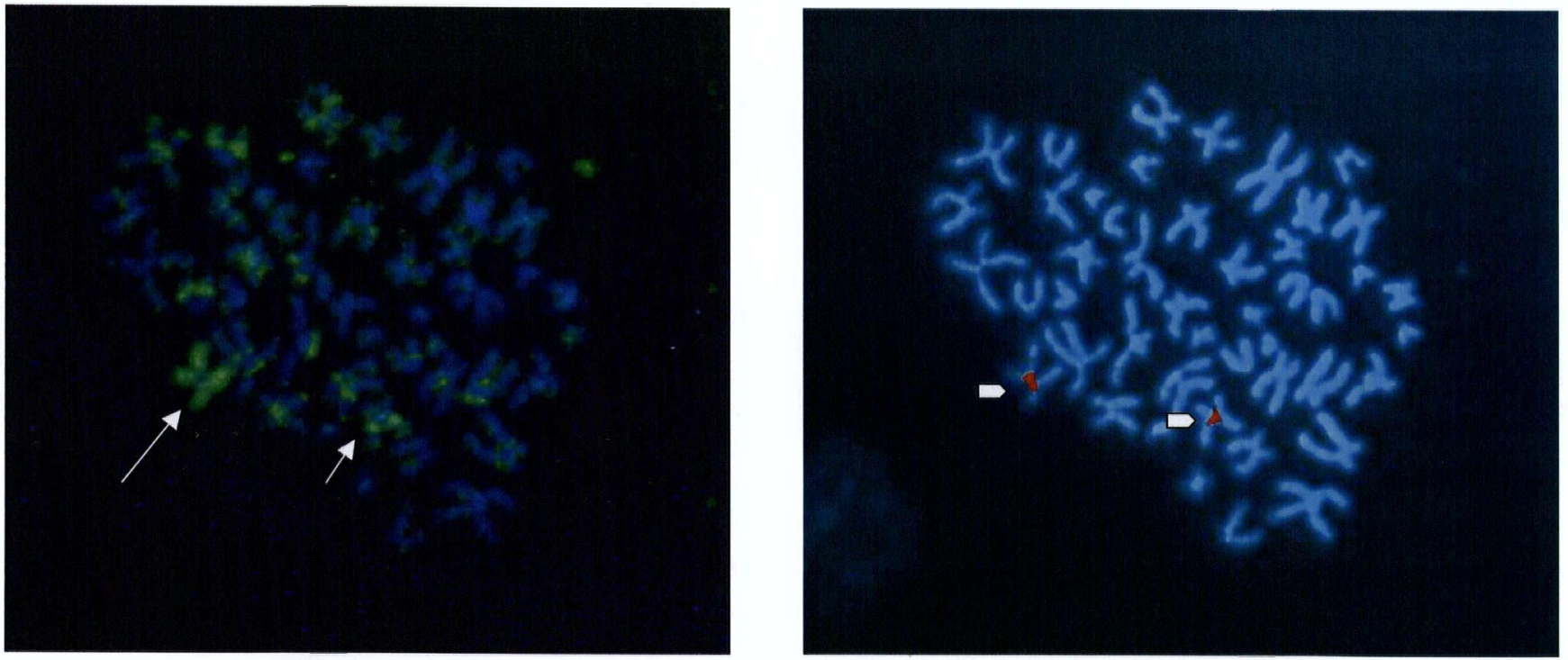
Methylation studies in term placental samples using the FMR1 assay

Given that all term placentae that were part of the study described in Chapter 3 were informative for the *AR* assay (see Chapter 3), none were initially tested with the *FMR1* assay. Based on the results from the CVS samples we decided to test whether hypomethylation of *FMR1* was also present in term placentae. In total, 4 samples of chorion, 13 samples of trophoblast and 10 samples of mesenchyme were examined and all but 1 sample of trophoblast,1 sample of mesenchyme and 1 sample of chorion showed hypomethylation for the 2 *HpaII* sites located in the 5' UTR of the *FMR1* locus. Eighteen of the 27 samples showed poor amplification of the digested sample (<30% but more than 0% amplification relative to the undigested one). These included 9 samples of mesenchyme, 7 samples of trophoblast and 2 samples of chorion. The remaining 6 samples, which showed complete absence of the gene product for the digested sample, included 5 samples of trophoblast and 1 sample of chorion. Fourteen of 15 controls (maternal blood, maternal decidua, cord blood and cord) showed normal methylation patterns for this marker and only 1 control showed some degree of hypomethylation (Table 5.1 and Figures 5.2 and 5.3). In every case the presence of amplifiable DNA was confirmed through previous PCR using the *AR* marker in the same digests and/or using the *XIST* 3':5' marker.

Presence of a late replicating X chromosome (BrdU-FISH)

The results from the BrdU-FISH are consistent with the presence of a late replicating X chromosome in all 4 samples tested. One of two X chromosomes was heavily labelled along the entire chromosome by the BrdU. Its homologous counterpart was also labelled but to a much lower extent and the signal was concentrated at proximal Xq. This finding confirms the presence of a late replicating X in CVS cultures (see Figure 5.7).

Figure 5.7 Metaphase spread of CVS-3JF after BrdU-FISH. Image on the left shows the late replicating X-chromosome heavily labelled with BrdU-antiBrdU-FITC (green fluorescence-long arrow) suggesting late replication. Some other regions in other chromosomes including the X homologue (short arrow) are also lightly labelled. Image on the right shows the same methaphase with both X chromosomes marked with a centromeric probe, also labelled with FITC and counterstained with DAPI, although the colour of the centromeric signal has been altered for contrast ("red" fluorescence- arrowheads).
(Image obtained by Jane Gair)



5.3 Discussion

In this study, the percentage of amplification of placental DNA after *Hpa II* digestion was well under 50%, whereas amplification of digested DNA derived from embryonic tissues showed on average >50% amplification, although there was substantial variability.

One explanation that could account for a percentage of amplification of the digested sample that is much lower or greater than the expected 50% is that this is an effect of a PCR artifact or bias that could affect different X-linked genes in different ways. Any difference in the PCR between digested and undigested samples could cause this effect. For example, the presence of the methylation sensitive restriction enzyme (*HpaII*) in the reaction and/or the fact that the DNA has been cut with this enzyme might result in differential amplification. It is also possible that this differential amplification is due to allele-specific amplification, which could have a larger effect for genes such as *FMR1* that are likely prone to secondary structure formation in the PCR product or the genomic DNA (Beever 2002). If this is the case, it is possible that allele-specific amplification would correlate with allele size, given that it has been demonstrated that secondary structure stability of the amplified alleles increases with trinucleotide repeat size (Mutter and Boynton 1995). However, no clear effect of allele size on percent amplification of the digested sample was observed for the placental or control samples tested with either the *AR* or *FMR1* assays (data not shown). Also, overamplification of DNA (ie. outside the linear range) can lead to >50% yield of the PCR product from the digested sample as compared to the undigested. Alternatively, a reduced yield of PCR product from the digested sample could be attributed to hypomethylation of the locus in question, making the genomic DNA of both alleles prone to digestion with methylation-sensitive enzymes.

It is possible that all of these factors are contributing to the variability seen in both placental and embryonic samples. Nonetheless, as the placental samples clearly showed less amplification upon digestion than controls it seems likely that gene hypomethylation is the most plausible explanation for these differences. Additionally, the methylation-based analysis yielded similar results for 6 of the 7 X-linked genes that were tested suggesting that hypomethylation does not affect specifically *FMR1,* but rather is a common characteristic to several X-linked genes. In addition, the effect is not clustered or localized to a specific region on the X chromosome, as hypomethylated genes mapped both to Xp and Xq and were located at various distances from the XIC, with no evidence of varying levels of methylation relative to their proximity to XIC (see Figure 1.1). Similarly, there was no obvious correlation between degree of gene methylation and gene location within G dark and G light bands on the X chromosome, although, this analysis was further complicated by the current imprecise mapping of certain X-linked genes to the human X-chromosome.

Hypomethylation of X-linked genes such as *G6PD* and *HPRT* has previously been demonstrated in human placenta (Migeon et al. 1985), while *HPRT* has shown extensive methylation of its 5'promoter region in all fetal tissues. Additionally, a study of CpG island methylation of the *FMR-1* gene showed evidence of hypomethylation in extraembryonic tissues such as the chorionic villi and "placenta", while normal methylation patterns were seen in brain, cord blood and lymphocytes (Iida et al. 1994). More recent studies have shown similar findings for the *AR* gene in human chorionic villous stroma (mesenchyme)(Goto et al. 1997; Looijenga et al. 1999).

Additionally, Kratzer and colleagues reported differences in the ability of the Xi DNA from murine yolk sac endoderm as compared to the Xi DNA from fetal cells, to be functional in gene transfer, likely reflecting the level of DNA modification in these two

tissues. In fact, it was shown that the DNA from the Xi from yolk sac endoderm can be very efficient in gene transfer, which could be indicative of reduced or no DNA modification in the chorionic villi (Kratzer et al. 1983). Using *HpaII* and *MspI* isoschizomers and cloned probes for several repetitive sequences in mice, Chapman and colleagues demonstrated that minor satellites and interspersed repetitive sequences are substantially undermethylated in all derivatives of trophoectoderm and primitive endoderm, in contrast with the highly methylated state of these same elements in primitive ectoderm derivatives and adult somatic tissues. They proposed that either inhibition of *de novo* methylation and/or specific demethylation may be involved and suggested that there may be gene regulation processes, dependent on DNA modifications, that are different in extraembryonic tissues (Chapman et al. 1984). Significant hypomethylation of *Sat2* DNA (satellite 2) from chromosome 1 was observed in various samples of human CVS and 1 sample of "placenta" as compared to postnatal somatic tissues which showed no evidence of hypomethylation (Ehrlich et al. 2001).

If we assume that lack of methylation at the promoter is generally associated with gene activity on the inactive X (Bird 2002), the consequence of gene hypomethylation would be expression of the X-linked gene in question from the inactive X chromosome. Lack of dosage compensation for several X-linked genes in placenta seems unlikely, although this possibility cannot be completely ruled out at this point. In fact, a study by Migeon and colleagues showed that many cultured diploid cells from human chorionic villi from 5 fetal and one term placenta expressed the *G6PD* locus on the allocyclic (late replicating) X chromosome (Migeon et al. 1985). Data from this study suggested that the level of expression from the locus on the inactive X is not the same as that of the active X, based on the ratios of homodimers and heterodimers in clones. It is possible that the presence of an

increased dosage of certain gene products is required for normal female development or that this difference is tolerated. Interestingly, it has been shown that in eutherian mammals (mouse, rat, cow and man) XY conceptuses are developmentally more advanced (and consequently larger) than XX conceptuses of equivalent gestational age. This developmental difference is already discernible in the preimplantation period and it has been suggested that the more advanced development of XY embryos relative to XX embryos may be a consequence of the preimplantation expression of Y chromosomal genes such as Sry or Zfy and the presence of two X chromosomes, which seem to have a retarding effect on embryo growth (Burgoyne et al. 1995).

There are, however, several situations where methylation status does not correlate with gene expression status. For example, murine ES cells deficient in DNA methyltransferase, which is the enzyme required for cytosine methylation, can undergo differentiation and inactivation of genes on the X chromosomes even when the genome remains in a hypomethylated state (Panning and Jaenisch 1996). Lack of correlation between XCI status assessed through the *AR* assay and expression studies (RNA based) using other X-linked loci was found in a study of human female neonates suggesting that at least in some cases methylation and expression are not necessarily correlated (El-Kassar et al. 1998). Also, in marsupials no methylation differences in the promoter regions of X-linked genes are apparent and overmethylation of the active X chromosome was found by in-situ nick translation of chromosomes in kangaroos using methyl-sensitive enzymes (reviewed in Graves 1996)

A more likely hypothesis is that XCI takes place in the absence of, or independently of, promoter methylation for several X-linked genes. Two lines of evidence support this hypothesis: first, the persistence of a late replicating X chromosome in CVS

samples as assessed in this study through BrdU-FISH and secondly, the persistence of *XIST* expression (likely indicative of the presence of an inactive X) as was found in a study involving 3 of the CVS samples studied in this chapter (Brown and MacDonald, personal communication). This hypothesis is supported by the finding of a persistent allocyclic X chromosome in the presence of incomplete dosage compensation for the *G6PD* locus in human chorionic villi (Migeon et al. 1985).

It is possible that an alternative mechanism of cellular memory stabilizes silencing in extraembryonic tissues and that methylation instead plays a different role in extraembryonic tissues relative to the one in embryonic tissues. There is evidence to suggest that imprinted XCI in mouse depends in part on the polycomb group (Pc-G) protein *Eed.* In fact, loss of this gene leads to reactivation of the inactive X chromosome in extraembryonic tissues but apparently has no effect in somatic cells (Wang et al. 2001). On the other hand, mutations of the *Dnmt1* gene (DNA methyltransferase) in mice lead to reactivation of the inactive X in embryonic tissues but not in the extraembryonic tissues (Sado et al. 2000). This suggests that perhaps methylation and the Pc-G (*Eed)* system are potentially interchangeable and that perhaps different tissues use either one to stabilize gene silencing (Bird 2002). Studies of the human homologue of mouse *Eed* (EED) have shown that repression of gene activity mediated by this gene involves histone deacetylation (van der Vlag and Otte 1999). Histone underacetylation in the inactive X seems to play a major role in the stabilization of the inactive state (Keohane et al. 1993).

Comprehensive studies of gene expression in both cultures and direct preparation of placental tissues are likely to shed some light into the correlation between hypomethylation and incomplete dosage compensation in human placenta. However, it is important to keep in mind that some variables as the potential for allele specific expression of

certain genes are likely to add to the complexity of the dynamics of the XCI process in extraembryonic tissues in humans.

Chapter 6: An association between skewed X-chromosome inactivation and abnormal outcome in mosaic trisomy 16 confined predominantly to the placenta.

Publication: *Peñaherrera MS, Barrett IJ, Brown CJ, Langlois S, Yong S-L, Lewis S, Bruyère H, Howard-Peebles PN, Kalousek DK, Robinson WP. (2000) An association between skewed X-chromosome inactivation and abnormal outcome in mosaic trisomy 16 confined predominantly to the placenta. Clin Genet 58:436-446.*

6.1 Introduction

A discrepancy between the chromosome complement of the embryo/fetus and the placenta, occurs in 1-2% of pregnancies analyzed by chorionic villus sampling (CVS) (Kalousek 1985; Wang et al. 1993). Typically, this involves the presence of a trisomic lineage in the placenta but only diploid cells in the fetus, a situation called confined placental mosaicism (CPM). This may arise either through trisomic zygote rescue, defined as the loss of a chromosome in the embryonic progenitors of a conceptus arising from a trisomic zygote (meiotic origin) or by somatic duplication (postzygotic origin) of a chromosome in the placental progenitors derived from a diploid zygote (Kalousek et al. 1996). Trisomy mosaicism found upon CVS and/or amniotic fluid (AF) testing (followed by the finding of trisomy in the placenta) can be difficult to confirm in the fetus, as it may in some cases be predominantly confined to the placenta, with only one or a few fetal tissues affected. In live born infants, typically only blood or in some instances skin is studied cytogenetically, therefore 'occult' mosaicism (limited to specific tissues) cannot be identified. The term 'occult' trisomy mosaicism was suggested to describe the situation in which the presence of an abnormal cell line is suspected from the data but has not been proven by laboratory tests (Benn 1998).

Although prenatally detected trisomy mosaicism is often a consequence of a postzygotic error arising in a chromosomally normal conceptus, mosaicism involving trisomy 16 is virtually always a consequence of trisomic zygote rescue (Robinson et al. 1997). Typically, for trisomy 16 mosaicism, very high levels of trisomy are seen in the placenta while subsequent amniocentesis or blood analysis may fail to show any evidence of the trisomy (Kalousek et al. 1993; Robinson et al. 1997; Benn 1998). Pregnancy outcome in these cases is often normal, but there is an increased risk for intrauterine growth restriction (IUGR) or abnormal outcome. The latter may include fetal malformations and intrauterine (IUD) or neonatal death (ND) (Kalousek 1994). Fetal growth may be impaired by a high population of trisomic cells in the placenta as a whole or in specific cell lineages within the placenta. However, some cases with high levels of trisomy in the placenta exhibit normal growth and outcome, and placental trisomy alone is unlikely to explain malformations and other abnormalities (Kalousek et al. 1993; Kalousek 1994; Robinson et al. 1997).

It is expected that in roughly 1/3 of trisomy rescue events, uniparental disomy (UPD) will be present in the diploid lineage (Robinson et al. 1997). UPD for some chromosomes can result in clinical abnormalities, although for many others including UPD16, it has no clear effect on the clinical outcome (Ledbetter and Engel 1995). The presence of fetal UPD is thus insufficient to explain the majority of clinical findings associated with trisomy 16 mosaicism, leading to the suggestion that the adverse outcome may frequently be due to 'occult' trisomy mosaicism in the fetus (Benn 1998).

Twelve mosaic trisomy cases (8 involving trisomy 16) were analyzed previously in our laboratory, to show that extremely skewed XCI was frequently found in the diploid fetal tissues of cases with mosaic trisomy predominantly confined to the placenta. It was hypothesized that this skewed pattern of XCI could have resulted from a reduction in the size

of the early embryonic cell pool, because of either poor early growth or subsequent selection against the trisomic cells (Lau et al. 1997). This thesis chapter extends this argument to suggest that cases showing extreme XCI skewing are likely to have had a greater number of trisomic cells in the embryonic precursor pool. Therefore, skewing will correlate with a higher risk for an abnormal outcome, due either to the presence of 'occult' trisomy mosaicism in the fetus (as not all the trisomic cells are necessarily eliminated from all fetal tissues) or secondary to early loss of a high percentage of trisomic cells by selection. To test this hypothesis I have evaluated whether XCI status in diploid fetal tissues correlates with outcome of mosaic trisomy 16 pregnancies and with the risk for 'occult' trisomy mosaicism. I chose to focus on mosaic trisomy 16 cases since these are the most frequently ascertained on CVS and because the great majority of cases are a consequence of trisomic zygote rescue (meiotic origin of the trisomy)(Robinson et al. 1997). Results on 6 mosaic trisomy cases involving other chromosomes were also included.

6.2 Methods and Results

Patient ascertainment

The study population consisted of 19 female fetuses or newborns with prenatally ascertained (on CVS or amniocentesis) trisomy 16 mosaicism. Six additional cases of trisomy mosaicism for chromosomes 2, 4, 7, 20 and 22 (2 cases) were also analyzed. As the previous study from our lab indicated that skewed XCI was only associated with mosaicism resulting from trisomic zygote rescue (meiotic origin of the trisomy) (Lau et al. 1997) this was used as criteria for inclusion in the present study. These cases were referred from various centers across North America. Informed consent was obtained for each of the cases involved in this study. Details on prenatal findings and outcome (clinical details) for the cases included in this

study are presented in Tables 6.1 and 6.2. The term IUGR is used for those situations in which the fetus weighed less than two standard deviations below the mean for gestational age (Bernstein and Divon 1997).

Table 6.1:
Prenatal and postnatal findings in patients with mosaic trisomy 16 predominantly confined to the placenta

Case	abnormal cell line	Prenatal [a] % trisomy CVS	AF	fetus [b]	Ultrasound findings [c]	PO [d] (GA wks)	BWg (SD)[e]	abnormalities [f]	follow-up [g]	previous publication
Patients with >90% skewed XCI										
93.43	47,XX,+16	NA	10.5	UPDmat	large placenta, severe IUGR, enlarged heart, possible septal defects.	TA (21)		severe IUGR, digitalization of thumbs, ulnar deviation of the hands, hypoplastic lungs, enlarged hypertrophic heart, multiple VSD and two vessel umbilical cord.		(Robinson et al. 1997)
95.28	47,XX,+16	100 c	0	UPDmat	9, 12,14 wks: IUGR, no fetal abnormalities	L B (38)	2000 (-3.1)	IUGR, small placenta.	22dy: cyanosis, partial anomalous pulmonary venous return, large ASD, tracheal narrowing Died at age 1mo.	(Robinson et al. 1997)
CPM16 -36	47,XX,+16	NA	50	BPD		TA		tetralogy of Fallot		
98.316	47,XX,+16	100 d	0	BPD	19-20wks: biometry indicates 16wks.	IUD (24)		IUGR		
99.143	47,XX,+16	NA	36	BPD		TA		small VSD		
93.73	47,XX,+16	100 c 100 d	0	BPD		L B (37)	2315 (-1.3)	birth length 45.6cm (-1.6SD) , no dysmorphic features	4yr: 94cm (-1.5SD); 13.6kg (1.6SD)	
94.21	47,XX,+16	100 c	0	BPD		L B (40)	3170 (-0.6)	No abnormalities.	1yr 5mo: weight 50-70%ile, length 75-90%ile, normal psychomotor development	(Robinson et al. 1997)
98.22	47,XX,+16	NA	40	UPDmat	16-17wks: normal fetus placenta with acute chorio-amnionitis	TA (19)	215 (NA)	small VSD		
98.234	47,XX,+16	NA	low level	BPD		L B (39)	2578 (-1.7)	no external malformations except for asymmetrically placed nipples.	normal psychomotor development,	

Table 6.1:
Prenatal and postnatal findings in patients with mosaic trisomy 16 predominantly confined to the placenta

Case	abnormal cell line	Prenatal [a] % trisomy CVS	AF	fetus [b]	Ultrasound findings [c]	PO [d] (GA wks)	BWg (SD)[e]	abnormalities [f]	follow-up [g]	previous publication
99.78	47,XX,+16	100 c 100 d	0	BPD	17wks: 9 days delayed , no fetal abnormalities, large placenta with sonolucent and cystic areas	L B (37)	2060 (-1.7)	no congenital anomalies		
CPM16-50	47,XX,+16	NA	100 93	BPD	18wks:biometry indicates 17 wks. Possible aortic stenosis 22wks: biometry indicates 20wks 5dy. Small VSD suspected. 27wks 5dy: biometry indicates 25wks 5dy. 30wks: biometry indicates 27wks 5dy. Mild aortic stenosis. 32 wks: biometry indicates 28w 5d. Weight below the 10%ile,oligohydramnios.	LB (32)	1080 (-2.5)	IUGR, possible ASD and aortic coarctation, small optic nerves and cloudy corneas, plagiocephaly, "sun setting sign" in sclera	6mo: weight and OFC (50%ile),length (10-25%ile). Repaired bilateral inguinal hernia. Mild craniofacial dysmorphism, plagiocephaly, abnormal sclera, developmental delay.	(Morris et al. 2001)
CPM16-51	47,XX,+16	100 d	0	UPDmat	12wks: normal 18wks: IUGR 31wks: enlarged cystic placenta, ASD, symmetrical IUGR absent end diastolic flow in one umbilical artery, increased resistance in the other umbilical artery, maternal hypertension without edema.	LB (35)	1530 (-2.0)	IUGR, ASD, no other obvious malformations.		

Table 6.1:
Prenatal and postnatal findings in patients with mosaic trisomy 16 predominantly confined to the placenta

Case	abnormal cell line	Prenatal [a] % trisomy CVS	AF	fetus [b]	Ultrasound findings [c]	PO [d] (GA wks)	BWg (SD)[e]	abnormalities [f]	follow-up [g]	previous publication
Patients with < 90% skewed XCI										
91.71	47,XX,+16	100 c 100 d	0	BPD	32wks: slowing of fetal growth 37wks: IUGR	L B (39)	1964 (-3.1)	IUGR, no physical abnormalities length 44.5cm (-2.7SD), OFC 32cm (-2.7SD)	2mo: no physical abnormalities, height and weight <5%ile.	case 3 (Johnson et al. 1993; Kalousek et al. 1993)
90.90	47,XX,+16	100 c 100 d	0	UPDmat	10,13,16,20,24,28 wks: normally developing fetus with appropriate interval growth between each U/S study.	L B (41)	3008 (-1.0)	No congenital anomalies.	18mo: normal growth and development.	case 5 (Johnson et al. 1993; Kalousek et al. 1993)
91.55	47,XX,+16	100 c	0	BPD		L B (40)	3320 (-0.3)	No congenital anomalies, length at birth 48 cm (-1.7SD)		case 7 (Kalousek et al. 1993)
97.20	47,XX,+16	100 c	NA	UPDmat	10wks: normal biometry	TA (12)		No fetal anomalies at 12 weeks gestation		(Stavropoulos et al. 1998)
CPM16-48	47,XX +16	100c	0	BPD	All U/S showed nornal biometry, no abnormalities were found	LB (41)	3632 (0.16)	No abnormalities noted length at birth 52 cm (-0.1SD)		
Patients uninformative for XCI										
91.14	47,XX,+16	100 c 100 d	0	UPDmat	21 wks: normal biometry, thick and large placenta containing echogenic regions	IUD (25)	410 (-2.3)	IUGR, no lesions to correlate with U/S findings in placenta, no fetal anomalies		case 2 (Kalousek et al. 1993)
98.92	47,XX,+16	NA	low level	UPDmat	17-18wks: IUGR, possible VSD 28 wks: biometry of 24 wks. 29 wks: IUGR	L B (29)	738 (-4.6)	IUGR, cardiac abnormalities, ASD.	9mo: repaired ASD, seizures, speech delay	case 10 (Hsu et al. 1998)

[a] NA= data not available; c = cultured; d = direct preparation [b] UPDmat = maternal uniparental disomy; BPD = biparental inheritance; NA = not available
[c] wks = weeks; IUGR = intrauterine growth restriction; VSD = ventricular septal defect
[d] PO = pregnancy outcome; GA = gestational age; wks = weeks; LB= live born; TA = termination of pregnancy; IUD= intrauterine death
[e] g = grams; SD =standard deviation based on (Usher and McLean 1969; Smith 1977)
[f] IUGR = intrauterine growth restriction; VSD = ventricular septal defect; ASD = atrial septal defect
[g] mo = months; yr = years; dy = days %ile = percentile (as reported by the attending physicians as no actual values were given)

Table 6.2: Prenatal and postnatal findings in patients with mosaic trisomy other than trisomy 16.

Case	abnormal cell line	Prenatal [a] % trisomy CVS	AF	fetus [b]	Ultrasound findings [c]	PO [d] (GA wks)	BWg (SD)[e]	abnormalities [f]	follow-up [g]	previous publication
Patients with >90% skewed XCI [h]										
95.66	47,XX,+22	100 c	0	UPDmat	11wks: IUGR 27wks: symmetric IUGR, oligohydramnios	L B (32)	1080 (-2.5)	IUGR, ASD, VSD, sub-aortic stenosis, mild aortic hypoplasia. Small asymmetric low set ear, preauricular pits, shallow orbits with proptosis, hypertelorism, rocker bottom feet, mongolian spots.	4mo: temperature instability, failure to thrive, abnormal CNS with increased tone, mild contractures (elbows, knees), abnormal renal U/S	(Robinson et al. 1997)
97.69	47,XX, +4	0 c 100 d	0	UPDmat	17wks: normal 29wks: IUGR, oligohydramnios, high impedance to flow in placental circulation, normal fetal circulation. 30 wks: cerebral reversal flow	IUD (31)		No external abnormalities noted but no data on autopsy findings.		(Kuchinka et al. 2001)
94.25	47,XX,+2	NA	NA	BPD	21wks: biometry indicates 19wks 32wks: small for gestational age.	L B (39)	1535 (-4.1)	IUGR, left preauricular pit, mild retrognathia, large anterior fontanelle	32mo and 4½yr: good catch-up growth, normal development.	
Patients with < 90% skewed XCI [h]										
91.53	47,XX,+7	100 c	0	UPDmat	17wks: mild oligohydramnios 29 wks: decreased fetal growth 31 wks: mild to moderate oligohydr.	L B (36)	1560 (-3.0)	IUGR	2yr: weight 9000g (-2.4 SD), no major abnormalities	case 4 (Kalousek et al. 1996)
98.190	47,XX,+20	NA	11	BPD	18wks: normal	L B (41)	3370 (-0.3)	No abnormalities noted	9dy: no evidence of abnormalities	
93.122	47,XX,+22	50 c	0	NA		L B		No outcome data available		

[a] NA= data not available; c = cultured; d = direct preparation; [b] UPDmat = maternal uniparental disomy; BPD = biparental inheritance; NA = not available
[c] wks = weeks; IUGR = intrauterine growth restriction; VSD = ventricular septal defect
[d] PO = pregnancy outcome; GA = gestational age; wks = weeks; LB= live born; IUD= intrauterine death
[e] g = grams; SD =standard deviation based on (Usher and McLean 1969; Smith 1977); [f] IUGR = intrauterine growth restriction; VSD = ventricular septal defect; ASD = atrial septal defect; [g] mo = months; yr = years; dy = days ; [h] XCI determined in blood, except for 97.69 for which skewed XCI was identified in tongue and liver.

The control population consisted of healthy adult females informative for the Androgen Receptor (*AR*) polymorphism used as the XCI test in this study. Their ages ranged between 29-49 years, with a mean age of 35.0 years and all had a term birth (Sangha et al. 1999). Although we are not using normal newborns as a control, it is known that the level of skewing tends to increase with age of the female and thus this is a conservative comparison group.

Molecular and cytogenetic studies

To determine percentage of trisomic cells, conventional cytogenetic studies were carried out by Irene Barrett in Dr. Dagmar Kalousek's laboratory on CVS samples, AF samples, fetal blood samples and placental samples, using standard techniques. Fluorescence in situ hybridization (FISH) on the same samples, was also performed by Irene Barrett as described previously (Henderson et al. 1996; Stavropoulos et al. 1998). Estimates of the percentage of trisomy in fetal tissues when available for study were also performed in the Kalousek laboratory using standard procedures (Lomax et al. 1994). In most cases, the presence of trisomy by DNA tests, the origin of trisomy and the presence or absence of UPD in the patient was determined in our laboratory by genotyping the child, parents and placental samples. Highly polymorphic microsatellite markers that mapped to the chromosome involved in the mosaic trisomy were used for this purpose, as described elsewhere (Robinson et al. 1997).

XCI assay

X inactivation status was assessed with the *AR* assay. The samples were classified into two groups using an arbitrary cut-off of 90% skewing: those with non-skewed XCI (<90% skewing) and those with extreme XCI skewing (≥90% skewing).

XCI skewing

The fetal tissues from a total of 19 cases of prenatally detected trisomy 16 mosaicism of meiotic origin were tested for XCI. Seventeen of these cases were informative at the *AR* locus (Table 6.3). Extreme skewing was seen in a total of 12 of 17 (71%) of these cases, which was significantly more than the control females (6/111 or 5%) ($p<0.001$; Fisher's exact test)(Table 6.3). In most fetal tissues from the 12 cases that were extremely skewed, there was no visually detectable amplification product from one of the two alleles after digestion of the sample with *HpaII*, indicating 100% inactivation of one allele. In contrast only 1 of the 111 controls was considered to show 100% skewed XCI. There was no evidence of a bias towards the inactivation of either the paternal or maternal allele (Table 6.3), nor was there a difference in skewing or outcome between cases with UPD or BPD (Table 6.1).

Table 6. 3:
X-chromosome inactivation tests and summary of data on patients with meiotic mosaic trisomy 16 predominantly confined to the placenta

Case	(% trisomy) AF [a]	fetal tissues	% trisomy (C) (F) [b]	presence of trisomy by DNA[d]	XCI skewing [e] fetus	summary of outcome [f]	placental tissues	presence of trisomy (C) (F) [b] or DNA[d]	XCI skewing [e] placenta
Patients with >90% skewed XCI									
93.43	10.5	intestine		-	100 (pat)	TA, IUGR, fetal abnormalities			
95.28	0	diaphragm	0 (C) 7.2(F) [c]	-	100 (pat)	ND, IUGR, fetal abnormalities			
CPM16-36	50	brain		-	100 (mat)	TA, tetralogy of Fallot			
		spleen		-	100 (mat)				
		AF	50 (C)	+	58 (mat)				
		skin	0 (C)	+	51 (mat)				
		kidney		-	79 (mat)				
98.316	0	heart		-	100 (mat)	IUD, IUGR	stroma	+	68 (mat)
		adrenal		-	100 (mat)		trophoblast	31(F)[c]	72 (pat)
		cord		-	100 (mat)				
		kidney		-	100 (mat)				
		lung	1.3 (F)[c]	-	100 (mat)				
		brain	80 (F)	+	60 (mat)				
99.143	36	kidney	9.0 (F)[c]	-	100 (nd)	TA,VSD			
		lung	18(F)	+	59 (nd)				
		liver		-	86 (nd)				
93.73	0	blood	0 (C)	-	100 (nd)	live born, normal			
94.21	0	blood	0 (C)	-	100 (pat)	live born, normal	chorionic villi 1 chorionic villi 2		70 (nd) 59 (nd)

Table 6. 3:
X-chromosome inactivation tests and summary of data on patients with meiotic mosaic trisomy 16 predominantly confined to the placenta

Case	(% trisomy) AF [a]	fetal tissues	% trisomy (C) (F) [b]	presence of trisomy by DNA[d]	XCI skewing [e] fetus	summary of outcome [f]	placental tissues	presence of trisomy (C) (F) [b] or DNA[d]	XCI skewing [e] placenta
98.22	40	blood		-	100 (mat)	TA, VSD	chorionic villi	81(F)[c], +	100 (mat)
		kidney		-	100(mat)				
		gonad		-	~90% (mat)[g]				
		adrenal		-	100 (mat)				
		skin		-	100 (mat)				
		spleen		-	100 (mat)				
		liver		-	~90%(mat)[g]				
		brain		-	99 (mat)				
		thymus		-	100 (mat)				
		lung	12 (F)	+	92 (mat)				
98.234	low level	blood		-	100 (pat)	live born, normal	amnion	27 (F)[c]	82 (pat)
							chorionic villi 2	35-43 (F)[c]	67 (pat)
							chorionic villi 4	35-43 (F)[c]	79 (mat)
							chorionic villi 5	35-43 (F)[c]	56 (mat)
							trophoblast 2	30-65 (F)[c]	56 (pat)
							trophoblast 4	30-65 (F)[c]	52 (pat)
							trophoblast 5	30-65 (F)[c]	76 (pat)
99.78	0	blood		-	100 (mat)	live born, normal			
CPM16-50	100 93	blood	0(C) 0(F)[c]	-	100 (nd)	live born, IUGR, possible ASD, various abnormalities			
CPM16-51	0	blood		-	99 (mat)	live born, IUGR, ASD	amnion 1	+	55 (pat)
							amnion 2	-	100 (mat)
							chorion	+	51 (mat)
							chorionic villi 1	+	87 (pat)
							chorionic villi 2	+	67 (pat)

Table 6. 3:
X-chromosome inactivation tests and summary of data on patients with meiotic mosaic trisomy 16 predominantly confined to the placenta

Case	(% trisomy) AF [a]	fetal tissues	% trisomy (C) (F) [b]	presence of trisomy by DNA[d]	XCI skewing [e] fetus	summary of outcome [f]	placental tissues	presence of trisomy (C) (F) [b] or DNA[d]	XCI skewing [e] placenta
Patients with < 90% skewed XCI									
91.71	0	blood		-	70 (pat)	live born, IUGR	amnion chorion chorionic villi	0 (C) 86 (C) 100 (C) 100 (F)[c]	61 (pat) 86 (pat) 98 (pat)
90.90	0	blood	0 (C) 0(F)	-	74 (nd)	live born, normal			
91.55	0	blood	0 (C) 0(F)[c]	-	83 (pat)	live born, normal	amnion chorion chorionic villi	0 (C) 0 (C) 0 (C) 0 (F)[c]	79 (pat) 60 (mat) 54 (pat)
97.20	NA	adrenal lung	1.2 (F)[c]	-	57 (nd) 53 (nd)	TA, normal at 12 weeks	amnion trophoblast stroma chorionic villi	+ 59 (F)[c]	66 (nd) 63 (nd) 53 (nd) 53 (nd)
CPM16-48	0	blood		-	52 (pat)	live born, normal			
Patients uninformative for XCI									
91.14	0	blood	0(C)	-	uninformative	IUD, IUGR			uninformative
98.92	low level	blood	0(C)	-	uninformative	live born, various abnormalities, IUGR			uninformative

[a] NA= data not available; [b] C = conventional cytogenetic studies; F = FISH studies; [c] These values were below our cut-off values for controls;
[d] - = negative for the presence of the trisomy; + = positive for the presence of the trisomy [e] Xi = inactive X; pat = paternal; mat = maternal;
nd= not determined; [f] IUGR = intrauterine growth restriction; TA = termination of pregnancy; IUD = intrauterine fetal death;
ND = neonatal death; VSD = ventricular septal defect; [g] estimated by eye.

XCI results in different fetal tissues

Fetal blood was used for the XCI tests in 11 of the 17 informative mosaic trisomy 16 cases, with significantly increased XCI skewing observed in 7 of these. In one of these cases (98.22) several fetal tissues were also tested and all showed extreme XCI skewing. In the remaining 6 informative cases, fetal blood was not available for testing, therefore, studies were performed in other fetal tissue(s) (see Table 6.3). Five of these 6 cases showed extreme XCI skewing (Table 6.3). Thus, XCI skewing does not seem to be limited to a specific tissue.

In the five cases (CPM16-36, 98.316, 99.143, 98.22 and 97.20), where it was possible to examine more than one fetal tissue, there was a high degree of correlation between XCI results from different tissues within one individual, and in every case skewing occurred in the same direction (Table 6.3).

Considering all 19 mosaic trisomy 16 cases included in this study, a combined total of 40 tissues (including fetal blood and other tissue samples) were tested. Thirty three of these tissues showed no evidence of the trisomic cell line by either cytogenetic or molecular methods and 25 of those 33 samples showed extremely skewed XCI. On the other hand, none of the 5 tissues that showed evidence of the trisomy, displayed 100% skewed XCI. The remaining 2 samples were diploid but uninformative for XCI (Table 6.3).

Correlation between X-chromosome inactivation skewing and outcome

A summary of XCI skewing and outcome for the mosaic trisomy 16 cases is given in Table 6.4. The frequency of fetal anomalies and/or intrauterine or neonatal death was increased in those cases that showed extremely skewed XCI (8 of 12 cases) as compared to those that were not significantly skewed (0 of 4 cases) ($p=0.038$; Fisher's exact test). IUGR was found in all cases for which this feature could be evaluated that had an 'abnormal'

outcome. However, the finding of IUGR alone (present in one case) was considered 'normal', as it is likely a consequence of placental dysfunction caused by high levels of placental trisomy. Most cases of trisomy 16 mosaicism show some degree of growth delay in utero and soon after birth (consistent with IUGR); however, follow-up data shows good catch-up growth postnatally in many of them, suggesting a potential role of the mosaic aneuploid placenta in limiting fetal growth in utero. One case (97.20) was eliminated from this comparison as it was terminated at 12 weeks gestation, and although developmentally normal, some malformations, IUGR and/or late IUD would not be apparent at this time of development.

Although our sample size for mosaic trisomy cases involving chromosomes other than 16 is still very small (Table 6.2), the results from these cases follow a similar trend to that of the mosaic trisomy 16 cases, and the correlation of skewed XCI with outcome was statistically significant if all cases were considered (11 of 15 cases are extremely skewed and showed an abnormal outcome vs. 0 of 7 that are not skewed and show an abnormal outcome; p=0.001 one-tail Fishers Exact Test). Specifically, three cases of mosaicism involving trisomy 2, 4, and 22 showed skewed XCI in the diploid fetal tissues. Of these, one resulted in an intrauterine death (trisomy 4), one had multiple developmental abnormalities (trisomy 22) and one had IUGR with minor anomalies (trisomy 2). In contrast three cases of trisomy mosaicism did not show skewed XCI. Of these, one case of mosaic trisomy 7, had poor growth, presumably as a consequence of having maternal UPD7. This individual was otherwise healthy. Mat UPD 7 has been associated with IUGR and post-natal growth restriction in the context of Silver-Russell syndrome (Kotzot et al. 1995; Bernard et al. 1999). The second case, a mosaic trisomy 20, was normally grown and developed and the third case,

a mosaic trisomy 22 individual resulted in a live birth but I was not able to obtain any other outcome information.

All cases with mosaic trisomy 16 for which trisomic cells were detected on amniocentesis (AF+) also had extremely skewed XCI, although the difference between the two groups was not significant (Table 6.5). Abnormal outcome was also more common when trisomy was present in amniocentesis although also not statistically significant probably due to small sample size (Table 6.6).

Table 6.4. XCI skewing vs. outcome in mosaic trisomy 16 cases

outcome	skewed (N=12)	not-skewed (N=4)[a]
Abnormalities and/or IUD/ND	8[b]	0[b]
IUGR only	0	1
Normal	4	3

[a] excludes case 97.20 with incomplete outcome information.
[b] p=0.038, Fishers exact test--comparing the frequency of cases with abnormalities/IUD/ND outcome between the two groups

Table 6.5. XCI skewing vs. AF results in mosaic trisomy 16 cases

AF results	skewed (N=12)	not-skewed [a] (N=4)
AF+	6	0
AF-	6	4

[a] excludes one case with no AF result (97.20).
p=0.125, Fishers exact test

Table 6.6. AF. results vs. outcome in mosaic trisomy 16 cases

outcome	AF+ (N=7)	AF- (N=11)[a]
Abnormalities and/or IUD/ND	6[b]	4[b]
IUGR only	0	1
Normal[c]	1	6

[a] excludes case 97.20 with incomplete outcome information
[b] p=0.052, Fishers exact test--comparing the frequency of cases with abnormalities/IUD/ND outcome between the two groups

XCI in extrambryonic tissues

Extraembryonic tissues from a few of the mosaic trisomy cases included in this study were obtained to allow comparisons of the patterns of XCI with the normal placentae. Additionally, it was of interest to assess whether XCI patterns in extraembryonic tissues in mosaic trisomy cases were consistent with those in embryonic tissues. Data on XCI was available for some placental and amniotic tissues from the mosaic trisomy cases studied in this project; unfortunately, as some cases were collected prior to the initiation of the present study, some samples of chorionic villi were not separated (enzymatically) into their trophoblastic and mesenchymal components before DNA extraction (Table 6.3). Trisomy was present in the majority (19 of 24) of the tissues examined, as assessed by either conventional cytogenetics (C), FISH (F) or DNA (+ or -). Skewing levels below the cut-off of $\geq$ 90% skewing were seen for the majority of samples (16 of 19) that showed evidence of the trisomy. In case CPM16-51, a correlation between absence of the trisomy by DNA and extremely skewed XCI, as opposed to presence of the trisomy by DNA and random XCI was clearly illustrated by the two amnion samples available for study in this case (Table 6.3). A few exceptions to this finding were seen in cases (91.71 and 91.55), although it is difficult to rule out possible difficulties with the detection of the trisomy by conventional cytogenetics.

6.3 Discussion

Skewed XCI and trisomy mosaicism

This study extends a previous finding of extreme skewing of XCI in cases of mosaic trisomy of meiotic origin that is predominantly confined to the placenta by including additional cases and evaluating clinical data (Lau et al. 1997). In total, extreme skewing has now been observed in diploid fetal blood/other tissue samples from 15 of 23 cases of trisomy

mosaicism including 12 of 17 cases (71%) with trisomy 16 mosaicism. This is significantly higher than the 5% extreme skewing observed in the adult control population, or the 2% observed in previously published series of healthy newborns (Busque et al. 1996; Sangha et al. 1999).

Skewed XCI was observed in both blood and other fetal tissue samples and there was a correlation of the level and direction of skewing in different tissues within the same individual. As blood was unavailable in most of the cases where multiple tissues were examined, these results cannot be used to determine if there is a correlation between the degree of skewing between fetal blood and other fetal tissues. However, the finding of significantly increased XCI skewing in these cases was not affected by the exclusion of the data from fetal tissues other than blood. The degree of skewed XCI is correlated between various tissues in mouse (Nesbitt 1971). The analysis of data published by Gale et al. (Gale et al. 1994) also shows a significant correlation in skewing between granulocytes, T-lymphocytes, E-negative cells, skin, muscle and colon cells in humans. However, in the latter study, only one of 5 hematologically normal females who showed >90% skewing in granulocytes and lymphocytes, also showed >90% skewing in skin and muscle. Non-random XCI limited to blood cells is likely due to selective differences between hematopoietic stem cells which have inactivated one or the other X-chromosomes or to a depletion of the stem cell pool over time, whereas our results suggest that skewing due to early trisomy mosaicism is more likely to affect all diploid fetal tissues.

Complete skewing, with no evidence of the other parental allele being active, suggests that the diploid fetal tissues may have derived from a single progenitor cell present at the time that an X-chromosome is designated to be inactivated. Normally, it is expected

that at least 10-20 fetal progenitor cells are present at this time (late blastocyst stage)(Puck et al. 1992), whereas the actual process of XCI does not occur until later, around the time of tissue differentiation (Tan et al. 1993). If all fetal cells were truly derived from just one of these progenitor cells, then one would expect 100% skewing in all diploid fetal tissues. Whereas, tissues in which trisomy mosaicism persists must be derived from 2 or more fetal progenitor cells and therefore would be less likely to show a tendency towards extreme skewing. For example, derivation of all diploid fetal tissues from a single blastocyst cell is likely in case 98.316, which showed 100% skewed XCI in all 5 diploid fetal tissues analyzed but was not skewed in brain, the only tissue that showed 80% trisomic cells. On the other hand, CPM16-36 and 98.22 showed 100% skewed XCI in some but not in all diploid fetal tissues, suggesting that there were at least two progenitor cells at the time an X is marked for inactivation. In this case skewing may have resulted from a reduced number of progenitor cells combined with the stochastic effects of sampling from the cell pool during tissue differentiation.

If it is assumed that the majority of the 10 to 20 embryonic precursor cells at this stage of the embryonic development are trisomic, then they must be eliminated from contributing to the developing fetus by selection (e.g. slower cell division rates and/or increased apoptosis). A similar mechanism involving selection and high cell death rate has been suggested to explain extreme XCI skewing in carriers of X-autosome translocations (Zabel et al. 1978). However, in this case cell selection is likely to affect about half of the cell population and not >95% as we suggest for the trisomy mosaic cases.

Very similar results to those described for the fetal tissues were found for the placental samples available for a subset of cases, indicating that similar dynamics apply to

the extraembryonic tissues. However, the limited sample size precluded any statistical comparisons in this case. In regards to a possible parental bias to XCI in these tissues, it is worth noting that 4 of 4 informative trophoblast samples tested (from 2 different individuals) showed preferential inactivation of the paternally derived X, while approximately half of the whole chorionic villi samples showed the maternal allele preferentially inactivated. Given that the main component of the chorionic villi is the mesenchyme, it is possible that results of the XCI study, obtained from whole chorionic villi, mostly reflect the mesenchyme. If this is true, the tendency (not significant) towards the inactivation of the maternally derived X seen in the mesenchyme of normal term placentae was not seen in this mosaic trisomy cases. Of interest is that 2 informative placentae (98.316 and 98.234) in the mosaic trisomy series showed preferential inactivation of the paternally derived X in the trophoblast as did 7/8 first trimester placentae in the study described in chapter 3. These were two of the few placentae for which isolated trophoblast was available for study. These cases were ascertained at 24 wks and 38wks of gestation respectively, which could indicate that this bias of XCI skewing in the trophoblast, if real, persisted on the second and third trimester of gestation.

Skewed XCI and correlation with outcome

In this study we found a positive association between skewed XCI and abnormal outcome ($p=0.04$), which was statistically more significant if data from all (not just trisomy 16) mosaic trisomy cases were considered ($p=0.008$). The poor outcome in these cases may have been a consequence of the failure to completely eliminate the trisomic cells from 'all' fetal tissues, resulting in tissue specific trisomy mosaicism or due to disruption to development caused by differential growth rates and/or cell death of the trisomic cells.

Unfortunately, it is difficult to evaluate the role of occult trisomy mosaicism as the tissues analyzed and the follow-up data for each case are not always identical.

Patients with a positive AF result in this study for trisomy 16 also showed an increased risk for poor outcome ($p=0.05$). This finding is also confirmed by a larger study of trisomy 16 (Yong et al. 2003). However, the lack of any trisomic cells in AF does not exclude fetal mosaicism as the cause of fetal abnormalities. This is particularly a consideration in the three cases of trisomy 16 mosaicism that were associated with skewed XCI and abnormal outcome, but were negative for the trisomy in AF. Mosaicism may be confined to just one tissue in the fetus and could be "missed" on amniocentesis for various reasons (e.g. preferential growth of certain cells over others in culture or presence of the trisomy only in lineages which do not contribute to amniotic fluid). Since in some cases it may be the elimination or poor growth of trisomic cells, rather than their persistence, which contributes to the observed developmental abnormalities, the absence of detectable trisomy in a particular fetal tissue does not exclude the possibility that trisomy has had an effect on the development of that tissue.

Previous studies have suggested a correlation between fetal UPD 16 and abnormal outcome, particularly IUGR (Yong et al. 2003). While there was no significant correlation between UPD and abnormal outcome or IUGR in the mosaic trisomy 16 cases included in this study, there was a trend towards worse outcome in the UPD cases (Table 6.1). Furthermore, it is likely that it is a combination of 'occult' tissue specific trisomy, cell death or different cell growth rates in early embryogenesis and potentially UPD that are ultimately responsible for the outcome in these cases.

As the present results are limited, and there may be chromosome specific differences, it would be premature to suggest that such an invasive test as cordocentesis be considered in mosaic trisomy cases for the sole purpose of determining the XCI status. Currently, our results cannot be extended to DNA analysis of amniocytes since: 1) there is little data on AF XCI status in either normal or mosaic pregnancies; 2) some cases in this study showed skewed XCI in fetal tissues but would have been unlikely to be detected in amniotic fluid as trisomic cells were present; and 3) absence of the trisomy as well as skewed XCI would need to be confirmed in uncultured amniotic fluid to avoid culture biases. The finding of IUGR or fetal abnormalities on ultrasound prior to 20 weeks gestation in this study's cases seems to be predictive of an abnormal fetal outcome (Table 6.1). This suggests that ultrasound remains one of the most reliable and non-invasive methods for prenatal assessment of fetal outcome in the presence of mosaicism. However, should further studies confirm the present study's results, XCI skewing in fetal cord blood may be used as an additional predictor of fetal outcome in cases of mosaic trisomy 16 of meiotic origin (predominantly confined to the placenta) and possibly other chromosomal trisomies, in conjunction with ultrasound and amniocentesis.

Chapter 7: Conclusion and Discussion

More than 40 years have elapsed since Lyon published the hypothesis to explain the mechanism of dosage compensation of X-linked genes in mammals (Lyon 1961). While most of the principles of that original hypothesis have stood the test of time, several exceptions have become apparent in the last four decades. Throughout this thesis work, I have explored the characteristics of this process in human embryonic and extraembryonic tissues, with a special emphasis on the latter and against the backdrop of extensive studies done in mice and marsupials. It is important to investigate and understand the similarities and potential differences of the XCI process between humans and other mammals if we are to explore our common ancestry and the evolutionary process that have set us apart. This is particularly relevant in light of the common use of the mouse as a model for understanding human disease.

This investigation began by trying to understand the XCI process in normal human placentae (Chapter 3). Given that DNA is readily obtainable from placental samples, methylation-based assays were used to assess XCI status in the different tissues. These assays have been extensively used by others to study XCI patterns in human somatic tissues. In order to get a more accurate idea of the XCI process in the placental disc as a whole, a reliable and representative method of placental sampling was used in this study and four different extraembryonic tissues were analyzed. Biases such as maternal contamination, and use of cultured material were excluded; these factors might have contributed to the lack of correlation of results from previous studies. Additionally, samples were collected from both 1st trimester and term placentae, to account for potential developmentally regulated differences in the XCI process. The overall idea was to perform a very comprehensive study of XCI in human extraembryonic tissues.

The following conclusions can be drawn from this initial study described in Chapter3:

First, XCI does not appear to be imprinted in human term placentae, in any of the 4 tissues that were analyzed (amnion, chorion, trophoblast and mesenchyme). In general, XCI is heterogeneous (preferential maternal X inactivation and preferential paternal X inactivation are present in different sites within the same placenta) and either parental X chromosome is capable of being inactivated in the placenta of a normal individual. Therefore, the sampling of multiple sites proved essential to understand the patchy nature of XCI in the human placenta.

Second, in first trimester placentae, a tendency towards the inactivation of the X^P was observed in the chorionic villi trophoblast, although it is not clear whether this trend represents a true effect or is due to small sample size. It is interesting however, that additional data on two informative placentae from a group of cases of mosaic trisomy preferentially confined to the placenta (Chapter 6) also showed this tendency in the trophoblast. These two placentae, however, were sampled on the 2nd and 3rd trimester of gestation and only 1 and 3 trophoblast samples were available for testing respectively. Thus, the significance of these findings is still unclear.

Imprinted XCI inactivation confined to the chorionic villi trophoblast has been reported in a previous study that assayed XCI using protein isoforms based assays (Harrison 1989). If this finding is to hold true, the question arises as to the nature of the mechanism that would allow an early imprint to be lost at later stages in gestation. Although the exact nature of such a mechanism is hard to predict, one can hypothesize that it bears similarities to the mechanism that makes the imprint non-absolute in murine extraembryonic tissues themselves. Huynh and Lee have proposed that only a subset of extraembryonic cells are

subject to imprinting, while some others escape imprinting, employing counting and choice mechanisms similar to those of the embryonic tissues (Huynh and Lee 2001). They propose that the paternal X chromosome is transmitted to the zygote in a pre-inactivated state, therefore the initiation and establishment of XCI takes place in the gamete, while the zygote is only in charge of maintaining this pre-established pattern. Studies in cloned mice seem to support this hypothesis as the XCI status of the X chromosome in the extraembryonic lineages of the recipient mice was found to be dependent on the transcriptional status of the X's in the donor nuclei and not their prior parent of origin (Eggan et al. 2000). As to what would predispose the paternally derived X to be preferentially inactivated, suggestions include a leaky effect of the highly methylated state in which the paternal X remains during spermatogenesis (Lifschytz and Lindsley 1972) or that preferential expression of maternally derived genes is favourable in early gestation and therefore it has been selected for throughout evolution.

Huynh and colleagues also proposed that the "maintenance" mechanism acting at the zygote stage according to their model, is imperfect, which causes some of the cells of the extraembryonic tissue to escape imprinting (Huynh and Lee 2001). In this context, it seems feasible that an imprint evident in humans at the early stages of embryonic development and perhaps only affecting the earliest tissues to undergo inactivation such as the trophoblast is not absolute and therefore is not adequately maintained throughout gestation. Imprint instability could lead to heterogeneous XCI later in development, as seen in the extraembryonic tissues of term placentae analyzed in this study.

Given that cytosine methylation is an epigenetic modification involved in genomic imprinting, it is possible that the mechanism mentioned above, involves loss of methylation followed by *de novo* methylation. It is also possible that the onset of XCI occurs

at different times in different cell populations within the human trophoblast, which would explain changing XCI patterns at different developmental stages. This would occur if some cell populations that have not inactivated an X chromosome persist or if XCI is unstable and is lost during gestation. There is evidence that XCI is less stable early in gestation in extraembryonic tissues (Migeon et al. 1986).

On the other hand, unlike the mouse, preferential inactivation of the paternally derived X is not likely a requirement for normal placental development in humans as suggested by cases of supernumerary X chromosomes and monosomy X (i.e. $X^MX^MX^P$ and X^PO). In these individuals, the parental origin of the extra or absent chromosomes apparently has no effect on placental development or fetal viability. In mice however, impaired cell differentiation and compromised viability due to failure of dosage compensation is often observed (reviewed in Huynh and Lee 2001).

From an evolutionary perspective, many agree that the data points towards an ancestral XCI mechanism that was likely unstable, incomplete and with preferential or exclusive inactivation of the paternally derived X (reviewed in Graves 1996). It is possible that the relaxed imprinting of extraembryonic tissues of mice represents a transitional stage in an evolutionary pathway that goes from an absolutely imprinted mechanism of XCI in marsupials to a random mechanism in human placenta (Huynh and Lee 2001). An alternate hypothesis proposes that XCI began as a randomly expressed gene cluster, on the undifferentiated proto-X chromosome in an ancestral mammal, which (under the influence of a controlling element) acquired a parent-specific imprint in rodents and marsupials, with the purpose of minimizing maternal-fetal incompatibilities (Ohlsson et al. 2001). The finding of imprinted XCI in extraembryonic tissues of bovines (Xue et al. 2002) might weaken the strength of this hypothesis as it seems less likely that imprinted XCI could have evolved in

parallel in so many different mammalian orders. It also suggests, that other species of mammals need to be studied in order to fully understand XCI in embryonic and extraembryonic tissues in mammals, from an evolutionary perspective.

The issue of XCI as a requirement for normal development was further explored in Chapter 4 of this thesis, through the study of a rare case of a paternally derived X/autosome translocation involving chromosomes X and 20 conceived through ICSI. In similar cases, a highly skewed XCI pattern favouring the inactivation of the normal X homologue (maternally derived X) is seen in the majority of embryonic tissues. A similar pattern was expected in the placental tissues if selection was acting to minimize genetic imbalance in this case, unless preferential paternal XCI was a requirement for normal embryo/placental development in humans. To my knowledge, there are no previous publications of XCI studies in the placenta of paternally derived X/autosome translocations. Our results show that the majority of placental samples (like their fetal counterparts) showed a bias towards the inactivation of the normal X-chromosome (maternally derived). This argues strongly against the requirement of preferential Xi^P for normal placental development, as no embryonic and extraembryonic abnormalities were found in the patient prenatally or on postnatal follow-up. However, it could still be argued that the strong selection in favour of the inactivation of the normal X (maternal) overrides a possible paternal imprint. On the other hand, unlike the fetal cord and cord blood which showed extremely skewed XCI in this case, many of the placental samples showed moderate levels of skewing and even instances of very small deviations from the 1:1 ratio. This finding is very interesting in that it suggests that the strong selection against cells that inactivate the translocated X clearly evident in the fetal tissues, is either "weaker" in extraembryonic tissues or perhaps is overridden by imprinted XCI in placenta. There is no evidence for strong selection against chromosomal

imbalances in placental tissues of mosaic trisomies either confined (CPM) or preferentially confined to the placenta. For example, a completely trisomic placenta of a normal disomic fetus, can in some cases support an apparently normal pre and/or postnatal development (eg: cases 94.21, 93.73, 99.78, 91.55 in Tables 6.1 and 6.2).

Based on the data of the balanced (X:20) translocation case, one can assume that there are some regions within this patient's placenta where the genetic imbalance generated by the inactivation of the translocated X is well tolerated, and apparently has no influence in the placental or fetal outcome. It appears that abnormal gene expression resulting from either possible spreading of inactivation into the autosomal segment or failure to inactivate part of the X is insufficient to influence cell viability and proliferation in placenta. It is also possible that functional disomy for certain X linked genes is not only tolerated but even a common characteristic of human extraembryonic tissues. Early studies on protein expression in human chorionic villi seemed to indicate that certain genes (i.e *G6PD*) are indeed biallelically expressed in placenta (Migeon et al. 1985). The evidence discussed in this thesis suggesting that XCI is incomplete for certain X-linked genes in CVS supports this idea.

The work presented in this thesis has shown that hypomethylation is not limited to *G6PD* and *HPRT* but affects other X-linked genes. Moreover, other studies have shown that globin sequences and repetitive DNA sequences are also undermethylated in trophoectoderm (van der Ploeg and Flavell 1980; Chapman et al. 1984). This suggests that some autosomal genes thought to be silent in this tissue may also be expressed if methylation plays a significant role in their regulation. I decided to look at chorionic villi stroma to determine the role of methylation in gene silencing in placenta. My findings (from a series methylation based assays) suggest that hypomethylation at the promoter regions of X-linked genes is a common characteristic of the mesenchymal component of the chorionic villi. *XIST*

was the only gene that consistently showed "normal" patterns of methylation. The finding of a "normally methylated" *XIST* gene suggests that XCI may be maintained regardless of the methylation status of individual genes. In order to investigate this possibility, BrdU based studies were conducted in 4 of these CVS samples and showed that the X chromosome remained allocyclic or late replicating, regardless of specific gene hypomethylation. Furthermore, expression studies of 3 CVS cases showed *XIST* expression in all of them (Brown and MacDonald, personal communication). These results are consistent with earlier findings that demonstrated that clonal populations from fetal and newborn chorionic villi, showing *G6PD* hypomethylation, retained a late replicating X-chromosome (Migeon et al. 1985).

Whether gene hypomethylation leads to derepression or lack of dosage compensation of certain X linked genes remains to be investigated. Expression studies using either single cell clones, single cell RT-PCR, RNA FISH or perhaps CVS cases with known extreme XCI skewing would be required to help us understand the status of gene activity in extraembryonic tissues in humans. Additionally, it would be important to study the correlations between gene expression status and replication status for the different X-linked genes, as well as the correlation with the replication status of the "inactive" X chromosome as a whole. Results from previous studies suggest that induction of transcriptional activity of certain X-linked genes is accompanied by the acquisition of early (isocyclic) replication of these genes in cells of trophoblastic origin (Migeon et al. 1986; Hansen et al. 1996).

Additionally, the data from Chapter 5 suggests that gene hypomethylation is not a feature confined to the chorionic mesenchyme (stroma) as the majority of samples of chorion, trophoblast and mesenchyme from term placentae also showed evidence of gene hypomethylation when tested with the *FMR1* assay. It is important to note that the findings in

term placental samples are not confounded by the potential culture biases that can be attributed to the CVS samples and yet they are remarkably consistent. This seems to suggest that gene hypomethylation is a feature shared by different placental tissues and leads me to speculate that biallelic expression of certain X-linked genes besides those that escape XCI (including those with Y-homologues), is perhaps required for normal development of female embryos. This argument may explain in part the finding that ~99% of XO individuals are spontaneously aborted (Thompson et al. 1991).

Because placental trophoblast and mesenchyme are major components of the 1st trimester chorionic villi, these tissues are of great significance in prenatal diagnosis when placental villi are used for biochemical, cytogenetic or DNA analysis. Only to the extent that the findings in samples of the chorionic villi reflect or are correlated with the genotype, epigenotype and phenotype of the fetus is a diagnosis accurate and useful. Therefore, the knowledge of the derivation and the properties of cells of the chorionic villi is very important. Additionally, the study of XCI patterns in embryonic/fetal tissues could be used as a tool to study some aspects of the dynamics of cell selection and precursor pool size. This idea led our laboratory to study XCI in pathological situations where conceptuses were ascertained on the presence of mosaic trisomy preferentially confined to the placenta. This initial study (in which I participated) found evidence of a significant level of extremely skewed XCI in diploid fetal tissues of mosaic trisomy cases of meiotic origin, compared to those cases where the trisomy was of somatic origin (Lau et al. 1997). In light of these findings, I decided to focus on those cases with a meiotic origin of the trisomy, and to further extend the study to include additional cases and all the available clinical information on each of the patients. Several conclusions can be derived from this study which results are summarized in Chapter 6:

First, there is a significant increase in the level of extreme XCI skewing in diploid fetal tissues of mosaic trisomy cases predominantly confined to the placenta and where the trisomy is of meiotic origin. It was hypothesized that this finding is largely due to cell selection which tends to eliminate the trisomic cells, preventing them from contributing to the embryo. The reduction in the embryonic precursor cell pool could therefore lead to extreme XCI skewing in diploid fetal tissues. Based on this model, either random or moderate XCI would be expected for embryonic tissues where the trisomy persists. The study findings described in Chapter 6 seem to fit this model with only a few exceptions that are addressed in the discussion of the corresponding Chapter.

Second, the availability of prenatal and postnatal clinical data on the majority of the cases allowed the establishment of correlations between the XCI status in diploid fetal tissues and the clinical outcome. An association between extremely skewed XCI inactivation and abnormal outcome was found in the study suggesting that poor outcome in these cases may be a consequence of the failure to completely eliminate the trisomic cells from "all" fetal tissues, resulting in tissue specific trisomy mosaicism (occult mosaicism), or due to disruption of development caused by differential growth rates and/or cell death of the trisomic cells. This association exists even in some cases where there is no evidence of the trisomy on amniocentesis. This is not hard to understand given that mosaicism may be confined to just one tissue in the fetus and could be "missed" on amniocentesis due to preferential growth of certain cells over others in culture or to the presence of the trisomy only in lineages which do not contribute to amniotic fluid. These findings highlight the potential for XCI studies to be used as a tool, in conjunction with ultrasound and amniocentesis, to predict fetal outcome in cases of mosaic trisomy 16 of meiotic origin (predominantly confined to the placenta) and possibly other chromosomal trisomies.

The study of XCI in embryonic and extraembryonic tissues in humans summarized in this manuscript has mostly managed to raise new questions about this complex developmental process. Future studies should seek to correlate the findings of this study and others, which are mostly reliant on gene methylation, to expression patterns of the different X-linked genes in extraembryonic tissues. As the extent of this correlation remains to be unravelled, any results based upon methylation should be analyzed with caution. Additionally, allelic expression analyses may be confounded by preferential allelic expression in certain genes. The trend towards imprinted XCI in the trophoblast should be further examined in first trimester placentae (preferably SABs in which the trophoblast is purified by microdissection) and additional species of mammals should be studied to achieve a clear understanding of the evolutionary characteristics of this process. The placental chorionic villi, and specifically the trophoblast in different mammalian species, provides an interesting model for exploring early events that program dosage compensation of X-linked genes in mammals.

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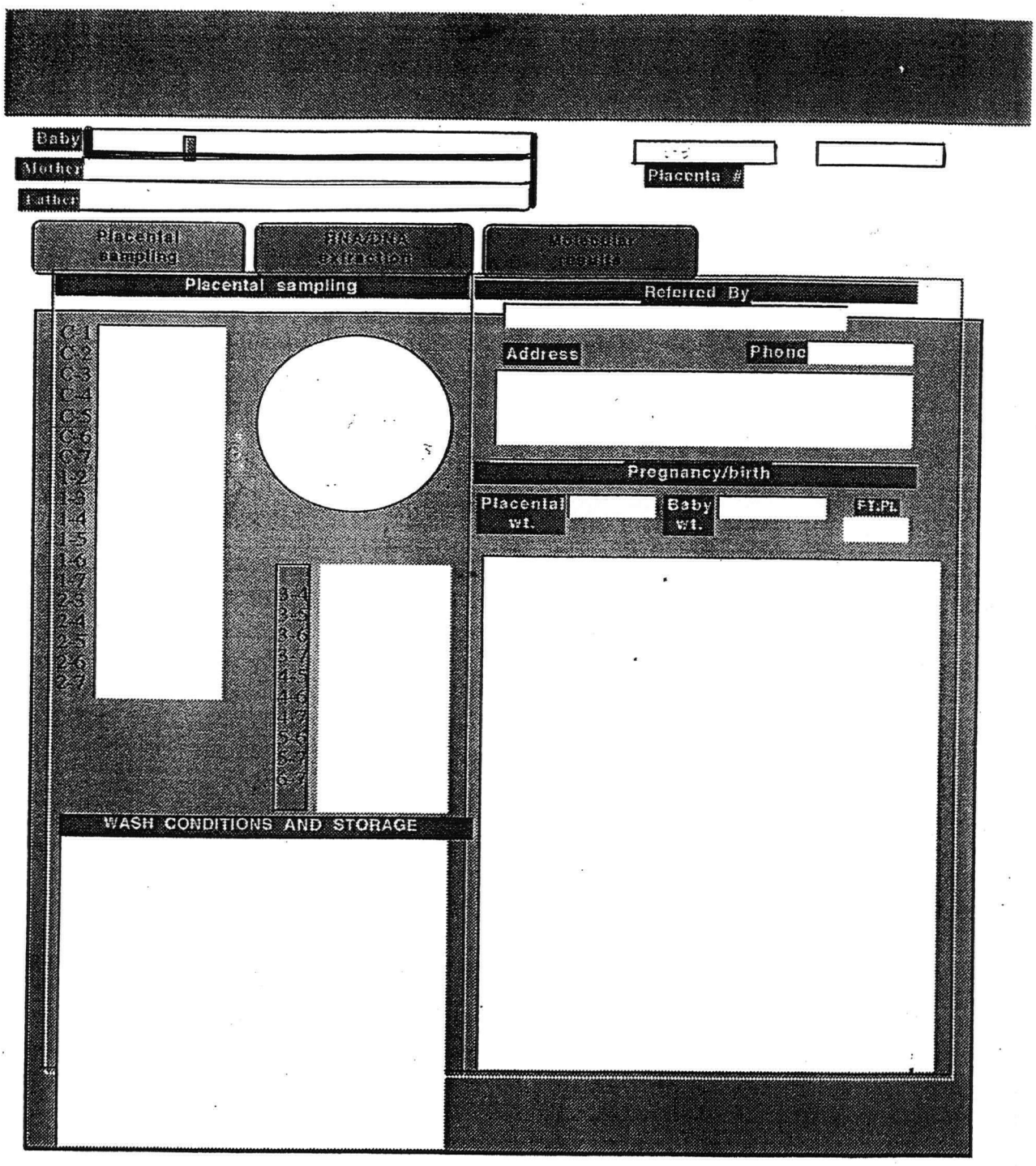
APPENDIX II


Baby
Mother
Father
Placenta #
Placental sampling
RNA/DNA extraction
Molecular results
Placental sampling
Referred By
Address
Phone
Pregnancy/birth
Placental wt.
Baby wt.
Fl.Pl.
WASH CONDITIONS AND STORAGE

APPENDIX III

PLACENTAL DETAILS

RC:_____ Name_____

Trimmed weight:_____

Diameter: _____

Thickness: _____

Shape: _____

Cord: insertion: _____

length: _____

no. of vessels: _____

Diagram showing sample sites:

Other comments: _____

_____

Samples taken:

cytogenetics tissue culture/FISH

amnion

chorion

villi (no. of sites)

blood

Histology

cord

placental wedge

amniotic membrane