GENETIC ANALYSIS OF EMBRYONIC AND FETAL TISSUES

by

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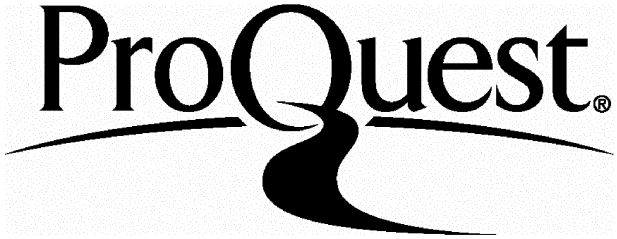

ABSTRACT

In this thesis, genetic analysis has been performed on embryonic and fetal material. Firstly, it was used to demonstrate the presence of fetal cells in the cervical mucus of pregnant women in the first trimester. Several studies have shown that fetal cells are present in the cervical canal but some reports have not identified these cells. In the present study, fetal genetic material was demonstrated in the cervical mucus from 66 of 193 (34%) women who underwent termination of pregnancy and from 7 in 37 (19%) on-going pregnancies. The polymerase chain reaction (PCR) was more efficient in detecting fetal cells than fluorescent *in situ* hybridisation (FISH).

Secondly, FISH was used to determine the level of the trisomic cell line in different tissues, including placenta, from first and second trimester trisomic fetuses obtained from termination of pregnancy. Several studies have suggested that there are correlations between the degree of mosaicism in a chromosomally abnormal fetus, the severity of the disease and the chance of survival to term. The level of abnormal cells obtained from this study appeared not to be correlated with the clinical manifestations and the survival potential. As a side-line of this work, the efficiency of FISH on metaphase and interphase nuclei from skin fibroblast cultures from a trisomic and a triploid fetuses was determined.

Finally, FISH was used to determine the mosaicism in human embryos on day 5 post-insemination, both arrested and blastocyst stage embryos. Mosaicism was found in 80% of the arrested embryos and 90% of the blastocysts. This may have an implication for preimplantation genetic diagnosis using blastocyst biopsy.

In conclusion, FISH and PCR were used to study fetal genetic material in cervical mucus; FISH was used to study percentages of abnormal cell lines in trisomic fetal tissues and blastomeres from day-5 embryos.

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To my late parents,
my dearest brother and sister,
and all the patients

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ABBREVIATIONS

μg	microgram
μl	microlitre
μM	micromolar
αMEM	α-modified minimum essential medium
6-FAM	6-carboxyfluorescein
AChE	acetylcholine esterase
AFAFP	amniotic fluid alpha-fetoprotein
bp	base pair
CCD	coupled charged device
CGH	comparative genomic hybridisation
cm^3	cubic centimetre
CPC	choroid plexus cyst
CPM	confined placental mosaicism
CRL	crown-rump length
CVS	chorionic villus sampling
DAPI	4', 6-diamidino-2-phenylindole
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxyribonucleoside triphosphate
DTT	dithiothreitol
E2	oestradiol
EDTA	ethylene-di-amine-tetra-acetic acid
FACS	fluorescent activated cell sorting
FBS	fetal blood sampling
FCS	fetal calf serum
FISH	fluorescent *in situ* hybridisation
FITC	flourescein-12-2'deoxuridine-5'triphosphate
FSH	follicle stimulating hormone
g	gram
GnRHa	gonadotropin releasing hormone analogue

h	hour
HB-EGF	heparin-binding epidermal growth factor
hCG	human chorionic gonadotropin
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid
HEX	4, 7, 2', 4', 5', 7'-hexachloro-6-carboxyfluorescein
HFEA	The Human Fertilisation and Embryology Authority
HLA	human lymphocyte antigen
hMG	human menopausal gonadotropin
HTF	human tubal fluid
ICM	inner cell mass
ICSI	intracytoplasmic sperm injection
IU	International Unit
IVF	*in vitro* fertilisation
Kb	kilobase
M	molar
MACS	magnetic activated cell sorting
M-FISH	multiplex or multifluor FISH
mg	milligram
min	minute
ml	millilitre
mm	millimetre
mM	millimolar
MSAFP	maternal serum alpha-fetoprotein
N	normal
NP-40	nonidet P-40
NT	nuchal translucency
NTD	neural tube defect
PAPP-A	pregnancy-associated plasma protein A
PBS	phosphate-bufferred saline
PCR	polymerase chain reaction
PGD	preimplantation genetic diagnosis
PHA	phytohaemagglutinin

pmol	picomole
pM	picomolar
PVB	preterm vaginal birth
QCCH	Queen Charlotte's and Chelsea Hospital
Rh(D)	rhesus blood group (D antigen)
RNA	ribonucleic acid
rpm	round per minute
SA	spontaneous abortion
SD	standard deviation
SDS	sodium dodecyl sulphate
SE	standard error
sec	second
SKY	spectral karyotyping
SRY	sex-determination region Y
SSC	standard saline citrate
SSR2	synthetic serum replacement 2
SSS	Synthetic Serum Substitute
STR	short tandem repeat
TA	therapeutic abortion
TAMRA	N, N, N', N'-tetramethyl-6-carboxyrhodamine
Taq	*Thermus aquaticus*
TCC sample	transcervical cell sample
TE	trophectoderm
TET	4, 7, 2', 7'-tetrachloro-6-carboxyfluorescein
TOP	termination of pregnancy
TRITC	rhodamine-4-deoxuridine-5' triphosphate
TVB	term vaginal birth
uE3	unconjugated oestriol
UPD	uniparental disomy
v/v	volume/volume
w/v	weight/volume
YAC	yeast artificial chromosome

CHAPTER 1
INTRODUCTION

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The ultimate goal of every pregnancy is that it should culminate in a healthy baby (or babies) and a healthy mother. Unfortunately, approximately 3% of live born infants have a congenital anomaly that requires medical attention (Shepard, 1986). Although non-genetic factors may cause malformations, genetic factors usually are responsible. Chromosome abnormalities are among those important factors. They are associated with a proportion of pregnancy loss. About 60% of first trimester spontaneous abortions, 5.6% of perinatal deaths and 0.6% of livebirths show a chromosome abnormality (Machin and Crolla, 1974; Boue *et al.*, 1975; Jacobs, 1977). Other genetic disorders also account for a further 1% of perinatal deaths (Machin and Crolla, 1974). Therefore, medical genetics has become important in reproductive medicine. Detection or recognition of such a disorder in a pregnancy can have a great impact on the antenatal care. Also, in some couples, detection before implantation can be beneficial. This chapter will generally describe chromosome abnormalities and detection techniques during pregnancy or before implantation both currently in use and in the research stage. Embryonic development related to the cells or structures involved in the sampling techniques for the chromosome analysis will also be reviewed. Finally, this chapter will include the aims of the thesis.

1.1 CHROMOSOME ABNORMALITIES

The advent of karyotyping, the technique used for chromosome analysis, has revealed the relationship between some diseases or syndromes with chromosome abnormalities. The abnormalities include all conditions with chromosomal changes that are visible under a light microscope. This usually affects a large number of genes and can cause a deleterious outcome in a conceptus such as failure to implant, spontaneous abortion, stillbirth, perinatal mortality and/or morbidity, or physical and mental abnormalities. Chromosome disorders can be categorised as numerical, structural, and other aberrations.

1.1.1 NUMERICAL ABERRATIONS

A normal human somatic cell contains 46 chromosomes (Tjio and Levan, 1956). There are 22 pairs of autosomes and a pair of sex chromosomes (XX in females and XY in males). This chromosome complement is termed diploid. Sex cells or gametes are haploid, *i.e.,* they contain one set of chromosomes (chromosomes 1-22 and a sex chromosome which is either X or Y). The term "polyploidy" is used when the chromosome number is an exact multiple of the haploid number and exceeds the diploid number. Aneuploidy is the term used to describe a chromosome complement that is not an exact multiple of haploid. Examples are autosomal trisomies and sex chromosome aneuploidies.

1.1.1.1 Polyploidy

Examples of polyploidy are triploidy (69 chromosomes) and tetraploidy (92 chromosomes). Complete tetraploidy is rarely seen in man.

Triploidy This condition occurs in 1% of all recognised conceptions (Niebuhr, 1974) and 20% of first trimester spontaneous abortions with chromosome abnormalities (Boue *et al.*, 1975). In most cases the extra set of chromosomes is paternally derived, in which case partial hydatidiform mole can occur (Jacobs *et al.*, 1982). Triploid livebirths are rare, almost all of which die within a few hours or days. The affected fetuses or infants show severe intrauterine growth retardation, low birth weight, neutral tube defects, craniofacial malformations, abdominal wall defects, skeletal abnormality of hands and feet, usually including syndactyly (Niebuhr, 1974).

1.1.1.2 Aneuploidy

Examples are autosomal trisomies or sex chromosome aneuploidies. Complete monosomies are incompatible with life except monosomy X.

Trisomy 21 (Down syndrome) The incidence of trisomy 21 is approximately 1 in 700 livebirths and increases with maternal age. The incidence at conception is higher but only 24% survive to term (Hassold *et al.*, 1996). It is a major cause of mental retardation. The facial appearance is characteristic with up-slant palpebral fissure, epicanthal fold, flat occiput, flat nasal bridge and micrognathia resulting in a protruding tongue in children. It is associated with premature ageing displaying similarities to Alzheimer's disease, with an increased risk of leukaemia and with low immunity resulting in frequent infections (Korenberg *et al.*, 1994). Other features include congenital heart disease, duodenal atresia, hypothyroidism, neonatal hypotonia, atlantoaxial joint instability, simian palmar crease, clinodactyly and a wide space between the first and second toes. Most cases (95%) are regular trisomy 21; in 85% the extra chromosome is maternally derived. In 3-4% of cases the extra chromosome

derives from a parent who is a balanced translocation carrier of chromosome 21. About 1-2% of cases are mosaics and not infrequently mosaicisms are found only in fibroblasts from a skin biopsy. The critical region on the chromosome is thought to be close to the marker D21S55, on the proximal part of 21q22.3 (Rahmani *et al.*, 1990).

Trisomy 18 (Edwards syndrome) This syndrome has an incidence of 1 in 10,000 livebirths and increases with maternal age. Again, the incidence at conception is much higher but 95% are spontaneously aborted and only 5% survive to term (Hassold *et al.*, 1996). There are more female cases than male in livebirths. This is presumed to be due to selection against male trisomic conceptions (Griffin *et al.*, 1996; Huether *et al.*, 1996). The condition is associated with a low birth weight and multiple dysmorphic features such as a small chin with a prominent occiput, low set ears, clenched hands with characteristic overlapping index and small fingers and rockerbottom feet. Frequently, there are cardiac or kidney malformations, or gastrointestinal tract abnormalities such as imperforate anus and Meckel's diverticulum and severe developmental delay. Most of the livebirths die within the neonatal period with the mean survival of 4-6 days and only 5% survive to 1 year (Goldstein and Nielsen, 1988; Root and Carey, 1994). In most cases (95%) the extra chromosome is maternally derived.

Trisomy 13 (Patau syndrome) The incidence is 1 in 20,000 livebirths, again, this increases with maternal age. Only 3% survive to term (Hassold *et al.*, 1996). The classical picture shows facial clefts, cerebral or ocular malformations and hexadactyly. Other features include low set ears with abnormal helix, haemangioma, rockerbottom feet and scalp defects. Mental retardation and some cardiac, gastrointestinal or renal abnormalities are common. Most of the livebirths die within the neonatal period with

the median survival of 2.5 days (Goldstein and Nielsen, 1988). In most cases (65%) the extra chromosome is maternally derived. In 20% of cases one of the parents carries a balanced translocation.

45,X (Turner syndrome) The incidence is 1 in 5,000-10,000 female births. This represents only a fraction of all conceptions with this condition, as 98-99% are spontaneously aborted and only 0.3% are born alive (Hassold *et al.*, 1996). The affected newborn infants may have a redundant neck skin and peripheral lymphoedema. The affected adults are usually short in stature, have a webbed neck, low hairline, shield chest with widely spaced nipples and wide carrying angle of the arms. Heart defects, coarctation of aorta and renal abnormalities are common. The important problem with this condition is varying degrees of ovarian degeneration which may occur *in utero*, causing primary amenorrheoa and infertility. Hormonal replacement therapy should be started at adolescence for development of secondary sex characteristics and long term prevention of osteoporosis. The life span and intelligence are normal. In 75-85% of cases the X chromosome is maternally derived (Larsen *et al.*, 1995; Jacobs *et al.*, 1997). Overall, 50% of patients have 45,X; 10-25% are mosaics; 20% have an isochromosome of the long arm of the X; 5% have a ring X and 5% have a short arm deletion of one X. In general, deletion of the short arm of the X is associated with the Turner phenotype, whilst long arm deletions alone produce streak degenerated ovaries without other features. In 4% of patients, mosaicism with a second cell line containing a Y chromosome is found. In this situation there is a risk of up to 20% that the streak gonad will develop gonadoblastomas and gonadectomy is crucial in these patients. Pregnancy is possible in 2% of Turner syndrome patients whose karyotypes have two copies of the Xq13-q26 region which contains the genes that are thought to control ovarian function.

However, the pregnancies are at high risk of chromosome anomalies and poor outcomes (Tarani *et al.*, 1998).

47,XXY (Klinefelter syndrome) The incidence is 1 in 800-1,000 males. There is an approximately equal chance that the extra X chromosome is maternally or paternally derived (Lorda-Sanchez *et al.*, 1992; MacDonald *et al.*, 1994). The syndrome involves testicular atrophy after puberty. The affected persons have gynaecomastia with poorly developed secondary sex characteristics and infertility. About 15% are mosaic 46,XY/47,XXY. There are rare cases of 48,XXXY which are more severely affected or cases of 49,XXXXY which have additional skeletal, facial and cardiovascular anomalies (Sepulveda *et al.*, 1999).

47,XYY The incidence of 47,XYY is 1 in 1,000 male births (Robinson and Jacobs, 1999). The affected persons are usually normal with a tall stature. Some have mild mental retardation or aggressive behaviour. The extra Y usually arises from the non-disjunction in paternal meiosis II (Robinson and Jacobs, 1999).

48,XXYY These rare cases have the clinical features of Klinefelter syndrome with the stature of XYY males and probably with a risk of congenital heart disease (Meschede *et al.*, 1995).

47,XXX The incidence is 1 in 800-1,000 female births. The affected persons appear normal but 10-15% have mild mental retardation. In 90% of cases the extra X is maternally derived (MacDonald *et al.*, 1994), the majority of which being an error at meiosis I with an association with advanced maternal age (May *et al.*, 1990). There are very rare cases of 48,XXXX or 49,XXXXX. In these cases, intelligence is reduced, and dysmorphic features and menstrual problems are frequent.

46,XX males The incidence is 1 in 20,000 males. This condition is usually caused by the transfer of a part of short arm of the Y chromosome, including the SRY (sex-determination region Y) gene or testis-determining factor, to the short arm of the X chromosome during paternal spermatogenesis (Ginsberg *et al.*, 1999). The affected persons have poorly developed secondary sex characteristics and usually have infertility problems similar to Klinefelter syndrome. In some cases, the Y material may be found on an autosome.

46,XY females These patients are apparently female but have a 46,XY karyotype. The production of androgen is normal but the target organs fail to respond to androgen due to deficiency or abnormality of androgen receptors. Deletions and point mutations of a gene on the X chromosome encoding these receptors have been identified. Testes may be in the abdomen or descend down to the inguinal region which may be the cause that brings patients for investigation. External genitalia are female with normal breast development but with no internal female reproductive tracts. Pubic and axillary hairs are sparse since these are androgen dependent.

About 15% of 46,XY females are caused by a mutation in the SRY gene and possibly by other mutations affecting the expression of SRY (Kwok *et al.*, 1996). Two XY females have been reported to be caused by the transfer of Xp to Yp in paternal meiosis (Leveilliers *et al.*, 1989).

PARENTAL-AGE-RELATED CHROMOSOME ANEUPLOIDIES

Trisomy 21 and other fetal aneuploid conditions associated with extra chromosomes (except XYY) increase markedly with maternal age (Hook, 1981; Hook *et al.*, 1983). This is found both in livebirths and at amniocentesis. On the other hand, Turner syndrome (45,X) decreases with maternal age (Ferguson-Smith and Yates, 1984; Hassold *et al.*, 1988). Advanced maternal age is the most common indication for prenatal diagnosis for chromosome disorders. It is proposed that the long duration to complete meiosis in oogenesis accounts for this maternal age impact by the wear and tear effect of the mitotic spindle, causing non-disjunction. The maternal age effect, however, shows different features among different chromosomes, including very small effects for the large chromosomes (group A and B), linear increases for chromosome 16, and exponential increases for chromosome 21 (Wyrobek *et al.*, 1996).

A review of the studies of effects of paternal age on the incidence of chromosome anomalies has found no strong evidence of any correlation, but there is evidence that the incidence of serious nonchromosomal birth defects, especially those arising from new autosomal mutations, increases with paternal age (Bordson and Leonardo, 1991). With spermatogenesis taking place from puberty continuously until death, the mature spermatozoa in an older man could well have undergone several hundred mitotic divisions, predisposing to mutation. Recently, findings from sperm studies suggest that there may be a small effect from paternal age for the sex chromosomes (Griffin *et al.*, 1995; Robbins *et al.*, 1995). A study of Klinefelter syndrome patients has found an increased paternal age in the cases where the extra X chromosome is paternally derived (Lorda-Sanchez *et al.*, 1992).

1.1.2 STRUCTURAL ABERRATIONS

In this condition, the structure of one or more chromosomes is changed. The initial event is chromosome breakage which produces two unstable sticky ends. With an improper repair process, an abnormal structure of the chromosome results. There are many possible structural aberrations such as:

Translocation A translocation is the transfer of chromosome material between two non-homologous chromosomes. This requires breakage of both chromosomes with the exchange of part of the chromosome between them. Usually there is no loss or gain of DNA in the genome and this is called a balanced translocation. The affected person is clinically normal but has a higher risk of producing abnormal gametes, resulting in reproductive problems. In a reciprocal translocation the chromosome material distal to the breaks in the two chromosomes is exchanged. This event results in twelve possible gametes, only one of which is normal, one will carry the balanced translocation and the rest carry various imbalances which can cause abortion, stillbirth, mental retardation and multiple congenital anomalies in the offspring. In a Robertsonian translocation, which involves two acrocentric chromosomes, the breakage takes place at or near the centromere of each chromosome and the acentric fragments will usually be lost. However, as this segment often carries only satellites from both original chromosomes, the individual will suffer no effect. Again, the problem is with gametogenesis of that individual. There are six possible gametes, one of which is normal, one balanced, and the other four with varying degrees of unbalanced gametes. A number of trisomic patients have a parent with a balanced translocation.

Deletions A loss of any part of a chromosome is a deletion. Individuals with visible deletions are rendered monosomic for a large number of genes. With autosomal

deletions, mental handicap and multiple congenital malformations are usual. Examples are cri-du-chat syndrome and Wolf-Hirschhorn syndrome which are deletions of the short arm of chromosomes 5 and 4, respectively.

Deletions of a size that cannot be detected with the light microscope are termed microdeletions. Some of these involve loss of a few genes at closely adjacent loci, giving rise to several disorders in the individual. These conditions, such as WAGR (Wilm's tumour, Aniridia, Genitourinary malformations, and Retardation of growth and development), a microdeletion of chromosome 11p13 (Gessler *et al.*, 1989), are called contiguous gene syndromes and molecular techniques have been developed to aid their detection (Fantes *et al.*, 1992).

In some deletions or microdeletions, the parental origin of the deleted region has a significant effect and results in different syndromes. The difference in expression of certain genes according to parental contribution is a phenomenon called genomic imprinting. Examples are Prader-Willi syndrome and Angelman syndrome. They are clinically different but both involve an abnormality at the same region of the long arm of chromosome 15 (Knoll *et al.*, 1989). The abnormality may be a deletion, a uniparental disomy or a mutation. In Prader-Willi syndrome, clinical features arise from the absence of the paternal contribution of this region whilst the absence of the maternal contribution causes Angelman syndrome (Cassidy, 1995).

Ring chromosomes A ring chromosome results from breaks in both arms of a chromosome. The terminal ends are lost and the two proximal sticky ends unite to form a ring. The most common ring chromosome is X(r); the female carrier manifests a Turner syndrome phenotype. Mental and behavioural problems have been reported (El Abd *et al.*, 1999).

Duplications These are more common than deletions and have less deleterious effects. Tiny duplications at the molecular level (repeats) contribute to genomic diversity. A parental carrier of balanced structural rearrangement accounts for 10-15% of cases of autosome duplication/deletion.

Inversions An inversion requires two breaks in a chromosome and a repair with the segment between the breaks rotating 180°. A pericentric inversion results when the rotated segment contains the centromere. A paracentric inversion occurs when the two breaks are on the same arm of the chromosome. A pericentric inversion has an incidence of 1 in 100 livebirths. Generally, the affected person has no clinical abnormalities but problems occur at the pairing of homologous chromosomes during gametogenesis and may result in unbalanced gametes.

Isochromosomes An isochromosome is an abnormal chromosome which has a deletion of one arm with a duplication of the other. It may arise from transverse division of the centromere during cell division or from an isochromatid break and fusion above the centromere. The commonest isochromosome in livebirths is an isochromosome of the long arm of the X chromosome. This results in the clinical features of Turner syndrome due to the effect of short arm deletion.

Marker chromosomes The incidence of a small additional chromosome of unknown origin (termed a marker chromosome) is 1:3,000 in the general population (Buckton *et al.*, 1985). In most of the cases, the marker is derived from the short arms and pericentromeric regions of acrocentric chromosomes, especially chromosome 15 (Friedrich and Nielsen, 1974; Buckton *et al.*, 1985). Parental karyotypes should be examined and an attempt made to identify the nature of the additional chromosome material using chromosome painting.

1.1.3 OTHER ABERRATIONS

Mosaicism A mosaic is an individual with two or more cell lines originated from a single zygote. For example, about 1-2% of patients with trisomy 21 are mosaic with normal and trisomic cell lines. Mosaicism arises after fertilisation. Usually the initial zygote is normal and a trisomic cell line arises at a subsequent mitosis by non-disjunction. In this event a cell line with monosomy 21 will also be produced and, due to nonviability, may be lost. If the abnormal cell line is confined to the gonads (gonadal mosaic) then an apparently normal parent may have a high risk of producing an abnormal offspring (Cozzi *et al.*, 1999). In a zygote with trisomy 21, in some instances a normal cell line is produced at a subsequent mitosis by the loss of one chromosome 21 (trisomic rescue). The presence of the normal cell line tends to ameliorate the clinical picture. Mosaicism can cause problems in prenatal diagnosis in cases associated with confined placental mosaicism **(section 1.4.2.1)**, or in cases where abnormal cells are confined to some tissues such as in tetrasomy 12p mosaics, diploid-triploid mosaics and some trisomy 21 mosaics which are only identified by a fibroblast culture from a skin biopsy.

Chimaerism A chimaera is an individual with two cell lines originated from two separate zygotes. This could arise by the early fusion of fraternal twin zygotes, by double fertilisation of the oocyte and a polar body or, more commonly, by exchange of haemopoietic stem cells *in utero* in dizygotic twins. Chimaerism is confirmed if a double distribution of maternal and paternal alleles can be demonstrated in the two cell lines.

Uniparental disomy Normally each parent contributes a haploid set of chromosomes to the offspring. In some instances, both copies of a chromosome pair are

inherited from one parent without the contribution of the other, resulting in uniparental disomy (UPD) (Cassidy, 1995). This can arise if the conceptus starts off being trisomic for the homologue by meiotic non-disjunction in one parent and later the homologue of the other parent is lost at an early cell division due to mitotic non-disjunction (Wolstenholme, 2000). This results in embryonic cells having two homologous chromosomes that come from the same parent. If the non-disjunction in the parent had occurred at the first meiotic division, the two chromosomes would come from both homologues of that parent, and uniparental heterodisomy will occur. If non-disjunction had occurred at the second meiotic division then both the chromosomes would come from the same homologue of that parent and uniparental isodisomy will be the result. In some cases, the loss of one chromosome in the embryo occurs late, giving rise to trisomic/diploid mosaicism, and modifying the clinical features of that trisomy in the affected person. In addition to the level and site of mosaicism, the presence of UPD can add to the modification of the outcome in some trisomies (Wolstenholme *et al.*, 1994).

Another mechanism that leads to UPD is the fertilisation of two abnormal gametes, one being nullisomy and the other being disomy for the same chromosome, or one being nullisomy and the other being normal but with later duplication of the same chromosome. The latter situation also results in isodisomy.

The karyotype in a cell with UPD is apparently normal. DNA marker analysis to determine the parental origin of the chromosomes is needed to detect these conditions (Webb *et al.*, 1995). Their clinical consequences can arise from genomic imprinting of certain chromosomal regions with consequent parent specific expression of alleles in these regions as previously mentioned in Prader-Willi and Angelman syndromes (Cassidy, 1995). Uniparental isodisomy can also result in homozygosity for mutant

genes on the chromosome involved and so result in offspring being affected with an autosomal recessive disorder despite the carrier status being found in only one parent (Wolstenholme, 2000).

Chromosome breakage syndromes A few inherited disorders are associated with an excess of chromosome breaks, a defective chromosome repair, and an increased risk of neoplasia on top of other clinical manifestations. Most are inherited as an autosomal recessive disorder. Examples are ataxia telangiectasia, Bloom's syndrome, Fanconi's anaemia and xeroderma pigmentosum (German, 1980).

In summary, chromosome disorders cover a wide range of abnormalities. Some can be detected during the prenatal period. Nevertheless, most of the techniques to retrieve the specimen for the analysis carry some risks for the pregnancy. Prenatal screening methods have thus been developed to identify high-risk cases justified for invasive prenatal diagnosis. These methods are described in the next section.

1.2 PRENATAL SCREENING

Prenatal diagnosis for chromosome abnormalities and other genetic disorders cannot be routinely used in every pregnancy since it has some risks and it is not cost-beneficial to do so. It should be carried out in cases with a risk of having an affected fetus. Prenatal screening strategies which can be performed on every pregnancy have been developed to cope with this problem. However, being "screening" tests, the results are not perfectly accurate. A positive screening test does not indicate that the fetus is affected but that the risk is high enough to proceed to invasive investigations. A negative result reduces, but does not eliminate the chance of the fetus being affected. The current screening tests are biochemical tests and ultrasound scanning.

1.2.1 BIOCHEMICAL TESTS

Biochemical tests of maternal blood can be used to screen for fetal chromosome abnormalities. The first analyte used for this purpose was alpha-fetoprotein in maternal blood (MSAFP, maternal serum alpha-fetoprotein). It had been used to screen neural tube defects before it was recognised to be useful for screening chromosome abnormalities.

1.2.1.1 Screening for neural tube defects (NTDs)

Neural tube defects (NTDs) are caused by a defect in the closure of the neural tube during early embryonic development. The aetiology is multifactorial or polygenic. Brock and Sutcliffe (1972) reported a high level of alpha-fetoprotein in the amniotic fluid of pregnancies affected with NTDs. This glycoprotein, synthesised by the fetal yolk sac in early pregnancy and later by the fetal liver, permeates from fetal capillaries

into amniotic fluid through the unkeratinised skin in the first trimester and later it enters the amniotic fluid *via* the fetal urine. It crosses the placenta and fetal membranes into the maternal circulation. MSAFP levels rise continuously throughout early pregnancy to a peak at 30 weeks of gestation and then declines towards term. Therefore the accurate gestational age is crucial for the interpretation. Apart from NTDs, significantly elevated MSAFP levels are also associated with other fetal abnormalities with skin defects or abnormal skin permeability such as anterior abdominal wall defects, fetal hydrops or fetal demise (Morrow *et al.*, 1993); or abnormal micturition such as congenital nephrosis. Feto-maternal haemorrhage can also cause an increased level of MSAFP. A further evaluation such as a detailed ultrasound scan is required after a high MSAFP level is detected.

1.2.1.2 Screening for chromosome abnormalities

Advanced maternal age has been used to identify high risk cases for pregnancies with chromosome abnormalities since its association with the risk of Down syndrome and some other chromosome abnormalities was recognised (Hook, 1981; Hook *et al.*, 1983). This approach, however, only detects a small portion of the total affected cases, as only 20-25% of these cases are born to older mothers (Merkatz *et al.*, 1984; Palomaki and Haddow, 1987).

After MSAFP had been used for screening for NTDs and other anomalies for some time, its low levels in fetuses with chromosome abnormalities were observed by Merkatz *et al.* (1984). Using MSAFP/age combination improved the efficiency of detection of Down syndrome pregnancies and could extend the screening to younger women (Cuckle *et al.*, 1987; Palomaki and Haddow, 1987; Zeitune *et al.*, 1991).

However, there was a marked overlapping between the value of MSAFP in normal and affected pregnancies.

Later, Bogart *et al.* (1987) found that an abnormal level of human chorionic gonadotropin (hCG) was associated with a high risk of fetal chromosome abnormality. The median hCG level was found to be higher in pregnancies with Down syndrome than in normal pregnancies (Wald *et al.*, 1988). Also, a low level of unconjugated oestriol (uE3) was found to be associated with Down syndrome (Canick *et al.*, 1988). These two markers have been added to the MSAFP/age protocol to improve screening performance. This combination can detect 64% of Down syndrome with a false positive rate of 6.6% (Haddow *et al.*, 1992). Another marker which may add more efficiency in detection of Down syndrome in the second trimester is inhibin-A. Its level was found to be high in affected pregnancies (Wald *et al.*, 1996a). The combination of all four analytes with maternal age has a Down syndrome detection rate of 76% with a 5% false positive rate (Wald *et al.*, 1999).

Trisomy 18 has been found to be associated with low levels of MSAFP, hCG and uE3 but the MSAFP level may be affected by the presence of an NTD or ventral wall defect associated with trisomy 18 (Palomaki *et al.*, 1995; Leporrier *et al.*, 1996; Sancken *et al.*, 1999). In cases with low levels of hCG and uE3, a detailed ultrasound scanning and fetal karyotyping are warranted (Palomaki *et al.*, 1992).

Maternal weight and some other maternal conditions must be considered in the interpretation of the results of these biochemical tests. A significant deviation of maternal weight from the average value tends to have some effect on the concentrations of the three markers which need adjustment before interpretation. Heavier women have a lower concentration of MSAFP compared with average weight women and it is *vice*

versa for lighter women (Haddow *et al.*, 1981). Insulin-dependent diabetes mellitus reduces the concentration of all the three markers and, without correction, this could lead to underdetection of NTDs or overestimated risks for Down syndrome or chromosome abnormalities (Wald *et al.*, 1992). Furthermore, MSAFP levels for blacks are higher than for whites (White, 1989).

Biochemical markers have also been studied in the first trimester. Pregnancies with a high level of free β-hCG and a low level of pregnancy-associated plasma protein A (PAPP-A) have a higher risk of being affected by Down syndrome (Spencer *et al.*, 1994). Using these two markers with maternal age has a 63-67% detection rate with around 5% false positive rate (Wald *et al.*, 1996b; Wheeler and Sinosich, 1998). Recently, Wald *et al.* (1999) reported the efficiency of an integrated result from screening in both the first and second trimesters to detect Down syndrome pregnancies. The first trimester screening included PAPP-A level and/or nuchal translucency measurement **(section 1.2.2.2)** and the second trimester screening included MSAFP, hCG, uE3 and inhibin-A. At the false positive rates of 5% and 1%, the detection rates for a Down syndrome pregnancy were 94% and 85% respectively. This integrated screening, with a low false positive rate and a high detection rate, should have a benefit of reducing pregnancy loss associated with an invasive procedure after positive screening results.

1.2.2 ULTRASONOGRAPHY

Ultrasound scanning can be performed at anytime of pregnancy but for screening purposes the two main times are at booking and at 18-20 weeks of gestation which is thought optimal to exclude anomalies. However, some abnormalities may manifest later than this time. The scanning at booking is performed to confirm the presence of an intrauterine pregnancy (and to exclude an ectopic pregnancy), gestational age, establish the number and viability of fetus(es), and to exclude gross anomalies. The scanning at 18-20 weeks is performed to examine the details of the fetal structures, fetal movement, placenta, amniotic fluid and umbilical cord. Abnormal results may be suggestive of genetic or non-genetic disorders. Some ultrasound findings may suggest a higher risk of the fetus being chromosomally abnormal.

1.2.2.1 Ultrasound markers for aneuploidies in the second trimester

Some fetal anomalies detected by ultrasound appear to be associated with abnormal karyotypes. Although these associations remain indefinite for individual markers because some can be found in normal fetuses, the more markers detected, the higher the risk of the fetus to be affected by chromosome abnormalities, and this warrants further investigation (Bilardo, 1996). Examples of these markers are: ventriculomegaly, microcephaly, holoprosencephaly, choroid plexus cyst, facial cleft, nuchal oedema, cystic hygroma, diaphragmatic hernia, cardiac defects, exomphalos, duodenal atresia, renal defects and abnormal extremities (Nicolaides *et al.*, 1992). A nuchal fold thickening of $\geq$ 6 mm, fetal pyelectasis, short humerus and femurs and hyperechogenic bowel are associated with Down syndrome (Benacerraf *et al.*, 1990, 1995; Nyberg *et al.*, 1990, 1993). The combination of nuchal fold thickening,

pyelectasis and short humerus has been reported to be useful for the detection of trisomy 21 (Vintzileos *et al.*, 1997). In addition, a second trimester Down syndrome fetus may have duodenal atresia or heart defects but these are usually seen in the late second trimester.

Choroid plexus cysts (CPCs) are found in 0.6-1% of prenatal ultrasound scanning (Peleg and Yankowitz, 1998; Chitty *et al.*, 1998; Choong and Meagher, 1999). The overall incidence of aneuploidy is 2%, but about 0.5-1% of fetuses with an isolated CPC and 38% of those with CPCs and an additional anomaly have an aneuploidy, 80% of which are trisomy 18 (Peleg and Yankowitz, 1998; Chitty *et al.*, 1998). The risk for aneuploidy also increases with maternal age (Chitty *et al.*, 1998). The high risk of aneuploidy when additional anomalies are found justifies considering amniocentesis but controversy arises in the management of isolated CPCs. The anomalies associated with trisomy 18 in a second trimester ultrasound scan are NTDs, ventral wall defect, heart defect, intrauterine growth retardation, polyhydramnios and clenched hands.

An echogenic intracardiac focus may be associated with trisomy 13 fetuses (Lehman *et al.*, 1995). It may also be associated with Down syndrome but this is controversial (Bromley *et al.*, 1995). In addition, a second trimester trisomy 13 fetus usually has craniofacial defects especially median clefts and holoprosencephaly, ventral wall defect, heart defect, hyperechogenic kidneys, cystic hygromata and polydactyly.

As reviewed by Chitty (1998), in one centre, a detection rate of all aneuploidies using a second trimester ultrasound in low-risk pregnancies was 22% (11.8%, 22% and 67% for trisomy 21, 18 and 13 respectively), and a detection rate for Down syndrome in referral centres in high risk pregnancies was 42.6-93%.

1.2.2.2 Ultrasound markers for aneuploidies in the first trimester

Recently, it has been feasible to perform ultrasound much earlier in pregnancy and some fetal structural anomalies can be identified. Some markers are used as clues for developmental abnormalities such as heart rate, the size and configurations of the yolk sac or amniotic sac (Hyett *et al.*, 1996; Jauniaux *et al.*, 1996; Stampone *et al.*, 1996). However, the most studied ultrasound marker in the first trimester is nuchal translucency (NT).

The cause of increased nuchal translucency in chromosomally abnormal pregnancies is not well understood. A pathological study showed oedema and dilatation of lymphatic capillary vessels which is probably related to an early disarrangement of lymphatic connections (Greco *et al.*, 1996). It was first reported to be associated with trisomy 21 (Szabó and Gellén, 1990) and has been reported to be associated with trisomies 18, trisomy 13, triploidy and 45,X (Nicolaides *et al.*, 1992; Pandya *et al.*, 1995; Jauniaux *et al.*, 1997). The cut-off thickness for an indication of aneuploidy mostly used is ≥ 3 mm or 95th centile or 99th centile for crown-rump length (CRL) or gestational age. This finding is also present in normally karyotyped fetuses, the majority of whom are associated with uneventful pregnancies (Cha'ban *et al.*, 1996). However, in chromosomally normal fetuses, there is an increased prevalence of major heart defects as the NT thickness increases (Hyett *et al.*, 1997) and there is a higher risk of other structural defects and genetic syndromes than the normal NT fetuses (Brady *et al.*, 1998).

The combination of first trimester screening using NT, maternal serum free β-hCG and PAPP-A has recently been shown to have a detection rate of 89% at a false-positive rate of 5% for Down syndrome (Spencer *et al.*, 1999).

In summary, prenatal screening measures for chromosome abnormalities are used clinically and are being refined for a better efficiency. For a more definite diagnosis, chromosome analysis must be performed on a fetal or embryonic derived specimen. This can be acheived most commonly *via* prenatal diagnosis or in certain cases *via* preimplantation genetic diagnosis (PGD).

1.3 EMBRYOLOGY RELATED TO PRENATAL AND PREIMPLANTATION GENETIC DIAGNOSIS (PGD)

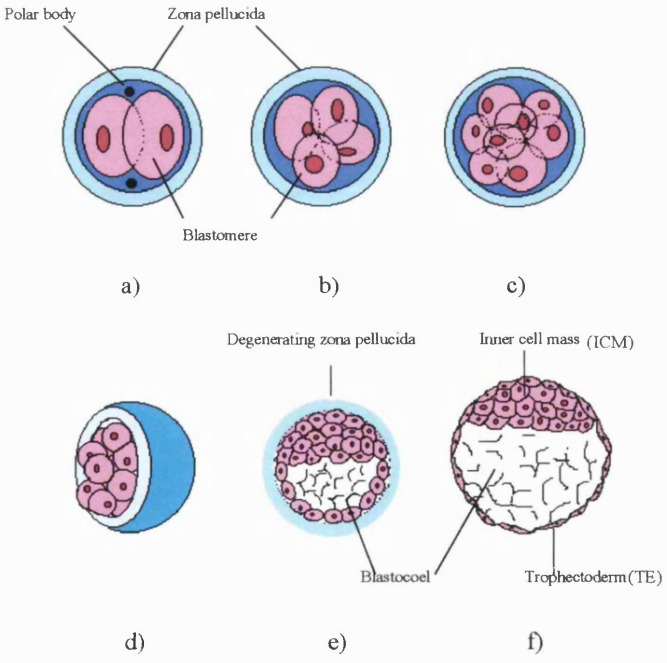
1.3.1 PREIMPLANTATION EMBRYONIC DEVELOPMENT

Naturally, human fertilisation usually takes place at the ampullar part of the fallopian tube. This process starts when the sperm enters the oocyte. The oocyte completes its second meiosis with the extrusion of the second polar body. Two pronuclei are formed from the nuclei of the sperm and the oocyte, each of which contains 23 chromosomes. The chromosomes replicate, giving rise to two chromatids for each chromosome. The pronuclei membranes disintegrated and this results in a single cell zygote with 46 chromosomes, each with 2 chromatids. The chromosomes subsequently divide by the separation of the chromatids into two daughter nuclei. Each chromatid becomes a chromosome of each new nucleus. The division of cytoplasm follows by the constriction of cell membranes, resulting in a 2-cell embryo with the identical set of 46 chromosomes in each cell **(Figure 1.1a)**. Further cycles of mitotic division follow, with DNA replication giving rise to a bivalent in each chromosome, which later separate to form a new chromosome in each new cell. Thus the chromosome number is constant. During the first few divisions, the total volume of the embryo is not changed while the cell number increases. This stage is called cleavage stage and is when cells are removed for PGD when the embryo has 6-10 cells **(Figures 1.1b, 1.1c)**. Meanwhile the embryo moves along the fallopian tube to the uterine cavity. With further cell divisions, beginning at the 12- to 16-cell stage, the embryo enters the morula stage during which the embryo forms a ball of cells with some of the cells situated inside and some at the outer layer **(Figure 1.1d)**. Compaction of the cells occurs as the

embryo begins to enter the uterine cavity. On day 5, fluid collection begins inside the embryo forming the blastocoel cavity and the embryo is called a blastocyst **(Figure 1.1e)**. The blastocyst is free floating in the uterine cavity for about 2 days before implantation. The fluid collection increases and the blastocoel expands, separating the cells in the embryo into two components. The first component is the outer cell layer which is called the trophectoderm (TE). This component will develop into the placenta and fetal membranes. The second component is called the inner cell mass (ICM), located in one pole of the embryo and destined to be the embryo proper **(Figure 1.1f)** but also has some contributions to the extraembryonic membranes.

Figure 1.1 Preimplantation embryo

Modified from Moore and Persaud (1998c) p. 40.



a) –c) Cleavage stage. d) Morula. e) Early blastocyst. f) Late blastocyst.

1.3.2 THE AMNIOTIC AND COELOMIC (CHORIONIC) CAVITIES

A human embryo begins the process of implantation on day 7 postfertilisation when it is at the blastocyst stage with the formation of the TE and the ICM. The TE at the embryonic pole is called the polar TE, and the TE at the opposite site is called the mural TE. The polar TE is the first site of embryonic attachment to the endometrium. During the process of implantation, which takes place in the second week, the trophoblast proliferates actively. The inner most cell layer is called the cytotrophoblast. This active proliferation also gives rise to outer multiple layers of erosive multinucleated cells called the syncytiotrophoblast. The syncytiotrophoblast gradually erodes maternal tissue and the blastocyst gradually embeds into the endometrial stroma. With active proliferation, several cavities, called lacunae, appear inside the syncytiotrophoblast and contain maternal blood and secretions from the disrupted maternal blood vessels and glands. These lacunae become the sources of nutrients and oxygen for the embryo. When the blastocyst is fully buried in the stroma, the epithelium of the decidua at the site of the implantation heals, forming the decidua capsularis, covering the embryo. Meanwhile, a small cavity, destined to be the amniotic cavity, appears in the inner cell mass, separating a cell layer from the inner cell mass to form a thin membrane called amnion which acts as the roof of the developing amniotic cavity. The rest of the inner cell mass differentiates into two cell layers: epiblast and hypoblast **(Figure 1.2a)**. The epiblast forms the floor of the amniotic cavity and continues peripherally with the amnion. The hypoblast proliferates giving a layer of cells lining the wall of the blastocoel to form the exocoelomic cavity or the primary yolk sac. The layer of cell lining is called the exocoelomic or Heuser's membrane **(Figure 1.2b)**. A thick, loose acellular material, called the extraembryonic reticulum, appears between

Heuser's membrane and the cytotrophoblast. It contains some cells of hypoblast origin **(Figure 1.2c)**. The epiblast, on day 12 or 13, proliferates peripherally, giving rise to two cell layers lining the inner surface of the cytotrophoblast and the outer surface of Heuser's membrane, called the extraembryonic mesoderm, trapping the extraembryonic reticulum in between **(Figures 1.2d, 1.2e)**. Several small spaces appear inside the reticulum and coalesce to form a single cavity, filled with fluid, called the chorionic cavity **(Figure 1.2f)**. On day 12, a second wave of hypoblast proliferation takes place, producing a new membrane lining the inner surface of the extraembryonic mesoderm, pushing the primary yolk sac to the abembryonic end of the chorionic cavity **(Figure 1.2g)**. This new layer becomes the endodermal lining of the definitive (secondary) yolk sac. The primary yolk sac breaks up and becomes a collection of vesicles at the abembryonic end of the chorionic cavity **(Figure 1.2h)**. These vesicles degenerate and the chorionic cavity enlarges, in which the embryo is suspended by a thick connecting stalk **(Figure 1.2i)**. The syncytiotrophoblast, the cytotrophoblast and the extraembryonic somatic mesoderm underneath the cytotrophoblast are collectively called chorion and will develop into the chorionic villi **(section 1.3.3)**.

Figure 1.2 Early formation of amniotic and chorionic cavities

Taken from Larsen (1997) pp 36, 37-40.

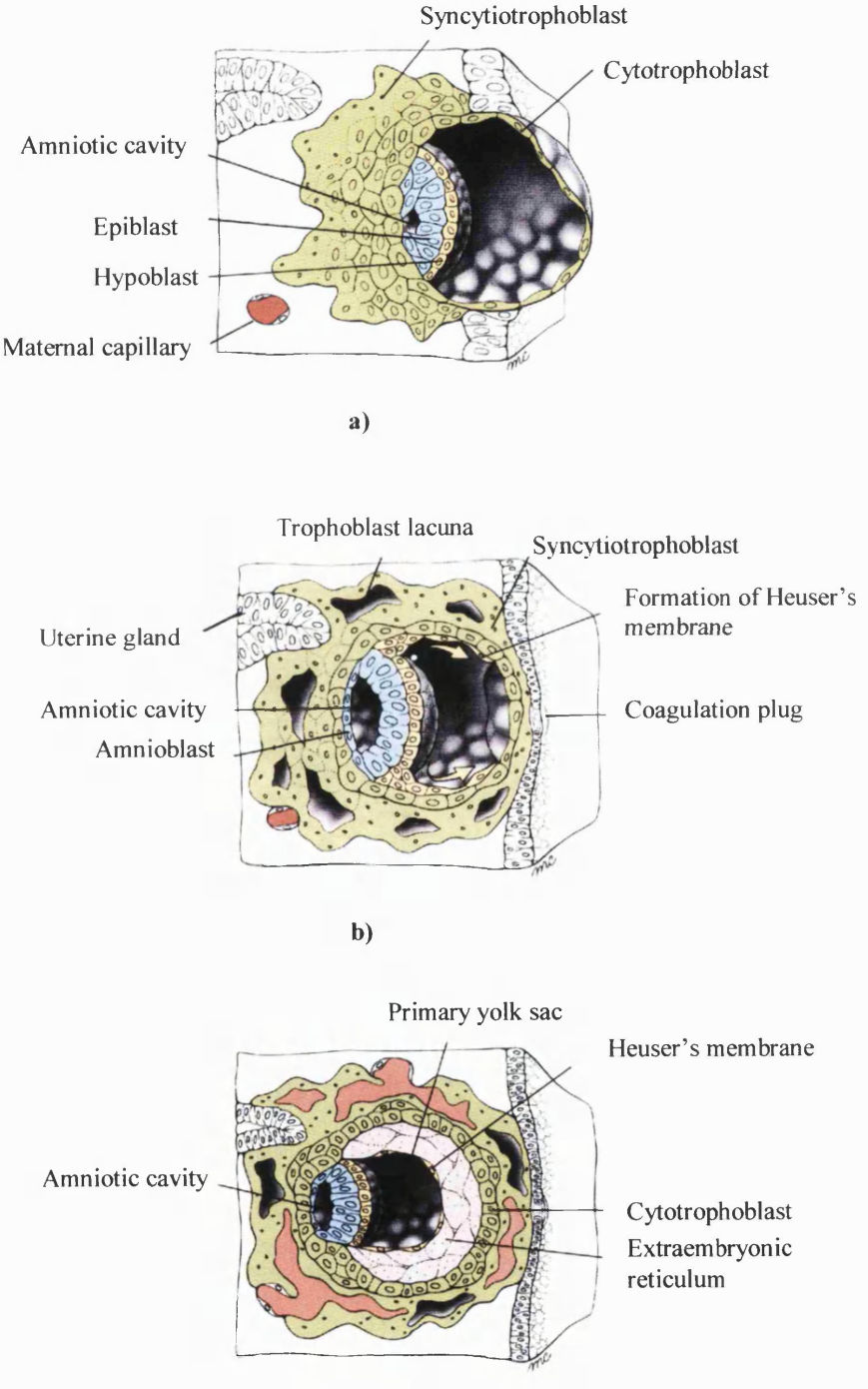
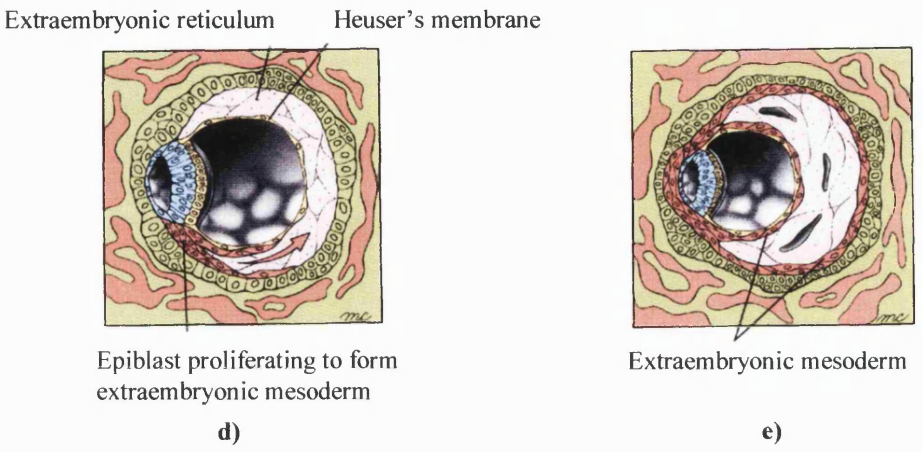
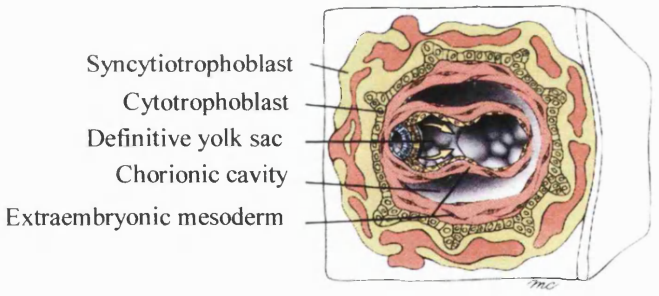


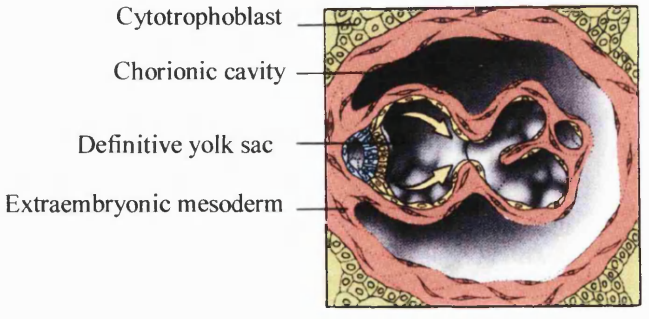
Figure 1.2 (continued)



d) e)

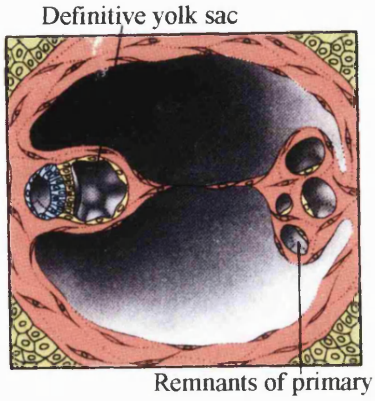


f)

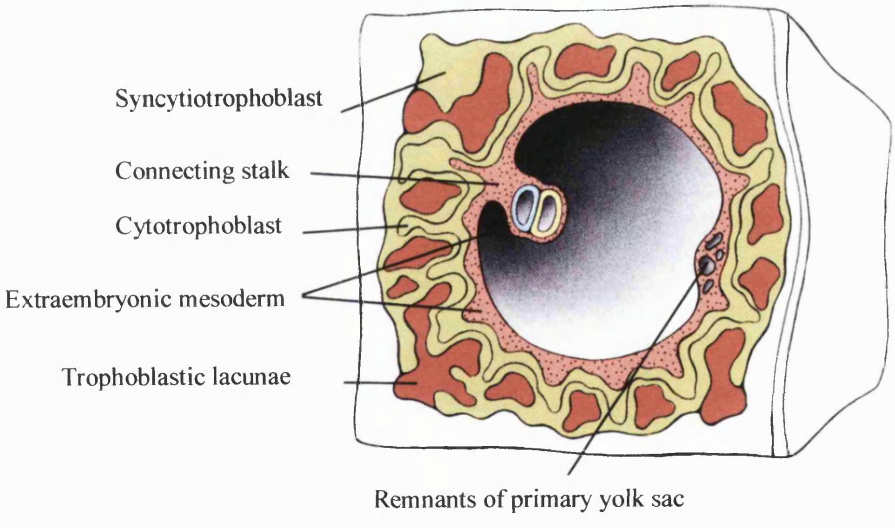


g)

Figure 1.2 (continued)



h)

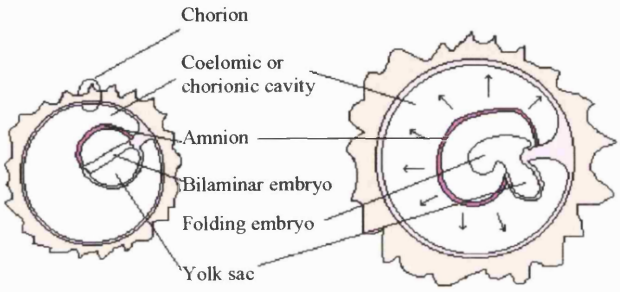


i)

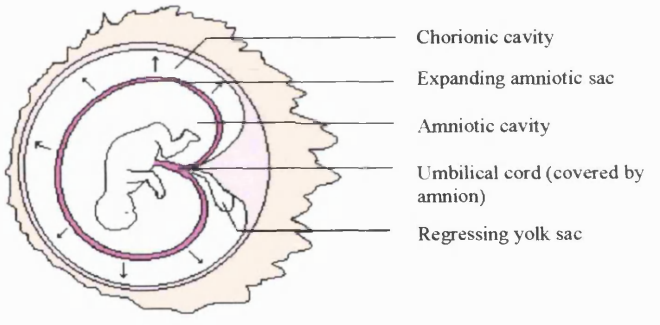
As the embryo develops, the amniotic cavity enlarges and expands, engulfing the folding embryo **(Figures 1.3a, 1.3b)**. As the margin of the embryonic disc grows ventrally during the development, the amniotic membrane also covers the regressing yolk sac and its associated extraembryonic mesoderm and a part of the later developed allantois, to form the umbilical cord. At about 12 weeks, the amniotic cavity expands such that it fills and obliterates the chrorionic cavity, formerly the extraembryonic coelom **(Figures 1.3c, 1.3d)**. Initially, most of the amniotic fluid is derived from the maternal interstitial fluid *via* diffusion. Before the keratinization of the fetal skin, tissue fluid from the fetus diffuses into the amniotic fluid. Later, fluid in the amniotic cavity is also derived from the respiratory tract secretion and the fetal urine. This is the specimen collected by amniocentesis for prenatal diagnosis.

Figure 1.3 Later development of amniotic and chorionic cavities

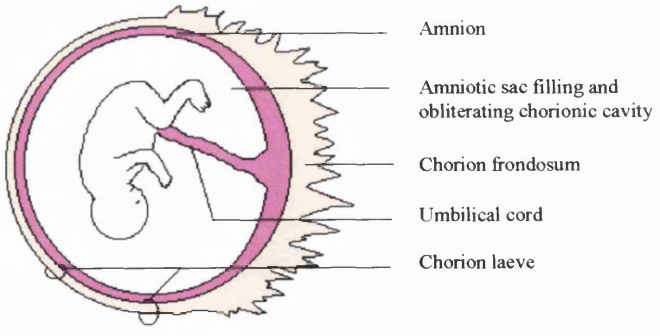
Modified from Moore and Persaud (1998b), p. 151.



a) at 3 weeks (5 weeks gestation) **b) at 4 weeks** (6 weeks gestation)



c) at 10 weeks (12 weeks gestation)



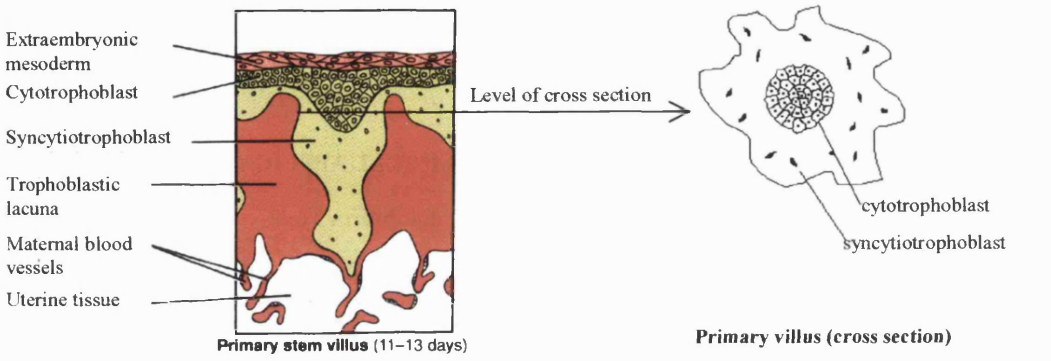
d) at 18-20 weeks gestation

1.3.3 THE CHORIONIC VILLI

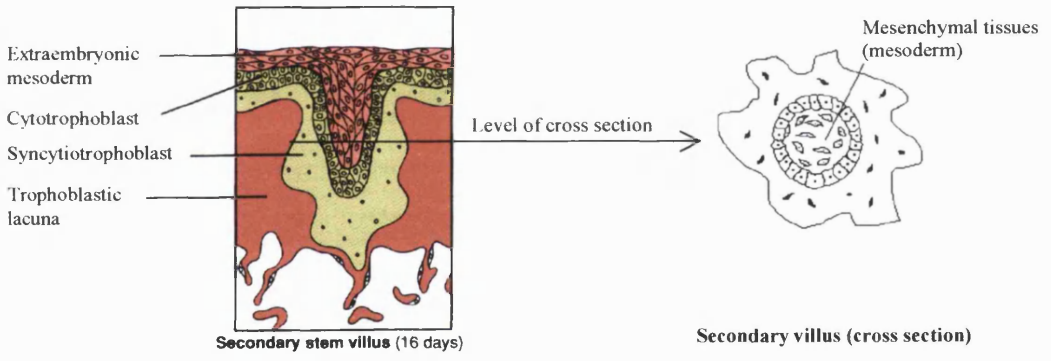
As the lacunae form, along with the proliferation of the cytotrophoblast, the primary villi develop, projecting like fingers into the lake of fluid-filled lacunae and decidual stroma. The cross section of the primary villi consists of cytotrophoblastic core, surrounded by the syncytiotrophoblast **(Figure 1.4a)**. During the third week, the mesenchymal cells from the extraembryonic mesoderm also proliferate into the villi with the formation of capillaries. Blood cells develop inside the blood vessels at the end of the third week. These lead to the development of the secondary and tertiary villi respectively **(Figures 1.4b, 1.4c)**. Some of these villi are free floating in the lacunae which contain maternal blood and maternal glandular secretion. Some of these villi have the very active proliferation of cytotrophoblast beyond the tip of the villi to the confronting decidual stromal as the trophoblastic column and this column of cells serves as a fastening point to the maternal tissue, hence the name "anchoring villus" for this type of villi. Cytotrophoblast cells at these points continue to proliferate and join cytrotrophoblastic cells from nearby cell columns, forming a shell called the trophoblastic shell. This shell along with the stromal cell layer at the maternal site forms "the basal plate" of the placenta. The trophoblastic cell layers associated to the embryonic/fetal site are collectively called "The chorionic plate". Spaces between anchoring villi are called intervillus space and are the points of the feto-maternal communication. Maternal blood spurts from the maternal capillaries into these spaces, bathing the villi and is drained back *via* endometrial veins **(Figure 1.5)**. This is thought to be a source of fetal cells in the maternal circulation. In addition, some cytotrophoblast cells are found in the decidual stroma and inside maternal capillaries.

Figure 1.4 The chorionic villi

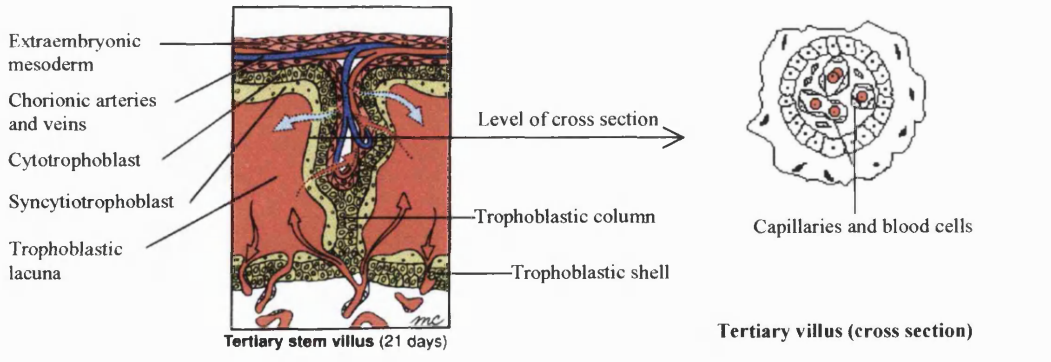
Modified from Larsen (1977) p. 41, Moore and Persaud (1998a) p. 53 and Moore and Persaud (1998b) p. 137



a)



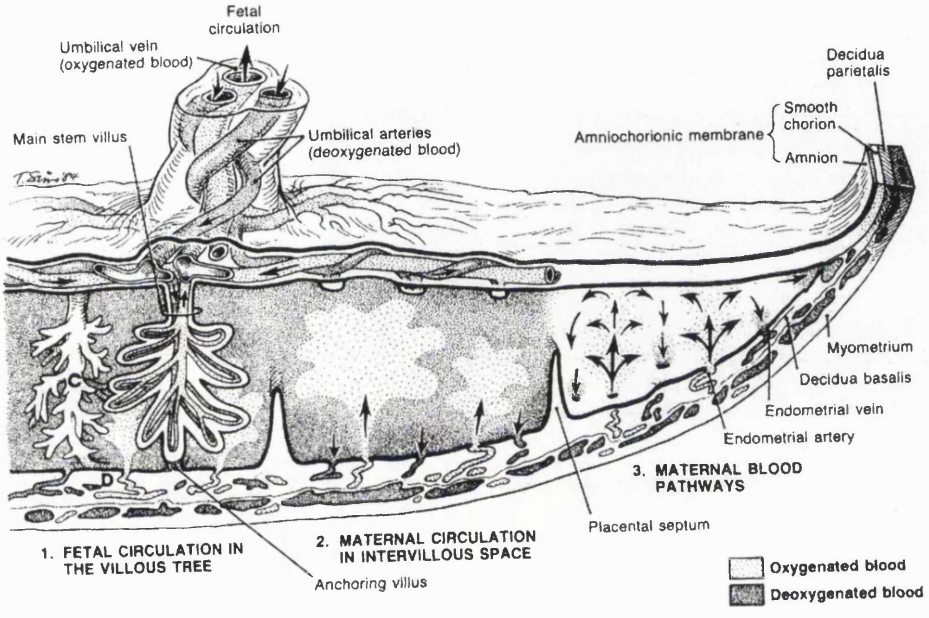
b)



c)

Figure 1.5 A full term placenta, showing the complete feto-maternal circulation

From Cunningham et al. (1997b)



The fetal deoxygenated blood flows through the umbilical arteries to the capillaries in the chorionic villi. The maternal oxygenated blood spurts into the intervillous spaces, bathing the villi and the gas exchange occurs. The maternal deoxygenated blood is drained back to the endometrial vein while the fetal oxygenated blood flows back to the fetus via the umbilical vein.

Initially, chorionic villi formation occurs around the entire chorionic sac. At the beginning of the eighth week (10 weeks gestation), the villi adjacent to the decidua capsularis are compressed as the conceptus enlarges. These villi soon degenerate, leaving a smooth membrane called chorion laeve. On the contrary, the villi adjacent to the decidua basalis proliferate rapidly, forming the chorion frondosum **(Figure 1.3d)**. This is the position for chorionic villus sampling (CVS) **(section 1.4.2)**. The degeneration of the villi at the position of the chorion laeve is thought by some to be a source of chorionic cells in the cervical mucus **(section 1.6.2)**.

1.4 PRENATAL DIAGNOSIS

With modern technologies, prenatal diagnosis is now able to detect a variety of genetic disorders, thus allowing informed decisions regarding the outcome of pregnancy. Prenatal diagnoses are based on the invasive procedures of amniocentesis, chorionic villus sampling (CVS) or fetal blood sampling (FBS). Some of these procedures also help in evaluating several non-genetic conditions.

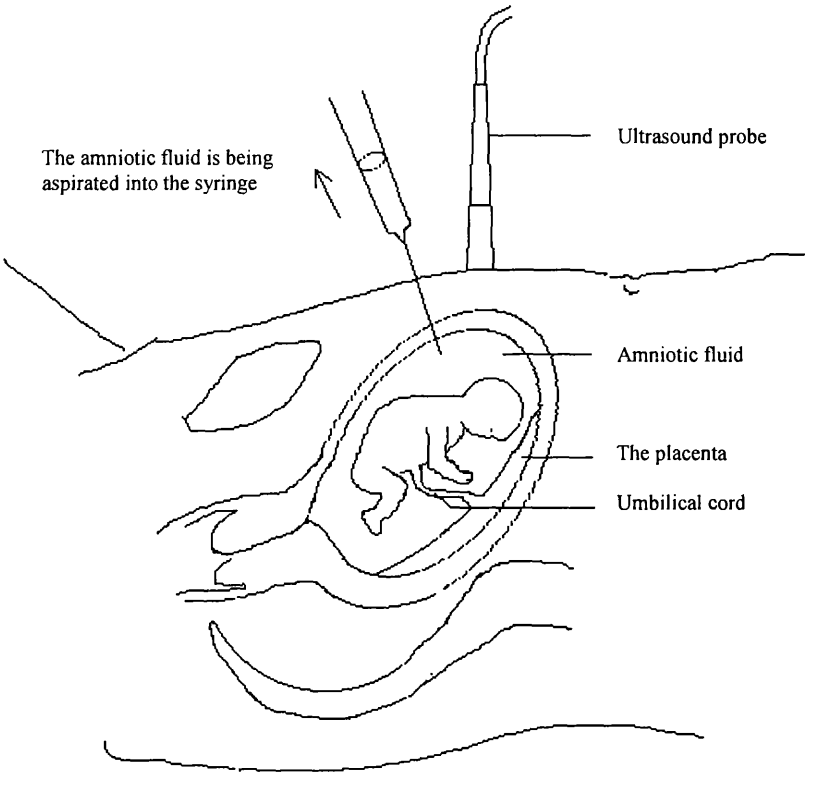
1.4.1 AMNIOCENTESIS

This procedure is usually performed at 15-16 weeks of gestation. After an ultrasound examination to determine the number of fetuses, viability, gestational age, possible malformations, site and appearance of the placenta and amount of amniotic fluid, a sterile needle is introduced into the uterine cavity preferably under ultrasound guidance or ultrasound monitoring. Amniotic fluid is aspirated (20 ml) and both cells and fluid can be used for genetic analysis (**Figure 1.6**).

Cells in the amniotic fluid, shed from different fetal organs, amnion and umbilical cord, are suitable for genetic studies. Cells can be cultured and karyotyped, allowing the diagnosis of chromosomal disorders. Molecular analysis of some inborn errors of metabolism can also be performed on these cells. The fluid can be used to diagnose NTDs using amniotic fluid alpha-fetoprotein and acetylcholine esterase (AFAFP and AChE), Rhesus isoimmunisation (Bevis, 1953) and can be used for enzyme analysis.

Figure 1.6 Amniocentesis

Modified from Cunningham et al. (1997a), p.31



However, amniocentesis carries with it some drawbacks. Introducing a needle into the pregnant uterus has some risks. Amnionitis occurs in about 0.1% of cases and at least one case of maternal death has been reported (Milunsky, 1979). Other minor maternal complications such as transient vaginal spotting or amniotic fluid leakage may occur; but fortunately these are usually self-limited. Other very rare complications include intra-abdominal viscus injury or haemorrhage. As this procedure may cause fetomaternal haemorrhage, prevention of Rh(D) isoimmunisation with anti-Rh(D) antibody should be given to all Rh(D)-negative mothers at the time of the procedure.

Concerning fetal risks, a randomised controlled trial of 4,606 women showed that the rate of spontaneous abortion following amniocentesis was 1.7% compared with 0.7% in controls (Tabor *et al.*, 1986). There have been some reports regarding fetal injuries associated with amniocentesis including scarring (Raimer and Raimer, 1984), limb or peripheral nerve injuries (Epley *et al.*, 1979; Holmes, 1997) and some more serious complications such as bowel injuries (Swift *et al.*, 1979), ocular injuries (Admoni and BenEzra, 1988) and even brain injuries (Youroukos *et al.*, 1980).

Another drawback is that this procedure is carried out in the second trimester and, with the additional time needed for the cell cultures, the prenatal diagnostic result will be available at 18-20 weeks of gestation. If the fetus is abnormal and termination of pregnancy (TOP) is required, performing TOP at this late stage of gestation involves more maternal risks and more emotional, social or religious problems. Some authors have tried early amniocentesis to avoid the problems of late TOP but the fetal loss rates are higher than CVS (Nicolaides *et al.*, 1994; Shulman *et al.*, 1994). Moreover, early amniocentesis may be associated with an increased risk of the baby being admitted into neonatal intensive care units (Greenough *et al.*, 1997). Fluorescent *in situ* hybridisation (FISH) has recently been introduced to overcome this problem, results being possible in 24 hours **(section 1.5.2)**.

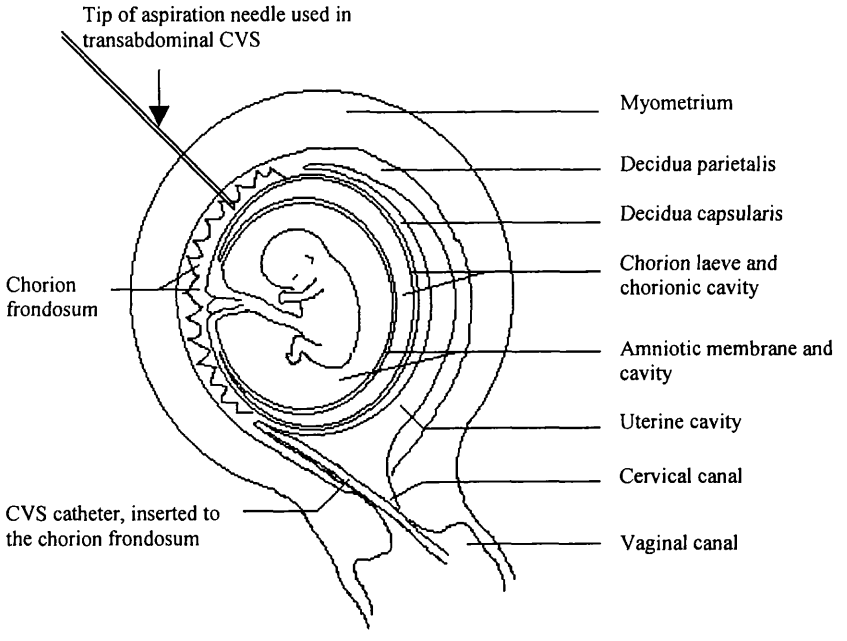
1.4.2 CHORIONIC VILLUS SAMPLING (CVS)

This procedure was developed in order to perform prenatal diagnosis at an earlier stage than amniocentesis. CVS can be performed at 9-11 weeks of gestation under ultrasound monitoring, either transcervically or transabdominally **(Figure 1.7)**. Like amniocentesis, ultrasound is performed before the procedure to determine the number of fetuses, viability, gestational age, possible malformations, amount of amniotic fluid and the placenta especially to establish the site of the chorion frondosum. The obtained chorion can be tested cytogenetically, tested for deoxyribonucleic acid (DNA) or for biochemical enzymatic analysis.

However, the procedure has some drawbacks since CVS may increase the chance of miscarriage over the background loss rate by more than 1% (Rhoads *et al.*, 1989). Transvaginal bleeding or subchorion haematoma can occur. Infection is reported in about 0.2-0.3% of cases. Delayed rupture of membranes can occur in 0.3% of cases (Hogge *et al.*, 1999). With this procedure, fetomaternal haemorrhage has consistently been reported (Blakemore *et al.*, 1986; Brambati *et al.*, 1986; Shulman *et al.*, 1990), therefore, anti-Rh(D) antibody should be administered to all Rh(D)-negative mothers undergoing CVS.

Figure 1.7 Chorionic villus sampling

Modified from Cunningham et al. (1997a), p. 934.



The figure shows two different approaches for chorionic villus sampling. At the top left corner of the figure, an aspiration needle is being inserted transabdominally. The tip of the needle is at the chorion frondosum. At the lower left corner, a transcervical approach for CVS is being shown by a catheter inserted through the cervical canal into the chorion frondosum.

Apart from the 1% procedure-related fetal loss rate, severe limb-reduction defects, oromandibular hypoplasia, and central nervous system anomalies have been claimed to occur in cases when CVS was performed earlier than 9 weeks of gestation (Firth *et al.*, 1994). It was suggested that these anomalies were consistent with incomplete morphogenesis. A vascular or placental disruption due to the procedure appears to be the most plausible explanation at present (Hoyme *et al.*, 1982; Evans and Hamerton, 1996). Damage to the vessels at the sampling site may cause release of vasoactive peptides or embolisation of fragments of trophoblast into the fetal circulation, or cause fetomaternal haemorrhage (Rodeck *et al.*, 1993). The resulting local hypoxia disrupts or damages organogenesis, causing the defects. Therefore, the procedure is not recommended before 9½ weeks of gestation.

1.4.2.1 Confined placental mosaicism

Another additional problem encountered in CVS is the interpretation of chromosome abnormalities diagnosed. Firstly, there are frequent discordances between direct preparations and cultures. Direct preparations are performed on the actively dividing cytotrophoblast cells **(Figure 1.4)** which arise from the trophectoderm. Cultures are carried out from the mesenchymal core of villi and are closer in line to the fetus proper, as mesenchyme arises from the inner cell mass. Secondly, there may be differences between the results of CVS and subsequent analyses, such as the results from amniocentesis, fetal blood, and postnatal tests, especially when a result of mosaicism is observed.

Mosaicism is defined as the presence of two or more cell lines, each with a different chromosome complement, in the same individual. This is found in 0.6-1.3% of

CVS samples in most of which the follow-up analyses reveal a normal fetal karyotype (Canadian Collaborative CVS-amniocentesis Clinical trial Group, 1989; Rhoads *et al.*, 1989; Vejerslev and Mikkelsen, 1989). Therefore, mosaicism in CVS results may reflect either true fetal mosaicism or so-called confined placental mosaicism (CPM) (Kalousek and Dill, 1983). A decision to terminate a pregnancy should therefore not be based entirely upon a CVS mosaic result. Further investigations such as amniocentesis or fetal blood sampling should be carried out. Cultures from amniocentesis samples display less mosaicism than CVS because the cells are usually shed from several fetal organs thus representing closer the fetal karyotype.

The reason for chromosome mosaicism being sometimes confined to only part of the conceptus is not clearly understood, but it might occur from some mitotic error. If the error occurs in the cleavage stage embryo, prior to the differentiation of the ICM and the TE, the mosaicism may be distributed into both the placenta and fetal tissues. If the error occurs at a later stage, the abnormal cells may be confined either to the placenta or to the embryo (Crane and Cheung, 1988; Simoni and Sirchia, 1994). Mosaicism occurs more frequently in chorion or placenta than in the embryo proper. There are several possible explanations for this. In a 64-cell mouse blastocyst, only 3-4 cells are destined to be the embryo and the remaining cells will develop into extra-embryonic tissue (Markert and Petters, 1978). Hardy *et al.* (1989) has shown that only 45 out of the 125 cells in the day-7 blastocyst make up the ICM, the remainder making up the TE. This gives more chance for an error to occur in the extra-embryonic tissue. In addition, there may be mechanism that preferentially allocates the abnormal cells to the TE (James and West, 1994; James *et al.*, 1995).

In a trisomic zygote, mitotic error may take place at any early cell division and may reduce the extra chromosome (post-zygotic aneuploidy correction or trisomic rescue). These disomic cells further replicate, resulting in a discrepancy between the fetus and the placenta (Kalousek and Vekemans, 1996; Sirchia *et al.*, 1999).

A "vanishing twin" may be the source of another cell line found in the placenta in cases of dizygotic twins (Reddy *et al.*, 1991) but this is not regarded as a true CPM.

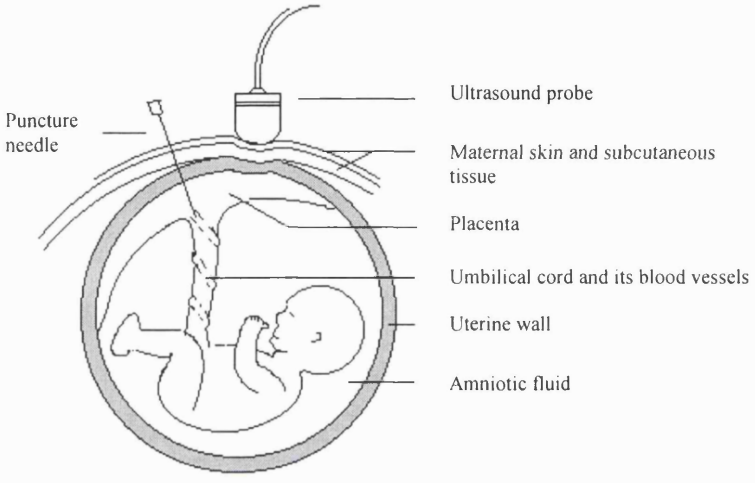
There have been several reports about the association of CPM and adverse pregnancy outcome, especially growth retardation (Johnson *et al.*, 1990; Wilkins-Haug *et al.*, 1995). However, contradictory studies have also been reported (Fryberg *et al.*, 1993; Roland *et al.*, 1994).

1.4.3 FETAL BLOOD SAMPLING (FBS)

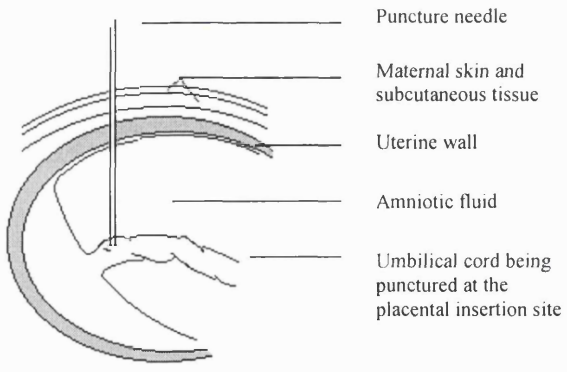
This procedure involves the collection of fetal blood from the umbilical cord either at the placental or fetal insertion site, or free loop, or even from the intrahepatic umbilical vein, under ultrasound visualisation **(Figure 1.8)**. Again, an ultrasound scan is performed to evaluate the general status of the fetus(es), the site of the placenta and the amount of amniotic fluid before the procedure. There is an increase risk of maternal cell contamination if the procedure is performed through the placenta. FBS has been used to diagnose thalassaemia and haemoglobinopathies in early studies by measuring the globin chain synthesis (Weatherall *et al.*, 1965); it is still occasionally performed for the diagnosis of other hereditary haematologic disorders such as haemophilia, thrombocytopaenia, and some immunodeficiency diseases.

Figure 1.8 Fetal blood sampling

Modified from Ryan and Rodeck (1993) p. 69.



a) A puncture through the placenta



b) A puncture without going through the placenta

Currently, the role of FBS in prenatal diagnosis for genetic disorders is declining, as the DNA analysis of CVS has become feasible (Morrison and Rodeck, 1997). It may still be used in patients who book late in pregnancy or for whom DNA analysis from CVS is not available or inconclusive. A fetal karyotype can be obtained from cultured fetal lymphocytes within 3 days, which is faster than from amniocytes. Other information such as acid-base balance, haematological status, and indices of infection can be acquired from FBS if indicated. FBS performed in the third trimester can give some information and guidance to the management of a complicated pregnancy in terms of time and method of delivery. However, FBS for this indication is gradually being replaced by Doppler studies (Morrison and Rodeck, 1997).

Regarding complications, Orlandi *et al.* (1990) reported 5% and 2.5% procedure-related risks at 12-15 weeks and at 19-21 weeks respectively. The fetal loss rate is 1.4% if the procedure is performed by an experience operator (Morrison and Rodeck, 1997). Nicolini *et al.* (1988) reported that fetomaternal haemorrhage occurred in 70% of cases where a similar procedure was used for intrauterine transfusion in which the placenta is transgressed. Prevention with anti-Rh(D) antibody is needed for Rh(D)-negative women at the time of the procedure.

1.5 LABORATORY TECHNIQUES

1.5.1 KARYOTYPING

Karyotyping, a method used to analyse the chromosome constitution of an individual, is carried out on metaphase chromosomes. These chromosomes can be obtained prenatally from cultures of chorionic villus sampling (CVS), amniocentesis samples or fetal blood, or from direct preparation of CVS samples. Occasionally samples from parents are also needed. The cells are cultured in a sterile medium, with additional phytohaemagglutinin for blood cultures to stimulate T lymphocyte division. Thymidine is used to bring more cells to metaphase simultaneously and colchicine is added to arrest cells at metaphase by blocking the formation of the mitotic spindle. Hypotonic saline is subsequently added to cause the cells to swell and the cells are fixed and mounted on a slide. The chromosomes are differentiated by their sizes, position of the centromere and the bands on their arms generated by special staining methods. The most commonly used method for banding is G (Giemsa) banding which gives each chromosome a characteristic and reproducible pattern of light and dark bands. The chromosomes are counted and examined for number and structural appearance under a microscope. At least 10-15 cells are counted to determine the chromosome number but if there is a suspicion of mosaicism, at least 30 cells are counted (Hook, 1977).

Using karyotyping, all the chromosomes in a cell can be analysed, both numerically and structurally. The drawbacks are the time needed for culture to obtain cells in metaphase especially in amniocentesis, the intensive work of analysis and a certain limit resolution of banding which is usually achieved at 4,000 – 8,000 Kb.

1.5.2 FLUORESCENT *IN SITU* HYBRIDISATION (FISH)

In situ hybridisation makes it possible to detect specific nucleic sequences in interphase as well as metaphase nuclei, eliminating the time needed for culture. The principle is the hybridisation of a labelled probe (a modified nucleic acid sequence) to its complementary sequence in the specimen under appropriate conditions. For FISH, the probes are labelled with a fluorochrome. This fluorochrome emits light of a certain wavelength after being excited by light of another certain wavelength (Griffin, 1994). The analysis is carried out by visualisation of fluorescent signals from the probe under a fluorescent microscope. The fluorochrome may be directly labelled onto the probe and the detection can be performed after the hybridisation and washing procedures. This so called direct FISH takes less time to perform than indirect FISH. With indirect FISH, the probe is labelled with an element called a hapten which enables the hybrids to be detected by its high affinity to another element which is fluorescently tagged and signals can subsequently be amplified by the affinity property. The amplification of signals makes indirect FISH more sensitive than direct FISH.

Three types of probes are commonly used in FISH.

1. Repetitive probes. These probes recognise repetitive targets such as the α-satellite sequences on the centromeric region, or those on the long arm of the Y chromosome. Most of the centromeric probes are chromosome specific but some are not; for example, probes for the centromeric region of chromosomes 13 and 21 which cross hybridise to each other.
2. Unique sequence probes. These probes recognise unique sequences on chromosomes. For example, probes cloned in yeast artificial chromosomes (YAC) can be used to differentiate chromosomes 13 and 21.

3. Paints or whole chromosome paints on metaphase spreads. These consist of a cocktail of probes for different parts of a particular chromosome. When the set of probes is used on a metaphase spread, the entire part of that chromosome fluoresces. This technique is useful to detect translocations, insertions, duplications, and to identify the origin of a marker or a ring chromosome.

1.5.2.1 Probe labelling (nick translation)

After DNA sequences for probes are cloned in the vector, they are purified and labelled by nick translation. The reaction of nick translation is shown diagrammatically in **Figure 1.9**. Deoxyribonuclease (DNase) is used to create free 3'-hydroxyl groups within the unlabelled DNA ('nicks'). The 5'→3'exonuclease action of *E. coli* DNA polymerase I removes the nucleotide unit from the 5'-phosphoryl terminus while its 5'→3' polymerase action catalyses the addition of a nucleotide residue with a free 3'-OH group to the 3'-hydroxyl terminus of the nick. The reaction moves along in a 3' direction. Fluorescently labelled deoxyribonucleoside triphosphates (dNTPs) are used as substrates in the reactions and are randomly incorporated into the new DNA strand, giving rise to a "labelled probe".

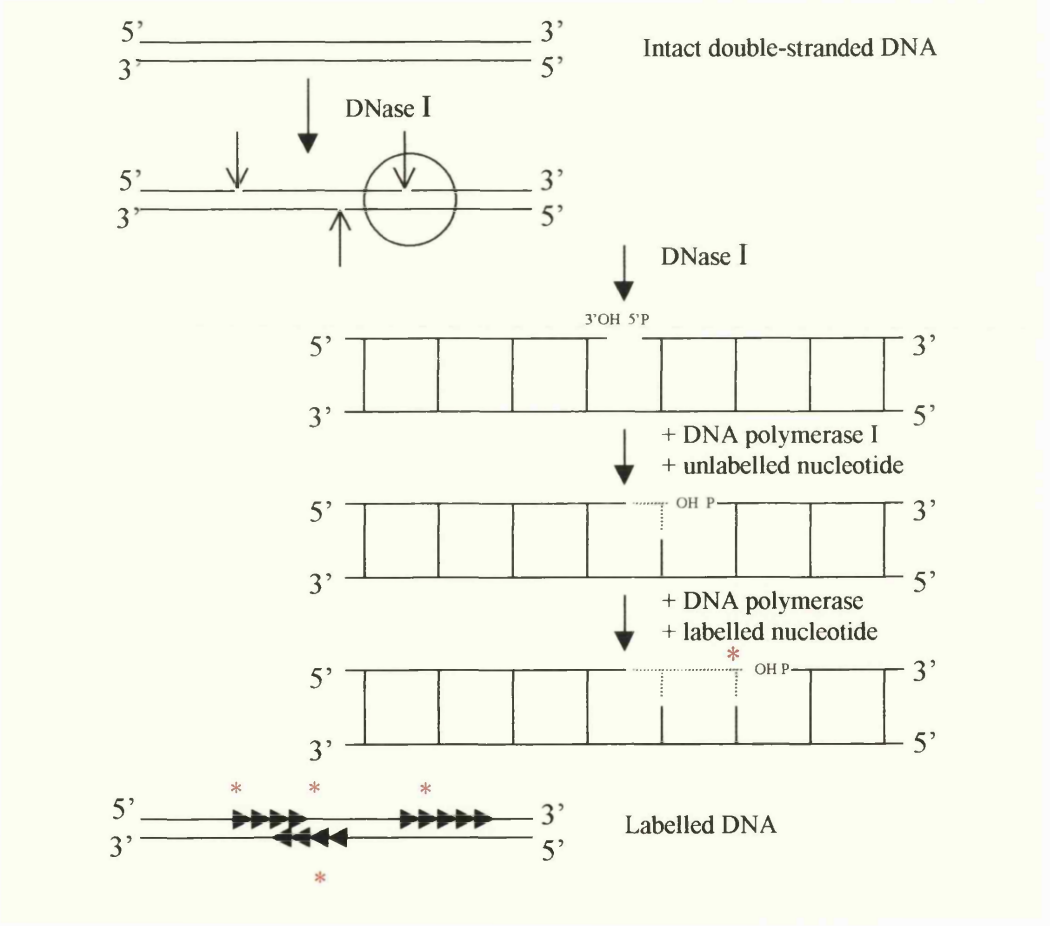
1.5.2.2 Fluorescent *in situ* hybridisation (FISH)

The specimens are pretreated by enzyme treatment so that the target sequences are accessible to the probes. Chromosome morphology is preserved by formaldehyde. Target and probe DNAs are denatured, followed by *in situ* hybridisation. After the hybridisation, unbound probes are washed off. Detection steps are performed for

indirect FISH. The specimens are mounted and visualised under a fluorescent microscope **(Figure 1.10)**.

Figure 1.9 Nick translation

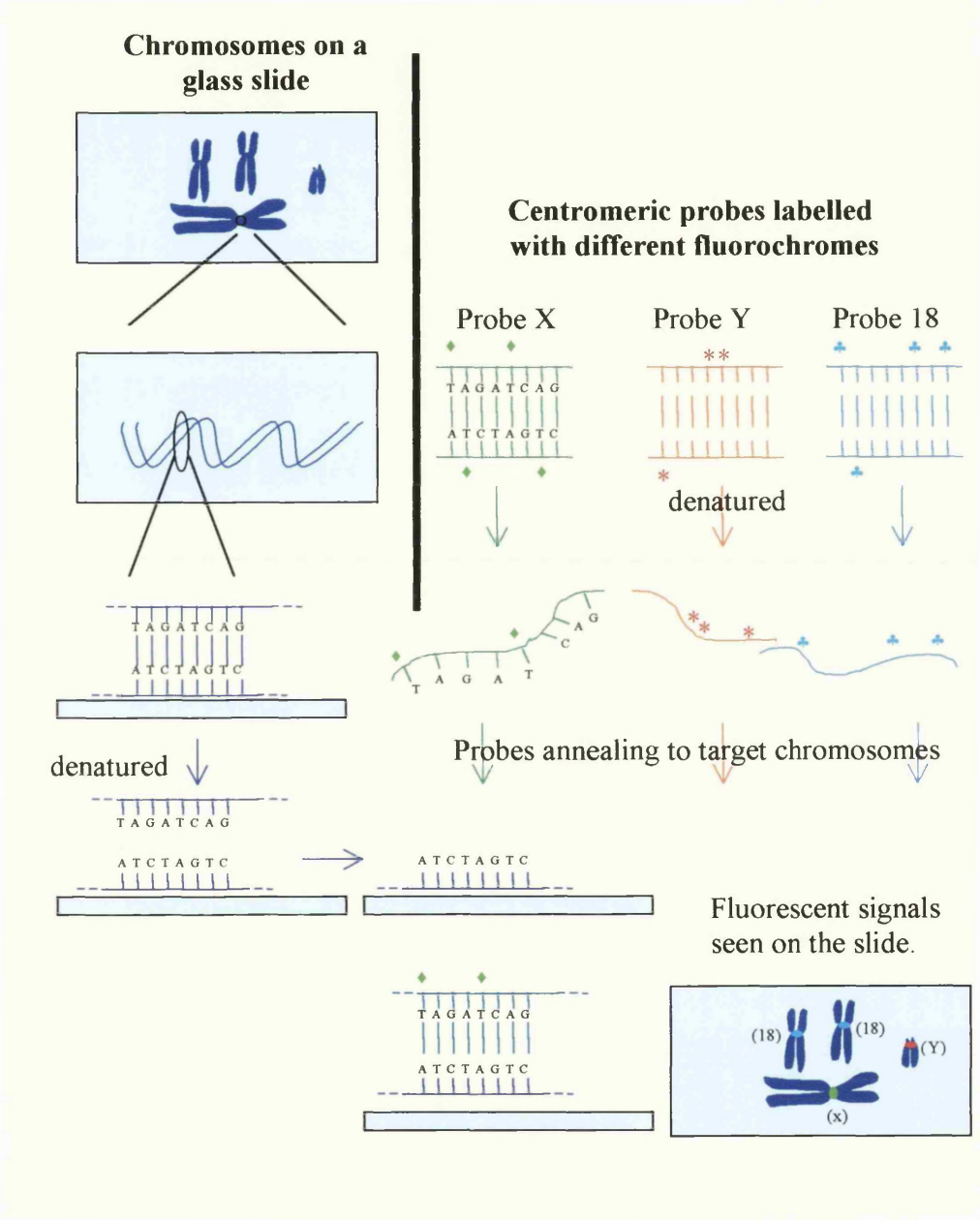
Modified from Arrand (1985), p. 31.



Free 3'-OH groups (nicks) are created within the unlabelled DNA by DNase I (thin arrow). DNA polymerase I activities are illustrated in one of the nicks (in the circle). Its 5'→3' exonuclease activity removes the nucleotide unit from the 5'-phospharyl terminus while its 5'→3' polymerase activity catalyses the addition of a nucleotide residue with a free 3'-OH group to the 3'-OH terminus of the nick. The reaction moves along in a 3' direction. Fluorescently labelled dNTPs are randomly incorporated, resulting in a labelled probe.

Figure 1.10 FISH procedure

Modified from Sirivatanauksorn (1999), p. 58 with permission



Probes and target DNAs are denatured. Single-stranded probes anneal to the complementary sequences on the target chromosomes. (Only chromosomes X, Y and 18 are shown in the figure, and only the annealing of probe for the X chromosome is illustrated.)

FISH can be performed on metaphase and interphase nuclei. This property is useful in situations where it is difficult to obtain metaphase spreads for karyotyping, such as cancer tissue (Gray *et al.*, 1994; Fox *et al.*, 1995; Popescu and Zimonjic, 1997), preimplantation embryos (Harper and Delhanty, 1996a; Munné *et al.*, 1998a, 1998b) and fetal material, for example after miscarriage or termination of pregnancy. Moreover, it takes less time than karyotyping. This can be useful when the results are needed quickly such as for a late booking case. Many studies have tried FISH on amniotic fluid to detect common aneuploidies (Isada *et al.*, 1994; van Opstal *et al.*, 1995; Gersen *et al.*, 1995). Recently, the efficacy of FISH in clinical practices has been evaluated by several groups. In a study using FISH on amniocytes in 315 high risk cases, 55 of which being more than 24 weeks of gestation, FISH was informative in 254 cases (80.6%), and all 21 aneuploid cases within this group were correctly identified with 100% accuracy (D'Alton *et al.*, 1997). Four cases with aneuploidies were within the 61 uninformative cases. The problems leading to samples being uninformative were poor quality or inadequate amount of amniotic fluid, inconclusive hybridisation pattern and technical difficulties (D'Alton *et al.*, 1997). From the experience of University College Hospital between October 1997 – July 1999, 812 FISH on uncultured amniocytes were performed, with 99% of samples being adequate for this test. Of 812 cases, 64 chromosome abnormalities were detected by karyotyping, 50 (78%) of which were detected by FISH. Most of the cases undetectable by FISH were chromosome structural abnormality cases. Considering only cases that should have been detected by the probes used (chromosomes 13, 18, 21, X and Y), only 3 (0.37%) cases would be counted as false negative. The causes of false negative results were maternal cell contamination and low level of mosaicism (Hastings, personal communication).

Recently, from a retrospective cohort of 27,407 samples, Lewin *et al.* (2000) reported a lower sensitivity of FISH for the detection of chromosome abnormalities of bad prognosis (89%), and for the detection of all chromosome abnormalities identified by karyotyping (71%). They defined bad prognosis cases as cases with trisomies involving chromosomes 21, 18, 13, aneuploidies involving the autosomes (except trisomy 20 mosaicism), and unbalanced structural abnormalities. This study shows clearly the necessity of karyotyping because, using FISH, only a few chromosomes can be analysed at a time due to the limited fluorochromes available and signal overlapping, and structural aberrations can be missed because they will need particularly designed probes for a particular aberration.

1.5.3 POLYMERASE CHAIN REACTION (PCR)

PCR is mainly used in single gene analysis. However, it is used to identify the origin of the chromosome of interest using polymorphic markers in informative cases **(Chapter 3)**. PCR can amplify a target DNA sequence selectively and specifically. The amount of the final DNA obtained can be a one-million fold increase from the starting concentration. This enables the target DNA sequence to be visualised or subjected to further analysis. Saiki *et al.* (1985) first described this procedure as a tool to diagnose sickle cell anaemia.

In order to perform PCR, short parts of the DNA sequences flanking the studied sequence on either side must be known. Complementary DNAs to these parts would be the primers used in the reaction. Two primers which have their 3'-termini facing each other are used in each studied sequence, forward and reverse.

The target DNA is denatured at a high temperature and, at the lower temperature, will reanneal with the primers. In the presence of all four nucleoside triphosphates and DNA polymerase, the primers will be elongated by the addition of nucleosides at the 3' end. The new 2 pairs of DNA (2 old strands and 2 new strands) would be separated again under a high temperature. A further round of cooling and extension will allow the binding of the new sets of primers to anneal to the four separated strands. The DNA polymerase used is isolated from a bacteria *Thermus aquaticus (Taq)* which resides in volcanic conditions and is thus stable at the necessary high temperature in the PCR procedure. Thus, the primers are extended to give rise to 4 more strands. From the first two rounds of amplification, the primers will act as boundaries of the new DNA strands giving copies of the sequence lying between the primers and all of the DNA strands would be templates for further amplification **(Figure 1.11)**. In each round of thermal cycling the amount of studied DNA doubles, leading to an exponential increase. Usually 20-30 rounds of amplification are performed. Using an automated thermocycling apparatus, the procedure can be completed in 2-4 hours. This is quicker than Southern blot techniques or dot blot analysis. However, PCR has some disadvantages. The sequences of the area flanking the studied DNA must be known, so that the appropriate primers can be designed and used. Due to the high sensitivity, meticulous precaution must be applied to prevent exogenous DNA contamination. After the PCR procedure, the amplification products can be directly visualised using ethidium bromide stained gels and viewed under ultraviolet light or subjected to other analyses. If the primer is labelled with a fluorescent substance, the products can be analysed by an automated laser DNA analyser.

Figure 1.11 The polymerase chain reaction

Modified from Newton and Graham (1994), p. 4

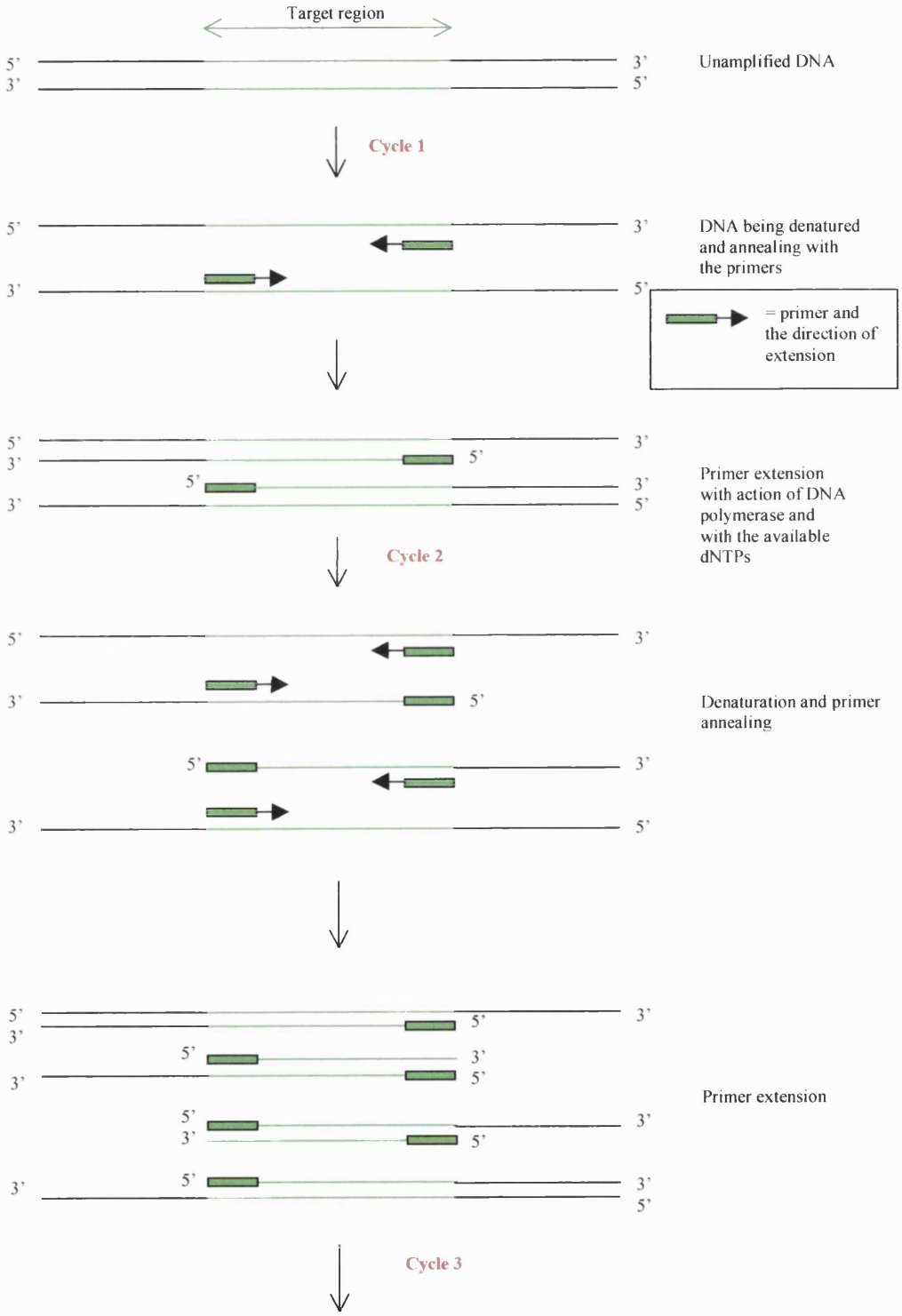
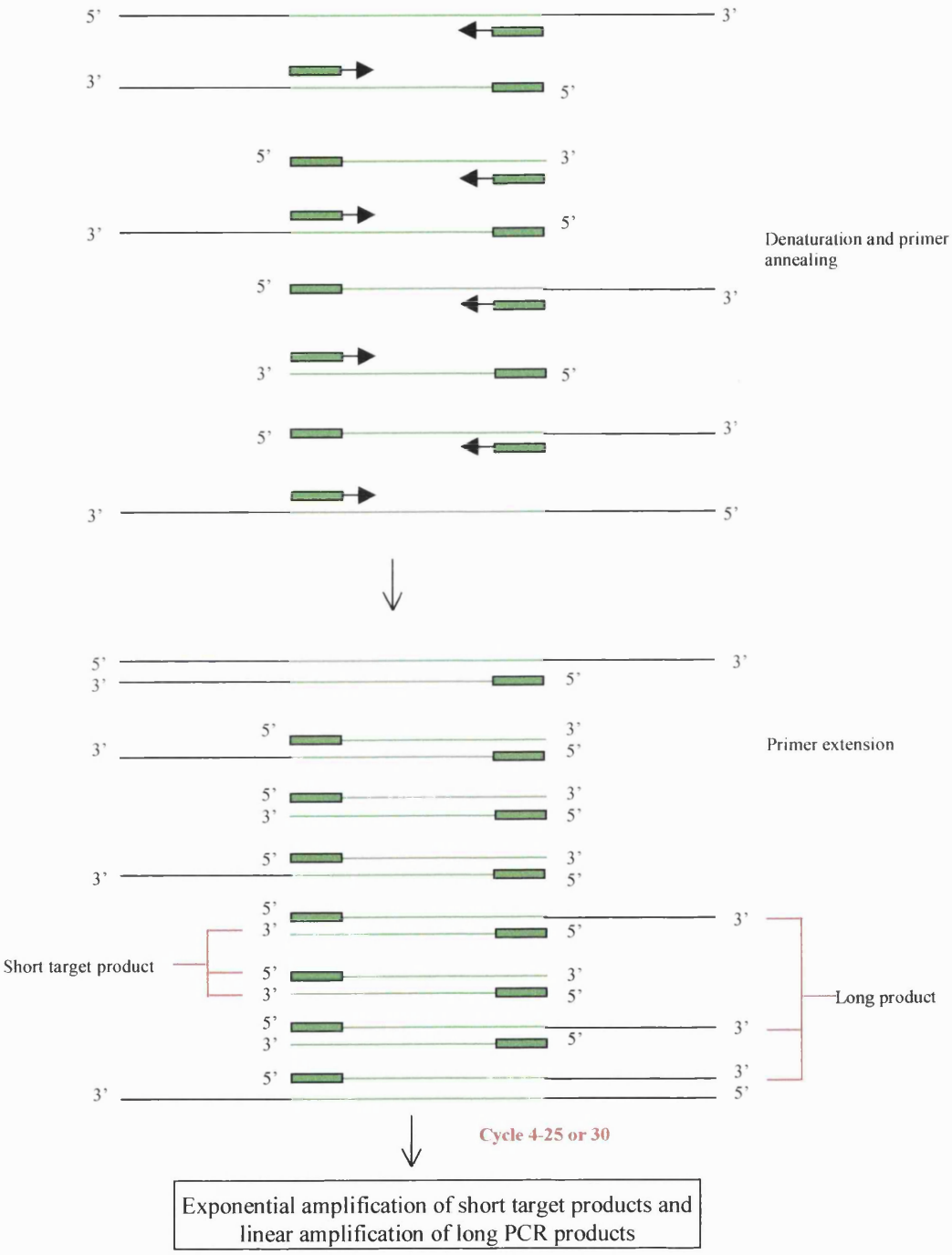


Figure 1.11 (continued)



PCR reaction. At a high temperature, the starting DNA templates are denatured. At a lower temperature, the primers anneal with the single-stranded DNA. The extension follows with the action of DNA polymerase and with the available dNTPs. Repetition of the cycles results in an exponential amplification of short target products and a linear amplification of the long PCR products.

1.6 NON-INVASIVE PRENATAL DIAGNOSIS

All the aforementioned prenatal diagnostic procedures have the disadvantage of being invasive. This has prompted several investigators to look for new less invasive techniques which can be performed at early stages of pregnancy. Two possible interesting sources of embryonic or fetal cells accessible in a non-invasive fashion are maternal peripheral blood circulation and cervical mucus.

1.6.1 FETAL CELLS IN MATERNAL BLOOD CIRCULATION

Placental villi are floating or anchoring in lacunae where they are in direct contact with the mixture of maternal blood and glandular secretion. Embryonic or fetal cells possibly get access into the maternal blood circulation via this contact. Many attempts have been made to detect, isolate and test fetal nucleated cells presented in the maternal peripheral blood circulation. These cells may be lymphocytes, trophoblasts, and nucleated erythroblasts (Adinolfi, 1992). In addition, Wessman *et al.* (1992) reported that granulocytes are also present in the maternal circulation but the value of this kind of cell for prenatal diagnosis seems to be limited. Fetal cells are present in a very low concentration in relation to maternal cells. Nakagome *et al.* (1991), using PCR for the amelogenin region, could not detect fetal cells at a level of 1 in 25,000. Fetal lymphocytes are present in less than 1:100,000 maternal lymphocytes (Yeoh *et al.*, 1991). Lymphocytes express HLA antigen and may be rapidly lysed by the maternal immune response, limiting the chance for them to be detected (Adinolfi, 1992). However, some reports have found fetal lymphocytes persisting in maternal blood long after delivery (Bianchi, 1998). As for trophoblastic cells, they are shed in the uterine vein and become trapped in the maternal lungs. Only a few of them reach the peripheral

circulation and perhaps only 2 out of 10 mothers have a few trophoblastic cells in their peripheral blood (Adinolfi, 1992). Moreover, these cells may be cleared from the circulation by 10-12 weeks of gestation (Beer *et al.*, 1994). Fetal nucleated erythroblasts are the most abundant nucleated cells in fetal blood, comprising ~10% of red cells in the 11-week fetus and 0.5% of the 19-week fetus (Simpson and Elias, 1995). This makes erythroblasts the most promising cell type to be studied in maternal blood. In a study designed to detect fetal nucleated erythroblasts, it has been calculated that there are only 10 μl of fetal blood in the total maternal circulation (Bianchi *et al.*, 1990). In a more recent study, the frequency of fetal cells in the maternal circulation is estimated to be 1 to 91 cells with the mean of 19 cells in 16 ml maternal blood (Bianchi, 1998).

Due to the low amount of cells present, several techniques have been used to isolate them from maternal blood. Fetal cells are different from maternal white blood cells in size, volume and DNA content. Based on these differences, density gradient centrifugation is used to isolate fetal cells from maternal blood. However, this technique cannot separate fetal cells from maternal nucleated red blood cells which are present in a higher number in the circulation than in the nonpregnant stage and other techniques are required to help purify the cells further. Fluorescent activated cell sorting (FACS) is a technique used to sort a specific subtype of cells from a suspension containing several types of cells. It has been used to sort fetal cells from maternal blood (Iverson *et al.*, 1981). The desired cells are labelled by a fluorescent-tagged antibody. The sorting or flow cytometry is carried out through a narrow tube. The cell suspension is passed through this tube and the fluorescently labelled cells give signals which are analysed using a computer according to the set criteria. The desired cells are then separated for the diagnosis. Magnetic activated cell sorting (MACS) is another technique that has

been used to sort fetal cells from maternal blood. It uses tiny metal spheres coated with a monoclonal antibody (immunomagnetic beads), specific to fetal cells to separate the cells from the cell suspension. The fetal cells bind to the beads and are sorted from the suspension by a magnet (Ganshirt-Ahlert *et al.*, 1992). The antibody used in FACS or MACS can also be specific for maternal cells and so the fetal cells would be in the negatively sorted portion. Using these strategies, several authors have reported successes in the detection of fetal cells in maternal blood by PCR and/or FISH (Price *et al.*, 1991; Yeoh *et al.*, 1991; Johansen *et al.*, 1995; Valerio *et al.*, 1997). Sometimes, after these "enrichment" techniques, identification of fetal cells may be needed where the analysis has to be performed on pure fetal cells. Identifying cells with fetal haemoglobin has been used for this purpose (Zheng *et al.*, 1993). Recently, a semi-automated microscopy procedure has been introduced to help scanning for the candidate cells (de-Graaf *et al.*, 1999).

Another approach to enrich fetal cells in maternal blood is cell culture. One of the early reports of fetal cells in maternal peripheral blood was the discovery of 46, XY cells in lymphocyte cultures from pregnant women (Walknowska *et al.*, 1969). For improvement of the result, a special condition that preferentially promotes proliferation of rare fetal cells in the vast majority of maternal cells is needed. Lo *et al.* (1994) and Valerio *et al.* (1996) demonstrated some success using erythropoietin-containing medium or separating and enriching cells with erythropoietin receptor before culture. Recently, Bohmer *et al.* (1999) used charcoal-treated serum to suppress growth of adult red cells and promote growth of fetal red cells. Although adult cell proportions were minimised, they could not be completely suppressed.

From maternal blood, several fetal chromosomal abnormalities have been detected such as trisomy 21, trisomy 18, Klinefelter syndrome, and 47,XYY as well as some Mendelian disorders or traits such as sickle cell anaemia and thalassaemia, HLA polymorphism and fetal Rhesus blood type (Simpson and Elias, 1995; Cheung *et al.*, 1996). Increased frequency of fetal cells in maternal blood is found in many cases where the fetus is abnormal (Simpson and Elias, 1995; Bianchi, 1998). The preliminary evaluation of a multi-institutional study has shown that up to 41% of cases of aneuploidy can be detected from maternal blood (Bianchi *et al.*, 1999).

However, many problems remain to be solved before fetal cells in the maternal circulation can be used for routine prenatal diagnosis. Identification and enrichment of fetal cells are not yet perfect. The monoclonal antibodies used are not absolutely fetal specific and there is high maternal cell contamination. Glycophorin A, which is one of the most commonly used antibodies, can cause agglutination of non-nucleated red blood cells leading to the change of light scatter and fluorescence signals of cells. Magnetic beads interfere with FISH because of the residual bead debris after sorting (Lewis *et al.*, 1996). Furthermore, it is not certain when the cells are shed and when they disappear from the maternal circulation. Thomas *et al.* (1995) found that the earliest detection of Y-chromosome specific sequences in male pregnancies was at 4 weeks and 5 days and they found no such sequences in any of the 18 male pregnancies in their study at 8 weeks after delivery. Moreover, no Y-chromosome sequences were detected in any of the female pregnancies (12 cases). From the recent review by Lamvu and Kuller (1997), looking at several cell types they stated that trophoblasts can be detected in the maternal circulation very early in pregnancy but they are cleared from the circulation by 10-12 weeks of gestation. Nucleated red blood cells reach the maternal circulation at 6 weeks

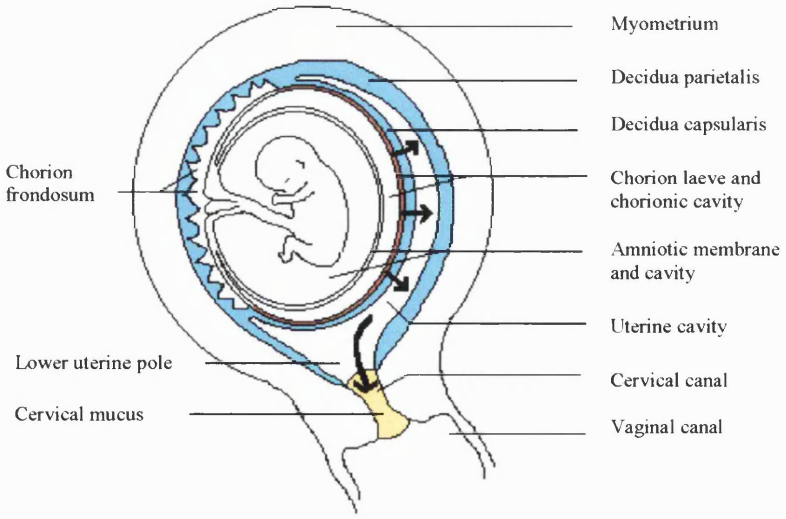
and are cleared from the circulation at 16 weeks of gestation. Leukocytes enter the maternal blood at 14 weeks of gestation and can persist for up to 5 years postpartum. This long persistence of fetal cells may interfere with the analysis in the next pregnancy. Bianchi *et al.* (1996) found male DNA in 4 of 13 pregnancies with female fetuses, 2 of the 4 women had prior pregnancies with a male fetus and the other 2 had prior abortions. In addition, they found male DNA in the blood of 6 nonpregnant women, one of them had given birth to her last son 27 years ago. This persistence of fetal DNA long after delivery can be a source of error if fetal cells in maternal blood are to be used in prenatal diagnosis. However, from the enrichment technique used in the study, it is thought that the persistent cells are lymphoid progenitor cells. It has been suggested that error from the persistent cells can be avoided by targeting other cell types such as erythroblasts (Simpson and Elias, 1995). Nevertheless, a recent study has shown that only half of the erythroblasts in maternal blood are of fetal origin (Troeger *et al.*, 1999). It has also been found that fetal DNA may be detected in maternal plasma and serum (Lo *et al.*, 1997). Fetal DNA in plasma is rapidly cleared from plasma at postpartum, giving less chance that the results are from a previous pregnancy (Lo *et al.*, 1999). Nevertheless, this will only help in DNA analysis. Finally, the important problem in using fetal cells in maternal blood is that the methods to purify the fetal cells are laborious, time consuming, sophisticated, expensive, and may not be suitable for practical use in clinical situations. This leads to some groups seeking other possible sources of fetal cells to be achieved non-invasively.

1.6.2 FETAL CELLS IN TRANSCERVICAL CELL SAMPLES (TCC)

Early in pregnancy, placental villi undergo different development according to their sites. The ones confronting the decidua basalis form a frond-like structure called the chorion frondosum and this will develop into the placenta. The villi covering the outer part of the gestational sac, facing the uterine cavity and covered by the decidua capsularis, is called the chorion laeve. The fate of this part of the villi is not clearly known. It has been suggested that they undergo degeneration, however, it is more likely that they invade through the decidua capsularis as they have invasive properties and fall into the uterine cavity (Rodeck *et al.,* 1995). Either way, this results in the accumulation of trophoblast cells in the lower uterine cavity and the cervical canal **(Figure 1.12)**. These cells can be collected for prenatal genetic studies.

Figure 1.12 Trophoblast from chorion laeve collecting in mucous plug

Modified from Adinolfi and Sherlock (1997)



(Blue colour indicates maternal decidua)

In 1971, Shettles used cotton swabs inserted into the endocervical canal of pregnant women to sample the mid-cervical mucus, and used a Y-chromosome fluorescent dye (quinacrine mustard) to detect male cells (Shettles, 1971). He correctly diagnosed the fetal sex of 10 fetuses, 6 males and 4 females. A year later, Warren *et al.* (1972) could also predict fetal sex in 50 women at all stages of pregnancy. However, Bobrow and Lewis (1971) tried to repeat Shettle's experiment and found no significant difference between the proportion of Y-positive cells in male and female pregnancies. Further studies also had failed to confirmed Shettle's results (Goldstein *et al.*, 1973; Tsuji and Sasaki, 1973; Manuel *et al.*, 1974).

Rhine *et al.* (1975) repeated Shettle's study. They correctly identified 18 male and 13 female fetuses in 23 male and 13 female pregnancies respectively. In 1978, being less successful in fetal sexing using the same technique, Amankwah and Bond (1978) suggested that the conflicting results could be due to the different sites of sampling. By personal communication they found that Rhine's group had obtained samples from the area of the internal cervical os. They expressed their concern about a risk of infection due to the disruption of the mucous plug (Amankwah and Bond, 1978). They also suggested that the analysis of the Y body in interphase cells might not be adequately reliable.

In 1977, Rhine *et al.* developed a device called the Antenatal Cell Extractor fitted with a syringe containing 5 ml of sterile normal saline to perform lower uterine flushing. TCC samples thus obtained were cultured. They found cells with trophoblast morphology in the culture and, from subsequent karyotyping, some cells were shown to be different from the maternal karyotype by the comparison of Q-band polymorphisms (Rhine *et al.*, 1977). A 46,XY chromosome spread had been found in a later study

(Rhine and Milunsky, 1979). However, these results were not confirmed by Goldberg *et al.* (1980) who detected only maternal cells in their cultures of TCC samples obtained by the same technique.

These conflicting results, along with the development of CVS and amniocentesis, made TCC studies less popular for some time. Later, advances in molecular technologies which enable genetic studies to be carried out on a very small amount of sample have prompted several groups to investigate the possibility of using TCC samples as a source of fetal genetic materials. Using PCR to amplify Y-chromosome sequences on samples obtained by cotton swabs, Griffith-Jones *et al.* (1992) correctly predicted fetal sex in 25 out of 26 pregnancies. Moreover, using immunohistochemical analysis, syncytial trophoblast fragments were identified in all TCC samples obtained by lower uterine flushing from another 7 pregnancies. On the contrary, in the same issue of the journal, Morris and Williamson (1992) reported high rates of false-positive and false-negative results using PCR on TCC samples. However, they admitted that the false-positive results might have been due to possible sperm contamination.

To reduce the chance of false-positive results in the PCR assay which might have arisen from contamination with spermatozoa, *in situ* hybridisation was employed by Adinolfi *et al.* (1993). This technique has advantages over PCR since 1) it can give the number of cells present in the sample, thus the frequency of fetal cells can be determined, 2) it can identify the haploid chromosome number of sperm, and 3) it can assess the copy number of the tested chromosome. In their study, lower uterine flushing or lavage with a flexible plastic tube connected to a 10-ml syringe filled with physiologic saline was used to retrieve the TCC samples. Y-derived sequence signals

were found in all 6 TCC samples collected from pregnancies with male fetuses, 4 of which had 18-30% of cells containing a Y signal. Also, in a case of trisomy 18 pregnancy diagnosed by CVS performed 8 days earlier, 26% of cells in TCC sample showed three signals for chromosome 18. Later, using both FISH and PCR for Y chromosome or DNA sequences that are present in fetuses but not in the mothers, more studies have confirmed the presence of fetal cells in TCC samples obtained either by lower uterine lavage or mucous aspirate before any invasive procedures. Examples of DNA sequences used in the studies are RhD sequence in RhD negative pregnant women (Adinolfi *et al.*, 1995a) or polymorphism of short tandem repeats (Adinolfi *et al.*, 1995b).

Subsequently, several studies have been able to detect fetal genetic material in TCC samples either from the lower uterine cavity or endocervical canal. Most of them have used molecular techniques such as PCR or FISH (Kingdom *et al.*, 1995; Briggs *et al.*, 1995; Kawamura *et al.*, 1995; Miller and Briggs, 1996; Daryani *et al.*, 1997) or immunohistochemical techniques (Bulmer *et al.*, 1995). Several groups have tried to increase or purify fetal cells for analysis by cell culture (Bahado-Singh *et al.*, 1995; Ishai *et al.*, 1995; Maggi *et al.*, 1996) or by micromanipulation, *i.e.,* selecting cell clumps that are likely to be trophoblast (Tutschek *et al.*, 1995). Various chromosome anomalies in agreement with the result from the fetus have been detected in TCC samples (Tutschek *et al.*, 1995; Adinolfi *et al.*, 1995c; Maggi *et al.*, 1996; Massari *et al.*, 1996; Sherlock *et al.*, 1997) as well as some haemoglobin mutations (Adinolfi *et al.*, 1997). With increasing evidences of the presence of fetal cells in TCC samples, some private practitioners in Taiwan even perform uterine lavage for fetal sexing which raises some moral concerns (Hsi and Adinolfi, 1997).

However, these promising results are not without a contradiction. In 1996, Overton *et al.* (1996) reported a less successful study, using three different PCR techniques to detect Y-derived DNA in 56 pregnancies and FISH to detect the Y chromosome in 36 pregnancies. They were able to detect Y-derived DNA in 79% of male pregnancies but also in 45% of female pregnancies. They correctly predicted fetal sex in 72% of cases using FISH, but fetal cells could be identified in less than 40% of male pregnancies. Generally speaking, the majority of investigators could demonstrate the presence of fetal genetic material in TCC samples obtained before termination of pregnancy. The results so far have not ensured that TCC samples can be used as a routine prenatal diagnosis or replace the more invasive procedures being currently employed. Variable frequency of fetal cells in the samples has been found. It can be as low as 2% and as high as 50% from lavage and 0.7-3% from aspirate before CVS (Adinolfi *et al.*, 1995c). The proportions of cases with fetal cells in the samples also vary. More work is needed to find a more efficient method for the collection and analysis of the samples, to enable TCC to be employed for practical non-invasive prenatal diagnosis in the future.

1.7 PREIMPLANTATION GENETIC DIAGNOSIS (PGD)

Preimplantation genetic diagnosis (PDG) can be regarded as an early form of prenatal diagnosis. As the name implies, the process is performed before the implantation of the embryos. This helps the couple to start a pregnancy free of the inherited disease at risk in their family thus avoiding the problems associated with TOP (Handyside and Delhanty, 1997). The whole process includes, after identification of the inherited disease at risk in the family, *in vitro* fertilisation (IVF), biopsy of cell(s) to be analysed and the genetic analysis.

1.7.1 *IN VITRO* FERTILISATION (IVF)

This process consists of ovarian stimulation, oocyte retrieval, and fertilisation with the sperm outside the body. Ovarian stimulation is usually accomplished by pituitary down regulation using a gonadotropin releasing hormone analogue (GnRHa) and stimulation with an external gonadotrophic hormone such as human menopausal gonadotropin (hMG), or synthetic follicle stimulating hormone (FSH). The woman must be monitored for the development of ovarian follicles using an ultrasound scan and/or blood oestradiol (E2) levels. Once there is an adequate number of suitable follicles, ovulation is stimulated by the administration of human chorionic gonadotropin (hCG) and the oocyte retrieval performed 34-36 hours after the administration just before the true ovulation takes place. Oocytes are cultured and inseminated with sperm and embryos are transferred on day 2 or 3 postinsemination.

1.7.2 OBTAINING CELL(S) FOR ANALYSIS

After fertilisation, the oocyte completes metaphase II and the second polar body is extruded. The zygote then undergoes mitotic divisions. From these processes, cell(s) from several stages can be utilised for genetic analysis. Biopsy can be performed on the polar body or cells from cleavage stage or blastocyst stage embryos. The most common technique currently in use is cleavage stage biopsy (ESHRE PGD Consortium Steering Committee, 1999).

1.7.2.1 Cleavage stage biopsy

The biopsy is carried out on 1-2 cells from 6- to 8-cell embryos. This is usually on day 3 postinsemination. A small hole is made in the zona pellucida using acid Tyrodes solution or a laser and the blastomere(s) aspirated through the hole. After the biopsy the embryos are kept in culture and the biopsied cell(s) tested. Only unaffected embryos are replaced into the mother. The biopsy is performed at this stage because the cells are still totipotent. The remaining cells usually survive and can still undergo further development (Hardy *et al.*, 1990). Biopsy at the 4-cell stage can retard cleavage (Tarin *et al.*, 1992). Soussis *et al.* (1996) reported the obstetric outcome of 16 pregnancies following PGD at the 8- to 10-cell stage (12 singleton and 4 twins), apart from 3 singleton pregnancies which were lost in first trimester, the remaining pregnancies resulted in 15 healthy babies.

1.7.2.2 Polar body biopsy

In order to avoid embryo biopsy, some groups have used polar body. Verlinsky *et al.* (1996) reported their experience in polar body biopsy for PGD. They tested for

common aneuploidies using FISH and for some single gene disorders in 187 clinical cycles. Three-quarters of the tested cycles resulted in embryo transfers which gave rise to 38 clinical pregnancies and 12 births of an unaffected child (Verlinsky and Kuliev, 1996).

An important drawback of this technique is that it is prone to misdiagnosis due to crossing over between non-sister chromatids in the first meiosis, loss of a single chromatid or a chromosome from the polar body or the primary oocyte, or some errors in meiosis II. In the light of this, sequential analysis of the first and second polar body has been introduced (Verlinsky *et al.*, 1997). This may reduce the chances of misdiagnosis but altogether the polar body analysis is technically and financially demanding. It may not be suitable for clinical PGD in general.

1.7.2.3 Blastocyst biopsy

Blastocyst biopsy is performed on day 5 or 6 when the embryonic cells become separated into the ICM and the TE with collection of fluid in the blastocoel. The technique involves a slit being made in the zona pellucida opposite the ICM and the embryo replace into culture. As the embryo expands, the TE herniates through the slit and can be partially removed for analysis (Dokras *et al.*, 1990). Laser biopsy has been introduced to increase the blastocyst recovery rate (Veiga *et al.*, 1997). Ten to thirty cells from the TE can be biopsied thus giving more cells available for analysis than biopsy at other stages. This could minimise the risk of error in analysis per se or error from mosaicism. Moreover, it should not affect the future embryo/fetus which arises from the ICM. However, with conventional embryo culture, a substantial number of

human embryos arrest before the blastocyst stage, thus hampering the use of blastocyst biopsy clinically.

1.7.3 GENETIC ANALYSIS IN PREIMPLANTATION GENETIC DIAGNOSIS

The next step in PGD is to test the biopsied cell(s). Currently, most centres performed cleavage stage biopsy from which only 1-2 cells are available. The tests carried out on one or two cells must be sensitive and accurate. In addition, the biopsied cells are unlikely to be in metaphase so it is difficult to assess a full karyotype. The development of FISH allows study of a certain chromosome or chromosomes to be performed on interphase nuclei. FISH has been employed for embryonic sexing in couples carrying X-linked diseases (Griffin *et al.*, 1994; Delhanty *et al.*, 1997) for the diagnosis of some chromosomal disorders in high risk couples (Conn *et al.*, 1998) and for age-related aneuploidy (Gianaroli *et al.*, 1999). PCR, which allows DNA analysis on a small amount of DNA, can be used for the diagnosis of single gene defects and triplet repeat disorders (Wells and Sherlock, 1998). However, some problems need to be considered in both techniques. In FISH the major concern is mosaicism. From previous studies, embryos with normal morphology may have sex chromosome or autosome mosaicism or a completely chaotic pattern (Harper *et al.*, 1995; Munné *et al.*, 1995; Delhanty *et al.*, 1997; Magli *et al.*, 1998). This could also cause a problem with PCR, especially for the diagnosis of dominant single gene disorders if the biopsied cell is haploid and carries the normal allele. Other concerns for PCR are contamination with exogeneous DNA and allele drop out (Findlay *et al.*, 1995; Ray *et al.*, 1996). Sometimes PCR is also used for embryonic sexing but this is less informative than

FISH since PCR does not give the copy number of the chromosomes (Harper and Delhanty, 1996b).

1.8 AIMS OF STUDIES

1.8.1 PRESENCE OF FETAL CELLS IN TCC SAMPLES

Several studies could demonstrate the presence of fetal genetic material in TCC samples obtained before TOP. A number of methods of collection have been employed **(Chapter 3)** and various results have been reported **(section 1.6.2)**. For the present study, a commercially available catheter was tested for the collection of TCC samples for analysis. The study would be a further contribution to the information about the presence of fetal cells in TCC samples.

1.8.2 FISH ANALYSIS ON TRISOMIC FETAL TISSUES

Chromosome mosaicism has been found in preimplantation embryos, fetuses, placenta, perinatal and postnatal patients. It may have a role in modifying the phenotype in some chromosomally abnormal cases. In some trisomies, the majority of the affected cases result in pregnancy loss while a small proportion of the affected fetuses can survive to term. This study on trisomic fetal tissues was to determine if there is a correlation of the survival potential of fetuses with the proportion of the trisomic cell line present.

1.8.3 FISH ANALYSIS ON DAY-5 EMBRYOS

Earlier studies on cleavage stage embryos have revealed a high frequency of abnormal chromosome patterns. The abnormality can be uniformly abnormal, mosaic or

without any specific pattern. FISH was used on day-5 embryos to study the chromosome patterns of blastocysts and arrested embryos. Several sets of probes were tried and resulting data from embryos were investigated.

CHAPTER 2

MATERIALS AND METHODS

CHAPTER 2
MATERIALS AND METHODS

Due to the different aspects of the studies in the thesis, sample collections and the rationales for the materials and methods will be described separately in subsequent chapters. This chapter will describe materials and methods used after sample collections, namely, PCR and FISH.

2.1 MATERIALS

Materials and stock reagents used in the study are listed below:

2.1.1 MATERIALS FOR DNA EXTRACTION

2.1.1.1 QIAmp™ Blood Kit (QIAGEN Ltd., UK)

The kit provided collecting tubes, spin columns, Buffer AL (12 ml) for lysis, Buffer AW1 (19 ml) and AW2 (13 ml) for washing, Buffer AE (12 ml) for elution, QIAGEN protease (24 mg) and 1.2 ml protease solvent. Before use, protease was mix with the solvent. Absolute ethanol 45 ml and 30 ml each was added to Buffer AW1 and AW2 respectively. All solutions were stored at room temperature except protease solution which was stored at -20°C.

2.1.1.2 Sodium dodecyl sulphate (SDS; BDH chemicals, UK)

2.1.1.3 Proteinase K (Sigma Chemical Company, USA)

2.1.1.4 Mineral oil (Sigma, USA)

2.1.2 MATERIALS FOR PCR PROCEDURE

2.1.2.1 **Primers** (Table 2.1, section 2.2.2)

2.1.2.2 ***Taq* polymerase** (10x; Promega, UK)

2.1.2.3 ***Taq* polymerase buffer** (10x; Promega, UK)

2.1.2.4 **Ultrapure dNTP set** (100 mM; Pharmacia Biotech, USA)

Stock as 5.0 mM by mixing 5 µl of each dNTP with 80 µl bidistilled water

2.1.2.5 **$MgCl_2$** (25 mM; Promega, UK)

2.1.3 MATERIALS FOR GEL ELECTROPHORESIS

2.1.3.1 **Agarose** (Sigma, USA)

2.1.3.2 **Ethidium bromide** (500 µg/ml; Sigma, USA)

2.1.3.3 **10xTBE**

0.89 M Tris Base, 0.89 M Boric acid and 2.5 mM EDTA

2.1.3.4 **Loading buffer** (10x)

40% sucrose, 0.025% w/v bromophenol blue; 0.025% w/v xylene cyanol

2.1.3.5 **1 Kb DNA ladder** (Gibco, Life Technologies Ltd., UK)

2.1.4 MATERIALS FOR ABI PRISM™ 310

2.1.4.1 **Deionized formamide** (Sigma, USA)

2.1.4.2 **Size standard** (Genescan 500-TAMRA; Perkin Elmer, UK)

2.1.5 MATERIALS FOR LYMPHOCYTE CULTURE

2.1.5.1 **GPS** (Gibco, UK)

Glutamine 200 mM, penicillin 300 mg/ml, streptomycin 500 mg/ml

2.1.5.2 **Iscoves modified Dulbeccos medium** (Sigma, USA)

2.1.5.3 **Fetal calf serum** (FCS; Gibco, UK)

2.1.5.4 **Phytohaemagglutinin** (PHA; Gibco, UK)

2.1.5.5 **Thymidine** (Sigma, USA)

2.1.5.6 **Deoxycytosine** (Sigma, USA)

2.1.5.7 **Colcimid** (Gibco, UK)

2.1.6 MATERIALS FOR EMBRYO SPREADING

2.1.6.1 **Poly-l-lysine** (Sigma, USA)

2.1.6.2 **Tween-20** (Sigma, USA)

2.1.7 MATERIALS FOR DNA PREPARATION FOR FISH PROBES

2.1.7.1 **LB (Luria-Bertani) medium**

10 g/l Bacto tryptone, 5 g/l Bacto yeast extract, 5 g/l NaCl, 1 g/l glucose (Bacto tryptone and Bacto yeast extract from Difco laboratories, USA)

2.1.7.2 **Wizard™ Maxipreps DNA purification system** (Promega, UK)

Contained Cell Resuspension Solution, Cell Lysis Solution, Neutralization Solution, DNA Purification Resin, Maxicolumns and Column Wash Solution.

2.1.7.3 **TE buffer**

10 mM Tris-HCl pH 8.0, 0.1 M EDTA pH 8.0

2.1.8 MATERIALS FOR NICK TRANSLATION (principle method)

2.1.8.1 **Nick translation buffer** (10x)

0.5 M Tris-HCl pH 7.5, 0.1 M $MgSO_4$, 1 mM dithiothreitol (DTT)

2.1.8.2 **Dithiothreitol** (DTT; Sigma, USA)

2.1.8.3 **dNTPs** (Pharmacia, Uppsala, Sweden)

0.5 mM 2'deoxyguanosine 5'-triphosphate

0.5 mM 2'deoxyadenosine 5'-triphosphate

0.5 mM 2'deoxycytidine 5'-triphosphate

0.1 mM 2'deoxythymidine 5'-triphosphate

2.1.8.4 **Fluorescein-12-2'deoxyuridine 5'-triphosphate**

(FITC, fluorogreen; Amersham, UK)

2.1.8.5 **Rhodamine-4 deoxyuridine 5'-triphosphate**

(TRITC, fluorored; Amersham, UK)

2.1.8.6 **DNA polymerase I** (10 unit/μl; Promega, UK)

2.1.8.7 **DNase I** (Boehringer Mannheim, Germany)

Stock as 1 mg/ml in 20 mM Tris-HCl, pH 7.6, 50 mM NaCl, 1 mM DTT, 50% glycerol

2.1.8.8 **Herring Sperm DNA** (10 mg/ml; Sigma, USA)

2.1.8.9 **Sodium acetate** (Fisons Scientific Equipment, UK)

2.1.8.10 **Hybridisation buffer**

60% formamide (BDH, UK)/2x SSC/20% dextran sulphate (BDH)

2.1.8.11 **Standard saline citrate (SSC, stock as 20x)**

175.3 g NaCl, 88.2 g sodium citrate in 1 litre of bidistilled water, adjust pH to 7

2.1.9 MATERIALS FOR NICK TRANSLATION (commercial kit)

Details in 2.3.2.2

2.1.10 MATERIAL FOR FISH

2.1.10.1 **Probes** (non-commercial and commercial; Table 2.2 and section 2.2.3)

2.1.10.2 **Pepsin** (Sigma, USA)

2.1.10.3 **Paraformaldehyde**

37% formaldehyde (Sigma, USA) was saturated with $NaHCO_3$ (BDH, UK) stored in the dark.

2.1.10.4 **Tween-20** (Sigma, USA)

2.1.10.5 **Vectashield** (Vector Laboratories, USA)

2.1.10.6 **4',6-diamidino-2-phenylindole** (DAPI; Sigma, USA)

2.1.10.7 **Nonidet P-40** (NP-40; Vysis Ltd., UK)

2.1.10.8 **Antifade II** (Vysis, UK)

2.1.11 OTHER CHEMICALS AND REAGENTS

Chemicals were from Sigma, USA; BDH, UK, and Fisons Scientific Equipment, UK.

2.1.11.1 **Phosphate buffered saline** (PBS; Sigma, USA)

One tablet was dissolved in 200 ml bidistilled water to obtain 0.01 M phosphate buffer, 0.0027 M KCl, and 0.137 M NaCl, pH 7.4

2.1.11.2 **Acetylcysteine** (Parvolex®, 200 mg/ml; Evan Medical Ltd., UK)

2.1.11.3 **Collagenase type IV** (Sigma, USA)

2.1.11.4 **Glycerol** (BDH, UK)

2.2 POLYMERASE CHAIN REACTION (PCR)

PCR was used in the TCC study. Specimens from each case consisted of maternal blood collected in an ethylene-di-amine-tetra-acetic acid (EDTA) blood tube, cervical mucus collected in sterile normal saline solution and the placenta, also collected in sterile normal saline solution. Maternal blood was prepared for PCR. Placenta and cervical mucus were prepared for PCR and FISH. Preparation for PCR included DNA extraction from samples, PCR procedure and analysis.

2.2.1 DNA EXTRACTION

2.2.1.1 DNA extraction from maternal blood

This was carried out using the QIAmp™ Blood Kit. Maternal whole blood (200μl) was placed in a 1.5 ml microcentrifuge tube, into which 20 μl of QIAGEN protease stock solution, and 200 μl Buffer AL were added and mixed immediately by vortexing for 15 seconds. The mixture was incubated at 56°C for 10 min after which 200μl of 100% ethanol was added and the mixture again vortexed.

A QIAamp spin column was placed in a 2 ml collecting tube and the mixture transferred into the spin column. The spin column, with the cap closed, together with the collecting tube, was centrifuged at 6,000 g (8,000 rpm) for 1 min. The spin column was placed in a new collecting tube and 500 μl Buffer AW1 was added. The spin column and the new collecting tube were centrifuged again at 6,000 g (8,000 rpm) for 1 min. The spin column was subsequently placed in a new collecting tube, 500 μl Buffer AW2 was added and the spin column, along with the collecting tube, was centrifuged at full speed for 3 min. The spin column was placed in a clean 1.5 ml microcentrifuge

tube. Buffer AE (200 µl) was added to elute the DNA. The spin column was left for 1 min at room temperature and centrifuged at 6,000 g (8,000 rpm) for 1 min. The filtrate was collected and the same spin column was placed in another microcentrifuge tube. The collected filtrate was added into the spin column and the spin column, in the new microcentrifuge tube, was centrifuged at the same speed for 1 min. The new filtrate (extracted DNA) was collected and stored at -20°C for PCR.

2.2.1.2 DNA extraction from the placenta (Method 1)

In a sterile laminar flow hood, a small piece of placental tissue (~0.5 cm^3) was rinsed several times with sterile phosphate buffered saline (PBS) and cut into as small pieces as possible with clean scissors. Under a dissecting microscope, a clump of placental cells (100-500 cells) in 2 µl of PBS was transferred into a 0.5 ml microcentrifuge tube for DNA extraction. The remainder of the cut placenta was prepared for FISH.

To the microcentrifuge tube with the placental cell clump, a mixture of 1 µl of 17µM sodium dodecyl sulphate (SDS) and 2 µl of 400 µg/ml proteinase K was added, followed by a drop of mineral oil. The tube was placed in the temperature cycling instrument (GeneAmp™ PCR System 2400, Perkin-Elmer, UK). DNA extraction was carried out for 30 min at 37°C and the enzyme activity was terminated by leaving the tube at 99°C for 15 min. The extracted DNA was stored at -20°C.

2.2.1.3 DNA extraction from the placenta (Method 2)

DNA extraction was carried out using the QIAmp™ Blood Kit. Approximately 100 mg of cut placenta was suspended in 200 µl PBS and the procedure was carried out in the same manner as blood DNA extraction.

2.2.1.4 DNA extraction from TCC samples

An aliquot of 1 ml TCC sample was transferred to a 1.5 ml tube and centrifuged at 13,000 g for 5 min. The supernatant was removed and the cell pellet resuspended in 200 µl PBS. If there was excessive mucus in the TCC sample, it was digested with acetylcysteine **(section 2.3.1.1)** and an aliquot of 200 µl of cell suspension in PBS was used for DNA extraction. DNA extraction was performed in the same manner as for maternal blood except that at the elution step, only 50 µl of buffer AE was used in order to increase the DNA concentration.

2.2.2 PCR PROCEDURE

PCR for the amelogenin region was performed for sexing. In later cases, multiplex fluorescent PCR reactions using labelled amelogenin and short tandem repeat (STR) primers were carried out. Details of the primers used in the study are shown in **Table 2.1**. STRs from chromosomes 21 and 18 and occasionally chromosome 13 were used as DNA markers. They were D21S11 or D21S1414, D18S535 and D13S631. The forward primer of D21S11 or D21S1414 was labelled with 6-FAM (6-carboxyfluorescein, blue), D18S535 with TET (4,7,2',7'-tetrachloro-6-carboxyfluorescein, green) and D13S631 with HEX (4,7,2',4',5',7'-hexachloro-6-

carboxyfluorescein, yellow). D21S1414 was used later as it gave more success in amplification than D21S11. Both primer sets cover the same STR region on chromosome 21 but the forward and reverse primers for D21S1414 are wider apart.

Table 2.1 Primers used in the study, their sequences, chromosome location, size and label*

Type of marker	Marker (F = forward primer) (R = reverse primer)	Sequence of Primers	Chromosome location	Size of the PCR product (bp)	Label (at 5' end of forward primers)**
Amelogenin	AMXY (F)[a] AMXY (R)	5'-ctg atg gtt ggc ctc aag cct gtg-3' 5'-taa aga gat tca tta act tga ctg-3'	Xp22.1-p22.3 Yp11	X - 1000 Y - 823	Unlabelled
	AMXY (F)[b] AMXY (R)	5'-ctg atg gtt ggc ctc aag cct-3' 5'-atg agg aaa cca ggg ttc ca-3'	Xp22.1-p22.3 Yp11	X - 432 Y - 252	6-FAM (blue) -
STRs	D21S11 (F)[b] D21S11 (R)	5'-tat gtg agt caa ttc ccc aag tga-3' 5'-gtt gta tta gtc aat gtt ctc cag-3'	21q21	172-264	6-FAM (blue) -
	D21S1414 (F)[b] D21S1414 (R)	5'-aaa tta gtg tct ggc acc cag ta-3' 5'-caa ttc ccc aag tga att gcc ttc-3'	21q21	330-380	6-FAM (blue) -
	D18S535 (F)[b] D18S535 (R)	5'-tca tgt gac aaa agc cac ac-3' 5'-aga cag aaa tat aga tga gaa tgc a-3'	18q12.2-q12.3	≥ 138	TET (green) -
	D13S631 (F)[b] D13S631 (R)	5'-ggc aac aag agc aaa act ct-3' 5'-tag ccc tca cca tga ttg g-3'	13q31-q32	≥ 209	HEX (yellow) -

*The table is modified from Sherlock *et al.* (1998) with permission.

**The fluorescent dyes were 6-FAM (6-carboxyfluorescein, blue), TET (4,7,2',7'-tetrachloro-6-carboxyfluorescein, green), and HEX (4,7,2',4',5',7'-hexachloro-6-carboxyfluorescein, yellow).

[a]The primers were supplied by Oswel DNA services (used in the earlier part of the study).

[b]The primers were supplied by Perkin-Elmer, UK.

2.2.2.1 PCR for the amelogenin region

PCR reactions were performed in a final volume of 25 µl. This consisted of 5 µl of DNA template, 15 µl of PCR reaction mix and 1.5 units of *Taq* polymerase in 5 µl of sterile water, the latter being added after the other components were heated to 94°C (hot start, see below). The PCR mix was prepared in a laminar hood using a specific set of Gilson pipettes and pipette tips under sterile conditions to minimise contamination. The mix consisted of 0.25 µl of 20 pmol/µl of each primer (forward and reverse), 1x *Taq* polymerase buffer, 1 µl of dNTP stock, 1.5 mM $MgCl_2$ and sterile water to make up to 15 µl. Each mix was put into a PCR microtube, followed by a drop of mineral oil. Each sample of DNA extract (5 µl) was added into the kit under the oil. A positive control from a normal male DNA and a negative control in which 5 µl of sterile water was used instead of DNA were prepared for each PCR performed. In placental samples that were extracted by method 1 **(section 2.2.1.2)**, the DNA was already in the tube under the oil, therefore the PCR mix was later added under the oil. All the tubes were heated to 94°C and the following PCR protocol was carried out:

Step 1:	94°C	5-15 min (hot start)	

At about 5 min after Step 1 was started, 1.5 units of *Taq* polymerase in 5µl of sterile water was added into each tube under the oil and PCR carried on to the next steps.

Step 2:	93°C	45 sec	
	65°C	45 sec	
	72°C	1 min	Step 2 for 35 cycles
Step 3:	72°C	5 min	

In cases where fluorescent PCR was used, the forward primer was labelled with 6-FAM (blue) (Perkin-Elmer) and the PCR product detected using the automated laser DNA analyser and the appropriate software.

2.2.2.2 PCR for short tandem repeats (STRs)

Due to the variability of PCR amplification among primer sets, primers were included at varying concentrations to ensure comparable amounts of PCR products. The final PCR mix consisted of the relevant primer sets, and other reagents as in non-multiplex reactions. Reactions were performed in a final volume of 25 µl. All samples underwent 35 repeating PCR cycles (step 2) at 93°C for 45 sec, 60°C for 45 sec, and 72°C for 1 min except maternal blood which underwent 25 PCR cycles. Step 1 and step 3 remained the same as PCR for the amelogenin region alone.

2.2.3 PCR ANALYSIS

2.2.3.1 Gel electrophoresis

This method of analysis was used in a few earlier cases only. A 1% agarose gel containing ethidium bromide (0.1 µl/ml) was prepared by mixing 0.5 g agarose in 50 ml of 1x TBE (stock as 10x, **2.1.3.3**). The mixture was heated in a microwave at full power until the agarose dissolved (approximately 2 min). Ethidium bromide (5µl) was mixed into the agarose mixture and the mixture poured into a gel tank with a 8 or 16 well-forming-comb and left to set at room temperature. The comb was removed. The set gel was immersed in 50 ml of 1x TBE. Each PCR product (8 µl), mixed with a one-tenth volume of loading buffer, was loaded into each well. One Kb DNA ladder was also loaded as a reference. Electrophoresis was performed for 30-60 min after which the gel was viewed under ultraviolet trans-illumination. The expected sizes of the PCR products of the Y and the X chromosomes were 823 bp and 1,000 bp respectively.

2.2.3.2 Automated laser DNA analyser (ABI Prism™310)

PCR products were separated and analysed using an automated laser DNA analyser (ABI Prism™ 310, Perkin-Elmer, UK) and the appropriate software (Genescan version 2.0.2). Each fluorescent PCR product (1μl), mixed with 12 μl deionized formamide and 0.5 μl of size standards (Genescan 500-TAMRA [red], Perkin-Elmer, UK) were run through a capillary (15 kVolts, 24 min at 60°C) after 2 min denaturation at 95°C. The specific PCR products were sized and STR alleles of the mother, placenta and TCC samples were compared **(Chapter 3)**.

2.3 FLUORESCENT *IN SITU* HYBRIDISATION (FISH)

FISH was used in all the studies in the thesis. The FISH process consists of:

- Sample or control lymphocyte cell suspension/slide preparation
- Probe preparation which consists of DNA preparation and nick translation or using commercially available probes
- FISH procedure and analysis

Sample preparation for FISH in each study differed slightly and will be described in separate sections.

2.3.1 SAMPLE AND CONTROL LYMPHOCYTES PREPARATION

2.3.1.1 Placental tissue and TCC preparation

Placental cell suspension for FISH

Approximately 0.5 cm^3 of the cut-up placenta was transferred into a conical centrifuge tube with 2-3 ml of PBS. To obtain a suspension of single cells, 1 ml of 0.2% collagenase type IV was added, and the suspension incubated at 37°C, with intermittent agitation for 15 min or until the tissue disintegrated. The suspension was centrifuged at 1,000 rpm for 5 min and the supernatant removed. The cell pellet was resuspended in 5 ml PBS and re-centrifuged. The supernatant was discarded and the washing procedure repeated. After removal of the supernatant in the last cycle, the cell pellet was resuspended in 5 ml 50 mM KCl which was pre-warmed to 37°C. The suspension was incubated at 37°C for 15-20 min after which 1 ml of fixative (methanol:glacial acetic acid, 3:1) was added and mixed. The solution was centrifuged at 1,000 rpm for 5 min and the supernatant discarded. The cell pellet was resuspended in 5 ml fixative,

recentrifuged at the same speed and duration and the supernatant discarded. The cell pellet was resuspended and centrifuged and the supernatant was discarded to make sure that the pellet was in fresh fixative. The cell suspension was stored at -20°C.

TCC cell suspension for FISH

If there was excessive mucus present in a TCC sample, or the mucus was very thick, 500 µl of acetylcysteine was added to the sample which was then incubated at 37°C for 30 min with intermittent shaking to dissolve the mucus and dislodge cells. PBS (2-3 ml) was added and mixed by pipetting. The sample was centrifuged at 1,000 rpm for 5 min and the supernatant discarded. The pellet was mixed in 5 ml PBS and centrifuged at the same speed and duration. The supernatant was removed and the pellet re-suspended in 5 ml 50 mM KCl which was pre-warmed to 37°C and subsequently treated in the same way as the preparation of placental cell suspension for FISH.

2.3.1.2 Fetal tissue preparation

In the early part of the study, fetal tissues were prepared into cell suspension for FISH in the same way as placental cell suspension in TCC study. Later in the study, the tissues were cut on the day when FISH was to be performed. The cut surface was directly dabbed on a clean slide and left to air-dry. The slides were flooded with fixative, allowed to dry, flooded with 70% acetic acid, allowed to dry and dehydrated through an ethanol series for subsequent FISH.

In addition, suspensions of skin fibroblast cultures from three fetuses with trisomy 13, 18 and 21 and another fetus with triploidy were kindly prepared by Professor Joy Delhanty, Department of Obstetrics and Gynaecology, University College

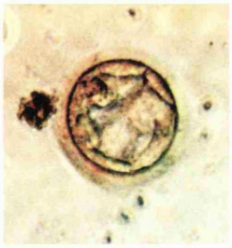
London, UK, to be used to determine the accuracy of the scoring in interphase compared to metaphase nuclei in trisomic or triploid specimens.

2.3.1.3 Day-5 embryo spreading

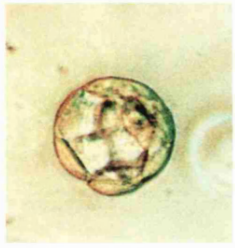
Poly-l-lysine coated slides Poly-l-lysine coated slides were used to enhance the fixation of nuclei on slides. They were prepared by placing glass slides in a mixture of 40 ml 100% methanol and 0.5 ml 1 N HCl for 5-10 min. The slides were left to air-dry and rinsed in 1:10 Poly-l-lysine in water v/v for 5 min. They were left at room temperature overnight and were stored at 4°C until needed.

The embryos were spread as described previously **(Figure 2.1)** (Harper *et al.*, 1994). In brief, the embryos were washed in PBS. If there were cumulus cells attached, they were removed by gentle pipetting. The embryos were transferred to a small drop of spreading solution (0.01 N HCl, 0.1% Tween-20) on a poly-l-lysine coated slide. The embryo was constantly observed under an inverted microscope and the spreading solution removed and replaced to dissolve the zona pellucida and the cytoplasm. The nuclei were washed by gentle agitation of the spreading solution until clear of cytoplasm. The slides were left to air-dry, washed in PBS for 5 min and dehydrated through an ethanol series (70%, 90% and 100% respectively). The nuclei were located using an England Finder (Graticules Ltd., UK) under phase contrast and a map was drawn. The slides were kept at room temperature for subsequent FISH.

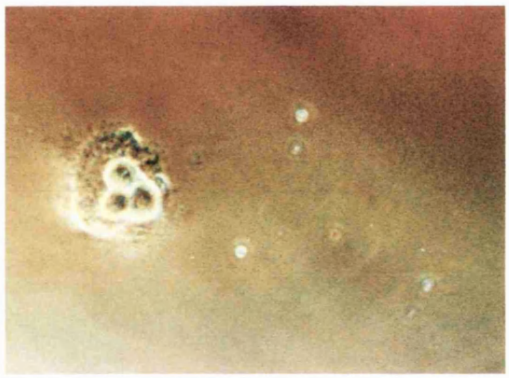
Figure 2.1 Embryo Spreading



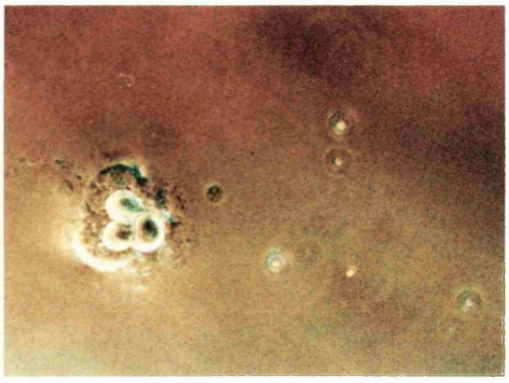
a)



b)



c)



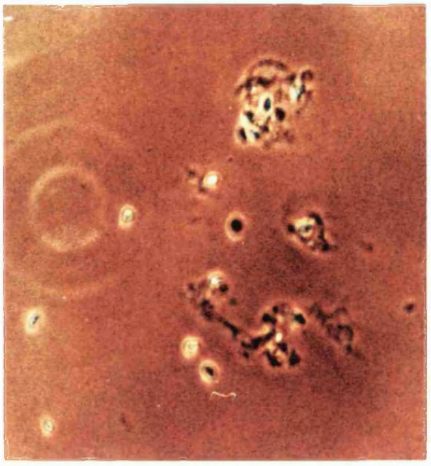
d)

a) A blastocyst with cumulus cells attached to its zona pellucida.

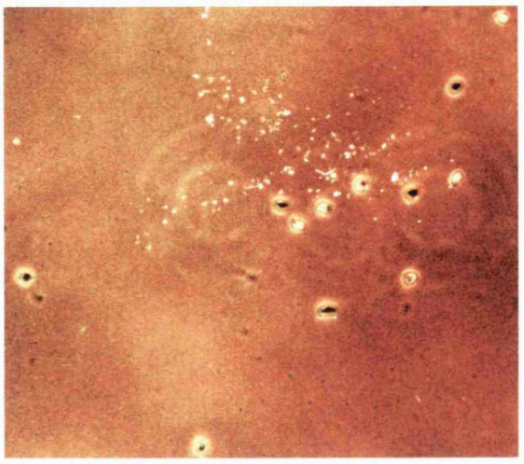
b) Blastocyst with the cumulus cells and the zona pellucida removed.

c) and d) The blastocyst beginning to lyse. Some of the single cells being spread out (focusing on different planes to show the already spread cells).

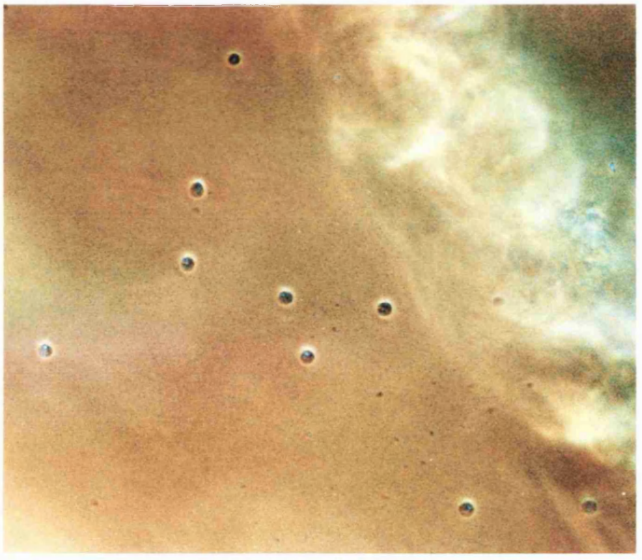
Figure 2.1 (continued)



e)



f)



g)

e) The blastomeres in spreading solution, cytoplasm still can be seen.

f) The cytoplasm clearing.

g) The nuclei clear of cytoplasm.

2.3.1.4 Control lymphocyte culture

In every FISH procedure, a control slide was processed to ensure the efficiency of the probes. Control slides were prepared from cultured lymphocytes from a normal male donor. The procedure for the culture is described below:

GPS (2 ml: glutamine 200 mM, penicillin 300 mg/ml and streptomycin 500 mg/ml) was added to 100 ml Iscoves modified Dulbeccos medium. This mixture was warmed to 37°C along with fetal calf serum (FCS). Donated blood was collected in a lithium heparin tube, 1 ml of which was put into a 50 ml culture flask along with 17 ml of the pre-warmed medium with GPS, 2 ml of the pre-warmed FCS and 200 µl phytohaemagglutinin (PHA) under aseptic conditions. The flask was shaken and incubated at 37°C for 48 hours during which the flask was shaken at 12-24 hour intervals. At 48 hours, 200 µl of thymidine (30 mg/ml) was added, and the flask incubated at 37°C. Eighteen hours later, 200 µl of deoxycytosine (0.277 mg/ml) was added to the flask. Four hours later, 200 µl of colcimid (10 µg/ml) was added and the flask, shaken and incubated at 37°C for another 20 min. The mixture was transferred to two 10 ml conical centrifuge tubes which were centrifuged at 1,000 rpm for 5 min. The supernatant was discarded and 10 ml of 75 mM KCl, pre-warmed to 37°C, was slowly added to resuspend the pellet and the tubes were left at room temperature for 15-20 min. The tubes were centrifuged at 1,000 rpm for 5 min. The supernatant was removed with a small amount left to resuspend the pellet. Fresh fixative was added 1-2 drops at a time while the tube was tapped to resuspend the pellet. When the contents stopped frothing, the fixative could be added more quickly up to a volume of 10 ml. The contents were centrifuged at the same speed and duration, and the supernatant was discarded. The

fixative was added a few ml at a time until 10 ml reached, and the contents centrifuged. This step was repeated until the pellet was white and the supernatant clear. The lymphocyte suspension was stored at -20°C until needed.

2.3.1.5 Slide preparation

For control lymphocyte culture and samples that were prepared in a suspension (TCC, placenta, some fetal tissues, and fetal skin fibroblast cultures), slides were made on the same day of the FISH procedure. Most of the fixative was removed and the pellet resuspended in fresh fixative according to the size of the pellet. After breathing on a clean slide, a drop of cell suspension was dropped onto the slide. The slides were warmed on the back of the hand to ensure nuclei spreading, and were left to air-dry. The slides were flooded in fixative for 10 seconds, left to dry, flooded in 70% acetic acid for 10 seconds and left to air-dry. The slides were inspected under a phase-contrast microscope for the distribution and appearance of nuclei, dehydrated through an ethanol series and left to dry.

For some fetal tissues that were directly dabbed on slides, the slides were treated similarly **(section 2.3.1.2)**.

For slides with blastomeres, they were already dehydrated thus FISH could be carried out straight away.

2.3.2 NON-COMMERCIAL PROBES

Non-commercial probes were used in the study of TCC (for chromosomes X and Y), the earlier part of fetal tissues study (for chromosomes X, Y, 1 and 18) and some parts of the study of day-5 embryos (for chromosome 16 only). The details of the probes are shown in **Table 2.2**.

Table 2.2 Details of probes used in FISH (non-commercial probes)

Probe	*Type*	*Insert size (kb)*	*Location*	*Label*	*Source*
pBam X5	alphoid	2.0	Centromeric region of the human X chromosome	Flourescein-12-2'deoxuridine-5'triphosphate (FITC)	(Willard *et al.*, 1983)
cY98	alphoid	3.77	Repeated sequence of the long arm of the human Y chromosome	Rhodamine-4-deoxuridine-5' triphosphate (TRITC)	Wolfe, personal communication
pUC 1.77	Satellite III	1.77	Centromeric region of the human chromosome 1	Either FITC or TRITC	(Cooke and Hindley, 1979)
pSE16	Alpha satellite	49.4	Centromeric region of the human chromosome 16	Either FITC or TRITC	(Greig *et al.*, 1989)

2.3.2.1 DNA preparation and purification

Non-commercial probes were prepared from bacterial cloned stocks. A bacterial colony was scraped from the top of a glycerol stock, inoculated into a 4 ml Luria-Bertani (LB) medium **(2.1.7.1)** supplemented with 100 μg/ml ampicillin or 25 μg/ml kanamycin, and incubated at 37°C for approximately 5-6 hours with continuous shaking. The contents were used to seed a 200 ml LB culture medium and incubated overnight at 37°C with continuous shaking.

Purification of DNA was performed on the following day using a Wizard™ Maxipreps DNA Purification System **(2.1.7.2)**. The culture was centrifuged at 5,000 g for 10 min at 22-25°C in a room temperature rotor. The cell pellet was carefully resuspended in 15 ml Cell Resuspension Solution. Cell Lysis Solution (15 ml) was added and mixed gently by stirring or inverting until the solution became clear and viscous. Neutralization Solution (15 ml) was added and gently mixed by inverting. The solution was centrifuged at 14,000 g for 15 min at 22-25°C in a room temperature rotor. The supernatant volume was measured and a half volume of room temperature isopropanol was added and mixed by inverting. The mixture was centrifuged at 14,000 g for 15 min at 22-25°C in a room temperature rotor. The supernatant was discarded and the pellet resuspended in 2 ml TE buffer. Wizard™ Maxipreps DNA Purification Resin (10 ml) was added. The solution was transferred into a Maxicolumn with its tip inserted into a vacuum source. A vacuum was applied to pull the solution through. Column Wash Solution (13 ml) was added to the bottle that had contained the DNA/Resin mix, the bottle was swirled and the solution poured into the Maxicolumn with vacuum applied. Another 12 ml of Column Wash Solution was added to the Maxicolumn, also with a vacuum applied. Ethanol (80%, 5 ml) was subsequently added to the Maxicolumn to rinse the Resin. A vacuum was applied to draw the ethanol through the Maxicolumn and allowed to draw for an additional 10 min. The Maxicolumn was placed in a 50 ml screw cap tube and 1.5 ml of preheated (65-70°C) water or TE buffer was added. After one minute, elution of DNA was carried out by centrifuging at 2,500 rpm (1,300 g) for 5 min. The DNA concentration in the filtrate was measured and the filtrate was stored at -20°C or 4°C.

2.3.2.2 Probe labelling (Nick translation)

Nick translation, Principle method

This method was described previously (Harper and Delhanty, 1996b). On ice, all the following reagents were mixed in a 1.5 ml microcentrifuge tube:

5	µl	10x nick translation buffer (0.5 M Tris-HCl, pH 7.5, 0.1 M magnesium sulphate, 1 mM dithiothreitol [DTT])
5	µl	0.1 M DTT
4	µl	Nucleotide mix (0.5 mM 2'deoxyguanosine 5'-triphosphate; 0.5 mM 2'deoxy adenosine 5'-triphosphate; 0.5 mM 2'deoxycytidine 5'-triphosphate; and 0.1 mM deoxythymidine 5'-triphosphate)
3	µl	1 mM label (Fluorescein-12-2'deoxyuridine 5'-triphosphate [FITC, green] or rhodamine-4 deoxyuridine 5'-triphosphate [TRITC, red])
x	µl	1 µg of DNA probe (volume depending on the concentration of the DNA stock)
2	µl	DNA polymerase I (10 unit/µl)
5	µl	1:1000 DNase I (1 mg/ml stock in 20 mM Tris-HCl, pH 7.6, 50 mM NaCl, 1 mM DTT, 50% Glycerol)
(50-x)	µl	Bidistilled water

The mixture was incubated at 15°C for two hours. After 1 hour, 5 µl of 1:1000 DNase I was added. At the end of the two hours incubation, 5 µl of 0.5 M EDTA was added and DNA precipitation followed. This was performed by adding 5 µl Herring Sperm DNA (10 mg/ml), 1/10 volume of 3 M sodium acetate (6 µl), and 1 ml ice-cold 100% ethanol. The tubes were inverted several times and incubated at -70°C for 1 hour or -20°C overnight. This was followed by centrifugation at full speed for 15 min. The supernatant was removed and the pellet left to dry in the dark and was resuspended in 50 µl hybridisation buffer **(2.1.8.10)** and stored at 4°C in the dark.

Nick translation using a commercial nick translation kit

This kit (Vysis(UK), Ltd.) contained:

- Nick translation enzyme mix
- 10x nick translation buffer
- dATP, dCTP, dGTP and dTTP (0.3 mM, each)
- Nuclease-free water

Fluorescent-labelled dUTPs used with the kit were SpectrumOrange-dUTP and SpectrumGreen-dUTP from Vysis (UK) Ltd.

Preparing the working solutions from the commercial kit:

- 0.2 mM SpectrumGreen or SpectrumOrange dUTP was prepared by adding 10 µl of 1 mM dUTP to 40 µl nuclease-free water.
- 0.1 mM dTTP was prepared by adding 10 µl of 0.3 mM dTTP to 20 µl nuclease-free water.
- 0.1 mM dNTP mix was prepared by mixing all the following together: 10 µl each of 0.3 mM dATP, 0.3 mM dCTP and 0.3 mM dGTP.

On ice, all of the following reagents were added in a 1.5 ml eppendorf tube: (17.5-X) µl of Nuclease-free water, X µl (1 µg) of extracted DNA, 2.5 µl of 0.2 mM SpectrumGreen or SpectrumOrange dUTP, 5.0 µl of 0.1 mM dTTP, 10 µl of 0.1 mM dNTP mix, and 5 µl of 10x nick translation buffer. The tubes were briefly centrifuged and mixed well. Ten microlitres of nick translation enzyme was added. The tubes were briefly centrifuged and mixed well. They were incubated at 15°C for 2 hours, after

which 5 µl stop buffer (0.3 M EDTA), 5 µl herring sperm DNA, 6 µl of 3 M sodium acetate and 1 ml of ice-cold 100% ethanol were added and mixed well. The tubes were incubated at -70°C for 1 hour after which they were centrifuged at high speed (13,000 rpm) for 10-15 min. The excess fluid was carefully discarded, leaving the pellet in the tube to air-dry. The pellet was resuspended in 100 µl hybridisation buffer **(2.1.8.10)** and kept at 4°C in the dark.

2.3.3 COMMERCIAL PROBES

Commercial probes were used in the later part of the fetal tissues study and the study of day-5 embryos.

AneuScreen Prenatal Aneuploidy Detection Panel [Vysis (UK) Ltd.]. This panel consisted of two probe mixtures with the details in **Table 2.3**. The hybridisation sites of the probes were shown in **Figure 2.2**.

Vysis™ MultiVysion™ PGT [Vysis, (UK) Ltd.]. This assay consisted of a 5-colour, 5-probe mixture for chromosomes 13 (red), 18 (aqua), 21 (green), X (blue) and Y (gold). The hybridisation sites of the probes were the same as the AneuScreen Prenatal Aneuploidy Detection Panel **(Figure 2.2)**.

Table 2.3 AneuScreen Prenatal Aneuploidy Detection Panel

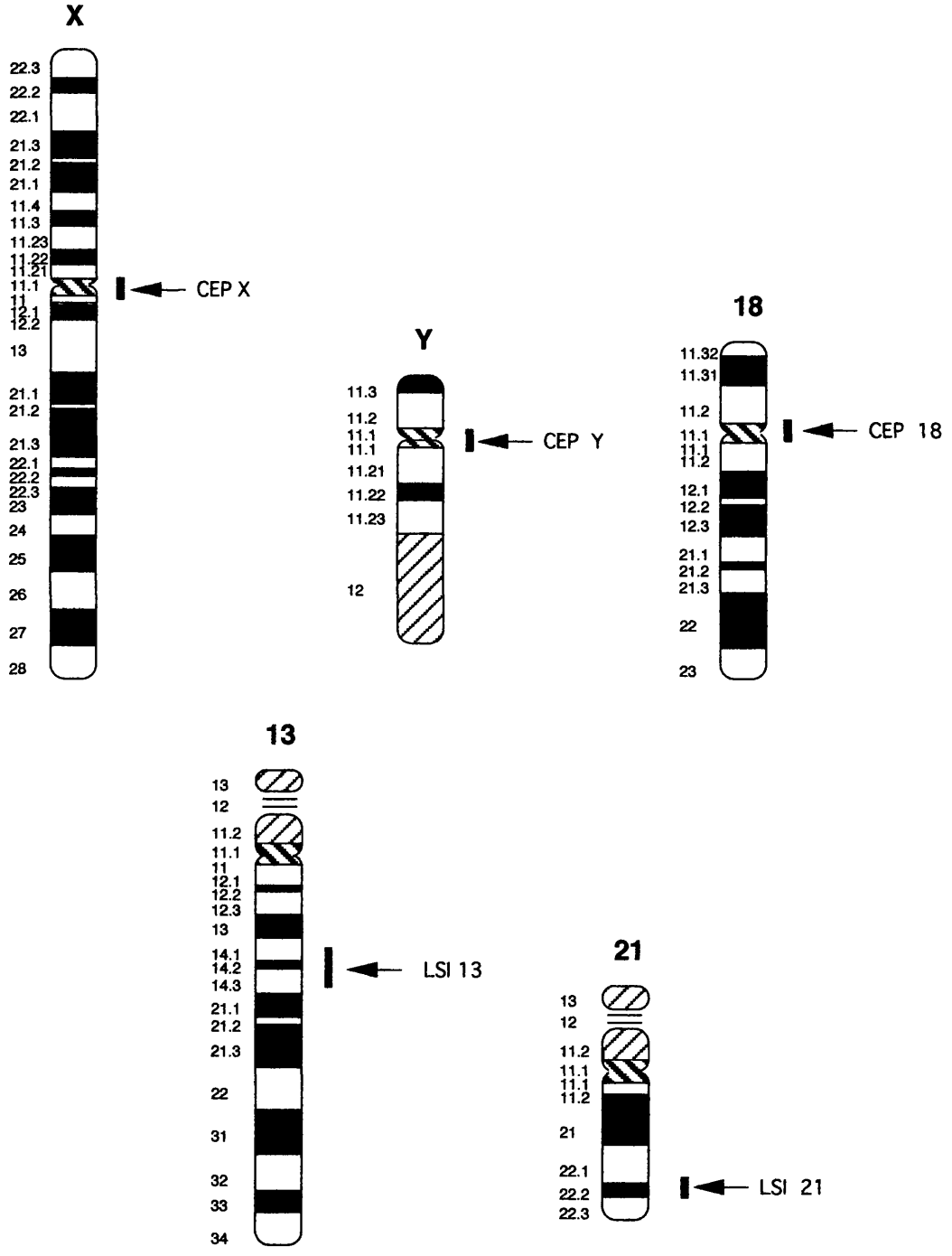
Probe mixture #1 (Alpha satellite probes for chromosomes X, Y and 18)

CEP18 SpectrumAqua	Alpha satellite D18Z1	centromere region of chromosome 18 (18p11.1-q11.1)
CEPX SpectrumGreen	Alpha satellite DXZ1	centromere region of chromosome X (Xp11.1-q11.1)
CEPY SpectrumOrange	Alpha satellite DYZ3	centromere region of chromosome Y (Yp11.1-q11.1)

Probe mixture #2 (Locus specific probes for chromosome 13 and 21)

LSI 13 SpectrumGreen	LSI 13 RB-1, 13q14
LSI 21 SpectrumOrange	Loci D21S259, D21S341, D21S342 (21q22.13-q22.2)

Figure 2.2 Positions of hybridisation on each chromosome for commercial probes



2.3.4 FISH PROCEDURE (Non-commercial probes)

The method used was as described previously (Harper *et al.*, 1994).

The slides were incubated in 100 μg/ml pepsin in 0.01 N HCl at 37°C for 20 min, after which they were washed in bidistilled water and PBS. The slides were incubated in 1% paraformaldehyde **(2.1.10.3)** in PBS at 4°C for 10 min, washed in PBS and washed twice in bidistilled water. The slides were dehydrated through an ethanol series and air-dried. Probes were applied to the slide under a cover-slip. The probes and target nuclear DNA were simultaneously denatured at 75°C for 3 min. The slides were incubated at 37°C in a moist chamber for 1 hour. The slides were washed at 42°C in 60% formamide/2x SSC (standard saline citrate, **2.1.8.11**) and 2x SSC for 5 min each before they were washed at room temperature in 4x SSC/0.05% Tween-20 twice, 5 min each. After the room temperature washes, the slides were dehydrated through an ethanol series, air-dried and mounted in Vectashield containing 1 μg/ml 4',6-diamidino-2-phenylindole (DAPI). The signals were visualised using a Zeiss Axioscope fluorescence microscope, captured using a cool coupled charged device (CCD) camera (photometrics) and analysed using Smartcapture software (Vysis (UK), Ltd.). FISH signals were scored according to Hopman *et al.* (1988). Paired spots with less than one spot's diameter apart were scored as one signal as they might be sister chromatids of a chromosome at the stage of DNA replication.

2.3.5 FISH PROCEDURE (Commercial probes)

Probes for chromosomes X, Y and 18 (alpha satellite probes)

These were already mixed in Probe mixture #1 in AneuScreen Prenatal Aneuploidy Detection Panel **(Table 2.3)**. FISH was carried out in the same way as non-commercial probes.

Probes for chromosomes 13 and 21 (locus specific probes)

FISH was carried out in the same way as for non-commercial probes up to the step of probe application. Probes were applied to the slide under a cover-slip, sealed with rubber cement. The probes and the target nuclear DNA were simultaneously denatured at 75°C for 5 min. The slides were incubated overnight at 37°C in a moist chamber. Post-hybridisation washing consisted of three washes in 50% formamide/2x SSC, three washes in 2x SSC at 45°C for 3 min each, followed by three washes in 4x SSC/0.05% Tween-20 at room temperature for 3 min each. After the room temperature washes, the slides were dehydrated through an ethanol series and air-dried. The slides were mounted and analysed in the same way as described for FISH using non-commercial probes.

MultiVysion™ PGT

FISH was carried out in the same way as for non-commercial probes up to the step of probe application. After paraformaldehyde fixation and dehydration, 3 µl of probe was applied on each slide under a cover-slip and sealed with rubber cement. The slides were denatured at 73°C for 5 min, followed by hybridisation at 37°C for 4-8 hours. Post-hybridisation washing consisted of one wash in 0.4x SSC/0.3% NP-40 at 73°C for 5 min followed by one wash in 2x SSC/0.1% NP-40 at room temperature for 1

min. The slides were left to dry in the dark and mounted in Antifade II solution. The slides were analysed using an Olympus fluorescence microscope, images captured by a cooled CCD camera and analysed using Quips™ Imaging Software (Vysis (UK), Ltd.).

CHAPTER 3

FETAL CELLS

IN TRANSCERVICAL CELL SAMPLES

CHAPTER 3
FETAL CELLS
IN TRANSCERVICAL CELL SAMPLES

3.1 INTRODUCTION

After implantation, the conceptus enlarges and stretches the covering decidua capsularis. This in turn compresses the chorionic villi underneath the decidua capsularis, leading to flattening of the villi. As a result, the chorionic sac in this region is smooth and is called the chorion laeve. With the further enlargement of the gestational sac, the area of implantation bulges into the uterine cavity and around the 12^{th} week or later, the decidua capsularis fuses with the opposite decidua parietalis, obliterating the uterine cavity. Before the fusion, it is thought that the trophoblastic cells at the chorion laeve either degenerate or invade through the decidua capsularis and fall into the uterine cavity, collecting in the lower uterine pole and in the cervical mucus. Some conflicting studies have supported or contradicted the presence of fetal material in the samples collected from the endocervical canal or the lower uterine pole (TCC samples). From these studies, the methods to collect TCC samples can be listed into 4 groups as follows (Shettles, 1971; Rodeck *et al.*, 1995):

1. Cotton swab **(Figure 3.1b)**. This method had been used in earlier studies. A cotton swab was inserted into the endocervical canal, rotated, and withdrawn. This technique was associated with some difficulties in cell retrieval from the swab itself and yielded poor results (Kingdom *et al.*, 1995).

2. Cytobrush. A cytology brush was used in the same manner as the aforementioned cotton swab. The end of the brush was cut off into a container which

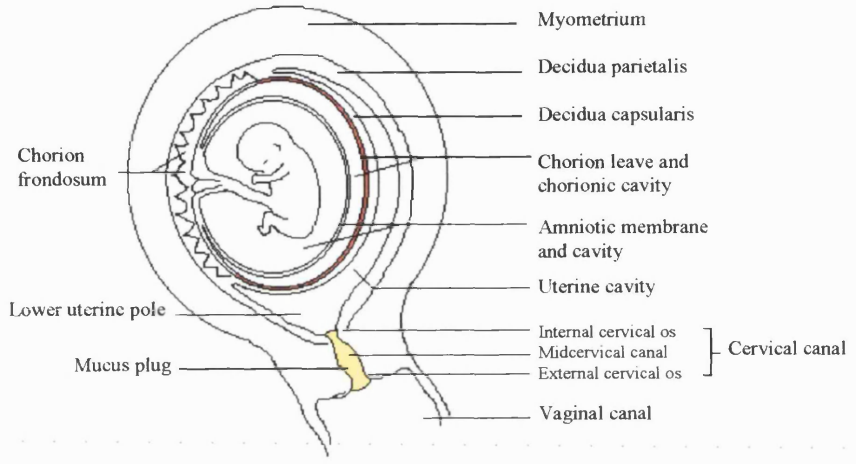
contained physiologic saline solution. The cells, mucus and debris were dislodged into the saline by shaking the container. With this device maternal cellular debris were also collected with the increased maternal cell content and this procedure was considered ineffective (Kingdom *et al.*, 1995).

3. Lavage/flushing/irrigation. This method has been carried out both in the lower uterine portion **(Figure 3.1c)** and the cervical canal **(Figure 3.1d)**. It usually involves a flexible catheter connected to a syringe filled with physiologic saline; after the catheter is inserted into the cervical canal, the fluid is gently injected and aspirated back. The catheter is then withdrawn and the fluid collected. The lower uterine lavage gives more chance of obtaining fetal material than collection at the lower level but there would be concern about the safety of injecting saline into the uterine cavity.

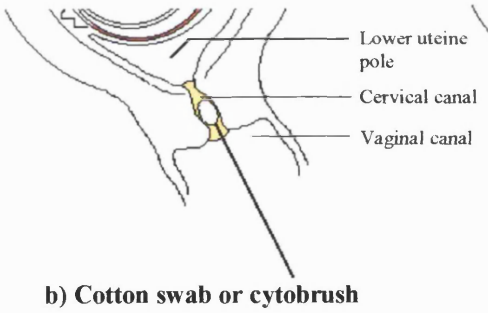
4. Aspiration. This technique is similar to lavage but without injection of fluid; instead, the mucus is directly aspirated into the catheter **(Figure 3.1e)**. It is performed in the endocervical canal or at the level of the internal os only.

Figure 3.1 Retrieval techniques of TCC samples

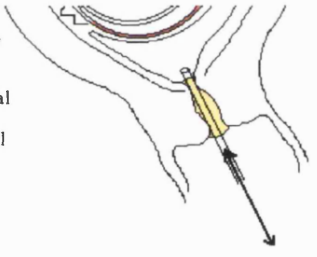
Modified from Rodeck et al. (1995)



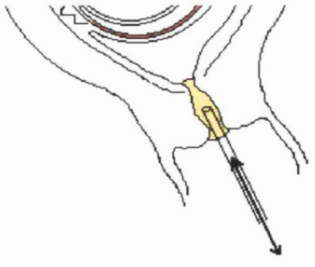
a) Positions of endocervical mucus and lower uterine pole in early pregnancy



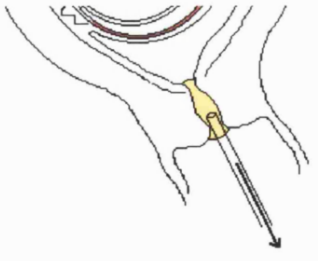
b) Cotton swab or cytobrush



c) Intrauterine lavage



d) Endocervical lavage



e) Aspiration of cervical mucus

Examples of methods for collection of TCC samples. The two-headed arrows indicate the direction of fluid being injected and aspirated. The single-headed arrow indicates the direction of the aspiration of the mucus.

Currently, the presence of fetal genetic material in cervical mucus is increasingly accepted (Adinolfi and Sherlock, 1997). Most studies use either lavage or aspiration techniques to collect TCC samples. Many studies have found lavage to be more efficient in retrieving samples with more fetal cells (Rodeck *et al.*, 1995; Adinolfi *et al.*, 1995b). With regard to safety, one follow-up study after aspiration showed no differences between the study group (n=113) and the control cases which were selected from a comparable group of pregnant women undergoing CVS without TCC sampling (n=121) in terms of miscarriages or stillbirths. The miscarriages in both groups occurred at 16 weeks or more, much later than when the procedures (CVS with or without TCC sampling) were performed (Rodeck *et al.*, 1995). At present, other collection techniques should be performed before termination of pregnancy only, due to concerns of the possibility of introducing infection. In addition, there was a report from Taiwan regarding a baby with severe limb reduction defects born to a mother who had undergone uterine lavage at 7-8 weeks' gestation for fetal sexing (Chou *et al.*, 1997). This could have been due to the catheter being inserted too high into the uterine cavity (Daryani *et al.*, 1997a).

With aspiration, usually the catheter is connected to a syringe to create negative pressure for the aspiration. A commercially available catheter (Pipelle de Cornier™ Neuilly-en-Thelle, France) used for endometrial sampling has its own piston which can be directly pulled back to create a negative pressure. The small caliber (outer diameter 3.14 mm) gives the advantage that it would practically impose little discomfort to the patient. The present study was to evaluate the results of retrieving TCC samples by 1) the Pipelle, 2) a catheter connecting with a syringe, and 3) a lavage technique.

3.2 MATERIALS AND METHODS

3.2.1 PATIENTS AND SAMPLE COLLECTIONS

TCC samples were collected from women undergoing TOP for non-medical reasons at gestational ages between 7-15 weeks and from pregnant women undergoing CVS. All the patients gave their informed consent, and the study was approved by the UCL ethics committee. Mucus samples were collected by an instrument inserted transcervically and were tested for the presence of fetal genetic material. Maternal blood and placental tissues were also collected to determine maternal and fetal genetic markers. FISH and/or PCR were used to test for the genetic make up of each sample. Several methods of mucus collection were examined.

3.2.1.1 Collection by the Pipelle catheter

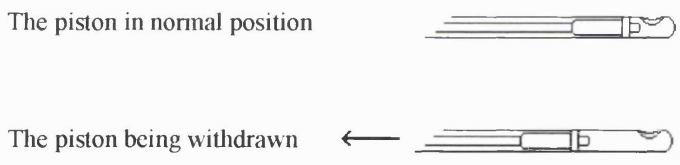
After general anaesthesia, 5 ml of maternal blood was collected into an ethylene-di-amine-tetra-acetic acid (EDTA) blood tube and transabdominal ultrasound scanning was undertaken to evaluate the presence of a viable pregnancy and gestational age. With the patient in the dorsal lithotomy position, a posterior vaginal speculum was inserted, and the vagina and the cervix were cleaned with antiseptic solution. Two pairs of tissue-grasping forceps were applied to the paracervical ligaments to help fix the cervix. A thin flexible polypropylene catheter with internal piston for endometrial biopsy (Pipelle de Cornier™ Neuilly-en-Thelle, France), was inserted up to the midcervical canal level under ultrasound guidance and the piston was pulled back, creating a negative pressure in the catheter, thus the cervical mucus was aspirated **(Figure 3.2)**. The catheter was withdrawn and the mucus collected into a conical centrifuge tube containing 2 ml of sterile normal saline solution. The TOP was then

performed as usual after which the placental tissue was collected and kept in normal saline solution.

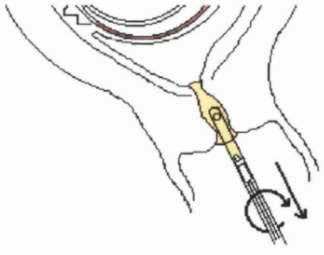
Figure 3.2 Pipelle catheter

The handle of the piston The tip of the catheter

a) Pipelle catheter



b) Tip of the catheter



c) Aspiration of the mucus

The pipelle catheter used in the study. There was a lateral hole at the tip of the catheter. During the aspiration, the catheter was rotated at the same time so that the hole collects mucus whilst being withdrawn.

3.2.1.2 Collection by lavage and syringe aspiration

For lavage and syringe aspiration methods, the techniques were carried out as previously described (Kingdom *et al.*, 1995; Rodeck *et al.*, 1995). For lavage, the catheter used was Trophocan™ (Portex Limited, Kent, UK). It was a thin catheter, 210 mm in length, used for CVS sampling, with inner diameter of 1.13 mm and outer diameter of 1.45 mm. The catheter was connected to a 20ml syringe containing 10 ml sterile normal saline solution. The syringes used for aspiration were 50ml in order to create more suction pressure (UCH mucus sampling set, Rocketmedical, R59100), even though only 5-10 ml sterile normal saline was injected in the cervical canal.

3.2.1.3 Collection from on-going pregnancies

Collection by Pipelle from on-going pregnancies was performed in the same way except that the pregnant women were not anaesthetised and the procedure performed without using the grasping forceps on paracervical ligaments. The collections were carried out before the CVS procedures.

3.2.2 FISH AND PCR

3.2.2.1 FISH

The presence of fetal genetic materials was assessed by FISH and/or PCR. For FISH, the placenta and the TCC sample were prepared as described **(section 2.3.1.1)**. FISH using probes for chromosomes X and Y was employed to determine fetal gender from the placenta and detect male cells in the TCC samples **(section 2.3.4)**. In addition, PCR using primers for the amelogenin region on the sex chromosomes was performed. To extend the study to cases where the fetus was a female, PCR for short tandem

repeats (STRs) was performed on maternal blood, placenta and the TCC samples. DNA extraction, PCR procedure and the analysis were described in Chapter 2 **(section 2.2)**.

3.2.2.2 PCR for the amelogenin region

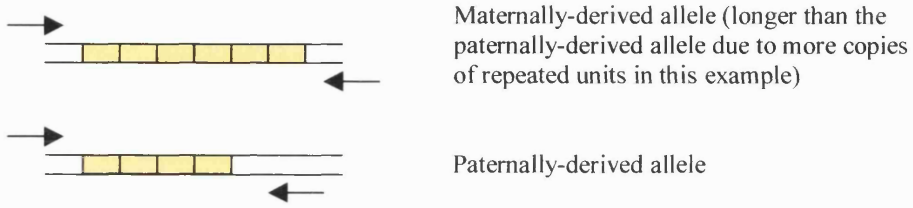
Amelogenin is a human homologous region on the sex chromosomes. During evolution, a deletion has occurred on the Y chromosome. Thus amplification of this sequence from a sample containing the X and the Y chromosomes results in 2 PCR products of different sizes (Nakahori *et al.*, 1991). With the primers used in PCR for gel electrophoresis in this study, the product from the X chromosome was 1,000 bp and the Y was 823 bp. With the labelled primers used in fluorescent PCR, the size of the X chromosome product was 432 bp and that of the Y chromosome was 252 bp.

3.2.2.3 PCR for the STRs

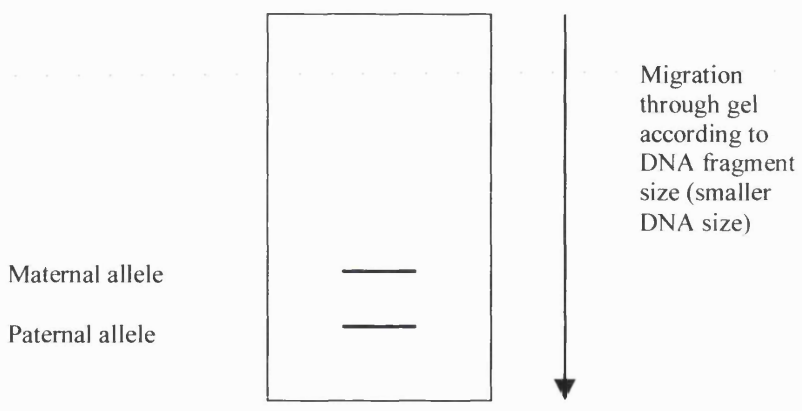
STRs are polymorphic repeated sequences of DNA. The polymorphism arises from different numbers of repeated units. These sequences are present on all chromosomes. The PCR products generated by primers flanking these regions can be of different sizes. For any specific STR, the more alleles (sizes) it has, the greater the possibility of heterozygosity, and the more useful that STR is as a marker (Mansfield, 1993; Pertl *et al.*, 1994, 1996) **(Figure 3.3)**.

Figure 3.3 PCR of the STRs

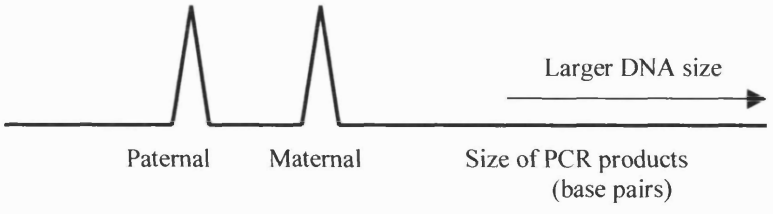
Modified from Sherlock (1998) with permission.



a) STRs being amplified with flanking primers



b) PCR products being run on standard gel electrophoresis



c) PCR products from fluorescently labelled primers being run on an automated DNA sequencer

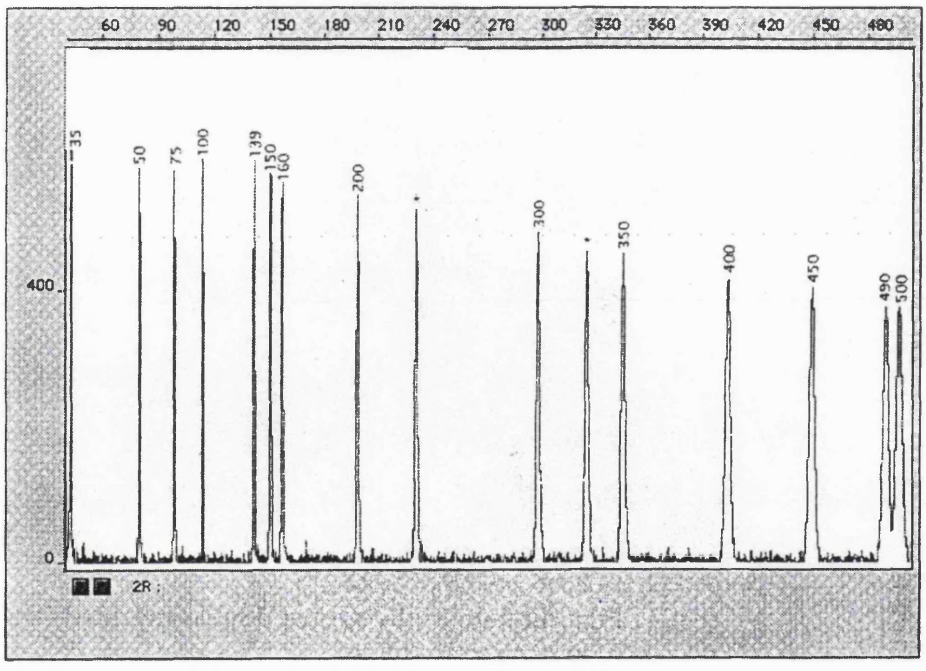
In this study, a number of early cases were analysed using agarose gel electrophoresis. In the remaining cases, fluorescent PCR was employed and the analysis was carried out on an automated laser DNA analyser (ABI Prism™ 310). The latter technique has several advantages. The primers are fluorescently labelled which enables different PCR products from different primer sets in a multiplex PCR that have the same or similar sizes to be analysed together, using different colours. The technique is more sensitive and has a better resolution for the PCR products from the same primer set that are only a few base pairs different in size.

Using fluorescent PCR, the size of any PCR product would be compared with the size standard by computer software. Size standard (Genescan 500-TAMRA [red], Perkin-Elmer) acts as a ladder in a gel electrophoresis. The size standard fragments and PCR products in the same tube run through a gel capillary according to their sizes and the fluorescence intensity at the time is detected by the machine. Figure 3.4 shows an electropherogram of the size standard fragments used in the study. These peaks were red but are shown in black in this figure.

In this study, STRs from chromosomes 21 and/or 18 and/or 13 were used as markers of fetal DNA in TCC samples. In most cases, two STRs were used. The STR alleles of the mother and the fetus were identified through the maternal blood and placenta. If the paternally derived allele differs from the maternal ones, its presence in the TCC samples could be used as an indicator of the presence of fetal DNA.

Figure 3.4 Electropherogram of GeneScan-500 (size standard)

Taken from Perkin Elmer. ABI Prism™ 310 Genetic Analyzer, GeneScan™ Chemistry Guide, 1995, p. 2-12



The peak pattern of size standard fragments. Sizing of PCR product fragments were obtained by comparison to these peaks. This figure is shown in black and white. However, in the actual results the size standard fragments would appear red.

3.3 RESULTS

3.3.1 RESULTS FROM TERMINATION OF PREGNANCY (TOP) CASES

TCC samples, maternal blood and placenta were collected from 193 TOP cases. Mean $\pm$ 2 S.D. of maternal age was 26.6 $\pm$ 12.2 years with the range of 15-44 years old (missing data 9 cases). The gestational age ranged from 6-16 weeks with the mean $\pm$ 2 S.D. of 10.9 $\pm$ 5.0 weeks (missing data 13 cases). All 193 cases underwent PCR analysis **(Table 3.1)**. In the early part of the study, the samples (56 cases) were amplified for the amelogenin only. Of these, 42 cases were collected by the Pipelle and 14 cases by lavage. FISH was also performed on 34 TCC samples in this group. It was not performed in all cases due to a period of probe contamination. PCR in the later part (137 cases) was performed for both the amelogenin and STRs. Samples in this group were collected by the Pipelle (77 cases), syringe aspiration (44 cases) and endocervical lavage (16 cases). FISH was performed in 16 TCC samples in this group and was subsequently discontinued due to the low success in fetal cell detection compared to PCR. Details of FISH results are discussed in the next section and PCR results are discussed subsequently.

Table 3.1 Details of experiments on 193 samples collected from TOP cases

PCR analysis	Number of cases collected by			FISH on TCC (cases)
	Pipelle aspiration	Syringe aspiration	Endocervical lavage	
Amelogenin only (56 cases)	42	-	14	34
Amelogenin and STRs (137 cases)	77	44	16	16

3.3.1.1 Cases with FISH results

In total, 50 TCC samples from TOP cases were tested by FISH using probes for the sex chromosomes. Five samples were collected by endocervical lavage, and the rest by aspiration using the Pipelle. Fetal sexing by FISH on the placenta revealed that these samples were from 26 male, 23 female and 1 XO pregnancies. Table 3.2 shows the results of FISH on the TCC samples grouped by fetal sex. For the simplicity of the Table, the XO pregnancy was included in the XX group. There was a wide variation in the quality of the FISH slides. Cell numbers available for scoring ranged from 0-450 cells. Only 13 out of 26 male cases and 13 out of 24 female cases had 50 cells or more for analysis while in 5 of male and 4 of female cases, no nuclei were obtained at all after cell suspension preparation. Endocervical lavage tended to yield a higher cell number.

From female control lymphocyte slides which were performed over 4 experiments, a total of 480 nuclei were scored, none of which had an XY signal. The presence of XY cells in the TCC slides was therefore regarded as real. In the slides obtained from female cases, no XY cells were found. Three out of 26 male cases had XY cells detectable in the TCC slides (detection rate of 11.5%). Figure 3.5 shows the appearance of signals in lymphocyte metaphase and placental interphase nuclei. The presence of a male cell in a TCC sample is also illustrated.

Table 3.2 Number of cells examined and cells with an XY signal from each TCC slide

XY placenta			XX placenta		
Slide no.	**Total cells**	**XY cells**	**Slide no.**	**Total cells**	**XY cells**
1	0	0	1 (XO)	0	0
2 (L)	0	0	2	0	0
3	0	0	3	0	0
4	0	0	4	0	0
5	0	0	5	1	0
6	1	0	6	2	0
7	3	0	7	5	0
8	5	0	8	5	0
9	7	0	9	10	0
10	12	0	10	10	0
11	14	0	11	12	0
12	20	0	12	20	0
13	40	0	13	40	0
14	50	0	14	60	0
15	60	0	15	70	0
16 (L)	60	0	16	80	0
17	**60**	**2**	17	80	0
18	76	0	18	100	0
19 (L)	80	0	19	120	0
20	80	0	20 (L)	150	0
21	85	0	21	150	0
22	100	0	22	200	0
23	250	0	23	400	0
24	**300**	**3**	24	400	0
25 (L)	**400**	7			
26	450	0			

(L) = TCC sample was collected by endocervical lavage

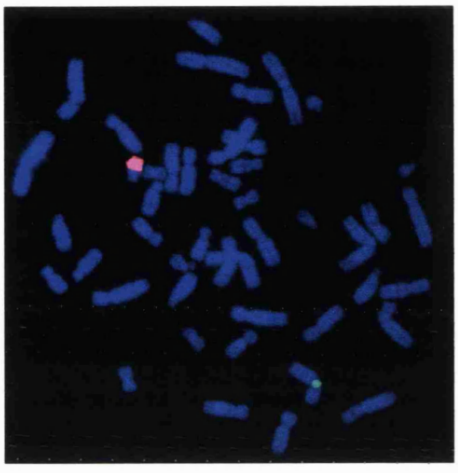
Bold = cases with positive male cells in the TCC

Bold lines mark where 50 cells or more were achieved for scoring in a case.

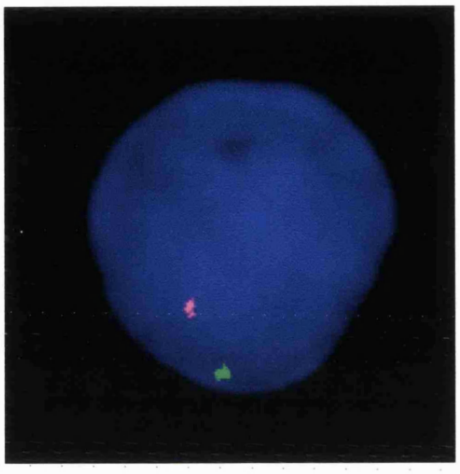
One case of XO placenta is included in the XX group for the simplicity of the Table.

From female lymphocyte control slides (on 4 experiments), a total of 480 cells were scored, none of them had a cell with an XY signal.

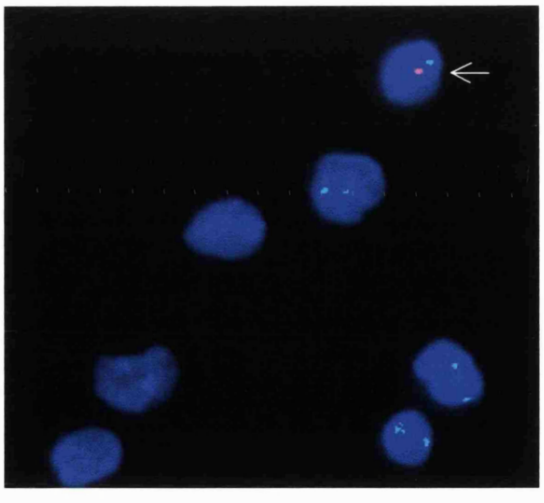
Figure 3.5 Various signals seen in the lymphocytes, placental nuclei and a TCC sample from a male pregnancy



a) a male lymphocyte metaphase nucleus



b) a male placental (interphase) nucleus



c) a male nucleus in the TCC sample

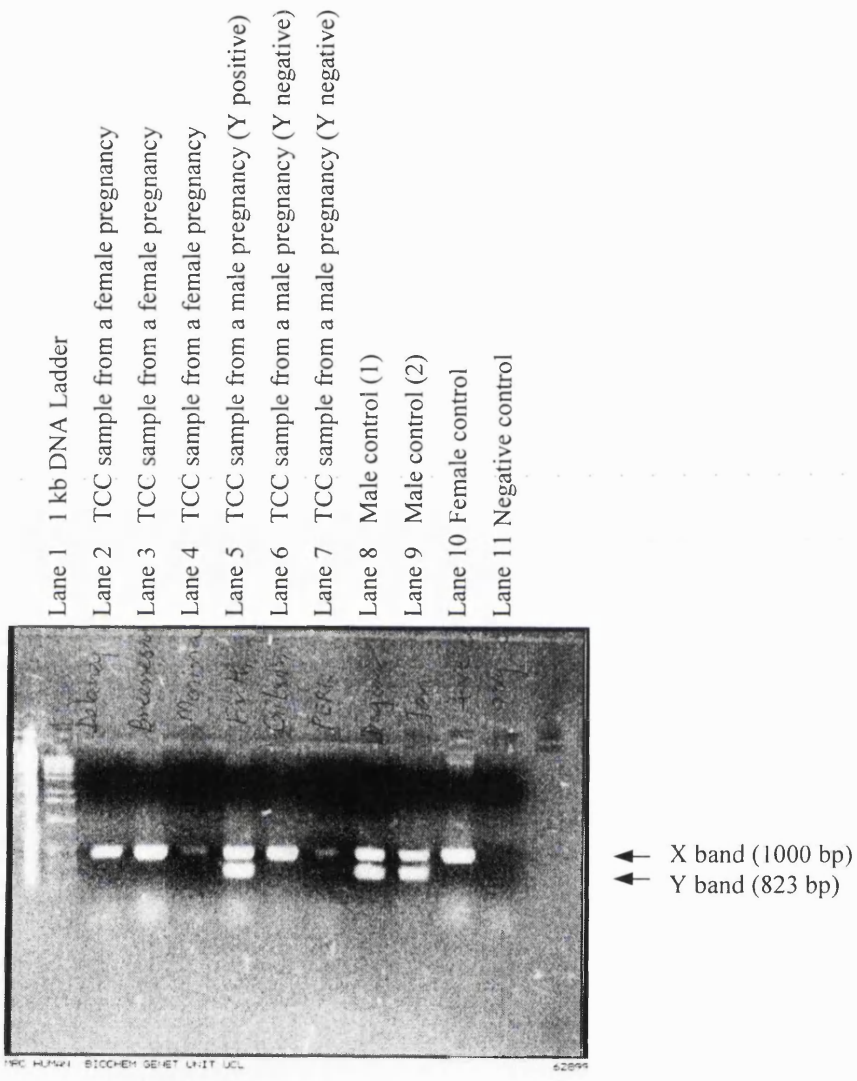
a) a metaphase nucleus from male lymphocyte control, showing a red signal for chromosome Y and a green signal for chromosome X.
b) an interphase nucleus from a male placenta, showing one red and one green signal for chromosomes Y and X respectively.
c) a male cell (arrow) in a TCC sample obtained from a male pregnancy. The other cells were maternal cells, several had XX signals out of the plane of focus.

3.3.1.2 Cases with PCR for the amelogenin region alone

In the early part of the study, PCR for the amelogenin alone was performed on an aliquot of TCC samples from 56 TOP cases in order to detect a Y-sequence in samples from male pregnancies. It was also performed in female pregnancies to assess a false positive rate. The fetal gender was determined from the placenta. In the later part of the study, PCR for STRs was also performed with the amelogenin (137 cases) and will be described later **(section 3.3.1.4)**.

Initially, TCC samples were analysed by gel electrophoresis, some of which are illustrated in Figure 3.6. TCC samples from female pregnancies showed only one band (X product). Two of the three male samples also had only one band which was interpreted as only maternal cells were found in the samples. One of the male TCC samples showed both X and Y products. This could be due to the presence of fetal cells in the TCC sample. Another possibility is the presence of sperm. However, from the results in female pregnancies (from the whole study), no Y product was detected. This implied that the chance of the Y product being from the sperm was negligible. It might be possible that the condensed sperm DNA was not adequately extracted by the technique used for TCC samples in this study. Although successful PCR of pure human sperm has been reported, more difficulty in DNA extraction from mouse-sperm DNA has been mentioned (Rank *et al.*, 1991). This can be the case in human sperm and with the presence of excess maternal cells, the less efficiently extracted sperm DNA may not be properly amplified.

Figure 3.6 Gel electrophoresis of a set of TCC samples



A gel electrophoresis of a set of TCC samples. The negative control showed no contamination. Two male positive controls showed two bands of PCR products while the female positive control showed only an X band. All TCC samples from female cases had only one X band. One of the TCC samples from male pregnancies had two bands, implying the presence of Y sequences in the sample. Other samples from male pregnancies failed to show the presence of the Y chromosome and were considered to be without fetal cells.

The initial 53 cases in the study were analysed by gel electrophoresis. Thirty-nine cases were collected by the Pipelle and 14 cases by lavage. The results are shown in Tables 3.3 and 3.4 for each collection technique.

Table 3.3 Results of PCR for the amelogenin region on TCC samples collected by the Pipelle and analysed by gel electrophoresis

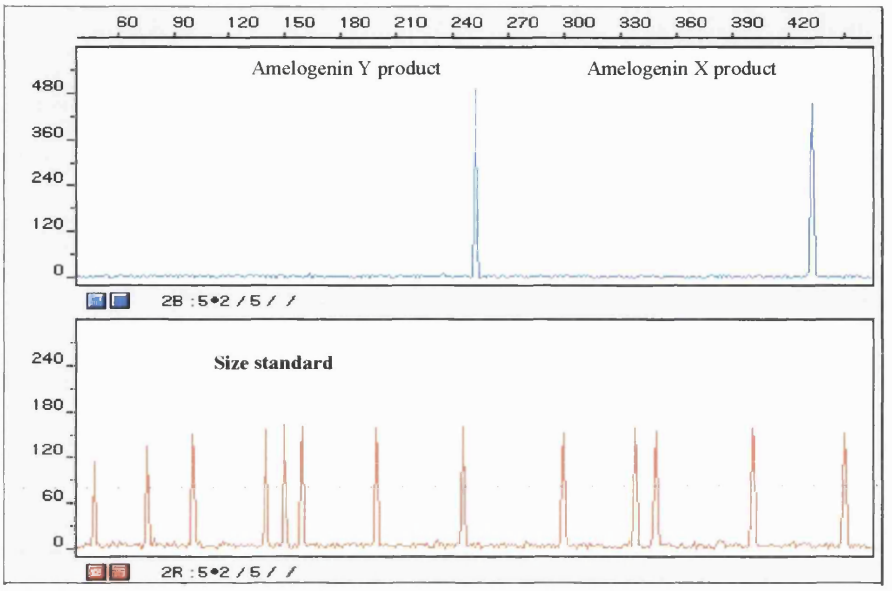
Male cases (18 cases)			Female cases (21 cases)		
No. cases with X product only	No. cases with XY products	No. cases with PCR failure	No. cases with X product only	No. cases with XY products	No. cases with PCR failure
10 (55.6%)	4 (22.2%)	4 (22.2%)	17 (81.0%)	0	4 (19.0%)

Table 3.4 Results of PCR for the amelogenin region on TCC samples collected by endocervical lavage and analysed by gel electrophoresis

Male cases (8 cases)			Female cases (6 cases)		
No. cases with X product only	No. cases with XY products	No. cases with PCR failure	No. cases with X product only	No. cases with XY products	No. cases with PCR failure
2 (25.0%)	1 (12.5%)	5 (62.5%)	4 (66.7%)	0	2 (33.3%)

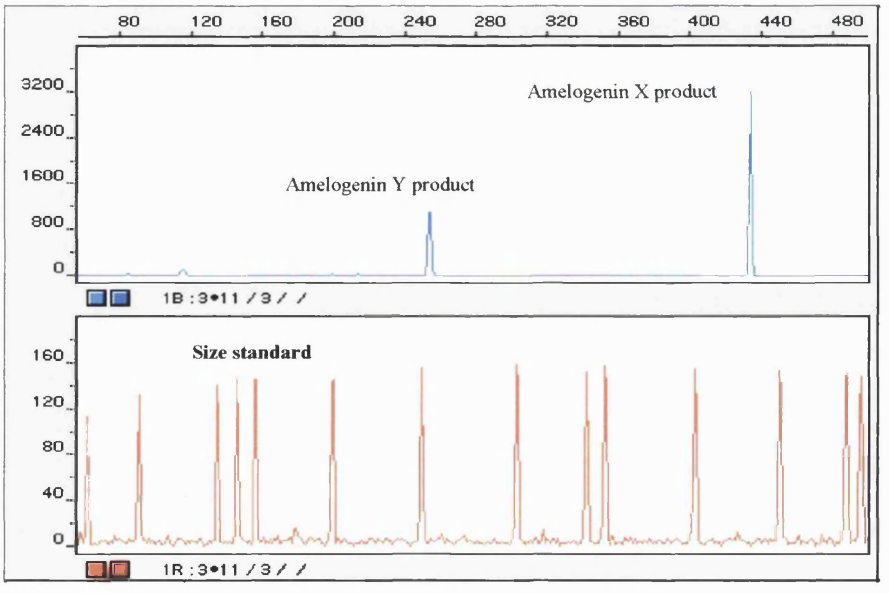
In an attempt to improve the amplification and the detection rate of the Y-sequence in male cases, fluorescent PCR of the amelogenin region was introduced into the study. The forward primer was labelled with 6-FAM (blue). In a sample that contained both the chromosomes X and Y, two peaks of amelogenin products of size 252 bp (Y) and 432 bp (X) were present **(Figure 3.7)**. In a sample that did not contain chromosome Y, only a peak of 432 bp was observed.

Figure 3.7 Electropherogram of amelogenin on samples containing male cells



a) A male placenta

The presence of two PCR products of 252 and 432 bp indicates the presence of both the X and Y chromosomes. Thus this placenta was judged a male. In a sample which was a female, only a peak of 432 bp would be observed.



b) A TCC sample containing male cells

A TCC sample obtained from a male pregnancy by the Pipelle, with a Y-sequence detectable, implying the presence of fetal cells in the sample.

It was found that, in several cases where PCR failed to amplify or failed to show a Y-sequence when analysed by gel electrophoresis, repeating with fluorescent PCR improved the results due to increased sensitivity. When repeating the positive cases analysed by gel electrophoresis, fluorescent PCR confirmed the results. Therefore, fluorescent PCR was used in 3 additional samples that were obtained subsequently.

Tables 3.5 and 3.6 summarise the results of PCR for only the amelogenin on TCC samples analysed by gel electrophoresis and the fluorescent PCR combined. With the additional 3 cases, there were 56 cases in total. Fetal sex was determined on the placenta by FISH in 42 cases and by PCR in 14 cases (due to a contamination problem of the FISH probes at the time). It is of note that sexing by PCR could not give the information about the copy number of each chromosome and could easily miss cases of sex chromosome aneuploidy. However, PCR was still informative in determining the presence of Y chromosome DNA and cases with X and Y sequences were presumed male while those with only the X sequence were presumed female. In total there were 42 cases collected by Pipelle, 20 males and 22 females, and 14 cases collected by endocervical lavage, 8 males and 6 females.

Table 3.5 Results of PCR for the amelogenin region on TCC samples collected by the Pipelle

Male cases (20 cases)			Female cases (22 cases)		
No. cases with X product only	No. cases with XY products	No. cases with PCR failure	No. cases with X product only	No. cases with XY products	No. cases with PCR failure
5 (25.0%)	14 (70.0%)	1 (5.0%)	18 (81.8%)	0	4 (18.2%)

Table 3.6 Results of PCR for the amelogenin region on TCC samples collected by endocervical lavage

Male cases (8 cases)			Female cases (6 cases)		
No. cases with X product only	No. cases with XY products	No. cases with PCR failure	No. cases with X product only	No. cases with XY products	No. cases with PCR failure
4 (50.0%)	4 (50.0%)	0	5 (83.3%)	0	1 (16.7%)

The results show that PCR could detect the presence of a Y-derived sequence in a higher percentage than FISH, including the 3 cases that were shown to contain XY cells by FISH. It also provided results in several cases where FISH failed. There were, however, two cases where there were cells on the FISH slides but PCR using both gel and fluorescent PCR failed to amplify. In the first case, the fetus was a male and there were 150 XX cells on the FISH slide. In the second case, the fetus was a female and there were 40 XX cells on the FISH slide. In both cases, fetal cells in the TCC samples could not be detected by FISH.

Using PCR, no TCC samples from female fetuses were found to have a Y-sequence, resulting in no false positives in the study. PCR for the amelogenin region, however, could not identify fetal cells in cases with a female fetus. With regard to this, PCR for STRs was introduced for the later cases in the study.

3.3.1.3 Patterns of fetal DNA evaluation using STR markers

There were 137 cases of TOP where the samples underwent PCR for the amelogenin and STRs. The STR markers used in each case were D21S1414 or D21S11 and/or D18S535 and/or D13S631 according to the availability at the time of the experiment.

In evaluating the presence of fetal DNA in TCC samples using an STR marker, four patterns can be expected: present, absent, possible, and non-informative (Adinolfi *et al.*, 1995c; Ruangvutilert *et al.*, 1998). If the paternal allele in the fetus is different from maternal alleles and is found in the TCC sample, the presence of fetal cells would be implied. If it is not found in the TCC sample, the absence of fetal cells would be implied according to that marker. A "possible" situation is classed when the paternal allele is present in a small amount and cannot be certainly distinguished from non-specific amplification. When the paternal allele is the same size as a maternal allele, it would be non-informative as no fetal specific marker would be available. These patterns are shown in the following examples:

Fetal DNA absent and non-informative patterns

Case A. TCC sample was collected by the Pipelle. PCR for STRs was performed on maternal blood, the placenta and TCC. The markers used were D21S1414, D18S535 and D13S631. Amelogenin primers were also included. Table 3.7 shows the allele sizes of each marker in each sample. The placenta had only X amelogenin product and the fetus was presumed female.

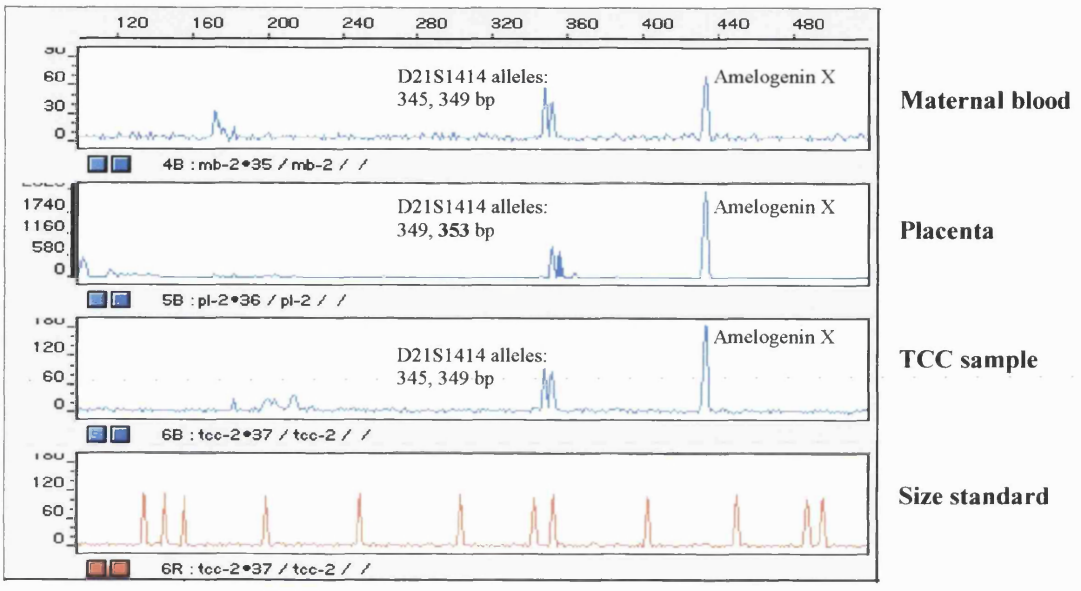
Table 3.7 Summary of alleles obtained from PCR in Case A

Sample	Amelogenin	D21S1414 (bp)	D18S535 (bp)	D13S631 (bp)
Maternal blood	X	345, 349	148, 152	195, 199
The placenta	X	349, **353**	**138**, 152	195, 199
TCC sample	X	345, 349	148, 152	195, 199

Bold = paternal alleles

From Table 3.7, D13S631 was non-informative as both the placental (fetal) alleles were the same size as maternal and it was not possible to identify paternally-derived allele for this marker. A different allele from maternal was observed for D21S1414 and D18S535 in the placenta but no such alleles were found in DNA extracted from the TCC sample, implying the absence of fetal cells. Figure 3.8 shows the electropherogram results form the same case by presenting the alleles from maternal blood, the placenta and the TCC sample from the same STR in the same panel.

Figure 3.8 Electropherogram of all the samples from Case A for each STR

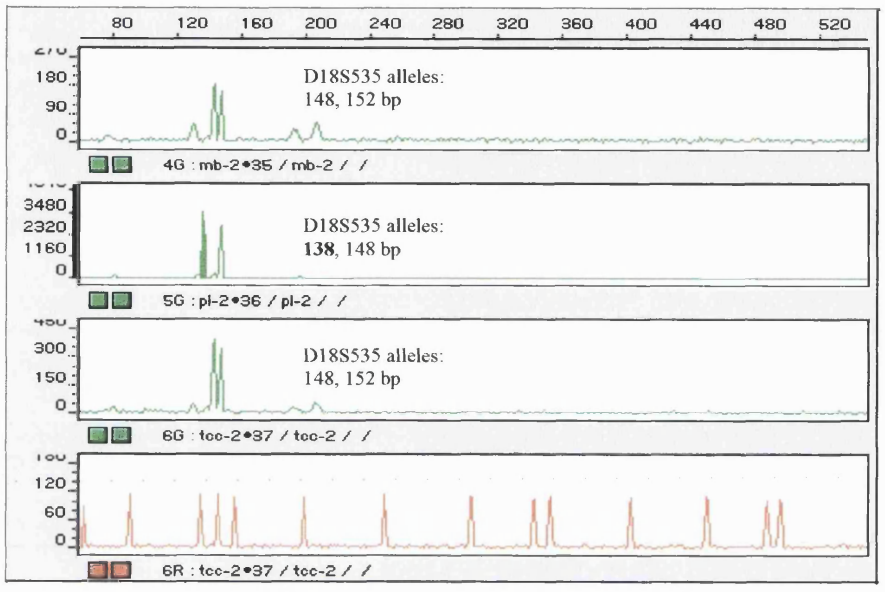


a) Panel for the D21S1414 and the amelogenin. Fetal DNA was absent in the TCC sample.

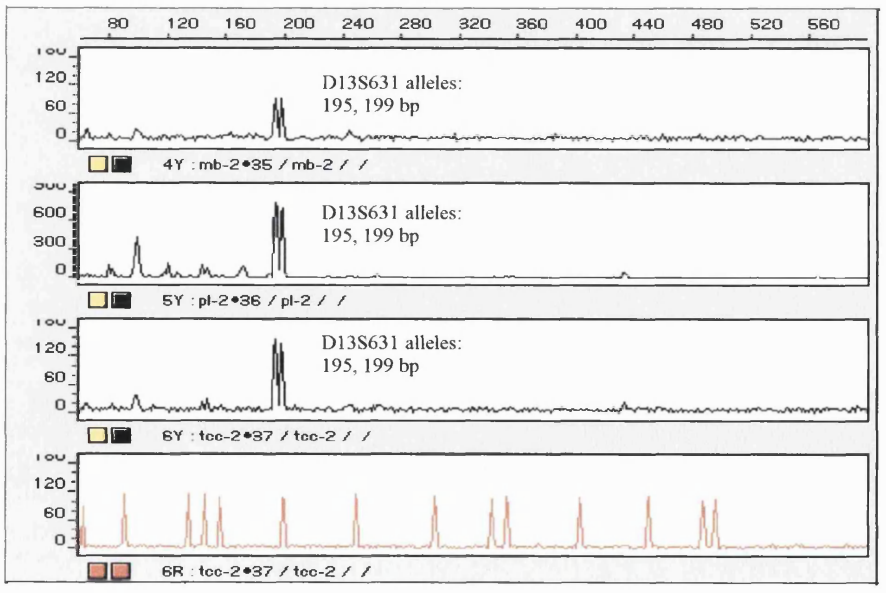
In the panel, the paternal allele in the placenta was presented by a filled peak. The size of the paternal allele was presented in **bold**. These formats were also used in the next panels of STRs. In this panel for D21S1414, the paternal allele was not identified in the DNA extracted from the TCC sample. Fetal DNA was negative (absent) according to this marker.

In the next page, panel b) also shows that fetal DNA was absent according to D18S535. However, in panel c), no fetal specific allele could be identified from D13S631. Therefore, this STR could not give information about the presence of fetal DNA in the TCC sample.

Figure 3.8 (continued)



b) Panel for the D18S535. Fetal DNA was absent in the TCC sample.



c) Panel for the D13S631 (non-informative)

Fetal DNA present (positive) pattern

Case B. TCC sample was collected by the Pipelle. PCR was performed on maternal blood, the placenta and the TCC sample, for amelogenin, D21S1414, and D18S535. The summary of all the alleles is presented in Table 3.8.

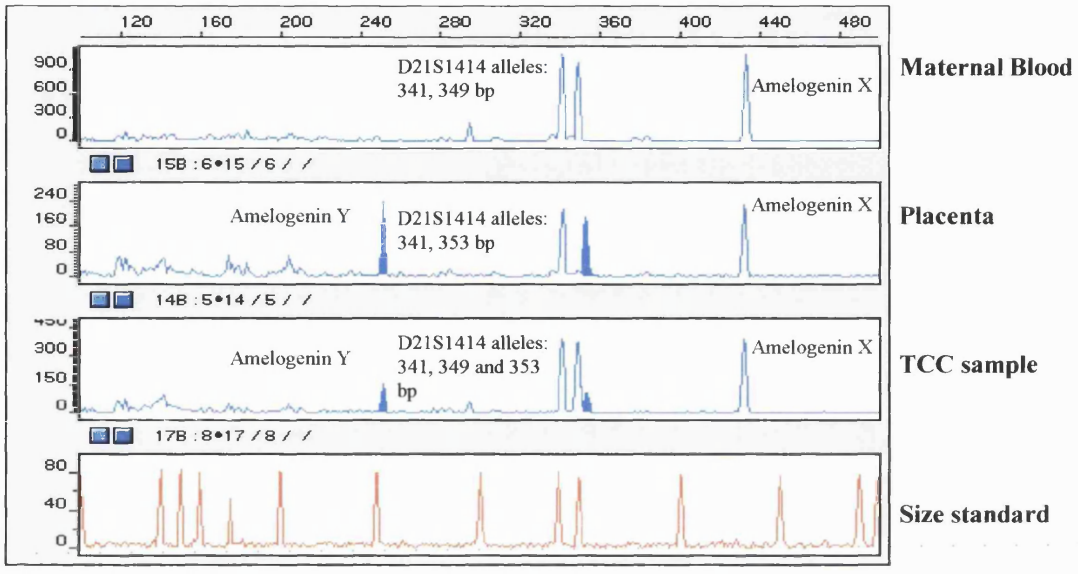
Table 3.8 Summary of alleles obtained from PCR in Case B

Sample	Amelogenin	D21S1414 (bp)	D18S535 (bp)
Maternal blood	X	341, 349	142 (homozygote)
The placenta	XY	341, **353**	**138**, 142
TCC sample	XY	341, 349, **353**	**138**, 142

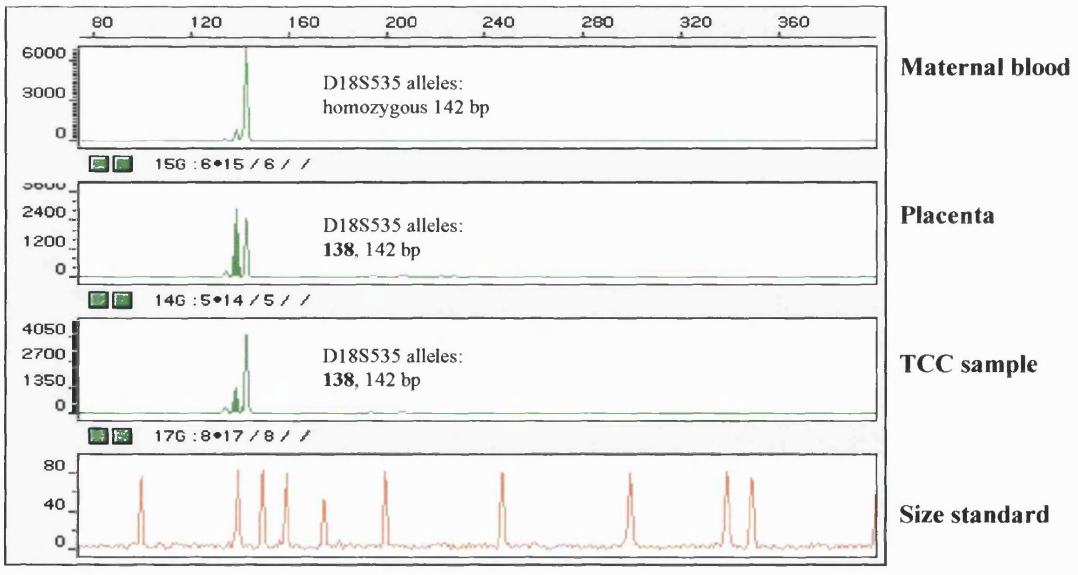
Bold = paternal alleles

The fetus was a male, judged from the result of the placenta. A paternal allele was observed for each STR marker. These alleles were present in the PCR results from the TCC sample, implying the presence of fetal cells. Electropherograms of PCR products from maternal blood, the placenta and the TCC from this case are illustrated in Figure 3.9.

Figure 3.9 Electropherogram of all the samples from Case B for each STR



Electropherogram for results from the amelogenin and D21S1414 primers. The fetus was shown to be a male from the presence of amelogenin Y product in the placenta. The fetal specific allele for D21S1414 product could be identified in the placenta. This allele and the amelogenin Y product were also identified in the TCC sample.

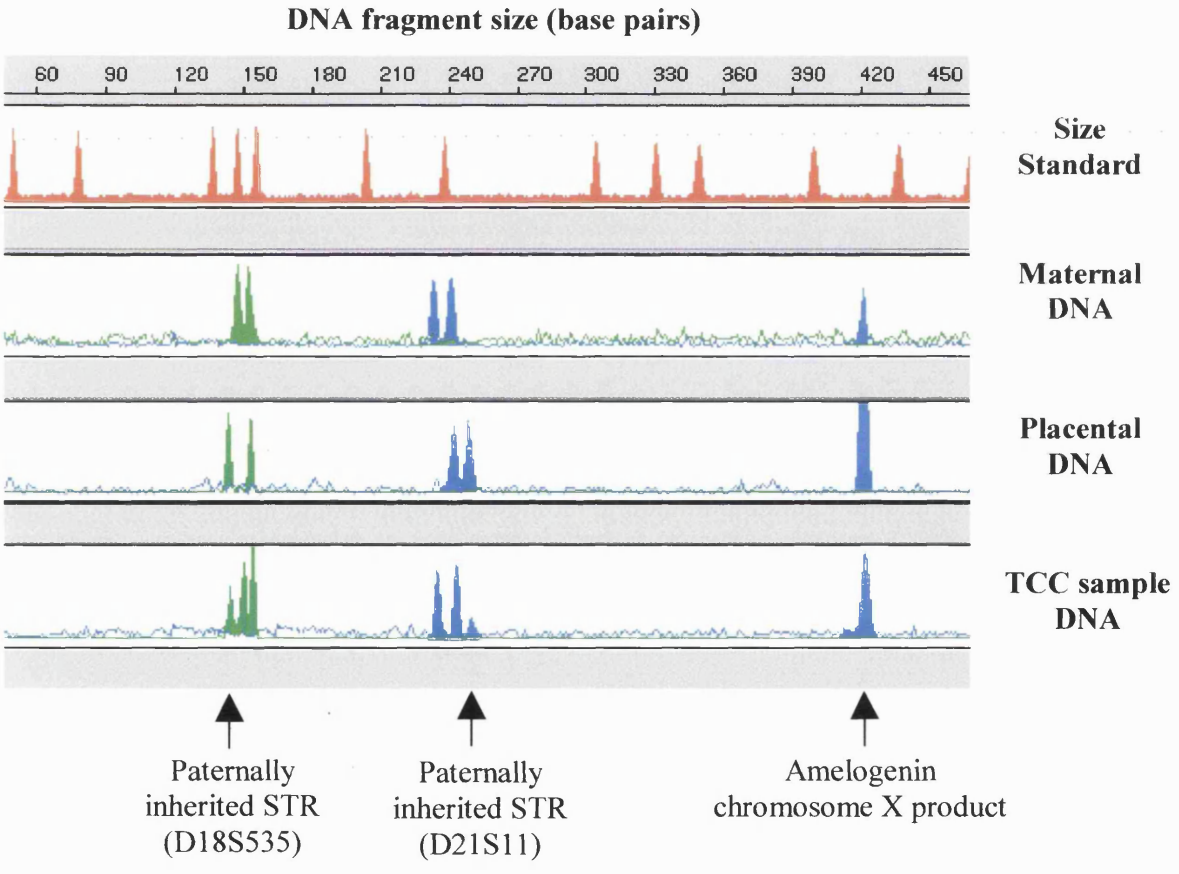


Electropherogram of results from D18S535 primers. The mother was homozygous for this STR and the fetal specific allele was identified with the size of 138 bp. This allele was also present in the TCC sample.

Figure 3.10 shows another case with the fetal DNA detected in the TCC sample. This case was a female pregnancy. The presence of fetal cells in this TCC sample would not have been detected without the STRs.

Figure 3.10 Electropherogram showing positive fetal DNA in a TCC sample

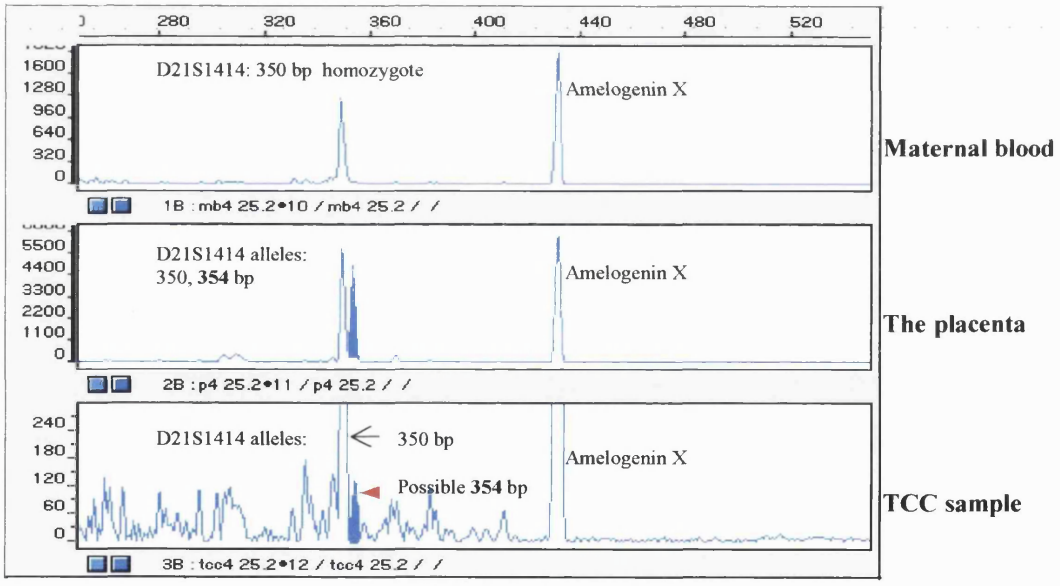
Taken from Ruangvutilert et al. (1998)



Possible pattern

This is a situation where a peak with the same size of paternal allele is present in an equal amount or smaller than non-specific amplification and its presence is not certain. Figure 3.11 shows this pattern with D21S1414 marker. The fetus was a female.

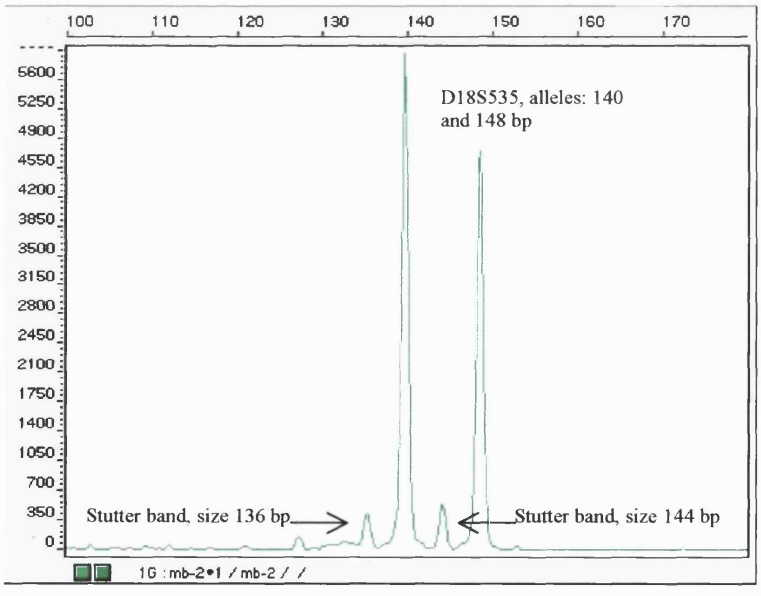
Figure 3.11 A possible STR pattern for the presence of fetal DNA



Electropherograms of maternal blood, the placenta and the TCC sample are illustrated for the PCR products of amelogenin and D21S1414. The TCC panel was zoomed in to show the background peaks. The presence of the peak of 354 bp (paternal allele) in the TCC sample (red arrowhead) was not definite. The fragment length matched the paternal allele but the height and the area of the peak did not exceed those of the nonspecific or background peaks. The presence of fetal DNA could not firmly established.

Apart from the four patterns of fetal DNA evaluation, an additional pattern, a "stutter band" can also be observed in an electropherogram of an STR (Sherlock, J., personal communication). This is generated by slippage of the amplification during the extension phase of PCR, resulting in a small amount of PCR product which differs from the expected size by the number of DNA bases in the repeat itself. For example if the repeat contains 4 bases, the stutter band will be 4 bases shorter than the size of the expected PCR product **(Figure 3.12)**, despite the absence of such DNA allele in the starting sample. This can be problematic in PCR with high background results and sometimes is not distinguishable from "STR possible" when the stutter band is the same size with the fetal specific allele.

Figure 3.12 Stutter bands



PCR for D18S535 from a maternal blood sample. Maternal alleles were 140, and 148 bp. Stutter bands from both alleles were present in this electropherogram with 4 bp shorter than the true alleles. If present in a TCC sample, a stutter band can cause confusion and sometimes it was difficult to distinguish from a possible presence of a fetal specific allele.

3.3.1.4 Cases with PCR for the amelogenin and STRs

Of the 137 cases of TOP where the samples were collected for the study using PCR for the amelogenin and STRs to detect the presence of fetal DNA, 77, 44, and 16 cases were collected by Pipelle aspiration, syringe aspiration and endocervical lavage using the Trophocan™ catheter respectively. The technique used for each patient depended on the instrument available on the day. Due to the less satisfaction achieved from the lavage technique compared to the others in the author's hand, it was used least.

3.3.1.4a The presence of fetal DNA in TCC samples

Table 3.9 shows the results of the fetal DNA in TCC samples collected by the Pipelle, syringe aspiration and lavage, grouped by the fetal sex for each technique. In the Pipelle group, there were 2 XO cases which were included in the female group due to the small number. Failure of amplification was observed in 12 (15.6%), 6 (13.6%), and 7 (25.0%) cases in the Pipelle, syringe aspiration and lavage groups respectively. There were more non-informative cases in female than in male pregnancies. This was because only STRs could be used in the female whereas in male, amelogenin could give additional results. Moreover, amelogenin appeared to be more sensitive to detect the fetal DNA. This is shown by more positive cases in male being found by amelogenin than by STRs **(Table 3.10)**. However, there were a few cases which were positive for STRs only and *vice versa*. Therefore, both markers were complementary to each other in detection of fetal DNA in TCC.

It is notable that the positive results obtained from male cases were lower than earlier cases in the series when only amelogenin was used both in the Pipelle aspiration (51% *versus* 70%) and endocervical lavage (14% *versus* 50%) **(Table 3.9 *versus***

Tables 3.5 and 3.6). For endocervical lavage, this discrepancy might have resulted by chance as the numbers of cases were small in both PCR for the amelogenin alone and PCR for the amelogenin with STRs. There was no clear explanation for the discrepancy in the results of Pipelle aspiration. It might have resulted simply by chance or by the reduced efficiency caused by multiplex PCR.

Table 3.9 Results of fetal DNA in TCC samples collected by Pipelle aspiration, syringe aspiration and endocervical lavage

Method	Fetal sex (cases)	Fetal DNA in TCC (number of cases and percentage)				
		Positive	Negative	Possible	Noninf.*	Fail
Pipelle aspiration (77 cases)	Male (35)	18 (51.4%)	12 (34.3%)	0 (0%)	0 (0%)	5 (14.3%)
	Female (42**)	12 (28.6%)	17 (40.5%)	2 (4.8%)	4 (9.5%)	7 (16.7%)
	Total (77)	30 (39.0%)	29 (37.7%)	2 (2.6%)	4 (5.2%)	12 (15.6%)
Syringe aspiration (44 cases)	Male (22)	9 (40.9%)	9 (40.9%)	0 (0%)	0 (0%)	4 (18.2%)
	Female (22)	6 (27.3%)	10 (45.5%)	1 (4.5%)	3 (13.6%)	2 (9.1%)
	Total (44)	15 (34.1%)	19 (43.2%)	1 (2.3%)	3 (6.8%)	6 (13.6%)
Endocervical lavage (16 cases)	Male (7)	1 (14.3%)	3 (42.9%)	1 (14.3%)	0 (0%)	2 (28.6%)
	Female (9)	2 (22.2%)	4 (55.6%)	0 (0%)	1 (11.1%)	2 (22.2%)
	Total (16)	3 (18.8%)	7 (43.8%)	1 (6.3%)	1 (6.3%)	4 (25.0%)

* Non-informative

** There were 2 cases of XO included

Table 3.10 The sensitivities of the amelogenin and STRs in fetal DNA detection in male cases, grouped by the collection technique

Total no. of male cases for each technique	No. of male cases with fetal DNA detectable in TCC for each technique	No. of male cases with fetal DNA detectable in TCC		
		by the amelogenin	by STRs	by both the amelogenin and STRs
Pipelle aspiration = 35 cases	18	15	9	6
Syringe aspiration = 22 cases	9	7	4	2
Endocervical lavage = 1 case	1	1	1	1

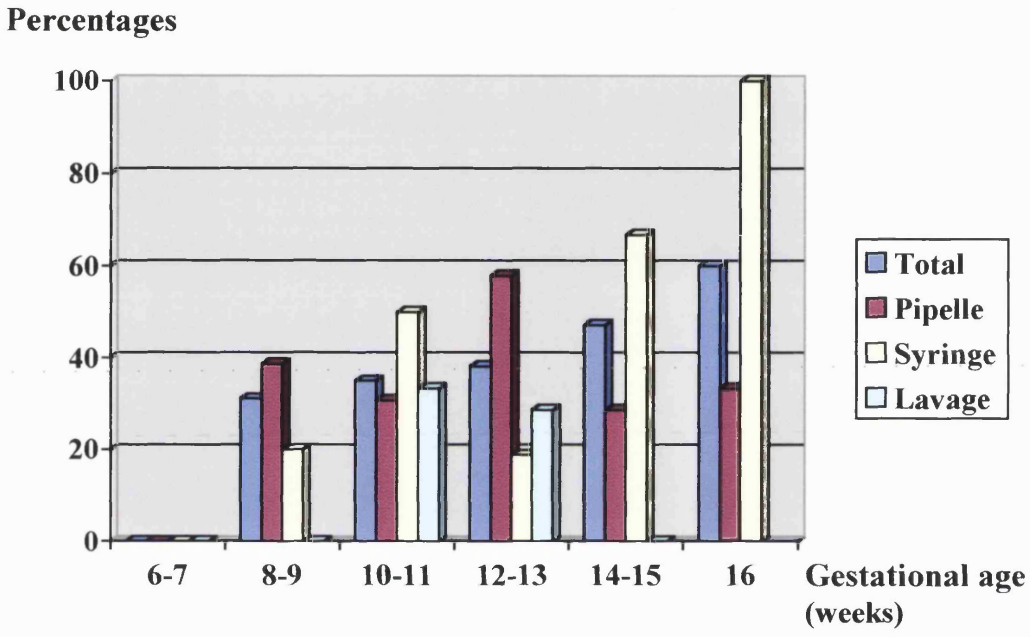
From the Table, the amelogenin was more sensitive than STRs in the detection of fetal DNA in the TCC samples

From all the aforementioned results, fetal genetic material was present in 34-39% of TCC samples collected by aspiration, and 19% by endocervical lavage. Judged by the male cases in the Pipelle aspiration, perhaps as high as 50% of TCC samples contained fetal cells.

3.3.1.4b Gestational age and the presence of fetal DNA in TCC samples

The effect of gestational age on the chance of finding fetal cells in TCC samples was investigated. When considering each method of collection separately, the number of cases for each gestational age in each method was small. Therefore, a combination of two weeks for each gestational age range was performed. Overall, in this data series, fetal DNA was undetectable from samples at the gestational ages of 6-7 weeks but was detectable in all gestational ages from 8-16 weeks. As illustrated in Figure 3.13, percentages of cases with fetal DNA positive rose gradually with gestational age from about one-third in the first trimester to about half of cases in the early second trimester. However, each collection technique did not yield a clear trend of success with gestational age. The Pipelle appeared to have a detection rate of one-third over these gestational ages except at 12-13 weeks when the detection rate was 58%. Regarding the syringe aspiration, this method seemed to have a better chance to detect fetal cells in a larger gestation. The detection rate at 16 weeks even reached 100%. However, this percentage was based on only 2 cases. As for endocervical lavage, the number of cases was too small to draw any conclusion.

Figure 3.13 Percentages of cases with fetal DNA found in the TCC samples at different gestational ages



Fetal DNA was not found at 6-7 weeks. Considering all the collection techniques, the percentages of cases with fetal DNA found in the TCC samples rose gradually from 8 weeks to 16 weeks. Using the Pipelle aspiration, fetal DNA was detected in about one-third of cases except at 12-13 weeks of gestation when fetal DNA was found in 58% of cases. Samples collected by the syringe aspiration method appeared to have a better chance to contain fetal DNA at a larger gestation. No conclusion could be drawn from the endocervical lavage.

3.3.1.4c Obstetric history and the presence of fetal DNA in TCC samples

To assess whether a previous vaginal birth had any effect on the chance of finding fetal DNA in the cervical mucus and failure rate, the previous obstetric history was obtained from the database in UCL antenatal clinic. Data were categorised into term vaginal birth (TVB), preterm vaginal birth (PVB), therapeutic abortion (TA), spontaneous abortion (SA), and G1 (primigravida), with the concept of the decreasing degree of previous stretching of the internal cervical os. A patient would be categorised according to the highest degree in the history. Table 3.11 presents a cross tabulation of the results of fetal DNA in TCC and the previous obstetric history. For patients with a history of TVB, all except two had the last delivery within the last 5 years. One delivered 9 years ago and the result of TCC showed a possible presence of fetal DNA; the other delivered 15 years ago with the result of non-informative for the presence of fetal DNA.

Table 3.11 Results of fetal DNA in TCC samples according to the previous obstetric history

Previous obstetric history	TCC results					Total
	Positive	Negative	Possible	Noninf.*	Fail	
TVB	14 (34.1%)	18 (43.9%)	1 (2.4%)	1 (2.4%)	7 (17.1%)	41 (100.0%)
PVB	2	1	-	1	-	4
TA	6	4	-	-	4	14
SA	3	1	1	2	-	7
G1	8 (32.0%)	12 (48.0%)	-	1 (4.0%)	4 (16.0%)	25 (100.0%)
Total	33	36	2	5	15	**91**

Noninf.* = non-informative
Note: Percentages of interest are shown out of the TVB and G1 categories (see text next page)

Excluding the possible and non-informative categories and using Pearson χ^2–test, there was no difference in the positive, negative and failure rate achieved from patients with different obstetric histories ($P = 0.67$). In addition, the two extremes (TVB and G1) of the obstetric history had similar positive, negative and failure rates, *i.e.*, 34%, 44% and 17% respectively for TVB and 32%, 48% and 16% respectively for G1.

3.3.1.4d The presence of fetal DNA in TCC and maternal age

Data of maternal age were available from 133 patients ranging from 15 to 44 years old (mean $\pm$ 2 S.D. = 27.6 $\pm$ 12.6). No correlation of the TCC results with the maternal age was found (Pearson χ^2–test, P = 0.27) **(Table 3.12)**. However, there appeared to be more chance to find fetal cells in TCC samples in younger age group.

Table 3.12 TCC results with maternal age group

Age group (years old)	TCC results					Total
	Positive	Negative	Possible	Noninf.*	Fail	
15-19	7 (41.2%)	5 (29.4%)	-	1 (5.9%)	4 (23.5%)	17 (100.0%)
20-24	18 (47.4%)	15 (39.5%)	2 (5.3%)	-	3 (7.9%)	38 (100.0%)
25-29	14 (41.2%)	12 (35.3%)	-	1 (2.9%)	7 (20.6%)	34 (100.0%)
30-34	6 (23.1%)	14 (53.8%)	1 (3.8%)	2 (7.7%)	3 (11.5%)	26 (100.0%)
35-39	3 (20.0%)	5 (33.3%)	1 (6.7%)	3 (20.0%)	3 (20.0%)	15 (100.0%)
40-44	-	2 (66.7%)	-	-	1 (33.3%)	3 (100.0%)
Total	48 (36.1%)	53 (39.8%)	4 (3.0%)	7 (5.3%)	21 (15.8%)	**133 (100.0%)**

* Non-informative

3.3.2 FETAL DNA IN TCC FROM ON-GOING PREGNANCIES

Thirty-eight TCC samples were collected from pregnant patients who underwent CVS procedure at the gestational age of 9-12 weeks. One sample, found to contain no cells in the fresh specimen, was discarded. Mean ± 2 S.D. of the age of the remaining patients was 31.7 ± 9.6 years old (range 21-39). Indications for CVS procedure are shown in Table 3.13.

Table 3.13 Indication for CVS procedure in the 37 patients

Indication	Number of cases
Thalassaemia trait	19
Sickle cell trait	9
Advanced maternal age	2
Other single gene disorders	7
Total	37

3.3.2.1 Results of fetal DNA in TCC samples

In this group of patients, fetal sexing was obtained by karyotyping results and/or PCR using amelogenin primers on the CVS samples. Of the 37 TCC specimens, three were amplified by amelogenin only. These three were from male pregnancies and a Y-sequence was found in one. The remaining 34 cases were amplified for both the amelogenin and STR primers, 15 were male and 15 were female fetuses including a case of 45,X and a case of 46,XX, inv(11)(p11.2q13). Sexing results were not available from 4 cases **(Table 3.14)**. Again, fetal DNA could be detected in one-third (5 cases) of male pregnancies with amelogenin positive in 4 of 5 and STRs in 2 of 5. In female pregnancies, fetal DNA was found in only 1 of 15 cases. This was similar to male pregnancies where fetal DNA was found in 2 of 15 cases by STRs.

Table 3.14 The presence of fetal DNA in TCC samples from 34 on-going pregnancies by PCR of amelogenin and STRs

Fetal sex (cases)	Fetal DNA in TCC				
	Positive	Negative	Possible	Noninf.	Fail
Male (15)	5*	7	-	-	3
Female (15**)	1	7	1	2	4
NK (4)	-	2	-	-	2

Noninf. = non-informative
- = no case in that category
NK = fetal sex not known
* = Fetal cell detection was achieved by amelogenin only in 3 cases, by an STR only in 1 case and by both amelogenin and STRs in 1 case.
** = including one 45,X and one 46,XX,inv(11)(p11.2q13)

Regardless of fetal gender and PCR analysis, the results show a high failure rate of 9 in 37 cases (24.3%). Fetal DNA was identified in a total of 7 cases (18.9%) including the male case which was amplified by amelogenin only, a proportion lower than samples from the TOP cases. The effect of gestational age was not investigated because all the patients were in the same range of gestation (9-12 weeks, except two cases which were at 13 weeks when the CVS was performed).

3.3.2.2 Safety of the procedure

Results from CVS revealed 7 affected pregnancies, 4 by β-thalassaemia, 2 by sickle cell disease and one by xanthine sulphate oxidase deficiency. Five of these cases underwent therapeutic abortion. Data were unavailable from the other two affected pregnancies, and from 8 unaffected pregnancies. Of the 22 unaffected pregnancies that had a complete follow-up, although one had a preterm delivery at 36 weeks, all resulted in a live-born baby. No adverse effect of the TCC sampling procedure was observed.

3.3.3 TOTAL CASES IN THE STUDY

Overall, there were 193 TOP cases and 37 on-going pregnancies included in the study. Considering the total positive cases from all techniques and analyses, TCC samples were found to contain fetal cells in 34% (66/193 cases) in TOP cases and 19% (7/37 cases) in on-going pregnancies. In the TOP cases, data were compiled from Tables 3.1, 3.5, 3.6 and 3.9 to obtain the overall percentages of cases with fetal DNA in TCC samples according to collection techniques. It was found that the Pipelle aspiration had a slightly higher percentage than the syringe aspiration and the endocervical lavage had the lowest percentage **(Table 3.15)**.

Table 3.15 Compiled results from TOP cases

PCR analysis	Number of total cases (number of positive cases)		
	Pipelle aspiration	Syringe aspiration	Endocervical lavage
Amelogenin only (56 cases)	42 (14)	-	14 (4)
Amelogenin and STRs (137 cases)	77 (30)	44 (15)	16 (3)
Total TOP cases in the study (193)	119 (44)	44 (15)	30 (7)
Percentages of positive cases for each collection method	**37%**	**34%**	**23%**

3.4 DISCUSSION

3.4.1 PRESENCE OF FETAL CELLS IN TCC SAMPLES

Currently prenatal diagnosis performed in early gestation is by CVS technique. The procedure carries with it rare but potentially serious complications. A less invasive prenatal diagnosis that can be performed in early gestation is desirable. Maternal blood and cervical mucus are attractive samples since they have been demonstrated by some studies to contain cells of fetal origin that are potentially useful for prenatal diagnosis.

The present study confirmed the presence of fetal DNA in TCC samples, albeit in a low frequency. A high success rate of 60-100% in detecting male cells in TCC samples have been reported by several groups (reviewed in Adinolfi and Sherlock, 1997). However, this study showed that a simple catheter could be used to collect samples with similar success rates to aspiration with a catheter connected with a syringe in the first trimester. To the author's awareness it was the first series to use the Pipelle catheter to collect TCC samples. It was more practical and less clumsy to use compared to syringe aspiration. As for endocervical lavage using Trophocan™ which is normally used for CVS procedure, due to the length and softness of the catheter, it was less easy to control the direction into the cervical canal, expel fluid and aspirate back the sample. There were a number of times when the fluid was lost and only a little was achieved. This could explain the lower success of the technique in this series.

Another finding was that fetal material could be found up to 16 weeks gestation, well beyond the time that the gestational sac fills the uterine cavity and the decidua capsularis fuses with the opposing decidua parietalis. This fetal material could be the cells previously trapped in the mucus before the closure of the uterine cavity. Because the study used PCR, these old cells could still be detected, as DNA would still be

analysable. Techniques relying on living cells such as culture might be less successful. However, at this gestational age, another option for prenatal diagnosis (amniocentesis) becomes available.

FISH appeared to have limited success in this series compared to several studies in recent years. Three positive cases were found in the series: two samples were collected by Pipelle aspiration and the other by endocervical lavage. In 50 TCC samples collected by intrauterine lavage form TOP cases, Ishai *et al.* (1995) reported concordant sexing results with the placenta in all cases using single- or dual-colour FISH for the Y chromosome or the X and Y chromosomes (29 males and 21 females). Kawamura *et al.* (1995) collected 24 TCC samples by intrauterine lavage from TOP cases and performed single-colour FISH for the Y chromosome. They correctly diagnosed fetal sex in all 13 male fetuses according to the FISH or karyotyping results from the placenta. No false positive FISH results were achieved from female fetuses. In addition, the frequency of nuclei with a Y signal in the male TCC samples was high, ranging from 5.8 to 23.5% in 3 missed abortion cases and from 7.4-21.5% in cases terminated for non-medical reasons, excluding 4 male cases with macroscopically visible villi. Recently, Daryani *et al.* (1997b) could detect 10 out of 12 male pregnancies using FISH on lavage samples. Regarding studies using FISH to detect male cells on aspiration samples, several groups were more successful than the present series. Two studies were able to detect 1 of 1 and 2 of 5 male pregnancies by FISH on aspirated samples (Adinolfi *et al.*, 1995b, 1995c). With aspiration followed by lavage, 12 out of 13 male pregnancies could be detected by FISH (Adinolfi *et al.*, 1995c). Another group was also successful in using FISH or PCR on aspirated samples, being able to detect 12 out of 13 male pregnancies with one false male prediction in 7 female pregnancies (Briggs *et al.*, 1995).

A less successful fetal sexing result on lavage samples using either dual-colour FISH or PCR was reported by Overton *et al.* (1996). They retrieved 87, 66 and 51 TCC samples by endocervical lavage, endocervical swab and swab from the lower uterine pole respectively. In the lavage samples examined by FISH, they were able to detect 6 out of 15 male pregnancies. In addition, they had one false positive result in 21 female pregnancies. A false positive result by FISH was not found in the present study.

The limited success of FISH in the present study was probably caused by the difficulty in obtaining cells in the solution. A number of slides contained very few cells or none at all. This was partly due to the limited experience in cell preparation, especially in cases where the mucus was very thick. Apart from causing cell lost, the thick mucus might also have affected the FISH efficiency. Less FISH problems were encountered with the placenta.

A higher fetal detection rate was achieved by PCR. With Pipelle and syringe aspirations, fetal DNA could be detected in 39 and 34% respectively, with as high as 50% in the male pregnancies when using Pipelle aspiration. Amelogenin was more sensitive than STRs. However, STRs should be included because some cases were positive for the STRs only while the amelogenin somehow failed to detect the Y sequence or even failed to amplify. Amelogenin is not useful in female cases. It should, however, be included to determine the false positive rate which was found to be zero in the current study.

Several possible factors affecting the fetal cell retrieval in the cervical mucus were investigated. They were considered from the cases where PCR was performed for both the amelogenin and STRs because this group comprised the majority of studied cases. In addition, all the three collection methods were included in this group. A

possible effect from gestational age on fetal detection rate in TCC samples was observed. This was not, however, observed when considering each method individually but was observed when considering the total cases. Considering each method separately, the number of each gestational age range was small and the effect might not discernable. In a previous study, it appeared that samples from aspiration improve in quality with gestational age (Rodeck *et al.*, 1995). For this study, the tendency of higher detection rates in the early second trimester was unlikely to be due to more shedding of fetal cells because by this time the uterine cavity was obliterated. The decidua capsularis and the decidua parietalis fuse at 14-16 weeks of gestation (Cunningham *et al.*, 1997b). The more frequency of finding fetal cells in TCC in the early second trimester was more likely due to the accumulation of previously shed cells trapped in the mucus. In a previous study collecting TCC samples by aspiration from beyond the internal cervical os and intrauterine lavage from 7-17 week pregnancies, no syncytia were observed under a microscope from gestational age of 14 weeks or more (Briggs *et al.*, 1995). In another study where the TCC samples were collected by mucus aspiration and cytobrush in TOP cases at 7-11 weeks gestation, no correlation with the gestational age was found (Falcinelli *et al.*, 1998). It is notable that in their study, fetal sex determination could be achieved on TCC samples from 7 weeks' gestation, earlier than the current study. This may be due to the highly sensitive inner primers that they developed for their nested PCR. The sensitivity was assessed to be able to detect one male in 600,000 female cells.

In the current study, detection rate in the ongoing pregnancies was lower than in the TOP cases. This was possibly due to the gentler maneuver being performed. This bias could inadvertently arise from concern about the safety of the pregnancy. In this

aspect, no adverse effect was found in the 22 on-going pregnancies where the outcome was available. This was in agreement with a previous larger study (n = 113) (Rodeck *et al.*, 1995).

With regard to the parity effect, some might speculate that previous cervical dilatation either by vaginal birth or intrauterine instrumentation might give more chance of fetal cell recovery. However, it is not the case as shown by the result in the current study. This is not surprising, considering that after a delivery or an abortion, internal cervical os will close.

The recovery rate of fetal cells using PCR in the current study was lower than a recent study. Miller *et al.* (1999) reported that in their study, Y-specific sequence DNA was found by PCR in 13 out of 26 (50%) flushings and 99 out of 154 (64%) aspirates. However, the TCC samples were obtained from the lower uterine pole and this might yield more chance to retrieve fetal cells than samples from the cervical canal collected in the current study.

The result of PCR for amelogenin only in the early part of the current study was comparable to a study by Falcinelli *et al.* (1998). Using nested PCR on TCC samples collected by mucus aspiration (n=27) and cytobrush (n=36) they could correctly determine fetal sex in 78% and 89% of cases respectively. In the aspirates, they were able to detect 15 out of 20 male TCC samples (75%) with one false positive (1 in 7 female cases). With cytobrush, they used two or three cytobrushes per case, one after another, to retrieve more material. This may explain the satisfactory results that they obtained with this technique. Also, they had reviewed four former studies (Kingdom *et al.*, 1995; Briggs *et al.*, 1995; Adinolfi *et al.*, 1995a, 1995c) and found the cumulative

sensitivity of male pregnancy detection to be 73%, similar to the early part of the current study.

3.4.2 POSSIBLE MECHANISMS OF THE FETAL CELLS BEING FOUND IN THE CERVICAL MUCUS

It is most likely that fetal cells in the cervical mucus come from the chorion laeve as this part of the chorion is nearest to the uterine cavity. As the conceptus grows, the gestational sac enlarges, bulging into the uterine cavity. This results in the decidua capsularis being stretched across the underlying chorionic villi, restricting the latter of blood supply. As a result, the chorionic villi in this region become flattened and avascular. However, the chorion laeve still survives and plays an important role in the maternal-fetal paracrine arm communication. For example, it expresses several hormones or factors such as placental alkaline phosphatase (Yeh *et al.*, 1989; Shih *et al.*, 1999), human placental lactogen (Yeh *et al.*, 1989), insulin-like growth factor-II (Han *et al.*, 1996), epidermal growth factor receptor (Bulmer *et al.*, 1989), integrin (Aplin, 1993), and HLA-G antigen (Hutter *et al.*, 1996). Trophoblast cells in the chorion laeve retain their proliferative capacity into late pregnancy (Bulmer *et al.*, 1988) but progressively disappear nearer term by apoptosis (Parmley, 1990). All show that the chorion laeve survives low oxygen environment. Indeed it has been proposed by a study group that the early part of gestation occurs in a low oxygen environment and an ambient oxygen tension may do harm to the syncytiotrophoblast (Watson *et al.*, 1998).

Under the stretching decidua capsularis, the chorionic villi may simply degenerate or be torn off by sheering force. With the chorion laeve being normally active under such a low oxygen environment, it may be the case that recently shed

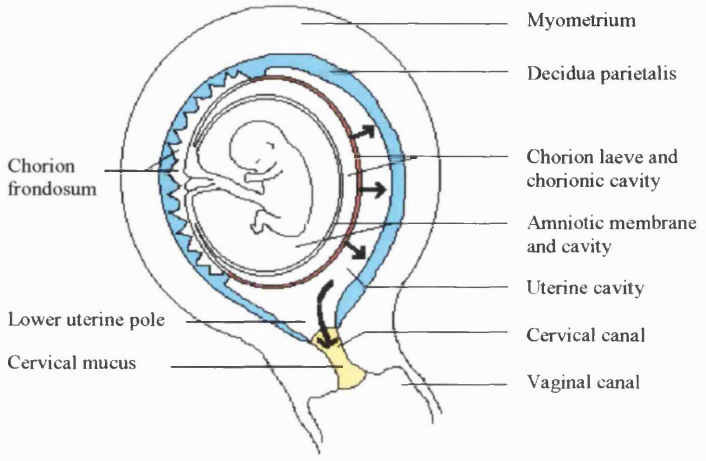
trophoblast cells still have the invasive properties, and from their position, the invasion should be directed to the uterine cavity. This may be the same mechanism of the initial implantation, where the trophoblast cells penetrate between the endometrial epithelial cells. Alternatively this can be the same as the migration and invasion of extravillous trophoblasts in the decidual stroma at the placental site (chorion frondosum). Having invaded through the endometrial epithelium to the uterine cavity, trophoblastic cells or fragments may then shed, collect and accumulate in the cervical mucus. The hypothesis of fetal cells invading or migrating through the decidua capsularis **(Figure 3.14b)** has been postulated by Rodeck *et al.* (1995). Their hypothesis differs from the view held by some earlier groups that the trophoblast cells degenerate, exfoliate and drop off into the uterine cavity. With this earlier view, it would appear that the chorion is directly exposed to the uterine cavity which is not true **(Figure 3.14a)**. In addition, the fetal cells found in the cervical mucus appear healthy and viable rather than degenerated cells (Rodeck *et al.*, 1995).

The second possibility causing the exposure of the trophoblasts in the chorion laeve to the uterine cavity is the rupture of the decidua capsularis which may result from the physical stretching over the conceptus. Again, the trophoblast cells or fragments may then collect in the cervical mucus.

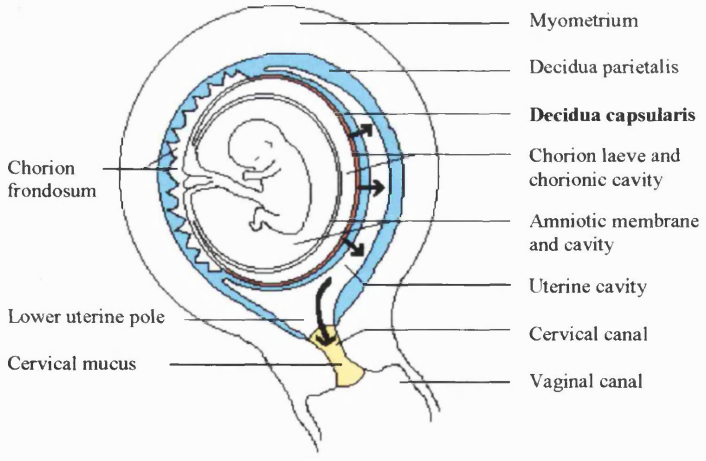
The third possibility is that these cells are trophoblast cells that might have been shed at the time of implantation before the total coverage of the endometrial epithelium **(Figure 3.15)**. This is unlikely as there were no positive cases at the gestational age of 6-7 weeks in the study. Also, this possibility would produce only a small number of cells and might not be detected.

Figure 3.14 Views of mechanism of the presence of fetal cells in cervical mucus

Modified from Rodeck et al. (1995)



a) Fetal cell degeneraion and exfoliation



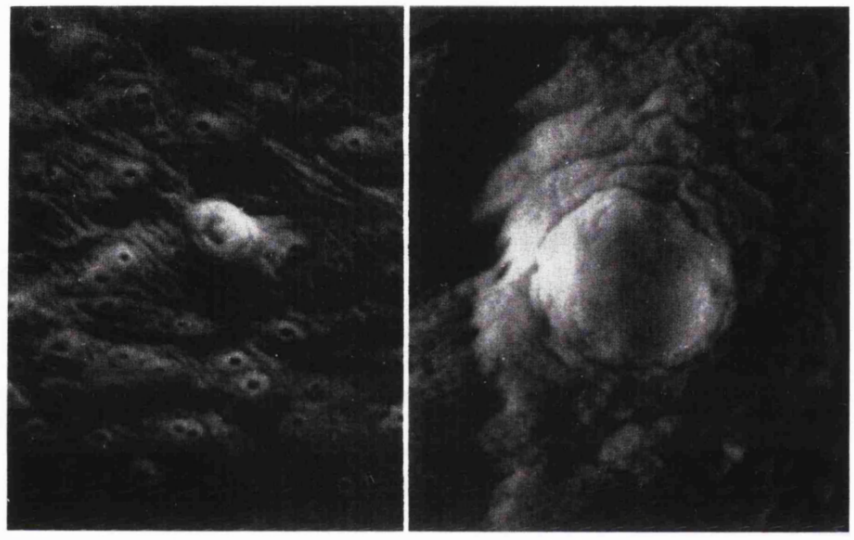
b) Fetal cell invasion and migration

a) Earlier view of fetal cell degeneration and exfoliation. Exfoliated cells drop off into the uterine cavity and collect in the cervical mucus. This mechanism is unlikely, considering the presence of the decidua capsularis.

b) The hypothesis postulated by Rodeck *et al.* (1995). Fetal cells invade and migrate through the decidua capsularis into the uterine cavity and collect in the cervical mucus.

Figure 3.15 Endometrial surface with an early implanted blastocyst

Taken from Cunningham et al. (1997c), p. 104.



Low- and high-power photomicrographs of surface view of an early implanted blastocyst fewer than 8 day after conception. Site was slightly elevated and measured 0.36x0.31 mm. Endometrial gland openings appear as dark spots surrounded by halos.

3.4.3 FUTURE WORK

TCC samples are demonstrated to contain fetal cells which are potentially useful for non-invasive prenatal screening. However, the frequency of fetal cell recovery from the samples varies greatly. There are a number of reasons for this variation such as the efficiency of the collection methods, the analytic technique used, the operator's experience and finally the true frequency that these cells are present in the lower uterine pole or the cervical mucus. In this study, even with the same operator performing the procedure, the success rate of PCR to detect a male pregnancy in the early part of the study was much higher than the latter part. Although multiplex PCR may explain the lower success in the latter, it is possible that the difference in the frequency of cases with fetal cells in the TCC sample between the two groups was real. The difference might have been due to a wide variation in fetal cells present in TCC samples from case to case. If a comparative study were to be performed in the future, a well designed and established protocol should be available for the collection techniques, the analysis techniques, the target cases, the operators and an adequate sample size.

Also, to be able to use TCC samples for diagnosis of a single gene disorder especially in a recessive disease, a technique to identify and isolate pure fetal cells should be developed and refined. For identification, several monoclonal antibodies for placenta have been employed. These, however, are not exclusively specific for fetal cells. Micromanipulation has been performed with some successful results (Tutschek *et al.*, 1995; Sherlock *et al.*, 1997; Adinolfi *et al.*, 1997; Adinolfi and Cirigliano, 2000).

Compared to fetal cells in maternal circulation, when positive, the frequency of fetal cells among maternal cells is higher in the TCC samples, judging from FISH results from previous studies and some positive cases in this study. Some enrichment

techniques used to enrich fetal cells in maternal blood may be applicable for the TCC samples if the mucus problem can be solved.

Another aspect that should be studied more is the effect of the TCC collection on the pregnancy. Cervical mucus is regarded as a protection against intrauterine infection and a disturbance with it may be harmful. However, from the limited number of the known outcome in on-going pregnancies in this study, no adverse effect was observed, even the procedure was performed before an intrauterine instrumentation (transcervical CVS). This was in agreement with a previous larger study where the outcome of pregnancies that had undergone TCC collection and had a complete follow-up (n = 113) was not different from general population (Rodeck *et al.*, 1995). Nevertheless, apart from the improvement of the technique, more reassurance should be achieved before the TCC collection for genetic analysis would be employed clinically.

CHAPTER 4
FETAL TISSUE MOSAICISM

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FETAL TISSUE MOSAICISM

4.1 INTRODUCTION

Chromosome abnormality is a major cause of pregnancy loss with more than half of the spontaneous abortions in the first trimester being karyotypically abnormal. Among these chromosomally abnormal abortuses, half are autosomal trisomy (Boue *et al.*, 1975). Despite high levels of spontaneous abortion, some trisomic fetuses survive to term as shown by the incidence at birth of trisomy 21, 1:700; trisomy 18, 1:5,000-10,000 and trisomy 13, 1:10,000-20,000 livebirths. It has been suggested that the presence of a normal diploid cell line found in placentae of some cases of trisomy could create an *in utero* environment that will maintain these trisomies to birth. In 14 cases of trisomy 13 and 18, a normal cell line, varying between 12-100%, in the cytotrophoblast alone was documented (Kalousek *et al.*, 1989). In addition, in other chromosome abnormalities such as Turner syndrome where 98-99% of affected embryos/fetuses abort, several studies suggest that the incidence of mosaicism is higher in the liveborn or phenotypically normal 45,X (Hassold *et al.*, 1992; Amiel *et al.*, 1996).

Studies using FISH on cleavage stage embryos have shown that a high frequency of chromosome mosaicism is observed even at this early stage of development (Munné *et al.*, 1994; Harper *et al.*, 1995; Delhanty *et al.*, 1997). The chromosome patterns observed at this stage have been categorized into 4 groups: uniformly normal (all cells diploid), uniformly abnormal (e.g., all cells aneuploid), mosaic (more than 1 cell line present) and chaotic (every cell shows a different chromosome complement) (Delhanty *et al.*, 1997).

Later in development, mosaicism is again evident from CVS samples which show an incidence of 1-2% mosaicism, most of which is CPM with a non-mosaic diploid fetus **(section 1.4.2.1)**. A phenomenon called trisomic zygote rescue probably plays an important role resulting in CPM in cases where the zygote starts as trisomic (Wolstenholme, 1996). However, in some cases, mosaicism is also present in the fetus and may persist to postnatal life.

From postnatal studies of dysmorphic patients, there have been several reports of different proportions of abnormal cell lines in lymphocytes and skin fibroblasts, sometimes with the abnormal cell lines being found in skin fibroblast only or *vice versa* (Reyes *et al.*, 1978; Pagon *et al.*, 1979; Metaxotou *et al.*, 1981; Bass *et al.*, 1982). In some instances, mosaicism is confined to a specific organ, or present in various percentages in different organs (Yokoyama *et al.*, 1992; Shashi *et al.*, 1996). In addition, karyotyping from lymphocyte cultures obtained at different ages in a mosaic patient showed a decrease in the number of abnormal cells with age (Reyes *et al.*, 1978; Bass *et al.*, 1982). Gravholt *et al.* (1991) found increases in the normal euploid cell line of >10% in 16 out of 32 chromosomal mosaics followed over 1-20 years. This possibly implies that there is a selective advantage for the euploid cell line with time.

In the present study interphase FISH has been used to examine different organs from fetuses with trisomies 13, 18 and 21 to evaluate the level of mosaicism in different tissues across gestation. The aim is to determine if there is any association between the level of mosaicism, if found, with the severity of phenotype and the potential survival to term with the idea that the older the gestational age, the more chance of survival to term.

During this study, two important aspects relating to the FISH technique were analysed. First, efficiencies of commercial and non-commercial probes were compared. Second, the efficiencies of the probes in interphase and metaphase in trisomic and diploid samples were compared. The second aspect was studied to establish a reference for non-mosaicism. Initially, percentages of disomic cells in tissues from normal fetuses were regarded as controls for non-mosaicism. After the work up, percentages of trisomic cells in non-mosaic fetal skin fibroblast cultures were used.

4.2 MATERIALS AND METHODS

4.2.1 TRISOMIC FETAL TISSUES

Trisomic fetal tissues from different organs were collected from therapeutic abortions at 11-26 weeks of gestation. Twenty-one cases were examined: 4 cases were trisomy 13, 10 cases were trisomy 18 and 7 cases were trisomy 21. Tissues collected were placenta, brain, lung, muscle, liver, kidney, intestine, heart, skin, spinal cord and adrenal tissues. Fetal tissues from diploid abortuses were also collected and studied in the same manner. Not all the tissues were collected from every pregnancy.

Fetal tissues were snap frozen on site and stored at -80°C. In the early part of the study, fetal tissues were prepared into cell suspension for FISH as described **(section 2.3.1.2)**. Later, the tissues were directly dabbed on a clean slide using the cut surface.

4.2.2 NORMAL FETAL TISSUES

Normal fetal tissues from termination of pregnancy for non-medical reasons were collected and studied in the same manner. There were one male and one female fetuses at the early part of the study. Later, some tissue types were available from more fetuses.

The collection of all fetal tissues for this study (obtained from Queen Charlotte's and Chelsea Hospital) had maternal consent and local ethical committee approval (Project registration 94/4290).

4.2.3 NON-MOSAIC TRISOMIC AND TRIPLOID SAMPLES

Skin fibroblast cultures were obtained from fetuses with trisomy 13, 18 or 21 and a triploid (69,XXY) fetus diagnosed by conventional karyotyping (courtesy of

Professor Joy Delhanty). The cells were prepared by standard methods for cytogenetic analysis and were kept in fixative solution at -20°C.

4.2.4 NORMAL MALE LYMPHOCYTE CONTROLS

To ensure probe efficiency a normal male lymphocyte culture was prepared as described **(section 2.3.1.4)** to be used in each experiment.

4.2.5 NON-COMMERCIAL AND COMMERCIAL PROBES

At the beginning of the study, FISH using non-commercial probes for chromosomes X, Y, 18 and 1 were performed for sexing and the study of chromosome 18. Details of probes are presented in **Table 2.2, section 2.3.2**, and the FISH procedure is described in **section 2.3.4**. The studies for chromosomes 13 and 21 and for trisomy 18 in later studies were performed with probe mixture #1 and #2 of AneuScreen Prenatal Aneuploidy Detection Panel [Vysis (UK), Ltd.] **(Table 2.3, section 2.3.3)** as described **(section 2.3.5)**.

4.3 RESULTS

4.3.1 FISH WORK UP

4.3.1.1 FISH work up on lymphocytes and normal fetal tissues using non-commercial probes for chromosomes 18, 1, X and Y

FISH using non-commercial probes for chromosomes 18, 1, X and Y were performed on lymphocyte controls and tissues from diploid fetuses to determine the probes performances on fetal tissues. The probe for chromosome 1 was used as an additional control. Experiments were performed using single-colour (only one probe on each slide), dual-colour (two probes for each slide) and multicolour FISH (using probes for chromosomes X, Y and 18 on each slide). All the tissues were prepared as cell suspensions as described in **section 2.3.1.1**.

Table 4.1 shows the results from single-colour FISH for chromosome 18 or 1 and dual-colour FISH for chromosomes X and Y. The fetal tissues were from a male fetus. The percentage of cells with two signals for each autosome and cells with an XY signal for the sex chromosomes are presented. At least 300 nuclei were scored per slide. Performances of the probes were similar for lymphocytes and fetal tissues, and similar for all the probes used.

Table 4.1 Single-colour FISH for chromosome 18 or 1 and dual-colour FISH for sex chromosomes on lymphocytes and a normal male fetal tissues

Samples	Single-colour FISH		Dual-colour FISH
	% of cells with 2 signals for chromosome 18	% of cells with 2 signals for chromosome 1	% of cells with XY signals
Lymphocyte	93.22 (316/339)	95.02 (305/321)	96.58 (339/351)
Fetal tissues			
Placenta	95.09 (310/326)	96.19 (303/315)	94.07 (349/371)
Liver	94.28 (346/367)	94.41 (304/322)	94.11 (447/475)
Lung	92.14 (293/318)	94.84 (294/310)	97.51 (353/362)
Brain	91.16 (330/362)	92.35 (338/366)	99.26 (405/408)
Average for fetal tissues	**93.17**	**94.45**	**96.24**

The numbers in parenthesis are number of cells with the expected signals/number of total cells counted.

For chromosome 18, the majority of the remainder of cells, i.e., 4.29, 5.72, 7.55, and 8.29% in the placenta, liver, lung and brain respectively, showed one signal. Similarly, the majority of the remainder of cells, i.e., 2.54, 3.73, 3.23, and 4.92% in the placenta, liver, lung and brain showed one signal for chromosome 1. For the sex chromosomes, 2.96% of cells in the placenta and 4.84% of cells in the liver had an XX signal which could have been due to maternal cell contamination. No XX cells were found in the lung and the brain. In the lung, the remaining cells were XYY (1.38%), XXY (0.55%) and Y (0.55%). In the brain, the remaining cells were XYY, XXY and Y at 0.25% each. These cells could have resulted from scoring errors.

Table 4.2 shows the results from dual-colour FISH for chromosomes 1 and 18. The number of cells showing a disomic signal for both probes was smaller than that for each probe singly. Counting disomic cells for chromosome 1 gave percentages similar to those obtained from single-colour FISH. Percentages of cells with a disomic signal for chromosome 18 was lower than those obtained from single-colour FISH.

Table 4.2 Dual-colour FISH for chromosomes 18 and 1 on lymphocytes and normal male fetal tissues

Sample (number of total cells counted)	Percentage of cells with 1, 1, 18, 18	Percentage of cells with 18, 18	Percentage of cells with 1, 1
Lymphocyte (327)	90.83	91.13	94.80
Lymphocyte (341)	90.03	90.91	94.72
Fetal tissues			
Placenta (320)	85.00	90.00	94.06
Liver (380)	87.89	90.53	95.26
Lung (323)	86.07	88.24	96.90
Brain (358)	84.92	91.06	91.34
Average for fetal tissues	**85.97**	**89.96**	**94.39**
Average for fetal tissues from single-colour FISH	-	**93.17** (from Table 4.1)	**94.45** (from Table 4.1)

Table 4.3 shows the results of multicolour FISH for chromosomes X, Y, and 18 using non-commercial probes. Probes for chromosomes X and Y were labelled in green and red respectively. The probe for chromosome 18 was a mixture of red and green in

equal proportion and the signal appeared yellow or orange. Fetal tissues were obtained from two fetuses, a male and a female. By scoring the signals for sex chromosomes and chromosome 18 separately, the percentages obtained were similar to or slightly lower than those achieved from single-colour FISH.

Table 4.3 Multicolour FISH for chromosomes X, Y and 18 on lymphocytes and normal fetal tissues

Sample (number of total cells counted)	Percentage of cells with** X, Y, 18, 18 or X, X, 18, 18	Percentage of cells with X, Y or X, X	Percentage of cells with 18, 18
Lymphocyte (319)	89.03	95.92	90.91
Male fetus			
Placenta (351)	87.75	96.01	90.88
Liver (374)	83.16	90.37	91.71
Lung (369)	88.08	96.48	88.89
Brain (365)	82.47	92.88	87.67
Average for male fetus	**85.37**	**93.94**	**89.79**
Female fetus			
Liver (362)	92.54	95.86	94.75
Lung (378)	88.89	96.03	92.86
Brain (347)	84.15	91.93	90.78
Muscle (311)	91.24	96.37	94.56
Average for female fetus	**89.21**	**94.91**	**93.24**
Average for male fetus from single-colour FISH*	-	**96.24** (from Table 4.1)	**93.17** (from Table 4.1)

* In this case, means single-colour FISH for chromosome 18 or dual-colour FISH for sex chromosomes.
**percentage of cells with X, Y, 18, 18 in case of lymphocytes and the male fetus and percentage of cells with X, X, 18, 18 in case of the female fetus

4.3.1.2 FISH work up on tissues from fetuses with trisomy 18 using non-commercial probes

FISH with non-commercial probes were performed on tissues from fetuses with trisomy 18. Single-colour FISH for chromosome 18 or 1 and dual-colour FISH for the sex chromosomes were used. Multicolour FISH was not selected, as it was occasionally difficult to distinguish the orange or yellow signals from red signals, especially when the signals were overlapping.

Tables 4.4 A-B show the percentages of cells with three signals for chromosome 18 or two signals for chromosome 1 from fetal tissues. While the percentages of disomic cells for chromosome 1 were slightly lower than disomic cells in normal samples, those of cells with trisomy 18 were much lower with a very wide range. The majority of the remainder showed disomic signals for chromosome 18 as shown in *italics* in Table 4.4A.

Table 4.4C shows percentages of common types of signals found in sexing of the tissues. In the female tissues, at least 91% of cells with an XX signal were found. The next most common cell type found in female tissues was XO. In the male tissues, 4 of the 5 tissues that were examined showed no or a small proportion of cells with an XX signal. Placenta of 18.6 however, had a substantial proportion of cells with an XX signal. This tissue was investigated further. Some male tissues also had a small proportion of cells with an XO signal. These XO cells in male and female tissues could have been due to hybridization failure although a low degree of mosaicism could not be excluded. In male cells, other types of cells (e.g., XXY, XYY, XXYY) could have been due to split signals.

Table 4.4A Percentages of cells with three signals for chromosome 18 on tissues of fetuses with trisomy 18 using non-commercial probes

Case*	Placenta	Brain	Lung	Liver	Muscle	Kidney	Intestine
18.1	70.06 (220/314) *27.07*	63.12 (190/301) *33.55*	61.67 (185/300) *36.33*				
18.2	44.77 (137/306) *22.73*				79.82 (250/352) *16.57*		
18.3	62.05 (188/303) *33.66*	78.00 (273/350) *18.57*			75.42 (227/301) *21.93*		
18.6	43.57 (183/420) *51.43*	75.94 (243/320) *20.63*	80.27 (297/370) *17.84*				
18.7	64.36 (195/303) *33.00*	62.93 (275/437) *34.32*	78.39 (312/398) *19.85*				
18.8	69.67 (209/300) *26.00*	51.00 (153/300) *45.00*			64.15 (204/318) *30.82*		
18.9				Poor quality			32.00 (96/300) *62.00*
18.10	46.01 (144/313) *45.36*	43.67 (131/300) *50.67*				52.33 (157/300) *43.00*	

*Tissues of fetuses 18.4 and 18.5 were not yet available at the time of these experiments.

The percentages of cells with two signals for chromosome 18 are given in *Italics*.

The percentage of cells with two signals for chromosome 18 on lymphocyte controls was 94.73, 91.11, 89.98, 90.59, and 90.26 from 5 experiments.

Table 4.4B Percentages of cells with two signals for chromosome 1 on tissues of fetuses with trisomy 18 using non-commercial probes

Case*	Placenta	Brain	Lung	Liver	Muscle	Kidney	Intestine
18.2	92.88 326/351				93.33 (308/330)		
18.6	89.29 (275/308)	92.62 (339/366)	91.90 (329/358)				
18.8	87.71 (264/303)	82.98 (250/302)			90.00 (270/300)		
18.9				89.67 (269/300)			85.52 (307/359)
18.10	85.33 (256/300)	85.33 (256/300)				89.67 (269/300)	

*Not all the available tissues were examined with probe for chromosome 1.

The percentage of cells with two signals for chromosome 1 in lymphocyte controls was 93.89, 95.02, and 95.26 from 3 experiments.

Table 4.4C FISH results for the sex chromosomes on tissues of fetuses with trisomy 18 using non-commercial probes

Sample (number of cells counted)		Percentage of cells with the indicated signal		
		XY	XX	XO
18.1	Placenta (321)	-	95.02	4.05
18.2	Placenta (312)	-	96.15	1.92
18.3	Placenta (306)	-	95.75	2.94
	Brain (378)	-	97.09	2.12
	Muscle (458)	-	98.03	0.87
18.7	Placenta (331)	-	94.86	3.32
	Brain (351)	-	96.01	3.13
	Lung (412)	-	95.39	2.91
18.8	Placenta (300)	-	91.00	7.33
	Brain (300)	-	92.67	6.00
	Muscle (300)	-	92.67	4.00
18.6	Placenta (434)	46.54	51.61	0.23
	Brain (359)	100.00	-	-
	Lung (374)	98.40	-	0.53
18.10	Placenta (not done)			
	Brain (300)	95.67	-	-
	Kidney (300)	96.33	0.33	0.67

- = no cells with the indicated signal found

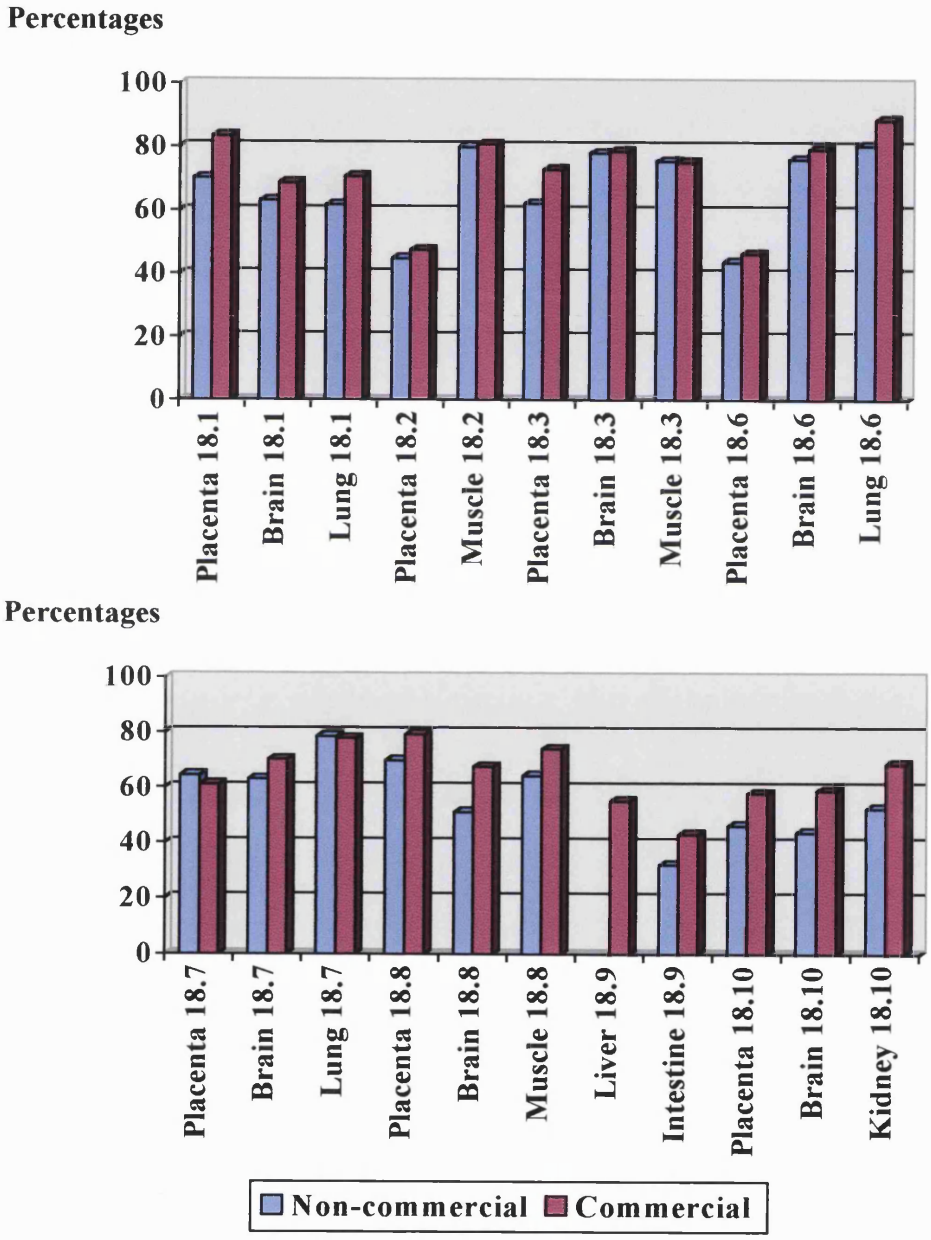
The low percentages of cells with three signals for chromosome 18 in trisomic fetal tissues might be explained by several possibilities:

1. Low probe efficiency. This may be the quality of the probes *per se*. However, the performance on lymphocyte controls and normal fetal tissues was acceptable and consistent. Some intrinsic factors in abnormal tissues may be responsible.
2. Maternal cell contamination. Because the majority of the remaining cells were disomic, maternal cell contamination was likely. This could be identified by FISH for sexing in a male fetal tissue. It was also evident in the placenta of fetus 18.6.
3. Trisomy 18 mosaicism. Disomic cells could be due to mosaicism in these fetal tissues. However, it was not expected that all the tissues were mosaic as the results might imply in **Table 4.4A**.

4.3.1.3 FISH work up on tissues from fetuses with trisomy 18 using commercial probes

FISH with commercial probes was performed on trisomic tissues to address the first possibility above. The results were compared to those previously obtained from non-commercial probes and were illustrated in Figure 4.1. The commercial probes were probe mixture #1 in the AneuScreen Prenatal Aneuploidy Detection Panel [Vysis (UK), Ltd.], which contained probe for chromosomes X (SpectrumGreen), Y (SpectrumOrange) and 18 (SpectrumAqua). Again, at least 300 cells were scored on each slide.

Figure 4.1 **Percentages of cells with three signals for chromosome 18 on trisomic fetal tissues obtained from non-commercial and commercial probe**



Percentages of cells with three signals for chromosome 18 on trisomic fetal tissues were obtained from non-commercial and commercial probes. Commercial probes either gave more or similar percentages of the trisomic cell line compared to non-commercial probes.

Overall, commercial probes appeared to have a better performance on trisomic tissues. They either gave more or similar percentages of the trisomic cell line compared to non-commercial probes. This made the results more realistic in terms of the number or the degree of tissue mosaicism. In addition, scoring signals for chromosome 18 and the sex chromosomes could be easily performed at the same time compared to multicolour FISH using non-commercial probes as the colour of chromosome 18 was aqua, not the combination of the red and green. Moreover, the study for chromosome 13 and 21 would have to be performed by commercial probes (probe mixture #2 in the same panel). Thus it was decided to use commercial probes for the whole study.

4.3.1.4 FISH investigation of maternal cell contamination

For maternal cell contamination, FISH using sex chromosome probes on male tissues would be informative. Table 4.5 shows results of FISH for chromosome 18 and the sex chromosomes, using commercial probes on the male fetal tissues. Various degrees of maternal cell contamination were evident in all placentae, and skin of case 18.4. Apart from kidney in case 18.10, all internal organs had no or a small number of maternal cells. Maternal cell contamination could explain the low percentage of cells with trisomy 18 when scored out of total cells in the cell suspensions. Nevertheless, male cells with two signals for chromosome 18 were also observed with a particularly high level in the placenta of case 18.6 and 18.10 and in the brain of case 18.10.

Table 4.5 Percentages of cells with indicated signals in male trisomic fetal tissues using commercial probes

Sample (total cell counted)		Percentage (number) of cells with the following signals out of total cell counted			Percentage (number) of cells with the following signals out of total cells with XY signal	
		3 (18)	XY	XX	3(18)XY	2(18)XY
18.4	Placenta (449)	59.47 (267)	69.04 (310)	30.51 (137)	83.55 (259)	13.87 (43)
	Lung (313)	80.19 (251)	99.68 (312)	-	80.45 (251)	17.31 (54)
	Heart (319)	79.62 (254)	99.37 (317)	-	79.81 (253)	17.67 (56)
	Intestine (305)	80.33 (245)	98.69 (301)	-	80.73 (243)	16.61 (50)
	Skin (310)	79.03 (245)	94.52 (293)	5.16 (14)	82.94 (243)	15.36 (45)
18.6	Placenta (307)	46.25 (142)	55.37 (170)	40.39 (124)	76.47 (130)	21.76 (37)
	Brain (312)	79.17 (247)	100.0 (312)	-	79.17 (247)	17.95 (56)
	Lung (337)	88.43 (298)	100.0 (337)	-	88.43 (298)	9.20 (31)
18.10	Placenta (324)	57.72 (187)	76.54 (248)	23.46 (76)	74.19 (184)	24.19 (60)
	Brain (300)	58.67 (176)	95.67 (287)	-	58.89 (169)	41.11 (118)
	Kidney (354)	68.36 (242)	84.75 (300)	15.25 (54)	80.00 (240)	16.33 (49)

3(18) = cell with three signals for chromosome 18, regardless of the result of signals for sex chromosomes

3(18)XY = cell with three signals for chromosome 18 and an XY signal for sex chromosomes, implying a male cell with trisomy 18

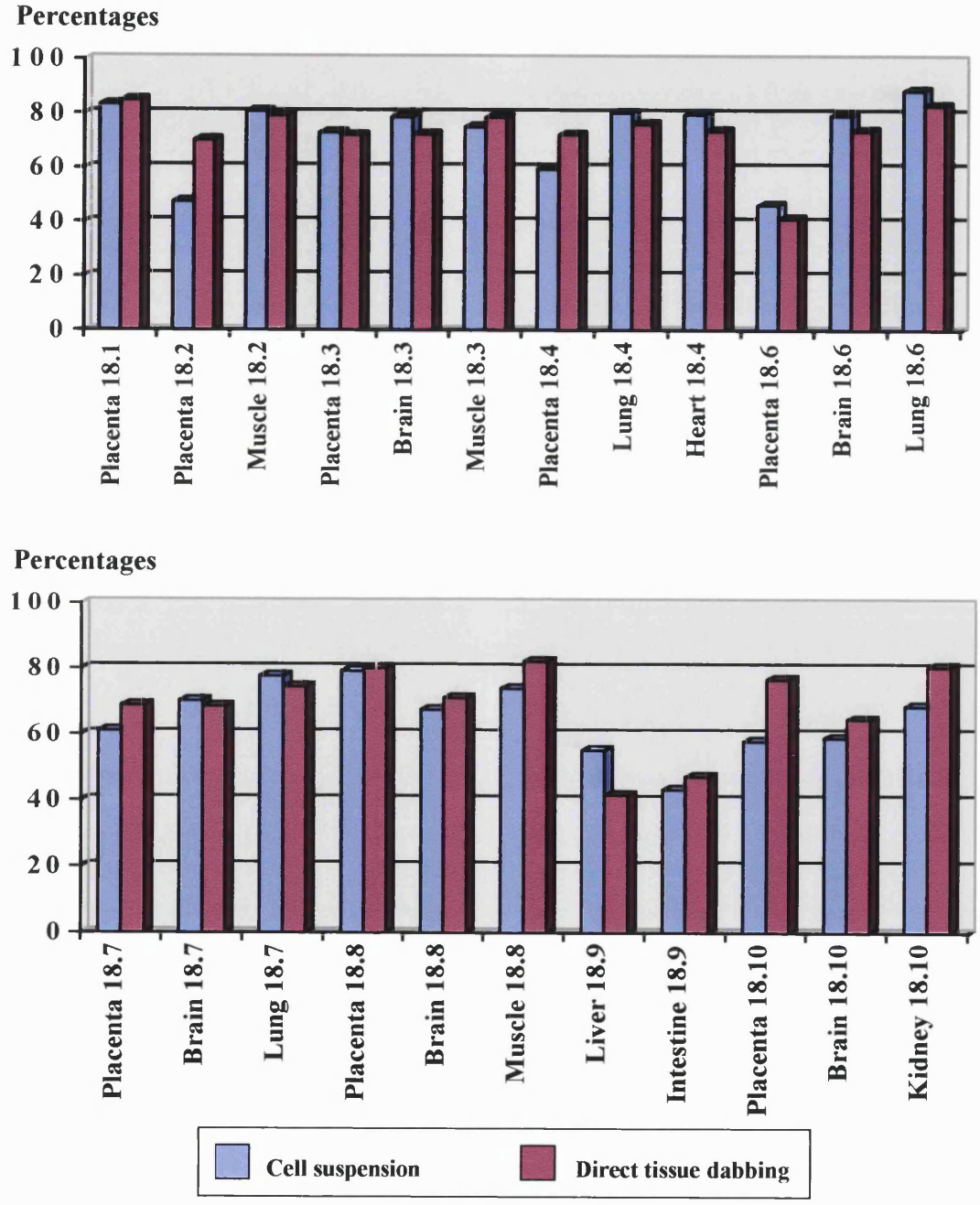
2(18)XY = cell with two signals for chromosome 18 and an XY signal for sex chromosomes, implying a male cell with disomy 18

FISH using commercial probes on slides prepared by direct tissue dabbing

All the previous results were obtained from FISH on tissues that were prepared into cell suspensions, and maternal cells could not be removed from the suspension. Subsequently, slide preparation by touching the cut surface of fetal tissues on to a slide was carried out with the idea that this technique might reduce maternal cells that possibly attached to the outer surface of the tissues. The first trial of this technique improved the percentage of cells with trisomy 18 in a number of tissues as shown in Figure 4.2.

With the direct dabbing technique, less maternal cell contamination was obtained judged by increased percentages of cells with trisomy 18. This benefit was observed in some male tissues previously shown to have maternal cell contamination such as the placenta of case 18.4 and 18.10 and the kidney of case 18.10 by the increasing of the trisomic cell line percentage of more than 10%. This technique also increased trisomic cell percentage in the placenta of fetus 18.2, in which maternal cell contamination could have caused the low percentage of trisomic cells when using cell suspension but could not be confirmed because the fetus was a female. Nonetheless, it is notable that this technique reduced the trisomic percentage in the liver of fetus 18.9. In other tissues the percentage of trisomic cells remained more or less the same. This implies that maternal cell contamination was not a problem or was not the cause of low percentages obtained for trisomic cells in most other tissues.

Figure 4.2 Comparison between percentages of cells with trisomy 18 from slides prepared by cell suspension and direct tissue dabbing



Percentages of cell with three signals for chromosome 18 were increased in Placenta 18.2, 18.4 and 18.10 and in Kidney 18.10 but was decreased in Liver 18.9 when using the direct tissue dabbing technique. In general, less maternal cell contamination was obtained in direct tissue dabbing technique.

Table 4.6 shows the details of FISH results using direct tissue dabbing on male fetal tissues that had previously shown maternal cell contamination in **Table 4.5**. The skin of fetus 18.4 was obtained as a flat piece of skin approximately 0.5 x 0.5 cm., hence was not applicable for dabbing using the cut surface. Apart from the persistent low percentage of trisomic cells and a high percentage of female cells in the placenta of fetus 18.6, Table 4.6 shows clearly that direct tissue dabbing could help reduce maternal cell contamination. Therefore, the study was performed using direct tissue dabbing with commercial probes and four experiments were performed on each tissue, with at least 300 cells scored per slide. Also, further attention was paid to the placenta of fetus 18.6 in further experiments.

Table 4.6 Results of FISH with direct tissue dabbing compared to results from cell suspension on male fetal tissues previously shown to have maternal cell contamination (except the skin of case 18.4)

Sample (total cell counted)		Percentage (number) of cells with the following signals out of total cell counted			Percentage (number) of cells with the following signals out of total cells with XY signal	
		3 (18)	XY	XX	3(18)XY	2(18)XY
18.4	Placenta (A)	59.47	69.04	30.51	83.55	13.87
	Placenta (B)	72.26	81.61	14.84	84.98	13.73
18.6	Placenta (A)	46.25	55.37	40.39	76.47	21.76
	Placenta (B)	41.23	56.17	**42.21**	66.47	31.79
18.10	Placenta (A)	57.72	76.54	23.46	74.19	24.19
	Placenta (B)	76.54	91.67	5.25	79.80	17.85
	Kidney (A)	68.36	84.75	15.25	80.00	16.33
	Kidney (B)	80.33	100.0	-	80.33	18.33

3(18) = cell with three signals for chromosome 18, regardless of the result of signals for sex chromosomes

3(18)XY = cell with three signals for chromosome 18 and an XY signal for sex chromosomes, implying a male cell with trisomy 18

2(18)XY = cell with two signals for chromosome 18 and an XY signal for sex chromosomes, implying a male cell with disomy 18

(A) = result from FISH using cell suspension
(B) = result from FISH using direct tissue dabbing

Bold shows the persistent high percentage of cells with an XX signal in the placenta of case 18.6 after using the direct tissue dabbing technique

Reduction of maternal cells in the placenta of fetus 18.6 by dissection of villi

After 4 FISH experiments on every tissue, a further assessment of maternal cell contamination was performed using average values in male tissues as shown in Table 4.7.

Compared to Table 4.5, reduction of maternal cell contamination was observed in almost all tissues previously showing this problem. However, the placenta of fetus 18.6 still had heavy maternal cell contamination judged from the presence of XX cells. When this placenta was examined under a microscope, maternal decidua was seen. When other placentae were examined in the same way, maternal decidua was present to a lesser extent. This might explain the improved results with the dabbing technique in other placentae. As for the placenta of fetus 18.6, maternal decidua was blended well into the placenta chunk so that the cut surface at the point used for dabbing still contained maternal cells.

Table 4.7 Mean percentages (n=4) of cells with indicated signals in male trisomic fetal tissues on direct tissue dabbing slides (except skin)

Sample (number of total cells from 4 experiments)		Percentage of cells with the following signals out of total cell counted (mean ± 2 S.D.)			Percentage of cells with the following signals out of total XY cells (mean ± 2 S.D.)	
		3 (18)	XY	XX	3(18)XY	2(18)XY
18.4	Placenta (1348)	73.06 ± 5.06	87.38 ± 7.73	11.22 ± 4.89	82.10 ± 6.43	15.11 ± 3.28
	Lung (1348)	80.02 ± 5.88	99.84 ± 0.38	-	80.16 ± 6.10	17.46 ± 4.22
	Heart (1278)	77.28 ± 6.91	99.61 ± 0.31	-	77.35 ± 6.91	19.60 ± 5.19
	Intestine (1366)	76.94 ± 4.69	98.64 ± 0.87	0.22 ± 0.61	77.70 ± 4.41	17.24 ± 3.11
	Skin* (1294)	75.71 ± 9.58	93.67 ± 7.76	4.69 ± 4.18	80.43 ± 4.37	15.88 ± 2.11
18.6	Placenta (1290)	**42.00 ± 3.85**	**55.35 ± 3.47**	**42.79 ± 1.44**	70.53 ± 7.22	26.84 ± 10.03
	Brain (1348)	75.68 ± 6.01	99.88 ± 0.48	-	75.71 ± 5.96	21.15 ± 5.65
	Lung (1472)	84.49 ± 5.72	99.65 ± 0.81	-	84.57 ± 5.61	13.29 ± 5.70
18.10	Placenta (1270)	70.81 ± 9.25	90.79 ± 1.37	7.60 ± 3.97	76.62 ± 7.77	20.77 ± 8.51
	Brain (1221)	61.18 ± 8.60	98.92 ± 4.33	-	61.24 ± 8.51	34.37 ± 10.95
	Kidney (1224)	81.37 ± 5.44	99.76 ± 0.62	-	81.40 ± 5.44	15.98 ± 5.28

3(18) =cell with three signals for chromosome 18, regardless of the result of signals for sex chromosomes

3(18)XY =cell with three signals for chromosome 18 and an XY signal for sex chromosomes, implying a male cell with trisomy 18

2(18)XY =cell with two signals for chromosome 18 and an XY signal for sex chromosomes, implying a male cell with disomy 18

*prepared in cell suspension

Bold = after 4 repeating experiments, a high percentage of XX cells was still persisting in this placenta, this might explain the low proportion of trisomic cells

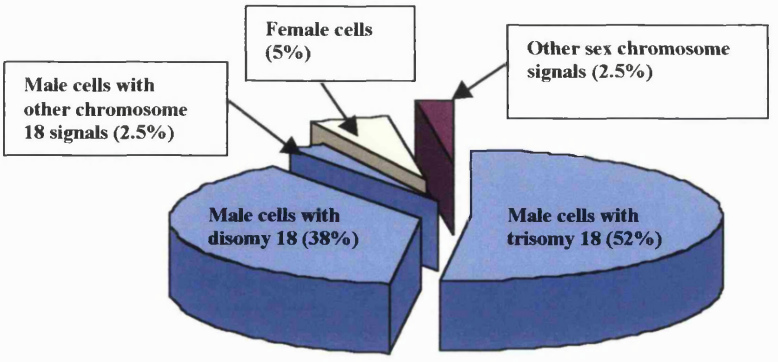
More dissection selecting only villi was performed from 4 different sites of the placenta of case 18.6 and prepared into four tubes of cell suspensions. Table 4.8 shows FISH results from 4 slides.

Table 4.8 FISH results from placenta of fetus 18.6 after dissection

Sample (number of total cells counted)	Percentage of cells with the following signals out of total cell counted			Percentage of cells with the following signals out of total XY cells	
	3 (18)	XY	XX	3(18)XY	2(18)XY
Slide no. 1 (306)	63.40	94.12	5.56	66.67	31.94
Slide no. 2 (354)	70.34	92.09	5.08	73.62	25.15
Slide no. 3 (300)	43.33	92.67	4.33	44.24	53.96
Slide no. 4 (309)	39.81	91.26	4.85	40.07	53.90
Mean ± 2 S.D.	**54.22 ± 29.90**	**92.53 ± 2.41**	**4.96 ± 1.02**	**56.15 ± 32.99**	**41.24 ± 29.83**

Using dissection for villi, maternal cells (XX) were reduced to a much less extent. The results that disomic cells were present in a substantial proportion of male cells were in agreement with the ones obtained previously **(Table 4.7)**. In addition, different proportions were found from different sites. This population of cells could well be the cause of the low proportion of trisomic cells in this placenta. From the mean values of Table 4.8, on average, disomic male cells comprised 38% and trisomic male cells comprised 52% of total cell population **(Figure 4.3)**. The results of this placenta will be considered again later.

Figure 4.3 Cell types in the placenta 18.6 (average values from 4 experiments)



The pie chart shows the average proportions of cells with different sex chromosome patterns. With dissection technique, the proportion of cells with an XX signal was much reduced. Trisomic male cells comprised 52% and disomic male cells comprised 38% of total cell population.

4.3.1.5 Results of disomic cells from normal fetal tissues using commercial probes

Initially, normal fetal tissues were used as references of non-mosaicism. After it was decided to use commercial probes on direct tissue dabbing slides on trisomic tissues, normal tissues were examined in the same way. By this time, more normal fetal tissues were available. Each tissue type was obtained from 1, 2 or 4 fetuses. Four slides were counted for each tissue type. If a particular tissue was obtained from 4 fetuses, 1 slide was prepared from each fetus. If a particular tissue was obtained from 2 fetuses, 2 slides were prepared from each fetus. If a particular tissue was obtained from only 1 fetus, all the 4 slides were from the same fetus. This approach was employed in order to have the results as general as possible. Table 4.9 shows results from these normal fetal tissues using commercial probes for chromosome 18 on slides

prepared by direct tissue dabbing technique apart from the skin which was prepared in a cell suspension.

Table 4.9 Percentages of cells with a disomy 18 signal in normal fetal tissues from 4 slides

	Placenta (n = 4)	Brain (n = 2)	Lung (n = 2)	Liver (n = 2)	Muscle (n = 2)
Slide no. 1	89.54	91.66	89.80	94.78	94.46
Slide no. 2	91.84	92.35	91.71	96.59	93.33
Slide no. 3	90.03	90.91	93.15	92.90	89.33
Slide no. 4	95.72	95.00	98.00	87.33	88.67
Mean ± 2 S.D.	**91.78 ± 5.62**	**92.48 ± 5.54**	**93.17 ± 7.01**	**92.90 ± 8.01**	**91.45 ± 5.75**
	Kidney (n = 4)	Heart (n = 4)	Skin (n = 1)	Intestine (n = 4)	
Slide no. 1	95.33	96.67	91.33	88.67	
Slide no. 2	90.67	89.67	87.33	92.33	
Slide no. 3	93.33	94.33	90.67	90.00	
Slide no. 4	88.67	90.67	90.33	91.33	
Mean ± 2 S.D.	**92.00 ± 5.86**	**92.84 ± 6.50**	**89.92 ± 3.55**	**90.58 ± 3.19**	

Number of n in the parenthesis was the number of fetuses with that kind of tissue available at the time of the experiments.

4.3.1.6 Summary of percentages of cells with trisomy 18 in trisomic fetal tissues and cells with disomy 18 in normal fetal tissues

Table 4.10 shows the summary of percentages of trisomic cells in tissues from fetuses with trisomy 18 compared with percentages of disomic cells from disomic fetal tissues. Results were from 4 experiments on each tissue using commercial probes for chromosomes X, Y, and 18 on direct tissue dabbing. Some tissues were prepared as cell suspensions, e.g., the skin of fetus 18.4 (not applicable for cut surface), the placenta of fetus 18.6 (selective dissection of villi) and the brain and the lung of fetus 18.1 (the tissues had been used up). In male tissues, the percentages of trisomic cells out of total cell number rather than out of the male cells were used in Table 4.10 so that the percentages in male and female tissues were in the same condition.

All the trisomic tissues had lower percentages of trisomic cells than those of disomic cells in normal fetal tissues and would all be classed as mosaic if the latter were used as references for non-mosaicism.

Table 4.10 **Percentages (mean ± S.E.) of cells with three signals from trisomy 18 samples and with two signals in the normal fetal tissues (repeats n=4)**

Case	Placenta	Brain	Lung	Liver	Muscle	Kidney	Heart	Skin	Gut
18.1	84.2±0.6	68.7**	70.6**						
18.2	74.0±2.0				80.2±0.6				
18.3	74.6±1.3	76.3±1.4			76.5±0.9				
18.4*	73.1±1.3		80.0±1.5				77.3±1.7	75.7±2.4	76.9±1.2
18.5	74.7±3.2				76.1±2.7				
18.6*	54.2±7.5	75.7±1.5	84.5±1.4						
18.7	67.1±2.3	69.6±0.6	77.4±1.1						
18.8	80.4±0.6	68.2±1.2			78.7±1.8				
18.9				45.6±3.2					44.9±1.0
18.10*	70.8±2.3	61.2±2.1				81.4±1.4			
Norm	**91.8±1.4**	**92.5±0.9**	**93.2±1.8**	**92.9±2.0**	**91.5±1.4**	**92.0±1.5**	**92.8±1.6**	**89.9±0.9**	**90.6±0.8**

Norm= percentages of disomic cells from disomic fetal tissues
* male fetus
** tissue only hybridised once due to scarcity

4.3.1.7 Results of FISH for fetal tissues with trisomy 13 or trisomy 21

The results for fetuses with trisomy 13 or 21, with the same strategy, i.e., the average percentage of trisomic cells from 4 experiments from each trisomic tissue comparing with the average percentage of disomic cells from 4 experiments on each normal fetal tissue, are presented in **Table 4.11 and 4.12**. The results presented in these two tables were obtained from Queen Charlotte's and Chelsea Hospital (QCCH).

Table 4.11 **Percentages (mean ± S.E.) of cells with three signals from trisomy 13 samples and with two signals in the normal fetal tissues (repeats n=4).**

Case	Placenta	Brain	Lung	Liver	Muscle	Kidney	Gut	Adrenal	Spinal cord
13.1	93.8±0.7	95.5±0.3							
13.2*	93.8±1.3	96.6±0.6		94.8±0.5				98.1±0.3	
13.3	40.4±3.5		92.2±0.3	91.2±1.5	94.6±0.6				
13.4			95.5±0.2	94.2±0.6		96.5±0.5	97.4±0.2		95.4±0.3
Norm.	**94.0±2.0**	**97.6±0.6**	**96.8±1.7**	**95.7±1.4**	**96.3±0.7**	**97.0±1.4**	**96.1±0.3**	**98.3±0.5**	

Norm. = percentages of disomic cells from disomic fetal tissues;
* male fetus

Table 4.12 **Percentages (mean ± S.E.) of cells with three signals from trisomy 21 samples and with two signals in the normal fetal tissues (repeats n=4).**

Patient	Placenta	Brain	Lung	Liver	Heart	Skin
21.1*	92.0±2.8	94.0±4.4	90.0±2.8	93.8		
21.2*	86.0±3.2	87.0±2.8				
21.3	86.7**	86.6**	86.8**		90.9**	
21.4	84.0±5.2	87.0±2.0	88.0±2.8		95.0±4.4	
21.5*		88.7±3.0		89.9±1.2	91.0±2.7	
21.6	92.0±0.5	92.0±1.5	90.0±1.3		89.0±2.8	
21.7*	91.0±3.2	95.0±2.4				90.9**
Norm	**94.3±1.5**	**95.0±1.7**	**95.5±0.6**	**94.0±0.9**	**96.6±0.6**	**95.0±0.5**

Norm. = percentages of disomic cells from disomic fetal tissues;
* male fetus; ** tissue only hybridised once due to scarcity

From Tables 4.10-4.12, generally the percentages of disomic cells in normal tissues were similar to the percentages of trisomic cells in the trisomic tissues for chromosome 13, less so for chromosome 21 and always higher for chromosome 18. If disomic cell percentages were to be used as references for non-mosaicism, all the tissues of the trisomy 18 fetuses would be mosaic. This was considered unlikely. Performances of probes on trisomic samples came into consideration and a study was set up to address this problem.

4.3.2 PROBE PERFORMANCES ON TRISOMIC OR TRIPLOID SAMPLES

This study was performed to determine the performances of probes on non-mosaic abnormal samples. FISH experiments were performed on normal male lymphocytes and skin fibroblast cultures from non-mosaic trisomic fetuses diagnosed by conventional karyotyping. Skin fibroblast cultures were chosen because they are considered stable in culture. These cultures were from fetuses with trisomy 13, 18 or 21 and a triploid fetus. To confirm non-mosaicism, at least 30 metaphases were also scored from each sample (Hook, 1977) using FISH. The mean percentages of interphase nuclei with expected signals were determined from each culture.

4.3.2.1 Results from normal male lymphocytes

Table 4.13 shows the mean percentages of interphase nuclei with the expected signals for each probe on a normal male lymphocyte culture. Ten FISH experiments were performed for chromosomes 13 and 21 probes, twelve for probe for chromosome 18 and six for probes for chromosomes X and Y. All were commercial probes. At least 300 nuclei were scored each time. Altogether more than 30 metaphases were counted

for each probe and, with 100% diploid signals of the tested chromosomes, this confirmed non-mosaicism in this lymphocyte culture in agreement with the karyotyping. In interphase nuclei, the results were shown as mean ± S.E. for each probe set. Most probes gave 96% of nuclei with the expected signals in interphase nuclei apart from probe for chromosome 18 which yielded only 91% of nuclei displaying two signals.

Table 4.13 **Percentages of cells with two signals or XY signals for each probe on normal male control lymphocytes**

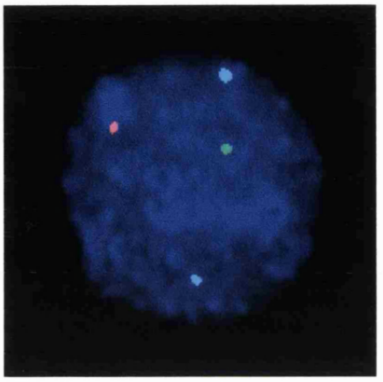
Signals (number of experiments)	Interphase nuclei		Metaphase nuclei	
	percentage mean ± S.E. (range)	total nuclei counted	percentage	total nuclei counted
2 x Chr. 13 (10)	95.73 ± 0.49 (93.67-98.67)	3000	100	88
2 x Chr. 18 (12)	91.11 ± 0.95 (85.71-96.94)	3819	100	96
2 x Chr. 21 (10)	95.90 ± 0.59 (93.33-98.67)	3000	100	88
XY (6)	95.73 ± 1.34 (89.33-98.47)	1957	100	70

2 x Chr. 13 = Two signals for chromosome 13
2 x Chr. 18 = Two signals for chromosome 18
2 x Chr. 21 = Two signals for chromosome 21

Figure 4.4 shows metaphase and interphase nuclei with expected signals from normal male lymphocytes. Two signals were expected from each autosome and an XY signal was expected for the sex chromosomes.

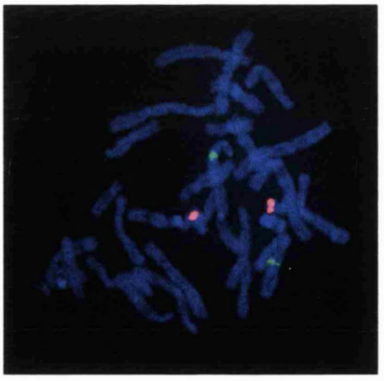
Figure 4.4 Disomic or XY signals in lymphocyte controls in metaphase and interphase nuclei

Metaphase

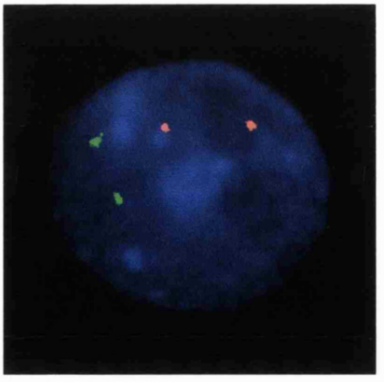


Interphase

a) Nuclei with XY and disomy 18



Metaphase



Interphase

b) Nuclei with disomy 13 and disomy 21

a) Green, red and aqua signals represent chromosomes X, Y, and 18 respectively.

b) Green and red signals represent chromosomes 13 and 21 respectively.

4.3.2.2 Results from skin fibroblast cultures from non-mosaic abnormal fetuses

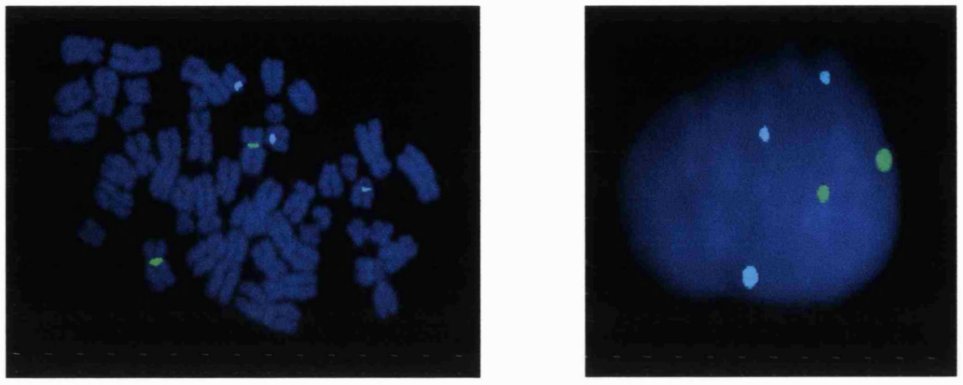
Table 4.14 shows the percentages of cells with three signals for the corresponding chromosome from each trisomic fetus. Eight FISH experiments were performed for each sample and scoring of interphase nuclei was performed on at least 300 nuclei each time. More than 30 metaphases from each sample were counted and this also confirmed non-mosaicism of each skin fibroblast culture. The results from interphase nuclei were shown as mean ± S.E. for each sample. Percentages of interphase nuclei with three signals were 88% for cultures from trisomy 13 and 21 fetuses while the probe for chromosome 18 gave a lower percentage (79%) of nuclei displaying three signals. Figure 4.5 shows the appearance of these signals in metaphase and interphase nuclei.

Table 4.14 Percentages of cells with the indicated signals from trisomic fibroblast samples

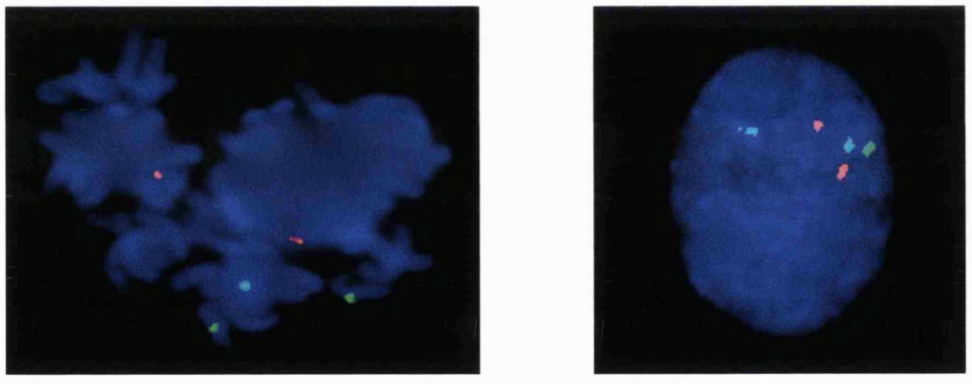
Signals (number of experiments)	Interphase nuclei		Metaphase nuclei	
	Percentage mean ± S.E. (range)	total nuclei counted	percentage mean ± S.D.	total nuclei counted
3 x Chr. 13 (8)	88.40 ± 1.41 (82.08-92.33)	2449	100	32
3 x Chr. 18 (8)	78.93 ± 1.67 (69.21-83.63)	2652	100	31
3 x Chr. 21 (8)	87.97 ± 1.19 (83.01-92.67)	2422	100	55

3 x Chr. 13 = Three signals for chromosome 13
3 x Chr. 18 = Three signals for chromosome 18
3 x Chr. 21 = Three signals for chromosome 21

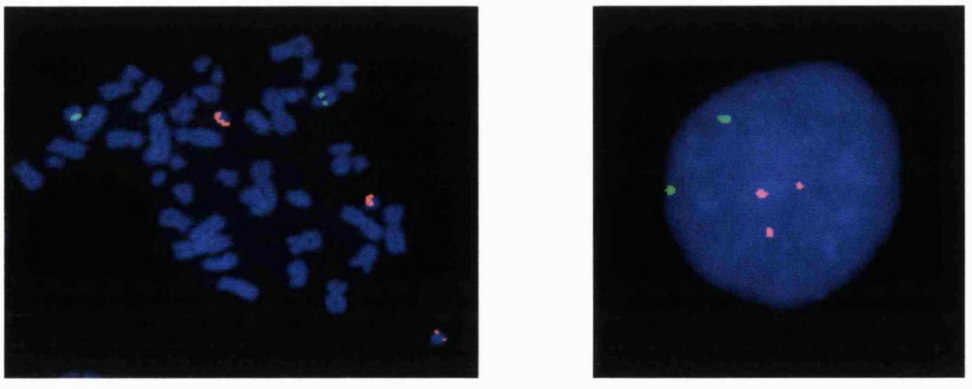
Figure 4.5 Signals from fetal skin fibroblast cultures in metaphase and interphase nuclei



a) Female nuclei with trisomy 18. Green and aqua represent chromosomes X and 18.



b) Nuclei with trisomy 13. Green and red signals represent chromosomes 13 and 21.



c) Nuclei with trisomy 21. Green and red signals represent chromosomes 13 and 21.

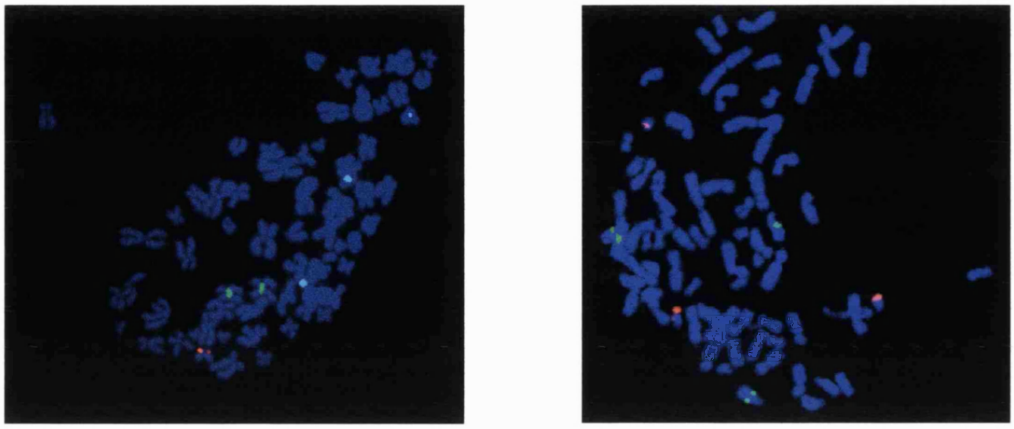
Table 4.15 shows the percentages of nuclei with three signals for the autosomal probes and nuclei with an XXY pattern for X, Y probes from the triploid fetus. Five FISH experiments were performed. The scoring was carried out on at least 300 nuclei each time. This triploid fetus showed slightly higher percentages of nuclei with the expected signals than the trisomic fetuses. The percentage of nuclei with three signals for chromosome 18 (84%) was again lower than for chromosome 13 (90%), chromosome 21 (91%) and sex chromosomes (90%). Figure 4.6 shows the appearance of triploid signals on metaphase nuclei.

Table 4.15 Percentages of cells with the indicated signals from the triploid fibroblast sample

Signals (number of experiments)	Interphase nuclei		Metaphase nuclei	
	percentage mean ± S.E. (range)	total nuclei counted	percentage mean ± S.D.	total nuclei counted
3 x Chr. 13 (5)	90.23 ± 1.27 (86.09-93.87)	1545	100	37
3 x Chr. 18 (5)	84.11 ± 1.33 (79.74-88.00)	1521	100	32
3 x Chr. 21 (5)	91.02 ± 1.33 (86.89-93.87)	1545	100	37
XXY (5)	90.15 ± 1.63 (86.14-94.12)	1521	100	32

3 x Chr. 13 = Three signals for chromosome 13
3 x Chr. 18 = Three signals for chromosome 18
3 x Chr. 21 = Three signals for chromosome 21

Figure 4.6 Metaphase triploid nuclei with the expected signals from FISH using commercial probes



a) b)

FISH results from the triploid skin fibroblast culture:
a) A metaphase nucleus with two green signals, one red signal and three aqua signals for chromosomes X, Y and 18 respectively.
b) A metaphase nucleus with three green and three red signals for chromosomes 13 and 21 respectively. Two pairs of green doublet signals can be seen for chromosomes 13 representing two chromatids.

From all the results of non-mosaic samples, the percentages of interphase nuclei with the expected signals were lower in the trisomic or triploid samples than in a diploid sample. Skin fibroblast cultures from trisomic samples were thus chosen to be references for the percentages of expected signals from trisomic fetal tissue samples to see whether the percentages obtained represented mosaicism.

4.3.3 FETAL TISSUES AND FETAL SKIN FIBROBLAST CULTURES

Each trisomic tissue was compared to the corresponding skin fibroblast culture to be classed as mosaic or non-mosaic. Non-parametric Mann Whitney *U*-test was used where possible, *i.e.*, where raw data from each scoring were available. Significant level was set at 0.05.

4.3.3.1 Trisomy 13

Table 4.16, which is a modification of Table 4.11 with the author's results added, shows the obtained percentages of trisomic cells from fetal tissues. The author's results (for case 13.4 and a repeat set of normal tissues) are presented in *italics.* The rest of the results were from the Queen Charlotte's and Chelsea Hospital (QCCH) for which raw data from each scoring were not available to be statistically compared to the result from skin fibroblast culture (88.40 ± 1.4). However, all the average percentages were higher than 90% with a narrow standard error apart from the placenta of fetus 13.3. This leads to the conclusion that all the tissues except this placenta were non-mosaic.

Table 4.16 **Percentages (mean ± S.E.) of cells with three signals from trisomy 13 samples and with two signals in the normal fetal tissues (repeats n=4).**

Case	Placenta	Brain	Lung	Liver	Muscle	Kidney	Gut	Adrenal	Spinal cord
13.1	93.8±0.7	95.5±0.3							
13.2*	93.8±1.3	96.6±0.6		94.8±0.5				98.1±0.3	
13.3	40.4±3.5		92.2±0.3	91.2±1.5	94.6±0.6				
13.4			95.5±0.2	94.2±0.6		96.5±0.5	97.4±0.2		95.4±0.3
13.4		*90.2±0.9*	*90.5±0.9*	*90.3±1.0*		*89.3±1.0*			
Norm.	**94.0±2.0**	**97.6±0.6**	**96.8±1.7**	**95.7±1.4**	**96.3±0.7**	**97.0±1.4**	**96.1±0.3**	**98.3±0.5**	
Norm.	***94.9±1.4***	***95.3±0.8***	***94.9±1.1***	***94.3±1.2***	***94.4±0.6***	***95.2±0.6***	***94.1±0.6***		

Norm. = percentages of disomic cells from disomic fetal tissues;
* male fetus
Percentages in italic = percentages scored by the author

Comparing percentages of cells with trisomy 13 between skin fibroblast culture and trisomic fetal tissues in this study, a possible inter-observer difference may arise because the skin fibroblast cultures were scored by the author whereas the results on trisomic tissues were obtained from a second operator at QCCH. This can be approximately calibrated using the data from some tissues of case 13.4 and normal fetal tissues which were scored by both operators. In the normal tissues, the author's results were slightly lower than the QCCH's. In the trisomic tissues, the QCCH's of approximately 95% would be equivalent to the author's 90%. After this calibration, all the tissues were still classed as non-mosaic apart from the placenta of fetus 13.3. For this fetus, the other tissues were all non-mosaic, thus the apparent mosaicism in the placenta could be confined placental mosaicism. However, maternal cell contamination cannot be definitely excluded. This fetus was a female therefore, FISH with sex chromosome probes was not informative. Also, the tissue was used up and dissection for villi or other further investigations were not possible.

In conclusion, from 4 fetuses with trisomy 13, confined placental mosaicism was possible in one fetus.

4.3.3.2 Trisomy 18

Table 4.17, which is a replication of Table 4.10, shows once more the results from fetal tissues with trisomy 18. These were compared to the mean $\pm$ S.E. of percentages of cells with trisomy 18 from the skin fibroblast culture (78.93 $\pm$ 1.67).

Table 4.17 Percentage (mean ± S.E.) of cells with three signals from trisomy 18 samples (repeats n=4).

Case	Placenta	Brain	Lung	Liver	Muscle	Kidney	Heart	Skin	Gut
18.1	84.2±0.6	**68.7****	**70.6****						
18.2	74.0±2.0				80.2±0.6				
18.3	74.6±1.3	76.3±1.4			76.5±0.9				
18.4*	**73.1±1.3**		80.0±1.5				77.3±1.7	75.7±2.4	76.9±1.2
18.5	74.7±3.2				76.1±2.7				
18.6*	**54.2±7.5**	75.7±1.5	84.5±1.4						
18.7	**67.1±2.3**	**69.6±0.6**	77.4±1.1						
18.8	80.4±0.6	**68.2±1.2**			78.7±1.8				
18.9				**45.6±3.2**					**44.9±1.0**
18.10*	**70.8±2.3**	**61.2±2.1**				81.4±1.4			

Bold = results judged as mosaic compared to the results from the skin fibroblast culture
* male fetus
** tissue only hybridised once due to scarcity

Statistically compared to the percentages from trisomic fibroblast culture, the bold numbers in Table 4.17 were classed as mosaic. Low-grade tissue specific mosaicism was possible in case 18.1 but with only one experiment available it was not certain. Also, low-grade tissue specific mosaicism was possible in cases 18.7 and 18.8. Strikingly low percentages, nonetheless, were present in both tissues of case 18.9, in the brain of case 18.10 and in the placenta of case 18.6. With regard to case 18.9, in the liver, the percentages of cells with three and two signals for chromosome 18 were 45.6 and 49.8% respectively. In the gut, these percentages were 44.9 and 49.6%. The fetus could be a case of generalised mosaicism with disomic cells. Nevertheless, maternal cell contamination was possible. This could not be absolutely ruled out, as the fetus was a female. However, high percentages of disomic cells were not observed in other fetuses, especially in internal organs. Also, maternal cell contamination was unlikely to occur repeatedly in a particular case. Generalised mosaicism was most likely in this case.

In case 18.10, a strikingly low proportion of trisomic cells (61%) was observed in the brain but not in the kidney. This fetus was a male, therefore maternal cell

contamination could be checked using the probes for sex chromosomes simultaneously. The details of 4 FISH results from brain tissue of this fetus using such probes are shown in Table 4.18. This illustrates that maternal cell contamination was not the case and the mosaicism was true. This fetus therefore had specific tissue mosaicism in the brain with 30-40% disomic cells.

Table 4.18 FISH results from the brain tissue of fetus 18.10

Sample (number of cell counted)	Percentage of cells with the following signals out of total cell counted			Percentage of cells with the following signals out of total XY cells	
	3 (18)	XY	XX	3(18)XY	2(18)XY
Slide no. 1 (300)	58.67	95.67	0.00	58.89	41.11
Slide no. 2 (303)	64.36	100.0	0.00	64.36	30.69
Slide no. 3 (308)	65.26	100.0	0.00	65.26	29.22
Slide no. 4 (310)	56.45	100.0	0.00	56.45	36.45
Mean ± 2 S.D.	**61.18 ± 8.60**	**98.92 ± 4.33**	**0.00**	**61.24 ± 8.51**	**34.37 ± 10.95**

In Table 4.17, as for the placentae of fetuses 18.4 and 18.10, whose percentages of trisomic cells (73% and 71%) were statistically different from the fibroblast culture controls (78.93%) (Mann-Whitney U test, P = 0.04 and 0.03 respectively), it was possible that maternal cell contamination could have affected this percentage. From Table 4.6 maternal cell contamination was evident in these placentae while it was minimal in other internal organs. Moreover, percentages of trisomic cells in the male cell population from the placentae of 18.4 and 18.10 were 82 and 77% respectively

which was not different form the fibroblast culture. These two placentae were therefore not regarded as true mosaic.

For case 18.6, while percentages of trisomic cells in the brain and the lung were consistent with non-mosaicism, the placenta was shown to have two major populations of male cells, one with a trisomic and the other with a disomic cell line for chromosome 18 **(Table 4.8 and Figure 4.3)**. Since other tissues were non-mosaic trisomy 18, this case could be confined placental mosaicism. Another less likely possibility was a vanishing twin.

PCR may be informative in a vanishing twin if it can demonstrate the presence of two individuals in the placenta in an apparent singleton pregnancy. If both parents are heterozygous for a polymorphic marker with different allele sizes between them, a vanishing twin can be confirmed if all the 4 alleles are present in the placenta. It is not conclusive, however, if less than 4 alleles are present. In the current study, the maternal DNA was obtained from the decidua but the paternal DNA was not available, therefore, several markers were randomly chosen. They were D21S1414, D18S535 and D13S631. Amelogenin was also included to confirm that the decidua was free from fetal cells. DNA extraction was performed on a piece of ~ 0.5 cm^3 of whole placental tissue, pieces of decidua for the maternal DNA, and some villi from different sites for fetal DNA. The latter two were obtained from dissection of the placenta. PCR were performed using fluorescent primers and analysed using the ABI Prism™ 310 **(section 2.2.3.2)**.

Table 4.19 shows the results from PCR from the decidua, the villi and the placenta of fetus 18.6.

Table 4.19 Allele sizes (bp) obtained from PCR of the decidua, the villi and the placenta of case 18.6

sample	Amelogenin	D21S1414	D18S535	D13S631
The decidua	X	345, 349	144, 148	195
The villi*	XY	345, **353**	140, 144	195, **203**
The placenta	XY	345, 349, **353**	**140**, 144, 148	195, **203**

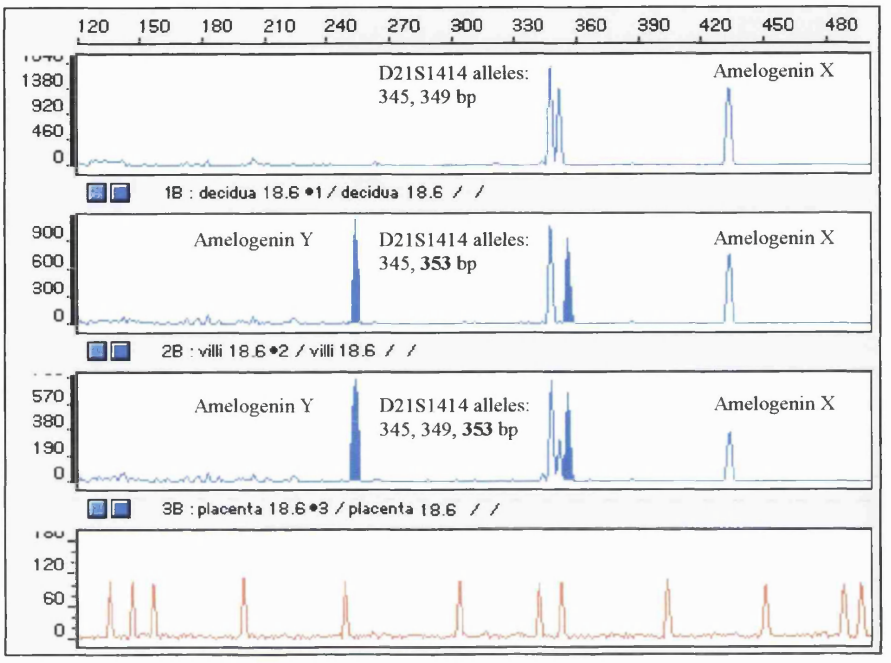
*Three experiments were performed for villi from different sites of the placenta. All STRs gave the same allele sizes. **Bold** means the fetal specific alleles.

From Table 4.19, at the most, only 3 alleles were obtained from the placenta which could be explained by maternal cell contamination which had been previously confirmed. A vanishing twin was also possible but not confirmed. This fetus was thus considered to have confined placental mosaicism rather than a vanishing twin. Also, there were only two alleles for chromosome 18. This was due to two of the three copies having the same allele size.

Figures 4.7-4.8 show electropherograms of the PCR products from the decidua, the villi, and the placenta as described in Table 4.19. The villi had a higher amount of allele 144 bp of D18S535 than allele 140 bp. Because 140 bp was the paternal allele, it may be deduced that the additional copy of the chromosome was derived from the mother.

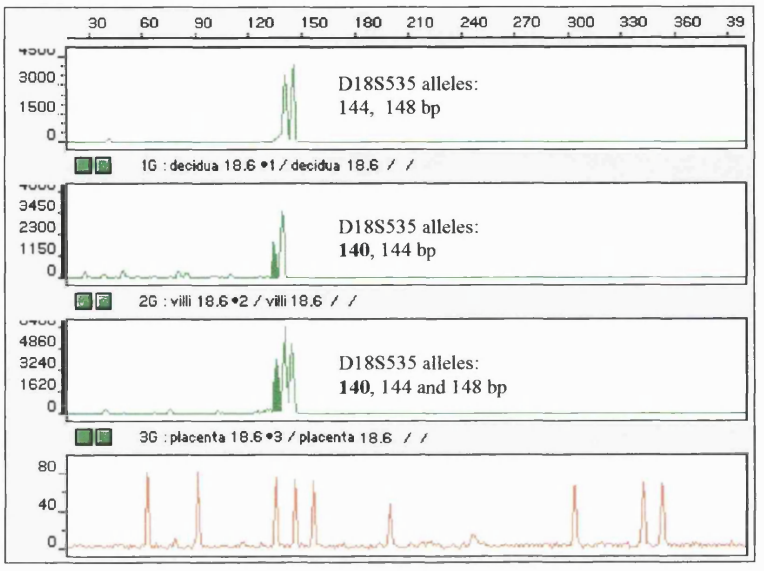
In conclusion, from 10 fetuses with trisomy 18, full trisomy was found in fetuses 18.2, 18.3, 18.4 and 18.5. Low-grade trisomy was found in fetuses 18.1, 18.7 and 18.8. Tissue specific mosaicism (the brain) was found in fetus 18.10 and confined placental mosaicism was found in fetus 18.6. Examples of the trisomic and disomic nuclei are illustrated in Figure 4.9.

Figure 4.7 PCR results of the amelogenin region and D21S1414 from case 18.6



The electropherogram shows PCR results from a multiplex PCR using amelogenin and D21S1414 primers. Fetal specific alleles are shown by the filled peaks and the **bold** numbers of DNA sizes. The dissection for decidua was successful as shown by no contamination from male cells (no amelegenin Y was seen). The villi and the placental DNA had both amelogenin X and Y products as expected. The decidua showed 2 alleles of 345 and 349 bp in length. The villi also had 2 alleles, with the sizes of 345 and 353 bp. Experiments on two more different dissections of villi had the same results. The results did not confirm the presence of another fetus. The PCR results from the placental DNA could be explained by maternal cell contamination with the presence of three alleles of D21S1414 which were presented in the decidua and the villi.

Figure 4.8 PCR results of D18S535 and D13S631 from case 18.6



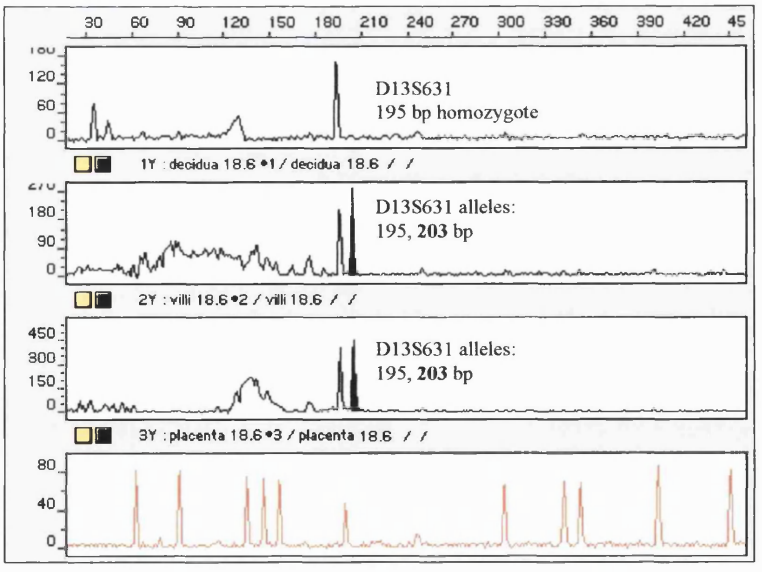
Decidua (maternal)

Villi (fetal DNA, from dissection)

Placental DNA (without dissection)

Size standard

For the D18S535, Two allele sizes were identified with approximately 1:2 ratio. This could be explained by the villi being trisomic with two chromosomes 18 with the same STR product size of 144 bp and one chromosome 18 with the size of 140 bp. Filled peaks and **bold** indicate fetal specific alleles.



Decidua (maternal)

Villi (fetal DNA, from dissection)

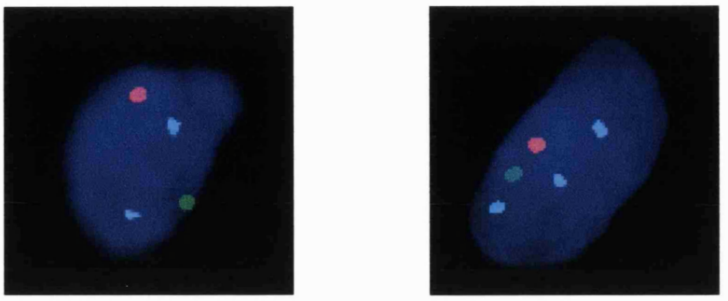
Placental DNA (without dissection)

Size standard

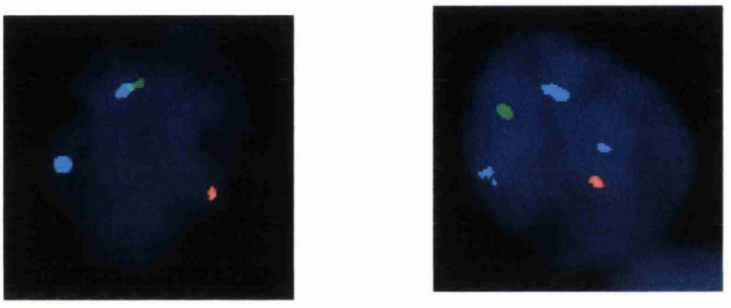
For D13S631, the mother was homozygous for this STR and a fetal specific allele could be identified (filled peaks and PCR sizes in **bold**).

Both STRs did not demonstrate the presence of a second fetus and the placental pattern could be explained by maternal cell contamination.

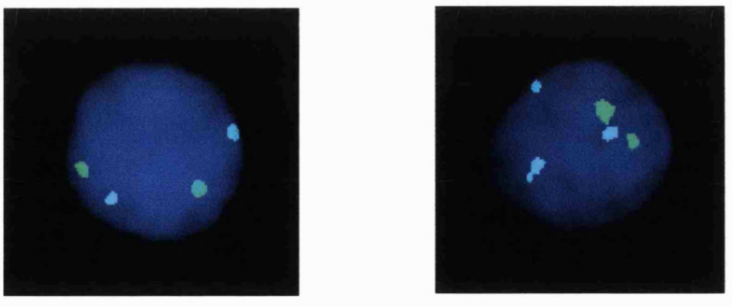
Figure 4.9 Disomic and trisomic nuclei from tissues of fetuses with trisomy 18



a) Placental cells of fetus 18.6



b) Kidney cells of fetus 18.10



c) Liver cells of fetus 18.10

Several nuclei from trisomic tissues are illustrated. Green, red and aqua signals represent chromosomes X, Y and 18 respectively. Cells on the left were disomic while cells on the right were trisomic.

4.3.3.3 Trisomy 21

Table 4.20 is a replication of Table 4.12 to show the percentages of cells with trisomy 21 in trisomic fetal tissues (results were from QCCH). These percentages were compared to the percentage obtained from the skin fibroblast culture (87.97 ± 1.19).

Table 4.20 **Percentages (mean ± S.E.) of cells with three signals from trisomy 21 samples (repeats n=4).**

Patient	Placenta	Brain	Lung	Liver	Heart	Skin
21.1*	92.0±2.8	94.0±4.4	90.0±2.8	93.8		
21.2*	86.0±3.2	87.0±2.8				
21.3	86.7**	86.6**	86.8**		90.9**	
21.4	84.0±5.2	87.0±2.0	88.0±2.8		95.0±4.4	
21.5*		88.7±3.0		89.9±1.2	91.0±2.7	
21.6	92.0±0.5	92.0±1.5	90.0±1.3		89.0±2.8	
21.7*	91.0±3.2	95.0±2.4				90.9**

* male fetus
** tissue only hybridised once due to scarcity

None of the tissues of fetuses with trisomy 21 showed mosaicism compared to the skin fibroblast culture. Percentages of nuclei with trisomy 21 in several fetal tissues were even higher than the average percentage from the full trisomic skin fibroblast culture. Again, this could be due to a slight inter-observer difference.

4.3.4 FISH FOR SEXING

Apart from the cases with maternal cell contamination in the placenta described in some cases of trisomy 18, all the tissues yielded > 90% of the expected signals for sexing and were all in agreement with the results from the prenatal diagnosis performed before the termination of pregnancy.

4.3.5 CLINICAL FEATURES

After establishing which fetuses were mosaic including degree of mosaicism, the results were correlated to the clinical features. Cytogenetic and clinical details of cases in the study are present in Table 4.21.

Table 4.21 Conventional cytogenetic results, FISH results and clinical features of the cases in the study

Case	maternal age	gestation (wks)	cytogenetic		FISH results	clinical data
			cell count	karyotype		
13.1	31	19	5 CVS	47,XX+13	FT	multiple abnormalities
13.2	37	20	15 AC	47,XY+13	FT	cleft lip, cleft palate, polydactyly, rockerbottom feet
13.3	42	23	5 CVS	47,XX+13	CPM	cleft palate, hydronephrosis, rockerbottom feet, exomphalos
13.4	26	26	10 FBS	47,XX+13	FT	multiple abnormalities
18.1	43	11	5 CVS	47,XX+18	LGM	no abnormalities seen at US or TOP
18.2	41	12	5 CVS	47,XX+18	FT	no abnormalities seen at US or TOP
18.3	46	13	5 CVS	47,XX+18	FT	no abnormalities seen at US or TOP
18.4	38	13	10 CVS	47,XY+18	FT	no data
18.5	44	13	7 CVS	47,XX+18	FT	no data
18.6	39	16	5 CVS	47,XY+18	CPM	multiple abnormalities on US
18.7	41	18	5 AC	47,XX+18	LGM	low set ears, rockerbottom feet
18.8	42	18	5 AC	47,XX+18	LGM	multiple abnormalities on US
18.9	45	18	5 CVS	47,XX+18	GM	mild phenotype, rockerbottom feet
18.10	33	20	5 CVS	47,XY+18	TSM	multiple abnormalities on US
21.1	36	13	10 CVS	47,XY+21	FT	abnormal US
21.2	38	15	10CVS	47,XY+21	FT	no data
21.3	31	17	15 CVS	47,XX+21	FT	no abnormalities detected by US
21.4	42	17	15 AC	47,XX+21	FT	no abnormalities detected by US
21.5	40	17	15 AC	47,XY, inv(8)(p23.1q23.2),+21	FT	no data
21.6	31	18	15 AC	47,XX+21	FT	a small ventricular septum defect
21.7	38	no data	10 CVS	47,XY+21	FT	no data

CVS = chorionic villus sampling AC = amniocentesis US = ultrasound
FBS = fetal blood sampling TOP = termination of pregnancy
FT = full trisomy CPM = confined placental mosaicism LGM = low grade mosaicism
GM = generalised mosaicism TSM = tissue specific mosaicism

Most of the pregnant women in the series were ≥ 35 years old. They were at higher risk to have a chromosomally abnormal fetus and this was the main indication for performing the prenatal diagnosis.

With regard to trisomy 13, all the cases survived at least to mid or late second trimester before termination of pregnancy albeit 3 out of 4 cases were full trisomic. Confined placental mosaicism seen in one of the cases appeared not to alleviate the severity of trisomic phenotypes. The fetus still had many anomalies.

With regard to trisomy 18, gestational ages of the fetuses ranged from 11 to 20 weeks. Mosaicism was found in all the older fetuses. Still, multiple anomalies were found in most of them. Full trisomy was more frequently found in younger fetuses. However, because the pregnancies were not spontaneously aborted, it was not possible to correlate to the survival potential. It is notable, however, that no abnormalities were seen. This might be associated with the young gestational ages. Some anomalies could have appeared later. In summary, for trisomy 18, mosaicism may have a small benefit to maintain a pregnancy late into the second trimester but no clear conclusion can be drawn.

With regard to trisomy 21 where most of the fetuses showed no serious anomalies and some fetuses were well advanced into 17-18 weeks, all the tissues were full trisomic.

4.4 DISCUSSION

Some individuals with some characteristics of a chromosome abnormality may have a normal blood karyotype but with further investigations, the expected chromosome abnormality can be found in other types of tissues or in a low proportion in the blood. On the contrary, some individuals with an abnormal karyotype may have a less severe phenotype than expected. For instance, although 45,X genotype is associated with a very high lethality with less than 1% of 45,X conceptuses surviving to term, it can be found in 1:10,000 female livebirths (Hook and Warburton, 1983). Several studies have shown that a higher incidence of mosaicism with a normal cell line is found in the liveborn 45,X than in the 45,X embryos or fetuses (Hook and Warburton, 1983; Hassold *et al.*, 1992). In some instances, the normal cell line is found only with further investigation. It has been suggested that the presence of a normal cell line may increase the survival potential and reduce the severity of the syndrome. A study using FISH on four fetuses from termination of pregnancies due to monosomy X diagnosed by conventional cytogenetics showed the presence of a normal cell line in three phenotypically normal fetuses. In all the tissues examined of the other 45,X fetus who had malformations, a normal cell line was not found (Amiel *et al.*, 1996). Similarly, although the majority of fetuses with trisomies 13, 18, or 21 abort, individuals with these trisomies surviving to term or beyond have been observed. Some rare liveborn trisomies reveal some degree of mosaicism or mosaicism confined to the placenta. It is of interest to see whether the fetuses with these trisomies have a different degree of mosaicism and if so, if there is any difference in the survival potential. To address these possibilities, FISH was used to examine frozen fetal tissues in this study.

4.4.1 FISH WORK UP

Earlier in the study non-commercial probes were used for the sex chromosomes and chromosome 18 with the probe for chromosome 1 as an additional control. On normal fetal tissues, the performances of these three probes in single-colour FISH for chromosome 18 or 1, and dual-colour FISH for the sex chromosomes were similar to those on lymphocytes **(Table 4.1)**. On lymphocytes, the percentages of cells having two signals for chromosome 18 or 1, or having an XY signal were 93, 95 and 97% respectively. These percentages were 93, 94 and 96% on normal fetal tissues. In dual-colour FISH, the number of cells which gave two signals for both chromosomes 1 and 18 simultaneously were lower than that of cells with two signals for chromosome 1 or 18 when consider one at a time. This was true for both lymphocytes and normal fetal tissues **(Table 4.2)**. On average, 90, 91 and 95% of lymphocytes had two signals for both chromosomes, for only chromosome 18 and for only chromosome 1 respectively. These percentages were lower in fetal tissues, whose percentages were 86, 90 and 94%. It is notable that the percentage of disomic cells for chromosome 18 was lower than for chromosome 1. It was observed during the study that the signals for chromosome 18 were more diffused and patchy. This different clarity was not due to the fluorochromes that were used to label these two probes since changing the fluorochromes (red and green) had the same results (data not shown).

In multicolour FISH using probes for the sex chromosomes and chromosome 18, again, the percentage of cells with normal signals simultaneously for all probes was lower than the percentage of cells with normal signals when considering either the sex chromosomes or chromosome 18 at a time **(Table 4.3)**. Single-colour FISH was therefore used.

However, when single-colour FISH for chromosome 18 was used for the trisomic tissues, the percentages of cells with three signals was unusually lower than expected, resulting in almost all tissues being classed mosaic. Several possibilities were considered, including the probe efficiency, maternal cell contamination and a non-mosaicism reference.

Regarding the probe efficiency, several tissues showed higher percentages when using commercial probes. This may be due to a better standard quality control in manufacturing the probes such as the DNA purification and labelling. Since it was possible to score the sex chromosomes and chromosome 18 at the same time with less confusion than using non-commercial probes, along with the fact that a parallel study for chromosome 13 or 21 was performed using commercial probes, AneuScreen probes were used for the further studies.

Regarding maternal cell contamination, it was a possible cause of low percentages of cells with trisomy 18 observed in several placentae. Early in the study, a cell suspension was prepared from each tissue. Maternal cells could attach to the conceptus during the termination of pregnancy. Not surprisingly, it was less prominent in the fetal internal organs than in the skin and the placenta. Direct tissue dabbing helped decrease this problem in the majority of the tissues. Although this technique was not applicable for the skin, the results from the skin were acceptable when prepared in cell solution. The problem was not alleviated using the dabbing technique for the placenta 18.6. At this stage, dissecting of the placenta under the microscope was introduced and proved useful. This technique should have been used in all the placentae from the start of the study as the decidua was easily included in the tissue. However,

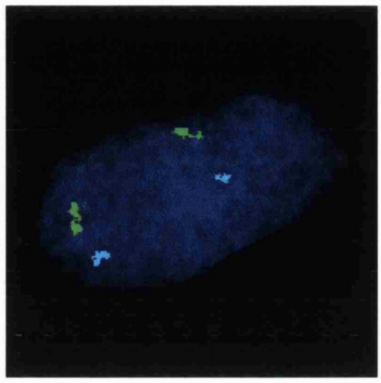
with the inspection of the placentae of other cases, the decidua was found in a very small proportion and was considered acceptable.

Using FISH to study copy number of chromosomes, a certain level of hybridization failure or scoring errors might be expected. In a non-mosaic sample, the expected signals are not present in 100% of interphase cells. Initially, FISH was performed on normal fetal tissues to establish the percentages of disomic cells in these tissues, which were then used as references for non-mosaicism. Percentages of trisomic cells in trisomic fetal tissues were compared to these control percentages to determine the degree of mosaicism in each trisomic tissue. It was found that with this strategy, almost every trisomic 18 tissue would be classed as mosaic. This result was thought to be due to the performance of probes on trisomic or triploid samples which might not be as efficient as on diploid samples since more signals in a nucleus were expected and this could increase the chance for scoring errors especially from overlapping signals. To address this problem, FISH experiments were performed on skin fibroblast cultures from non-mosaic trisomic or triploid fetuses compared to normal male control lymphocytes. It was confirmed that FISH on trisomic nuclei was less accurate than on disomic nuclei. The trisomic samples gave lower percentages of nuclei with the expected chromosome copy number. Overlapping signals seems to be the major cause as two signals were seen in most of the nuclei that did not produce three signals. For this reason, the percentages from fetal skin fibroblast cultures were used as references. Fetal skin fibroblasts were used because they are considered stable in culture. Also, non-mosaicism in each culture was confirmed by scoring at least 30 metaphases. With this, a level of mosaicism of 5% or higher can be excluded with 95% confidence limits

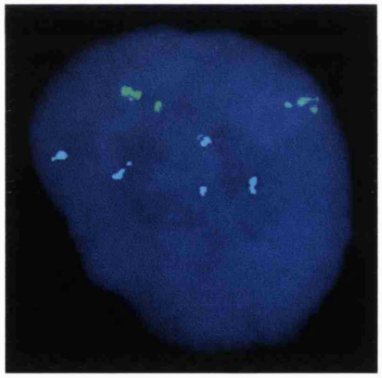
(Hook, 1977). In FISH, good quality metaphases should have the same results in terms of chromosome copy numbers as karyotyping.

Another problem encountered in the study was the clarity of FISH signals in some of the fetal tissues. The problem appeared to be most prevalent for the α-satellite probes especially the probe for chromosome 18. The signals were diffused and looked as if the DNA was stretched **(Figure 4.10)**. This problem was less frequent in signals for a locus-specific probe, possibly due to the nature of the hybridization site. It was also less frequent in the lymphocyte cultures.

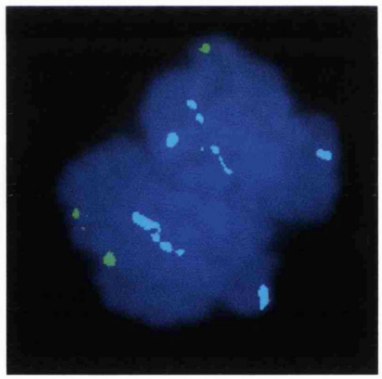
Figure 4.10 Problematic signals encountered in the study



a) String of DNA and stange-shaped signals in interphase trisomic skin fibroblast cultures



b) Small, fragmented signals in fibroblast culture



c) String DNA in placental cells

Various types of problematic signals encountered in the study. Shown here are problems of alpha satellite probes. Green signals represent chromosomes X and aqua signals represent chromosome 18.

4.4.2 RESULTS OF THE STUDY

4.4.2.1 Trisomy 13

In 4 fetuses with trisomy 13, although all survived to the late second trimester, all had multiple abnormalities. Three fetuses were full trisomic and only one had a possible CPM. From the available clinical details, this fetus did not appear to have any obvious advantage from CPM compared to other fetuses. As reviewed by Delatycki and Gardner (1997), trisomy 13 mosaicism is rare, perhaps with the presence of a normal cell line in about 5% of all trisomy 13. They reviewed 33 cases over a 33-year period and found a wide range of phenotypes from a severe form similar to Patau's syndrome to physical and mental normality although unimpaired intellect was rare. They also found that there was a poor correlation of the percentage of trisomic cells in lymphocytes and the phenotype. They suggested that the broad phenotype spectrum might reflect the difference in the proportion and tissue distribution of the trisomic cell line.

4.4.2.2 Trisomy 18

In 10 cases of fetuses with trisomy 18, a case with generalised mosaicism based on the two available tissues was found (18.9). One fetus had a mosaicism specific to the brain tissue (18.10) and one fetus had CPM (18.6). Three fetuses had possible low-grade mosaicism (18.1, 18.7, and 18.8). Most of the fetuses with mosaicism were in the second trimester before the termination of pregnancy. It was not definite that mosaicism helped these fetuses to have a better viability than the non-mosaic fetuses in this study because the non-mosaic pregnancies were not spontaneously terminated. In addition, most of the fetuses with mosaicism also had visible abnormalities while no

abnormalities were seen in the non-mosaic cases. The reason for the latter case may be due to the earlier gestation when some abnormalities may not be clearly visible.

4.4.2.3 Trisomy 21

In 7 fetuses with trisomy 21, details about fetal manifestation were not available in some cases. However, different severity can be seen from the available data. The cases with a lesser extent or no anomalies could not be explained by mosaicism as every fetus was full trisomic, i.e., no association of mosaicism with a less severe anomaly or a better survival potential could be demonstrated. It has also been noted by Kalousek (1993) that trisomy 21 can be viable after 20 weeks of gestation without the presence of CPM.

4.4.2.4 Mosaicism

Mosaicism is a condition where there are two or more cell lines in an individual originating from a single zygote. It results from postzygotic mitotic error, giving rise to several conditions including generalised mosaicism, CPM, and confined embryonic mosaicism. In this series, excluding cases with low-grade mosaicism, a case of generalised mosaicism (18.9) was found. In this condition, usually the mitotic error occurs before the formation of the ICM and the TE and the mosaicism is distributed to both compartments. In case 18.9, the placenta was not available for the study and confined embryonic/fetal mosaicism was possible. The latter condition, however, is rare.

From all 21 fetuses, 2 cases of CPM were found (cases 13.3 and 18.6). This condition is observed in 1-2% of CVS samples (Kalousek *et al.*, 1992). It was first

described in human conceptions by Kalousek and Dill (1983). It results from postzygotic error in the blastocyst stage when the formation of the TE and the ICM occurs. The TE cells give rise to cytotrophoblast and syncytiotrophoblast whereas the ICM cells give rise to the embryo/fetus proper, mesenchymal core of trophoblastic villi and amnion. A study in mice has demonstrated that 3 cells in a 64-celled blastocyst are the source of all adult tissues (Markert and Petters, 1978). Therefore, the chance of the mitotic error is greater in the progenitors of the placenta than of the embryo itself. This results in CPM. There is another theory, however, regarding the origin of CPM. When a mitotic error occurs, the abnormal cells will be allocated to the TE or extraembryonic tissue (Everett and West, 1996).

Three types of CPM have been observed (Kalousek *et al.*, 1992). CPM I is defined as mosaicism presenting in the cytotrophoblast. CPM II is defined as mosaicism presenting in the villus stroma (mesenchymal tissue). In CPM III, mosaicism is present in both components. These reflect the type of progenitor cells where mosaicism occurs. It has been found that liveborn non-mosaic trisomy 13 and 18 infants are associated with CPM type I (Kalousek *et al.*, 1989). The trisomic zygote has a mitotic error occurring in the TE, resulting in a disomic cell line in the cytotrophoblast. This is thought to be able to support the trisomic fetus to term in liveborn cases (Kalousek, 1993). In another study of placentae from 11 pregnancies with trisomy 18 (2 spontaneous abortions, 5 terminations due to the trisomy and 4 deliveries as either stillborn or liveborn), 7 were found to be associated with CPM I (Harrison *et al.*, 1993). This type of CPM may be the case of fetus 18.6, whose placenta was shown to be mosaic with the cells prepared by collagenase digestion which yields cytotrophoblast cells in the suspension for FISH (Henderson *et al.*, 1996).

4.4.2.5 Vanishing twin

Another possible cause of the discrepancy between the placental (mosaic) karyotype and the fetal/embryonic karyotype is a vanishing twin. It should be considered especially when the two cell lines in the placenta could not have derived from each other as this suggests chimaerism (Reddy *et al.*, 1991; Falik-Borenstein *et al.*, 1994). This condition is rarer than CPM. From a collaborative study of 7595 CVS samples, 4 cases of presumptive vanishing twin were observed (Association of Clinical Cytogeneticists Working Party on Chorionic Villi in Prenatal Diagnosis, 1994). Usually a twin pregnancy has been identified in a first trimester ultrasound scan and one twin disappears in the later scan, only a single baby is delivered or a single abortus is seen in an abortion. The examination of placenta may reveal remnants of the other conception which may be only a nodule, an area of fibrosis or another fetus itself can be seen in various appearances such as fetus papyraceus or macerated. In the current series, a possible CPM was observed in the placenta 13.3 and 18.6. Further investigation was performed on the placenta of case 18.6 only. The placenta of case 13.3 was not available. The clinical history of case 18.6 did not reveal any previous diagnosis of a twin pregnancy. Also, it was not possible to examine the placenta for the evidence of another conceptus as it was partially obtained. PCR with several polymorphic markers was performed and failed to demonstrate the presence of two individuals in the placenta. Although a vanishing twin cannot be definitely excluded, it is very unlikely as no supportive evidence could be illustrated. In addition to the lower incidence, when it takes place, the vanished twin is usually the abnormal one. Therefore fetus 18.6 was more likely a case of CPM.

In conclusion, from this series of fetuses, mosaicism was present in some fetal tissues without advantages for fetal phenotypes. Mosaicism might slightly improve fetal viability of trisomy 18 fetuses in this series but had no impact on the other two trisomies. Also, some tissues with mosaicism were found despite the non-mosaicism in prenatal diagnosis. However, the results were based on karyotyping of 15 cells or less which may miss mosaicism (Hook, 1977).

4.4.3 FUTURE WORK

This study was performed on a small number of fetuses and the correlation of the mosaicism with the fetal phenotype or viability can only be relatively evaluated. A definite conclusion cannot be drawn and extrapolated to all trisomic gestations. More cases are needed with several cases for each range of gestational ages. This may be difficult, however, as with the appropriate management for each case of these pregnancies, including the couple's option and consent which obviously are the priority, only a limited number of cases is expected. It may be possible with more collaboration and a longer period of time. In addition, other chromosomal trisomies can possibly be studied.

Another aspect that has not been performed in the study is to establish the type of CPM more clearly. The preparation of chorionic stroma was not carried out. A method to produce separate single cell suspensions of cytotrophoblast and chorionic villus stroma suitable for FISH using collagenase digestion has been developed (Henderson *et al.*, 1996). The author was unaware of this possibility at the time and the experiment was not performed for these two cell types separately. As for the preparation of the stroma for FISH by culture which has been employed in previous studies

(Harrison *et al.*, 1993; Webb *et al.*, 1998), it was thought not applicable because the work was carried out on frozen placenta. With future study, this aspect may yield more information about the type of CPM and the fetal phenotype or viability.

PCR should be used more to obtain information about the origin of aneuploidy in these common trisomies especially when the parental samples are available. It would be interesting to study the disomic and trisomic cell populations from fetal tissues separately using polymorphic markers to study the incidence or characteristics of uniparental disomy (UPD) in these fetuses. To date, no UPD effects or imprinted genes have been clearly identified on these chromosomes (Wolstenholme, 2000).

A long-term study of mosaic trisomies is also interesting. Several studies have shown a reduction of the percentage of trisomic cell line with age (Reyes *et al.*, 1978; Bass *et al.*, 1982; Gravholt *et al.*, 1991; Petit and Fryns, 1994). Further studies may reveal the possibility of the selection against the abnormal cell line with time and the effects on phenotype. This, however, may be difficult or impossible for data on the distribution of cells in different tissues. Perhaps at least data from lymphocytes are feasible. Another difficulty is the time needed to follow-up. Obviously a study in this aspect needs more work, time and resources.

CHAPTER 5
FISH ANALYSIS OF HUMAN EMBRYOS ON DAY 5 POST-INSEMINATION

CHAPTER 5
FISH ANALYSIS OF HUMAN EMBRYOS ON DAY 5 POST-INSEMINATION

5.1 INTRODUCTION

In vitro fertilisation (IVF) treatments have been developed to help infertile or subfertile couples to have a family. They result in an overall clinical pregnancy rate of 15-20% (Bongso *et al.*, 1991). Despite the improvement in ovarian stimulation protocols and the advent of intracytoplasmic sperm injection (ICSI) to treat male factor problems, IVF outcomes have not improved, resulting in implantation rates of only 5-30% (Gardner *et al.*, 1998). One of the causes of these poor results is thought to be the unnatural timing that the embryos are exposed to the uterine cavity. Currently, most IVF centres perform an embryo transfer on day 2-3 post-insemination when embryos are at the cleavage stage (around 4-10 cells) when, in a natural cycle, the embryo would be in the fallopian tube. Transfer later, however, is not routinely performed since prolonged culture *in vitro* results in developmental arrest of most embryos, leaving a small number available for transfer. With conventional embryo culture media, such as Earle's balanced salts solution or human tubal fluid (HTF), only 25-30% of embryos reach the blastocyst stage and less than 10% implantation rates are achieved (Bongso *et al.*, 1991; Bolton *et al.*, 1991). This may be due to artefacts in the culture conditions.

Another possible cause of the disappointing results of IVF is the high frequency of chromosome abnormalities. This has been found in cleavage stage embryos both by karyotyping (reviewed in Zenzes and Casper, 1992) and FISH (Delhanty *et al.*, 1993; Munné *et al.*, 1994; Harper *et al.*, 1995). Chromosome abnormalities in embryos are

found in both patients with and without known genetic risk in the family. Furthermore, they cannot be readily detected by examining embryos morphologically (Zenzes and Casper, 1992; Jamieson *et al.*, 1994; Harper *et al.*, 1995). This makes it difficult to decide which embryos to select for transfer at the cleavage stage.

Normally, embryos reach the uterine cavity at the early blastocyst stage. In the light of this, blastocyst transfer should be beneficial in terms of a better synchronisation between embryos and endometrium. Moreover, it is speculated that blastocyst culture offers a better selection of embryos for transfer (Ménézo *et al.*, 1992, 1997; Desai *et al.*, 1997; Gardner, 1998). The embryos that have a genetic abnormality would probably be eliminated as the activation of the embryonic genome occurs between the four- and eight-cell stages (Braude *et al.*, 1988; Jones *et al.*, 1998). Improvements in *in vitro* culture, however, are needed to overcome the cleavage arrest seen in conventional culture.

In 1989, Bongso *et al.* observed improved cleavage with less fragmentation and a higher rate of cavitation of human embryos when co-cultured with human ampullary cells. In 1990, Ménézo *et al.* developed a blastocyst culture system using co-culture with Vero cells. Both groups suggested that the feeder cells provided some embryotrophic factors and helped remove some toxic substances from the culture. Later, Ménézo *et al.* (1992) reported the results of embryo transfers using blastocysts developed in co-culture with Vero cells without serum. They obtained 55-60% blastocyst formation in co-culture compared to 17% in simple culture. In addition, a high pregnancy rate per transfer (44%) was achieved for patients with previous repeated failures of embryo transfer without a known risk. In a randomised prospective study, however, no benefit was demonstrated from co-culture (Sakkas *et al.*, 1994). Co-culture

needs tissue culture expertise and there is a risk of exposure to cells/proteins with potential for infection. The necessity of this technique was questioned by Desai *et al.* (1997). Citing the work of Noda (1992) who obtained 60.5% blastocyst formation in untransferred embryos using α-modified minimum essential medium (αMEM), they postulated that an appropriate basal medium and protein supplement may replace co-culture. In an experiment in the mouse they found no difference in blastocyst formation rates using αMEM alone or with co-culture. With a further study on human spare embryos using αMEM in conjunction with commercially available Synthetic Serum Substitute (SSS) as the protein supplement, they achieved 45% blastocyst formation with a high rate of blastocysts with expansion or a discernible inner cell mass (Desai *et al.*, 1997). In another study, heparin-binding epidermal growth factor (HB-EGF) was used to promote embryonic growth instead of co-culture (Sargent *et al.*, 1998). The technique resulted in 71% blastocyst formation with 81% hatching.

A preimplantation embryo *in vivo* is exposed to a changing environment from the fallopian tube to the uterine cavity. This naturally results in the embryo having a changing physiology and metabolism. According to this concept as well as attempts to reduce embryonic cellular stress from the culture conditions, sequential culture media have been developed to suit embryos at different stages (Gardner, 1998). For example, glucose impairs early cleavage stage development. A medium at the cleavage stage should be glucose-free or should contain amino acids and EDTA to suppress glycolysis. Later embryonic development requires glucose, and this should be included in the medium for blastocyst culture. With the use of different media for different stages of development, blastocyst formation rates of more than 50-60% with a high viability have been reported, sometimes with group culture with the reasoning that embryos would be

metabolically beneficial to one another (Gardner, 1998; Jones *et al.*, 1998; Behr *et al.*, 1999). These results are similar to that obtained with co-culture (Ménézo *et al.*, 1998).

In conclusion, *in vitro* culture of embryos is being refined with improving results which may result in blastocyst transfer being more routinely performed. Together with the feasibility of blastocyst biopsy **(section 1.7.2.3)**, this may affect PGD strategies in the future. With this respect, basic genetic information in blastocyst stage embryos should be available. Chromosome patterns in cleavage stage embryos have been classified into 4 groups: uniformly normal, uniformly abnormal, mosaic and chaotic (Harper and Delhanty, 1996a; Delhanty *et al.*, 1997). While chromosome abnormalities have been frequently found in cleavage stage embryos (Delhanty *et al.*, 1993; Munné *et al.*, 1994; Harper *et al.*, 1995), little is known about chromosome complements in embryos at the blastocyst stage. Clouston *et al.* (1997) karyotyped nuclei from blastocysts and their results suggested that all four groups of chromosome patterns are also observed at this stage. The present study aimed to use FISH to analyse chromosome complements of human embryos on day 5 post-insemination in both arrested and blastocyst stage embryos. This approach has the advantage that a result is obtained from almost all nuclei of the embryos.

5.2 MATERIALS AND METHODS

5.2.1 SAMPLES

5.2.1.1 Fresh embryos from Assisted Conception Unit, University College Hospital

Surplus embryos were donated from patients undergoing routine IVF treatment at University College Hospital with written consent. The study was approved by UCL ethical committee and the Human Fertilisation and Embryology Authority (HFEA).

Superovulation

The superovulation regime was as described previously with some modification (Ranieri *et al.*, 1998). After general clinical evaluation, patients underwent an ultrasound scan on cycle day 2 to rule out the presence of ovarian cysts. The pituitary down regulation was achieved by the administration of gonadotropin releasing hormone analogue (GnRHa) nasal spray (Buserelin, Suprefact; Hoechst, UK) 1,200 μg/day starting on cycle day 2 for 10-13 days. Ovarian stimulation was commenced using 225 IU/day recombinant follicle stimulating hormone (FSH) (Gonal-F; Serono, UK) or human menopausal gonadotropin (hMG; Menogon, Ferring GmBH, Germany). Patients were monitored by vaginal ultrasound regularly starting on day 6 of the stimulation and the dose of FSH was adjusted accordingly. Between days 12 and 14 of the stimulation, patients who had ≥ 5 follicles of 16 mm in diameter were given 10,000 IU of human chorionic gonadotropin (hCG) (Profasi; Serono, UK) to trigger ovulation. Thirty-six hours later, follicular aspiration was carried out under transvaginal ultrasound guidance.

Embryo culture (All media used were from Medi-Cult (UK) Ltd.)

Oocytes were retrieved using flushing medium (Earle's Balanced Salt Solution supplemented with 0.8mM sodium pyruvate, synthetic serum replacement 2 (SSR2), $NaHCO_3$ (1,100 mg/l), HEPES 25 mM, 0.1% Human Serum Albumin), incubated in 5% CO_2 in air at 37°C, inseminated and cultured in 100 μl droplets of universal IVF medium (Earle's Balanced Salt Solution supplemented with 0.8 mM sodium pyruvate, SSR2, $NaHCO_3$ 2,200 mg/l, 1% Human Serum Albumin). On day 1, they were assessed for the number of pronuclei and transferred into a fresh microdrop of the same medium. On day 3, the best 2-3 embryos were selected for transfer and suitable spare embryos were frozen. The remaining embryos were cultured in M3 medium (a modification of Ham's F10 and F12 media composed of amino acids, vitamins, inorganic salts and D-glucose with SSR2, 0.2% Human Serum Albumin) until day 5 when they were assessed and spread for FISH. The spreading procedure was described in **section 2.3.1.3**. Only the embryos from a bipronucleate zygote were included in the study.

5.2.1.2 Frozen-thawed embryos from Australia

Already spread frozen-thawed embryos were mailed from Australia for FISH work-up. No patients' clinical data were available for this group of embryos.

5.2.2 FISH WORK UP

5.2.2.1 One-round 5-colour FISH for 5 chromosomes on lymphocytes and embryos

Five-colour FISH using MultiVysion™ PGT **(section 2.3.3)** was performed as described **(section 2.3.5)** on four fresh embryos from two patients and lymphocyte controls.

5.2.2.2 Two-round FISH for 6 chromosomes on lymphocytes and embryos.

Two-round FISH was performed for chromosomes X, Y and 18 in the first round and chromosomes 13, 21, and 16 in the second round. In the first round, probe mixture #1 in the AneuScreen Prenatal Aneuploidy Detection Panel **(section 2.3.3)** was used as described **(section 2.3.5)**. In the second round, probe mixture #2 from the same panel was used in conjunction with a non-commercial probe for chromosome 16 **(section 2.3.2)**. The old signals and counterstain were washed off in 4 x SSC/0.05% Tween-20 at room temperature twice for 10-15 min each wash. The cover slips were gently removed at the first wash. The slides were rinsed in PBS for 5 min, dehydrated through an ethanol series and left to dry. Probes were applied and the slides were denatured. The subsequent procedure was carried out as described for the commercial probe set for chromosomes 13 and 21 **(section 2.3.5)**. For the analysis, the nuclei were identified by their positions in a map previously drawn. The position of the signals was compared with the first round signals to ensure they were not old signals. This protocol was performed on four fresh embryos and lymphocyte controls.

5.2.2.3 Two-round FISH for 5 chromosomes on lymphocytes and embryos

This procedure was performed in the same manner as **section 5.2.2.2** except that in the second round, FISH was performed without the probe for chromosome 16. This protocol was performed on 7 frozen-thawed embryos from Australia, 40 fresh embryos from UCL and lymphocyte controls.

5.3 RESULTS

5.3.1 RESULTS FROM FISH WORK UP

5.3.1.1 Results of one-round five-colour FISH

Five-colour FISH was performed on four arrested embryos in two experiments. A high efficiency was achieved with one embryo which showed good signals (Embryo 1) but most of the embryos and lymphocytes had a low signal to noise ratio and analysis was impossible **(Table 5.1, Figure 5.1)**. Subsequently, this FISH protocol was discontinued.

Table 5.1 One-round 5-colour FISH for 5 chromosomes on embryos and control lymphocytes

Control lymphocytes (from two experiments combined)

Cell type	Number of nuclei	percent
X, Y, 2(18), 2(13), 2(21) (Expected signals)	128	70.33
X, Y, 1(18), 2(13), 2(21)	4	2.20
X, Y, 3(18), 2(13), 2(21)	2	1.10
X, Y, 2(18), 2(13), 3(21)	2	1.10
X, Y, 2(18), 2(13), 1(21)	1	0.55
X, Y, 2(18), 3(13), 2(21)	1	0.55
X, Y, 2(18), 1(13), 2(21)	1	0.55
X, Y, 2(18), 2(13), 1(21)	1	0.55
Cannot be determined (due to high background)	42	23.08
Total	182	100.0

Embryo 1 (7 nuclei)

Cell type	Number of nuclei	percent
X, Y*, 2(18), 2(13), 2(21) (Expected signals)	7	100.0
Total	7	100.0

* The Y signal in one nucleus was fainter than in other nuclei

Table 5.1 (continued)

Embryo 2 (18 nuclei)

Cell type	Number of nuclei	percent
X, Y, 2(18), 2(13), 2(21) (Expected signals)	5	27.78
Nuclei with signals from one or two pairs of chromosomes unable to be determined	10	55.56
Nuclei with signals from all chromosomes unable to be determined	13	16.67
Total	18	100.0

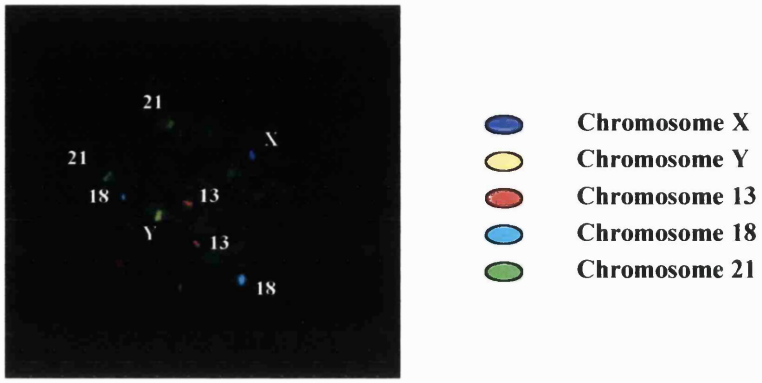
Embryo 3 (10 nuclei)

Cell type	Number of nuclei	percent
X, Y, 2(18), 2(13), 2(21) (Expected signals)	0	00.00
Nuclei with signals from one or two pairs of chromosomes unable to be determined	6	60.00
Nuclei with signals from all chromosomes unable to be determined	4	40.00
Total	10	100.0

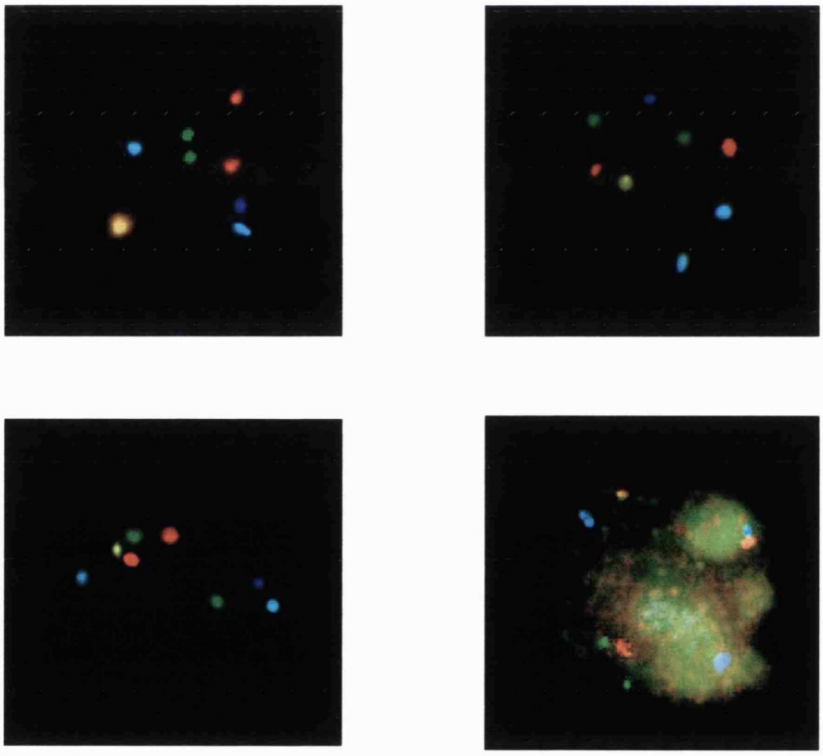
Embryo 4 (23 nuclei)

Cell type	Number of nuclei	percent
X, Y, 2(18), 2(13), 2(21) (Expected signals)	14	60.87
Nuclei with signals from one or two pairs of chromosomes unable to be determined	6	26.09
Nuclei with signals from all chromosomes unable to be determined	0	00.00
Nuclei lost	3	13.04
Total	23	100.0

Figure 5.1 One-round 5-colour FISH using MultiVysion™ PGT



a) A metaphase in control male lymphocytes



b) Blastomeres

Five-colour FISH using MultiVysion™ PGT. a) In control male lymphocytes, each cell contains one blue, one yellow, two red, two aqua and two green signals for chromosomes X, Y, 13, 18 and 21 respectively. b) FISH worked well on the first three blastomeres with normal signals for male nuclei. In the last blastomere there was low signal to noise ratio and the analysis was impossible.

5.3.1.2 Results of two-round FISH for 6 chromosomes

Two experiments using this FISH protocol were performed on a total of four embryos. Results from lymphocyte controls and the four embryos yielded low percentages of nuclei being analysable for chromosome 16 signals **(Figure 5.2a)**. Many nuclei gave no signals for this chromosome **(Figure 5.2b)** or gave high background and signals could not be analysed. This might have been caused by the possible incompatibility between locus specific probes and α-satellite probes. **Table 5.2** shows the summarised results of the two experiments using this protocol. Due to the low accuracy of the results judged from the lymphocyte controls, the probe for chromosome 16 was removed from the study.

Table 5.2 Two-round FISH for 6 chromosomes on embryos and lymphocytes

Control lymphocytes (from two experiments combined)

Cell type	Number of nuclei	Percent
X, Y, 2(18), 2(13), 2(21), 2(16) (Expected signals)	122	54.46
X, Y, 2(18), 2(13), 2(21)	61	27.23
X, Y, 2(18), 2(13), 2(21), 1(16)	4	1.79
X, Y, 1(18), 2(13), 2(21), 1(16)	3	1.34
X, Y, 2(18), 2(13), 1(21), 2(16)	2	0.89
X, Y, 2(18), 3(13), 1(21), 2(16)	1	0.45
X, Y, 2(18), 2(13), 3(21), 1(16)	1	0.45
X, Y, 2(18), 2(13), 3(21), 2(16)	1	0.45
2(X), Y, 2(18), 2(13), 2(21)	1	0.45
2(X), Y, 1(18), 2(13), 2(21)	1	0.45
X, Y, 2(18), 1(13), 2(21), 1(16)	1	0.45
2nd round cannot be determined (due to high background and a loss of one nucleus)	26	11.61
Total	224	100.0

Table 5.2 (continued)

Embryo 1 (results available for chromosome 16 in 18 of 54 nuclei = 33.3%)

Cell type	Number of nuclei	Percent
2(X), 2(18), 2(13), 2(21), 2(16)	15	27.78
2(X), 2(18), 2(13), 2(21)	28	51.85
2(X), 1(18), 2(13), 2(21), 2(16)	1	1.85
2(X), 2(18), 2(13), 1(21)	1	1.85
2(X), 2(18), 2(13), 2(21), 1(16)	1	1.85
1(X), 2(18), 2(13), 2(21)	1	1.85
2(X), 1(18), 2(13), 2(21)	1	1.85
4(X), 4(18), 4(13), 4(21)	1	1.85
2(X), 2(18), 3(13), 2(21), 2(16)	1	1.85
2(X), 2(18), 1(13), 2(21)	1	1.85
2nd round cannot be determined (due to high background)	3	5.56
Total	54	100.0

Embryo 2 (results available for chromosome 16 in 13 of 21 nuclei = 61.9%)

Cell type	Number of nuclei	Percent
X, Y, 2(18), 2(13), 2(21), 2(16)	9	42.86
X, Y, 2(18), 2(13), 2(21)	4	19.05
X, Y, 1(18), 2(13), 2(21), 2(16)	2	9.52
X, Y, 2(18), 2(13), 2(21), 1(16)	2	9.52
X, Y, 1(18), 2(13), 2(21)	1	4.76
2nd round cannot be determined (due to high background)	3	14.29
Total	21	100.0

Table 5.2 (continued)

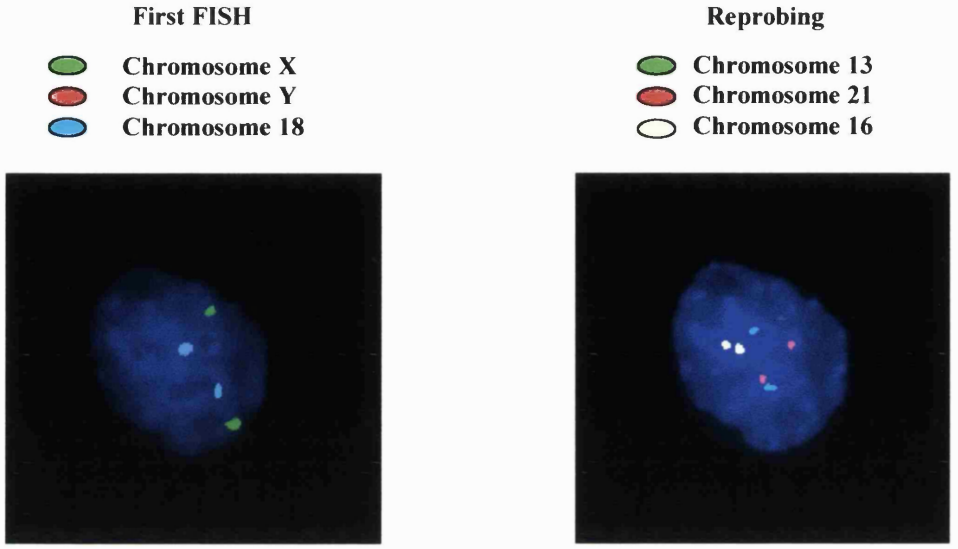
Embryo 3 (results available for chromosome 16 in 9 of 14 nuclei = 64.3%)

Cell type	Number of nuclei	Percent
2(X), 2(18), 2(13), 2(21), 2(16)	7	50.00
2(X), 2(18), 2(13), 2(21)	3	21.43
2(X), 2(18), 2(13), 2(21), 1(16)	2	14.29
2(X), 2(18), 2(13), 1(21)	1	7.14
2(X), 2(18), 3(13), 2(21)	1	7.14
Total	14	100.0

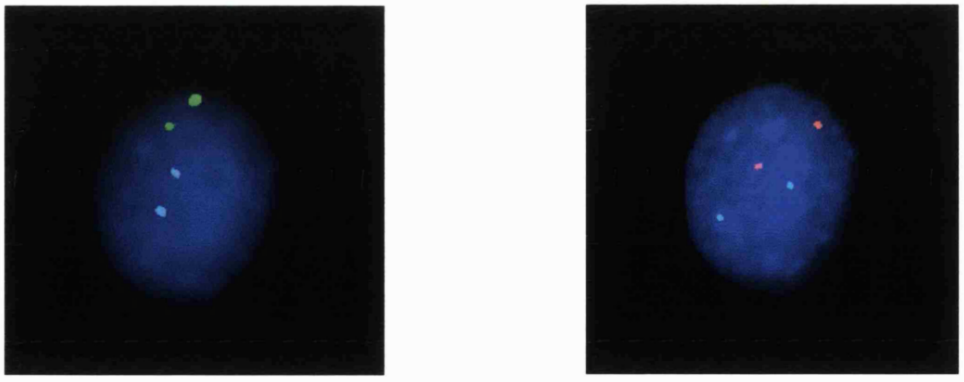
Embryo 4 (results available for chromosome 16 in 53 of 84 nuclei = 63.1%)

Cell type	Number of nuclei	Percent
2(X), 2(18), 2(13), 2(21), 2(16)	34	40.48
2(X), 2(18), 2(13), 2(21)	23	27.38
2(X), 2(18), 2(13), 2(21), 1(16)	7	8.33
2(X), 1(18), 2(13), 2(21), 2(16)	5	5.95
2(X), 2(18), 2(13), 1(21), 1(16)	2	2.38
1(X), 2(18), 2(13), 2(21), 2(16)	2	2.38
2(X), 2(18), 1(13), 2(21), 2(16)	1	1.19
2(X), 2(18), 1(13), 2(21), 1(16)	1	1.19
2(X), 2(18), 3(13), 2(21)	1	1.19
2(X), 1(18), 2(13), 2(21)	1	1.19
2(X), 2(18), 2(13), 0(21), 1(16)	1	1.19
1(X), 2(18), 2(13), 2(21)	1	1.19
3(X), 2(18), 2(13), 2(21)	1	1.19
2nd round cannot be determined (due to high background)	4	4.76
Total	84	100.0

Figure 5.2 Two-round FISH for 6 chromosomes



a)



b)

Results from the same embryo using probes for chromosomes X, Y and 18 in the first round; and probes for chromosomes 13, 21 and 16 in the second round.

a) Signals for chromosomes 16 can be seen.

b) Signals for chromosomes 16 cannot be seen.

5.3.1.3 Results of two-round FISH for 5 chromosomes on frozen-thawed embryos

Three experiments of two-round FISH for 5 chromosomes were performed on 7 frozen-thawed embryos, already spread on slides and mailed from Australia. While expected signals were achieved from about 87% of lymphocyte controls for all three experiments, results from the embryos were problematic. Many nuclei had a high background and/or poor signal quality **(Table 5.3, Figure 5.3)**. Some of the results from the first round were inadequate and reprobing was not performed.

Table 5.3 Two round FISH for 5 chromosomes on frozen-thawed embryos and lymphocytes

Control lymphocytes (results from three experiments combined)

Cell type	Number of nuclei	percent
X, Y, 2(18), 2(13), 2(21) (Expected signals)	262	87.04
X, Y, 1(18), 2(13), 2(21)	16	5.32
X, Y, 3(18), 2(13), 2(21)	7	2.33
X, Y, 2(18), 2(13), 1(21)	4	1.33
X, Y, 2(18), 1(13), 2(21)	4	1.33
X, Y, 1(18), 1(13), 2(21)	2	0.66
X, Y, 2(18), 3(13), 2(21)	2	0.66
X, Y, 2(18), 2(13), 3(21)	1	0.33
X, O, 2(18), 2(13), 3(21)	2	0.66
2(X), Y, 2(18), 2(13), 3(21)	1	0.33
Total	301	100.0

Table 5.3 (continued)

Embryo 1 (25 nuclei)

Cell type	Number of nuclei	percent
2(X), 2(18), 2(13), 2(21) (Expected signals)	3	12.00
2(X), 1(18), 2(13), 2(21)	4	16.00
2(X), 2(18), 1(13), 2(21)	2	8.00
2(X), 2(18), 2(13), 1(21)	1	4.00
No signals analysable from both rounds	5	20.00
No signals analysable from the first round	2	8.00
Signals for chromosome 18 were not analysable	8	32.00
Total	25	100.0

The signals were not analysable mostly due to speckle or broken signals

Embryo 2 (12 nuclei)

Cell type	Number of nuclei	percent
2(X), 2(18), 2(13), 2(21) (Expected signals)	7	58.33
2(X), 1(18), 2(13), 2(21)	3	25.00
2(X), 2(18), 2(13), 1(21)	1	8.33
2(X), ?(18), 2(13), 1(21)	1*	8.33
Total	12	100.0

*This nucleus had signals for chromosome 18 broken into 4 tiny dots

Embryo 3 (4 nuclei)

Cell type	Number of nuclei	percent
2 (18)	4	100.0
Total	4	100.0

For this embryo, no XY results were analysable and reprobing was not performed

Table 5.3 (continued)

Embryo 4 (15 nuclei)

Cell type	Number of nuclei	percent
X, Y, 2(18) (Expected signals from the first round)	8	53.33
X, Y, 4(18)	1	6.67
X, Y, 1(18)	1	6.67
2(X), 2(Y), 4(18)	1	6.67
0(X), 1(Y), 2(18)	1	6.67
2(18)*	3	20.00
Total	15	100.0

*Three nuclei had not available XY signals. Also, reprobing was not performed

Embryo 5 (11 nuclei)

Cell type	Number of nuclei	percent
2(X), 2(18)	2	18.18
No signals analysable from probes for sex chromosomes	4	36.36
No signals analysable from probes for chromosome 18	4	36.36
No analysable signals from chromosomes X, Y and 18	1	9.09
Total	11	100.0

Reprobing was not performed for this embryo due to only 2 nuclei were analysable from the first round

Embryo 6 (37 nuclei)

Cell type	**Number of nuclei**	**Percent**
X, Y, 2(18)	24	64.86
X, Y, 1(18)	2	5.41
Cannot be determined properly (due to high background or broken, granular signals)	11	29.73
Total	37	100.0

Reprobing was not performed.

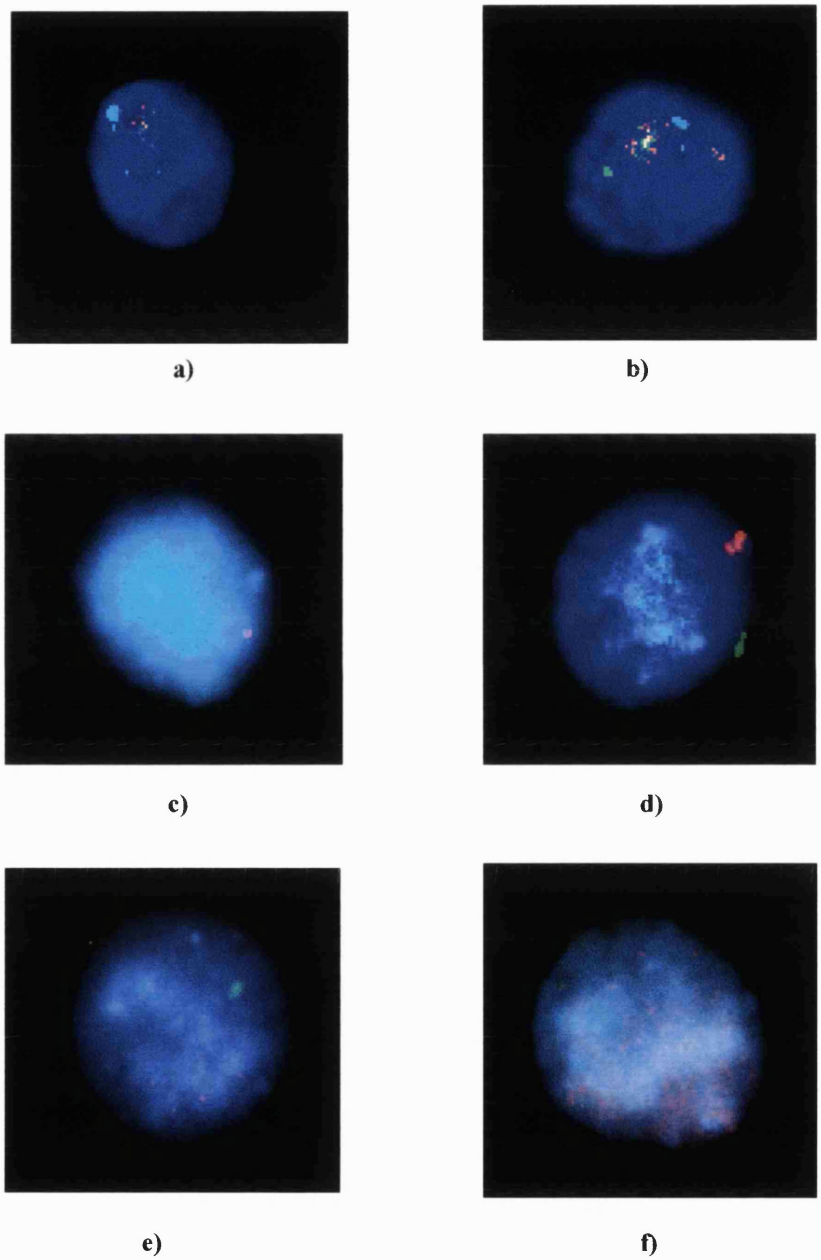
Table 5.3 (continued)

Embryo 7 (19 nuclei)

Cell type		Number of nuclei	percent
X, Y, 2(18)		4	87.04
No signals for chromosomes X, Y		5	5.32
No signals for chromosome Y		3	2.33
No signals for chromosome X		2	1.33
No signals for chromosome 18		3	1.33
No signals for chromosomes X,Y and 18		2	0.66
	Total	301	100.0

Reprobing was not performed

Figure 5.3 Two-round FISH for 5 chromosomes on frozen-thawed embryos



FISH on frozen-thawed embryos. These pictures show the results from the first round using probes for chromosomes X, Y, and 18 in green, red and aqua respectively.

a)-b) show poor signal quality

c)-f) show high background. In both situations analysis was not possible

5.3.2 TWO-ROUND FISH FOR 5 CHROMOSOMES ON DAY-5 EMBRYOS

Due to the poor results from the aforementioned protocols, and better results from two-round FISH for 5 chromosomes on fresh day-5 embryos which will be described subsequently, the main study was based on the latter.

5.3.2.1 Patients' clinical details

Table 5.4 shows the clinical data of 14 couples in the study. Two couples required an oocyte donor. In these cases, the donor's age was used in the calculation of maternal age. In all cases, the husband's sperm was used. Four couples presented with male factor and intracytoplasmic sperm injection (ICSI) was performed. In these cases, the husbands had a normal karyotype. None of the couples had a known genetic risk in the family or a history of ≥ 3 IVF treatment failures. Means ± S.D. for maternal and paternal ages were 32.6 ± 3.9 and 36.8 ± 5.8 respectively.

Table 5.4 Patients' clinical details

Patient	Age	Partner age	Partner condition	Diagnosis	Procedure
A	37 (32)*	35	normal	Ovarian cancer stage 1a, BSO	IVF (donor's oocytes)
B	28	40	normal	tubal factor (adhesion)	IVF
C	31	33	obstructive azoospermia	male factor	IVF+PESA +ICSI
D	34	53	failure vasectomy reversal	male factor	IVF+ICSI
E	47 (29)*	NA	normal	ovarian failure	IVF (donor's oocytes)
F	36	29	normal	tubal factor (hydrosalpinx)	IVF
G	37	36	normal	tubal factor (adhesion)	IVF
H	32	33	normal	tubal factor + fibroid	IVF
I	27	39	oligoasthenospermia	male factor	IVF+ICSI
J	35	36	normal	tubal factor (endometriosis)	IVF
K	33	35	normal	tubal factor	IVF
L	28	41	oligospermia	male factor	IVF+ICSI
M	33	35	normal	unexplained primary infertility	IVF
N	41	34	normal	tubal factor (post tubal sterilisation)	IVF

BSO = bilateral salpingo-oophorectomy
IVF = *in vitro* fertilisation
PESA = percutaneous epididymal sperm aspiration
ICSI = intracytoplasmic sperm injection
NA = data not available
*For patients A and E, the oocyte donor's ages are given in the parenthesis.

5.3.2.2 Results from lymphocyte controls

Using FISH to analyse the chromosome constitution, some problems have to be considered. Scoring errors may arise from signal overlapping, split signals, diffused or patchy signals, hybridisation failure, and decreased probe efficiency (Ruangvutilert *et al.*, 2000). As a baseline for these errors, lymphocyte cultures from normal donors are used as references as it is not possible to obtain a "standard, normal" embryo as a control. **Table 5.5** shows the results from male and female lymphocytes from 11 experiments that were performed alongside the embryos and were reprobed in exactly the same way. Reprobing may be a source of errors with possible cell loss or failure of hybridisation due to suboptimal nuclear quality in the second round. However, in total from 11 experiments, two-round FISH results could be obtained from 98.6% (3071/3116 nuclei) and 99.1% (2437/2460 nuclei) of the starting nuclei for male and female controls. Normal diploid signals for all probes tested were achieved in 86% and 85% of male and female nuclei respectively. Furthermore, if reduced hybridisation efficiency had resulted in the second round, it would have given a high proportion of monosomic 13 or 21. This was not observed.

Initially, it was intended that when a reference was needed to compare with an embryo, either male or female control lymphocytes would be used according to the sex of the embryo in case there were any sex-related differences in hybridisation efficiency especially with the sex chromosomes. However, an increased aneuploidy rate in cultured lymphocytes with advancing age could be found in older women particularly the loss of an X chromosome (Jacobs *et al.*, 1963; Fitzgerald and McEwan, 1977; Richard *et al.*, 1993). This was probably the case with the presented results. In the first 10 experiments, male controls were obtained from several donors but female controls

Table 5.5 Percentages of nuclei with various signals from control lymphocytes obtained from two-round FISH for 5 chromosomes

Control		Total cell analysed	normal	mono 18	mono 13	mono 21	tri 18	tri 13	tri 21	XO	XXY	XYY	YO
Male	1	171	87.13	3.51	1.75	3.51	0.58	0.58	0.58	0.00	0.58	0.58	1.17
	2	300	88.00	5.33	2.33	2.33	0.67	0.33	0.33	0.67	0.00	0.00	0.00
	3	300	85.00	6.00	3.00	3.00	0.33	0.00	0.33	1.00	0.67	0.33	0.33
	4	301	81.40	8.97	3.32	3.65	0.66	0.33	0.00	1.33	0.33	0.00	0.00
	5	293	84.98	8.53	3.41	1.71	0.00	0.34	0.00	0.34	0.00	0.00	0.00
	6	308	84.74	7.79	1.62	3.25	0.00	0.65	0.32	0.00	0.65	0.32	0.00
	7	300	85.33	6.33	3.67	2.67	0.33	0.33	0.00	0.67	0.00	0.67	0.00
	8	296	85.14	7.43	2.36	3.04	0.68	0.34	0.34	0.34	0.00	0.00	0.34
	9	296	86.49	5.07	1.35	2.70	1.01	0.00	0.34	1.01	1.01	0.34	0.00
	10	304	87.50	5.59	2.63	2.63	0.66	0.66	0.00	0.00	0.33	0.00	0.00
	11	202	90.10	5.94	0.50	1.98	0.50	0.00	0.99	0.00	0.00	0.00	0.00
	mean		**85.98**	**6.41**	**2.36**	**2.77**	**0.49**	**0.32**	**0.29**	**0.49**	**0.33**	**0.20**	**0.17**
	S.D.		2.25	1.63	0.98	0.61	0.31	0.25	0.30	0.48	0.36	0.26	0.36

Control		Total cell analysed	normal	mono 18	mono 13	mono 21	tri 18	tri 13	tri 21	XO	XXY	XYY	XXX
Female	1	93	86.02	3.23	2.15	1.08	0.00	0.00	1.08	5.38	0.00	0.00	1.08
	2	101	84.16	7.92	0.99	1.98	0.99	0.00	0.00	1.98	0.00	0.00	0.99
	3	103	80.58	5.83	2.91	1.94	0.00	0.97	0.00	7.77	0.00	0.00	0.00
	4	104	84.62	8.65	1.92	1.92	0.00	0.96	0.96	0.00	0.00	0.00	0.96
	5	300	89.33	3.33	1.67	1.67	0.33	0.00	0.00	3.00	0.00	0.00	0.33
	6	308	85.71	6.49	0.97	1.95	0.65	0.00	0.65	2.92	0.00	0.00	0.65
	7	306	84.97	5.88	1.31	1.96	0.98	0.65	0.00	3.27	0.00	0.00	0.65
	8	308	82.79	7.47	2.27	1.30	0.97	0.00	0.32	3.57	0.00	0.00	1.30
	9	300	84.33	6.00	2.33	2.33	0.33	0.33	0.33	3.00	0.00	0.00	1.00
	10	298	85.23	4.70	2.68	2.01	0.67	0.34	0.34	3.02	0.00	0.00	0.67
	11	216	91.20	3.70	0.00	0.93	0.46	0.00	0.93	0.93	0.00	0.00	0.46
	mean		**85.36**	**5.75**	**1.75**	**1.73**	**0.49**	**0.30**	**0.42**	**3.17**	**0.00**	**0.00**	**0.74**
	S.D.		2.88	1.85	0.86	0.44	0.39	0.39	0.42	2.07	0.00	0.00	0.38

Numbers of total nuclei analysed from male and female controls were 3071 and 2437 respectively. These were obtained from 3121 male and 2460 female nuclei in total at the start of the nuclei mapping. The results were obtained from 98.6 and 99.1% of the initial male and female nuclei.
(Some results with a very low percentage were not included in the table.)

were obtained from the same donor due to the unavailability of more female lymphocyte cultures at the time. The only available information for this female lymphocyte culture was that the blood had a normal karyotype. Abnormal signals for sex chromosomes in the male control were negligible. In the female control, however, as high as 4% of nuclei (on average from the first 10 experiments) displayed one signal for the chromosome X, and 1% displayed three signals. There were no significant differences for other kinds of signals between the male and female controls and the difference in sex chromosome signals was initially thought to be due to the sex difference. In the 11^{th} experiment where a different female control was used, however, the sex chromosome signals from the male and female controls were not different. This female lymphocyte culture was obtained from a young donor. It had been reconsidered that the first female control culture might have come from an advanced age donor and that this culture might not be a good control. With the possibility of this, and because other signals were not different between male and female controls, it was decided to use only male lymphocyte cultures as controls.

Overall, 86% (range 81-90) of male lymphocyte controls displayed normal signals for all five probes used. Nuclei with one signal for chromosomes, 18, 13 and 21 were found in 6.4% (3.5-9.0), 2.4% (0.5-3.6) and 2.8% (1.7-3.7) respectively. The chance of overlapping signals was higher with the chromosome 18 probe due to the large and diffuse nature of the signal. Nuclei with three signals for autosomes or abnormal signals for the sex chromosomes were found in less than 0.5%.

5.3.2.3 Results from embryos

Embryo categorisation

Forty day-5 embryos were available from 14 couples. These embryos were divided into 2 groups depending on the number of nuclei: arrested embryos (<30 nuclei) and those that reached or had a potential to reach the blastocyst stage (more than 30 nuclei). The nuclei number of 30 was arbitrarily chosen based on the findings of Hardy *et al.* (1989) that 42% of normally fertilised human embryos *in vitro* reached the blastocyst stage on day 5 or 6 with the number of cells on day 5 ranging from 24 to 90 cells (mean = 58 cells). In the current study, morula/blastocysts with 30 nuclei or more on day 5 were therefore regarded as normally developed and were collectively classed as the blastocyst group. Morula with less than 30 nuclei and embryos at an earlier stage on day 5 were regarded as being arrested. It was also found that no embryos with less than 30 nuclei in this study had formed a blastocoel cavity. Altogether, 21 arrested and 19 blastocysts were available. **Table 5.6** shows the embryos in the order of nuclei number and their morphological grading. **Figure 5.4** shows arrested and blastocyst stage embryos.

Table 5.6 Embryos with nuclei number and morphological grading

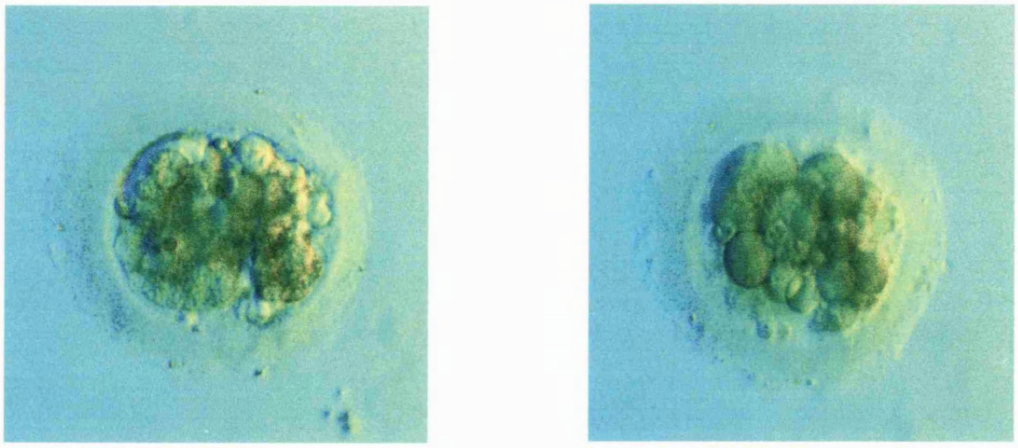
Arrested Emb. ID.	Arrested embryo group		Blastocyst ID.	Blastocyst group	
	Fresh nuclei number	grade		Fresh nuclei number	grade
H1	7	ii	L1	31	B
J2	7	ii	J3	34	A
A1	7	ii	L2	35	B
I2	8	iii	I3	36	C
G2	8	ii	F1	37	iii
K3	8	ii	I1	43	C
G1	9	ii	H4	51	D
K2	9	ii	N4	54	C
F2	10	ii	E1	57	D
J1	12	ii	C3	66	iii
H2	13	iii	N1	68	D
D2	14	iii	A3	76	C
H3	15	iii	N2	79	D
A2	16	iii	C1	86	iii
K1	19	iii	N3	89	D
D4	20	iii	N5	93	D
D1	23	iii	C2	113	C
J4	24	iii	B1	136	D
D3	26	iii	B2	144	D
M1	28	iii			

Embryos with the same initial letters in the I.D. came from the same patients.

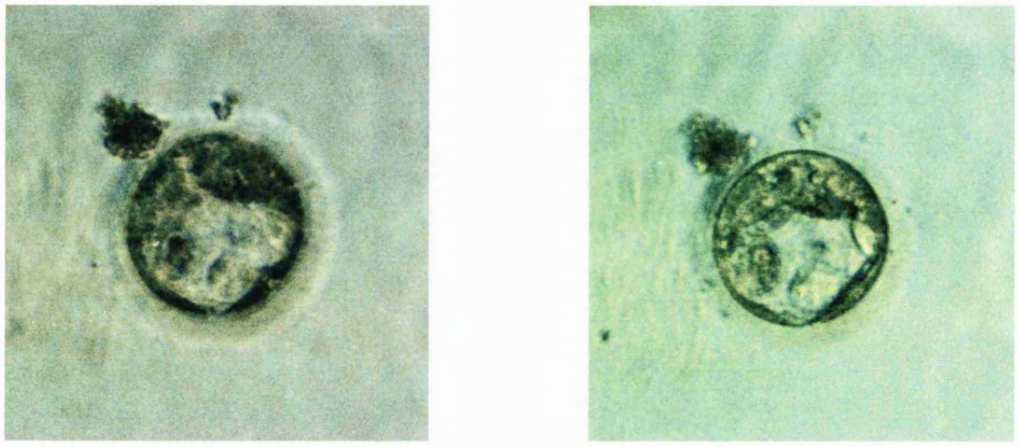
Grade = Voorberg grading system for day-5 embryos (http://www.ivf.nl/Grading_systems.htm)

- i Degenerating embryo: embryo shows signs of degeneration *e.g.*, fragmentation, *etc.*
- ii Developmental arrest: embryo has not developed further than 8-16 cell-stage without signs of fragmentation.
- iii Morula or compaction; embryo has proceeded to the morula or compaction stage without any sighs of cavitation.
- A Just cavitating embryo
- B Single cavity up to half of the embryonic volume
- C Single cavity occupying most of the embryonic volume
- D Expanding blastocyst

Figure 5.4 The same embryos at 2 different planes



a) An arrested embryo (embryo ID H3)



b) A blastocyst (embryo ID H4)

a) An arrested embryo with fragmentation and uneven cell sizes. No blastocoel cavity was formed.

b) A blastocyst with cumulus cells attached at the zona pellucida. A blastocoel cavity can be clearly seen.

Eleven FISH experiments were performed on these embryos. In each experiment, embryos from one or more patients were analysed along with the corresponding controls previously presented in **Table 5.5**. Table 5.7 describes case(s) in each experiment with their available embryos. Data of maternal age and the proportion of blastocysts will be discussed later **(section 5.3.2.4)**.

Table 5.7 Patients, ages, available embryos, proportion of blastocysts and number of the corresponding experiment

Experiment no.	Patient	Age	Number of arrested embryos	Number of blastocysts	Proportion of blastocysts
1	A	32	2	1	0.33
2	B	28	0	2	1.00
	C	31	0	3	1.00
3	D	34	4	0	0.00
4	E	29	0	1	1.00
5	F	36	1	1	0.50
	G	37	2	0	0.00
6	H	32	3	1	0.25
7	I	27	1	2	0.67
8	J	35	3	1	0.25
9	K	33	3	0	0.00
10	L	28	0	2	1.00
	M	33	1	0	0.00
11	N	41	1*	5	0.83
Total			**21**	**19**	

*FISH results were not available from this embryo.

Signals from the arrested embryo of patient N which had 7 nuclei were not satisfactorily analysable due to poor nuclei quality. Consequently, FISH results were available from 20 arrested embryos and 19 blastocysts. Like the lymphocyte controls, the two-round FISH did not cause a significant loss of results in embryos. This was shown by the fact that results from the 2nd round of FISH were available from 96.8% and 95.8% of the starting number of nuclei from the arrested embryos and the

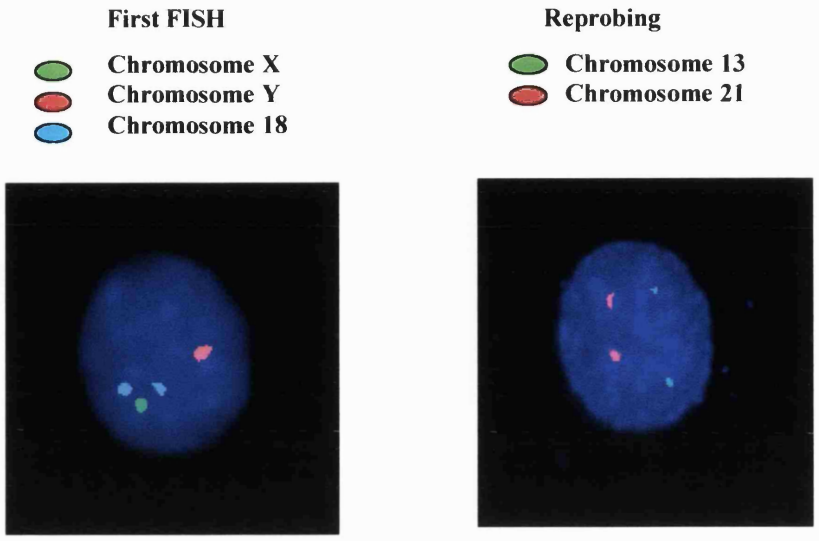
blastocysts respectively **(Table 5.8)**. Examples of signals encountered in the study are shown in Figure 5.5.

Table 5.8 Number of nuclei with available FISH results from the 1st FISH and 2nd FISH out of the initial nuclei number on fresh slides

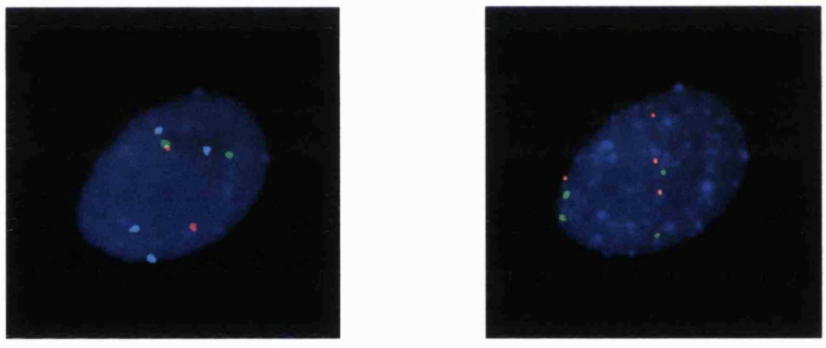
Arrested Emb. ID.	Nuclei number			Blastocyst ID.	Nuclei number		
	Fresh	1st FISH	2nd FISH		Fresh	1st FISH	2nd FISH
A1	7	7	7	A3	76	76	74
A2	16	16	16	B1	136	131	131
D1	23	23	22	B2	144	141	137
D2	14	14	14	C1	86	86	84
D3	26	26	24	C2	113	110	101
D4	20	19	18	C3	66	63	63
F2	10	10	9	E1	57	57	54
G1	9	9	9	F1	37	36	35
G2	8	8	8	H4	51	51	48
H1	7	7	6	I1	43	43	41
H2	13	13	13	I3	36	36	36
H3	15	15	15	J3	34	34	33
I2	8	8	8	L1	31	31	31
J1	12	12	12	L2	35	32	32
J2	7	7	7	N1	68	67	66
J4	24	24	23	N2	79	79	77
K1	19	19	19	N3	89	88	87
K2	9	9	9	N4	54	52	52
K3	8	8	8	N5	93	91	90
M1	28	27	27				
Total	**283**	**281**	**274**	**Total**	**1328**	**1304**	**1272**
Percent		**99.3**	**96.8**	**Percent**		**98.2**	**95.8**

Percent = percentage of nuclei number with an available FISH result after the first or second round out of the initial nuclei number in fresh slides.

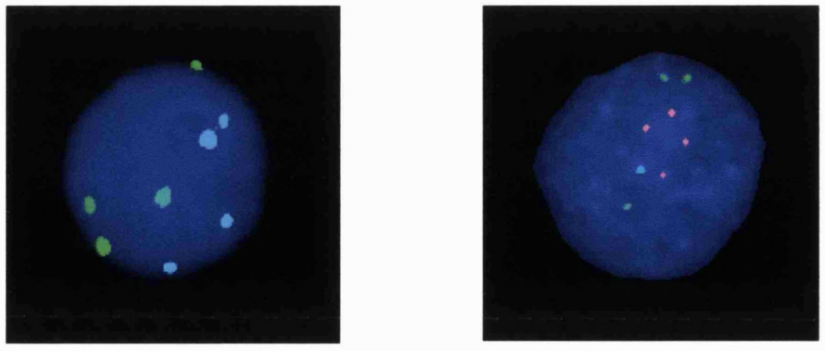
Figure 5.5 Various types of nuclei from embryos according to FISH signals



a) A diploid male nucleus (embryo ID B2)

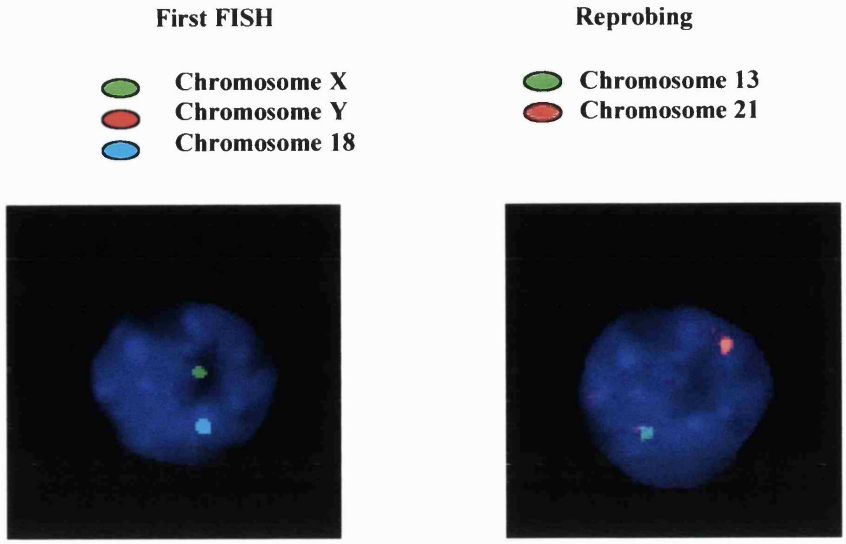


b) A tetraploid male nucleus (embryo ID N3)

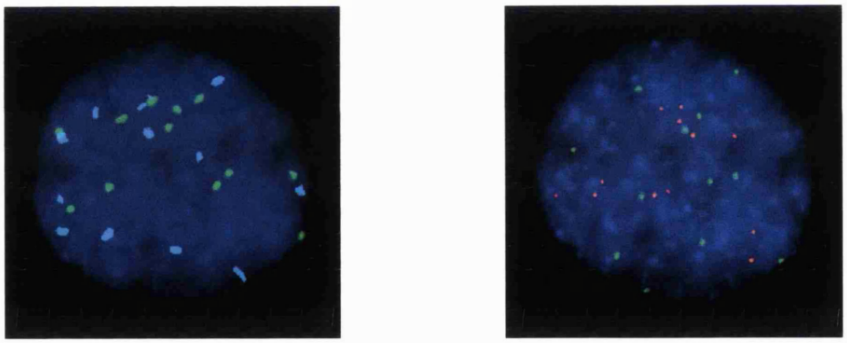


c) A tetraploid female nucleus (embryo ID H1)

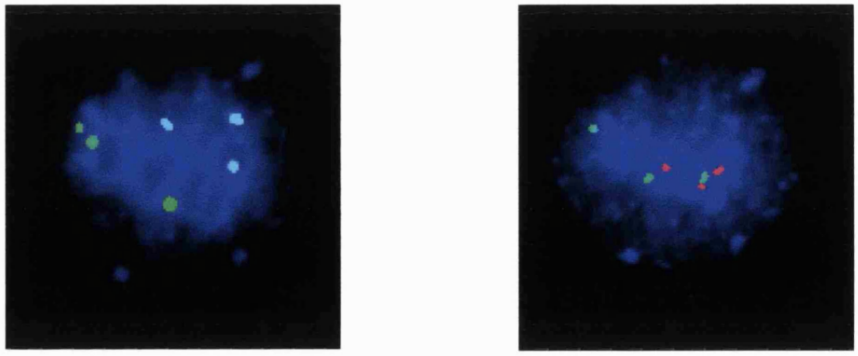
Figure 5.5 (continued)



d) A haploid nucleus (XO) (embryo ID G1)

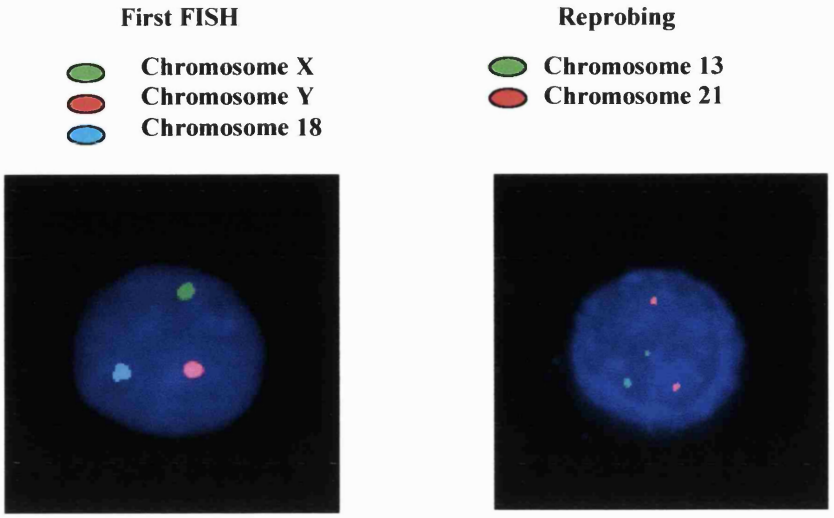


e) A female nucleus with 12n chromosome constitution (embryo ID N4)

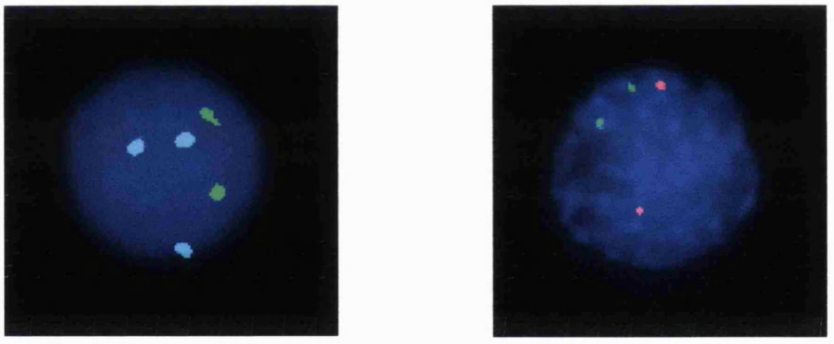


f) A triploid female nucleus (embryo ID C3)

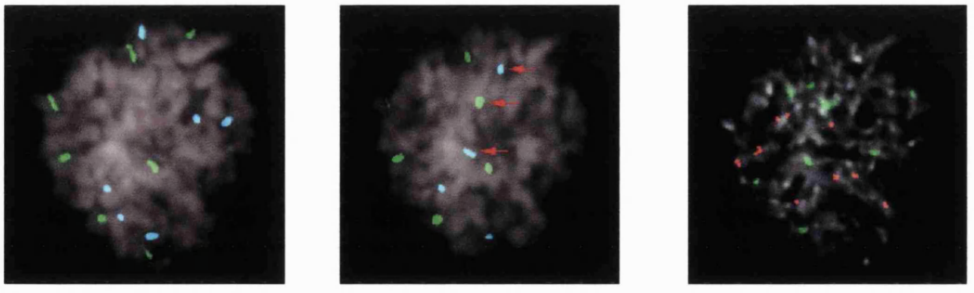
Figure 5.5 (continued)



g) A monosomy 18 male nucleus (embryo ID B2)



h) A trisomy 18 female nucleus (embryo ID C3)



1st image 2nd image 3rd image

i) A late prophase octaploid nucleus (embryo ID N2). For this nucleus, in the first round, two images were captured because all of the signals could not be taken in one plane. In the first image, 7 green and 8 aqua can be seen. In the second image, the additional signals were shown (red arrows). Altogether signals from the first round represent 8(X) and 8(18). The third image revealed 8 green and 8 red signals for chromosomes 13 and 21 respectively. Split signals for chromosomes 21 can be seen.

Tables 5.9A and B show FISH results of 274 and 1272 nuclei from arrested embryos and blastocysts. One concern in FISH analysis of embryos is how to classify an embryo to be mosaic or non-mosaic. Chromosome patterns compatible with polyploidy or haploidy are unlikely to be FISH artefacts. For example, blastocyst no. 2 (B1) was classified as mosaic despite the fact that only 1 cell in 131 was tetraploid. The same applies with chromosome patterns compatible with non-disjunction. These patterns were considered true and their presence in a diploid embryo categorised the embryo mosaic. Examples are arrested embryo no. 14 (J1), blastocysts no. 6 (C3) and no. 12 (J3). However, apparent aneuploid signals may be caused by an artefact or error due to signal overlapping, split signals, diffused or patchy signals, failure or suboptimal efficiency of hybridisation. Arrested embryos were classed as mosaic aneuploidy only when at least two nuclei with the same aneuploidy were found since the result from only one cell could be an artefact. For example, arrested embryo no. 19 (K3) was classed as non-mosaic. Blastocysts were classed as mosaic aneuploidy when the proportion of the aneuploid nuclei was significantly different from controls. In addition, due to the variability of percentages which might have resulted from a slight variation in conditions between FISH experiments, control results from each experiment, instead of mean values, were used specifically as references for the embryo results from the same experiment. For example, in blastocyst no. 4 (C1) which was studied in the 2nd FISH experiment, the presence of 6 apparent monosomy-18 nuclei out of 84 (7.1%) was compared with the proportion from the male control in same experiment (16 out of 300 nuclei, 5.33%). These two proportions were not significantly different (Fisher's exact test, $P = 0.595$) and this blastocyst was considered non-mosaic.

Table 5.9A FISH results from the arrested embryos

Case no.	Embryo ID*	Nuclei analysed	Sex	Results			Conclusion
				Types of cell	Nuclei number	Percent diploid	
1	A1	7	M	Dip	7	100.0	Uniformly diploid
2	A2	16	F	Dip	16	100.0	Uniformly diploid
3	D1	22	F	Dip/XO	15/7	68.2	Mosaic aneuploidy (sex)
4	D2	14	M	Dip	14	100.0	Uniformly diploid
5	D3	24	F	Dip/XO	22/2	91.7	Mosaic aneuploidy (sex)
6	D4	18	M	Dip/hap	17/1	94.4	Mosaic haploidy
7	F2	9	F	Dip/tet	5/4	55.6	Mosaic polyploidy
8	G1	9	M	Dip/hap	8/1	88.9	Mosaic haploidy
9	G2	8	M	Dip/mono 21	6/2	75.0	Mosaic aneuploidy
10	H1	6	F	Dip/tet	2/4	33.3	Mosaic polyploidy
11	H2	13	F	Dip/tet/trip	10/2/1	76.9	Mosaic polyploidy
12	H3	15	M	Dip/chaotic	8/7	53.3	Mosaic chaotic
13	I2	8	F	Dip/mono 18	6/2	75.0	Mosaic aneuploidy
14	J1	12	M	Dip/mono 18/ tri 18	10/1/1	83.3	Mosaic aneuploidy (NDJ)
15	J2	7	F	Dip/tet/oct/trip	3/2/1/1	42.9	Mosaic polyploidy
16	J4	23	M	Dip/chaotic	7/16	30.4	Mosaic chaotic
17	K1	19	F	Dip/tet	16/3	84.2	Mosaic polyploidy
18	K2	9	M	Dip/XO (XY/XO)	5/4	55.6	Mosaic aneuploidy (sex)
19	K3	8	F	Dip/mono 18	7/1	87.5	Uniformly diploid**
20	M1	27	F	Dip/tet/oct	22/3/2	81.5	Mosaic polyploidy
Mean±SD		13.7 ± 6.5				73.9 ± 21.9	

dip = diploid; tet = tetraploid ; hap = haploid; trip = triploid; oct = octaploid; mono 18 = monosomy 18; tri 18 = trisomy 18;

(sex) = (sex chromosomes); (NDJ) = (nondisjunction)

*The embryos with the same initial letter in the Embryo ID came from the same patients.

** This embryo was regarded as non-mosaic as only one nucleus showed monosomic signal.

Table 5.9B FISH results from the blastocyst stage embryos

Blastocyst No.	Blastocyst ID	Cells analysed	Sex	Results			Conclusion
				Types of cell	Cell number	Percent diploid	
1	A3	74	F	Dip/tet	65/9	87.8	Mosaic polyploidy
2	B1	131	M	Dip/tet	130/1	99.2	Mosaic polyploidy
3	B2	137	M	Dip/mono 18/tet/XXYY	114/17/4/2	83.2	Mosaic polyploidy and aneuploidy
4	C1	84	F	Dip/mono 18*	78/6*	92.9	Uniformly diploid*
5	C2	101	F	Dip/tet/chaotic/hap/ mono 21	74/12/5/2/8	73.3	Mosaic (complex)
6	C3	63	F	Dip/mono 18/tri 18/trip	14/28/20/1	22.2	Mosaic aneuploidy (NDJ) (plus one triploid cell)
7	E1	54	F	Dip/tet/trip	52/1/1	96.3	Mosaic polyploidy
8	F1	35	F	Dip/tet	34/1	97.1	Mosaic polyploidy
9	H4	48	M	Dip/tet	43/5	89.6	Mosaic polyploidy
10	I1	41	M	Dip/tet	36/5	87.8	Mosaic polyploidy
11	I3	36	M	Dip/tet	31/5	86.1	Mosaic polyploidy
12	J3	33	M	Dip/tri 18/mono 18/ XYY/XO	29/1/1/1/1	87.9	Mosaic aneuploidy (NDJ)
13	L1	31	F	Dip	31	100	Uniformly diploid
14	L2	32	M	Dip/mono 21/tri 13	25/4/3	78.1	Mosaic aneuploidy
15	N1	66	F	Dip/tet/chaotic/trip/oct	49/10/4/2/1	74.2	Mosaic polyploidy and chaotic
16	N2	77	F	Dip/tet/chaotic/oct	55/20/1/1	71.4	Mosaic polyploidy and chaotic
17	N3	87	M	Dip/tet/mono 18*/XYY	72/11/3*/1	82.8	Mosaic polyploidy and NDJ of sex chromosomes
18	N4	52	F	Dip/tet/12n/mono 18*	43/5/1/3*	82.7	Mosaic polyploidy
19	N5	90	M	Dip/tet/XXY	82/6/2	91.1	Mosaic (complex)
Mean±SD		66.9 ± 32.1				83.4 ± 17.1	

dip = diploid; tet = tetraploid ; hap = haploid; trip = triploid; oct = octaploid; mono 18 = monosomy 18; tri 18 = trisomy 18;

NDJ = nondisjunction

*The presence of the aneuploid nuclei was not significantly different from the corresponding normal controls.

From Tables 5.9A and B, a high prevalence of mosaicism is found in both groups; 80% (16/20) in the arrested embryos and 89.5% (17/19) in the blastocysts. In the arrested group, the number of nuclei ranged from 6 to 27 per embryo with the percentage of diploid nuclei ranging from 30-94% in the mosaic embryos. Three embryos (no. 7, 10 and 17) were diploid/tetraploid mosaic, in 5 embryos (no. 6, 8, 11, 15 and 20) nuclei with other ploidies were seen and in 5 embryos (no. 3, 5, 9, 13 and 18) a monosomic line was present. Embryo no. 14 had probably undergone mitotic non-disjunction of chromosome 18. The remaining two embryos (no. 12 and 16) contained chaotic nuclei. In all, tetraploid nuclei were found in 14 of the 16 mosaic arrested embryos.

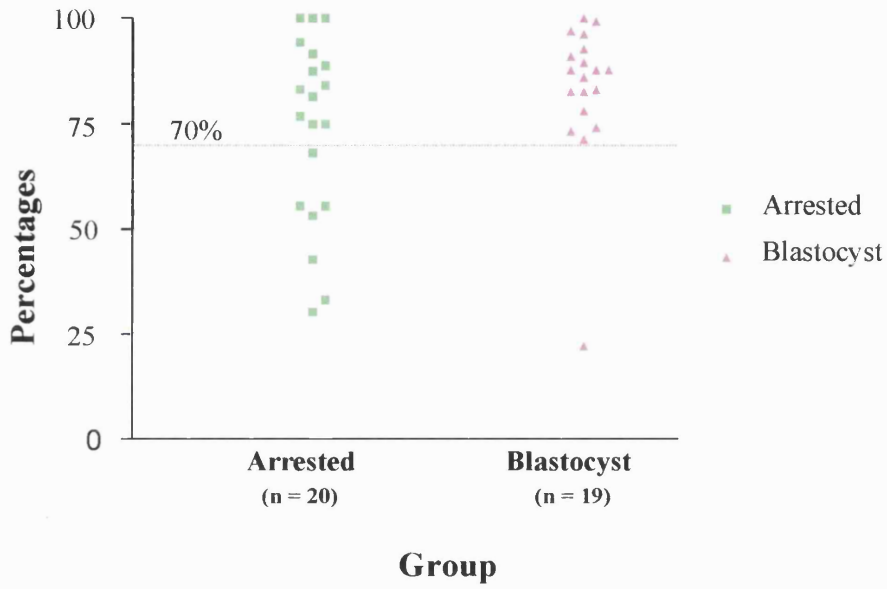
In the blastocyst group, the number of nuclei ranged from 31 to 137 per embryo. As previously mentioned, 17 out of 19 blastocysts were mosaic. However, with only one exception (blastocyst no. 6), at least 71.4% of the nuclei in each mosaic blastocyst were diploid. The next frequently found chromosome arrangement was tetraploid which was present in 14 out of 17 mosaic blastocysts. Other ploidies and some aneuploidies were also seen.

In both groups, haploid and triploid nuclei were present in some embryos in a low proportion, mostly one nucleus per embryo. Of the total 274 nuclei analysed in the arrested group, there were only 2 haploid (0.7%) and 2 triploid (0.7%) nuclei in 4 embryos (no. 6, 8, 11 and 15). Of the total 1272 nuclei analysed in the blastocyst group, only 4 triploid (0.3%) and 2 haploid cells (0.2%) were found in 4 blastocysts (no. 5, 6, 7 and 15). These two types of nuclei have been found more frequently in cleavage stage embryos.

Most of the blastocysts had a high diploid percentage whereas in the arrested embryos the diploid percentages varied widely **(Figure 5.6)**. The mean and median of the diploid percentage in the arrested embryos were 73.9% and 79.2% while those of the blastocysts were 83.4% and 87.8%. The differences between the two groups were not statistically significant in this study due to the small number of samples. However, almost all of the blastocysts (18/19 = 94.7%) had higher than 70% of their nuclei diploid compared to 13 out of 20 (65%) of arrested embryos (Fisher's exact test, P = 0.044). In 8 embryos with lower than 70% diploid nuclei in this series, 7 were arrested. This may imply that embryos with a low proportion of the diploid cell line (*i.e.*, a high degree of mosaicism) have a lower developmental potential. It is of interest that some of the arrested embryos also had a high percentage or even 100% of apparently diploid nuclei. However, it should be noted that only 4 chromosome pairs have been monitored; many of the apparently diploid cells may be aneuploid for other chromosomes. On the contrary, blastocyst no. 6 had only 22.2% diploid nuclei, with a high proportion of monosomic and trisomic lines due to an early mitotic non-disjunction event followed by clonal expansion of the aneuploid cell lines.

The most frequently found type of abnormal cell in both groups of embryos was tetraploidy. Overall they constituted 7.3% (113 out of total 1546 nuclei analysed), broken down to 6.6% (18 out of 274 nuclei) in the arrested embryos and 7.5% (95 out of 1272 nuclei) in the blastocysts. Other ploidies had also been found. One nucleus with twelve signals for each pair of chromosomes was found in a 52-cell blastocyst (no. 18, N4) **(Figure 5.5)**. Chaotic nuclei were found in two arrested embryos and in three blastocysts **(Tables 5.10A and 5.10B)**. Some of the chaotic nuclei appeared to be derived from chaotic division of a tetraploid cell line.

Figure 5.6 Percentages of diploid nuclei in each embryo in both groups



Red dashed line represents 70% level. Eighteen out of 19 blastocysts had ≥ 70% diploid in their nuclei compared with 13 out of 20 arrested embryos. If the two groups are considered together, 7 out of 8 embryos with ≤ 70% were arrested.

Table 5.10A Details of two arrested embryos which contained chaotic nuclei

Arrested embryo no. 12 (H3) total 15 nuclei

Diploid (XY) 8 nuclei
Chaotic 7 nuclei as follow:

Chaotic nuclei no.	X	Y	18	13	21
1	1	1	2	3	3
2	1	0	2	1	2
3	0	0	1	3	3
4	2	2	3	1	1
5	6	2	5	7-8	4-5
6	2	2	4	3	4
7	0	0	0	1	1

Arrested embryo no. 16 (J4) total 23 nuclei

Diploid (XY) 7 nuclei
Chaotic 16 nuclei as follow:

Chaotic nuclei no.	X	Y	18	13	21
1	2	1	3	0	0
2	2	2	4	3-4	3-4
3	0	1	3	2	2
4	2	2	4	3-4	3-4
5	2	2	4	3	3
6	1	0	1	1-2	1-2
7	1	2	2	3	3
8	1	2	4	4	4
9	1	1	1	2	2
10	2	1	2	2	2
11	1	4	3-4	2	2
12	1	1	3	2	2
13	1	0	1	1-2	1-2
14	2	1	0	2	2
15	1	0	0	2	2
16	1	1	1	2	2

Table 5.10B Details of three blastocysts which contained chaotic nuclei

Blastocyst no. 5 (C2) total 101 nuclei

Diploid (XX)	74	nuclei
Tetraploid	12	nuclei
Haploid	2	nuclei
Monosomy 21	8	nuclei
Chaotic	5	nuclei as follow:

Chaotic nuclei no.	X	Y	18	13	21
1	4	-	4	3	2
2	4	-	4	2	2
3	2	-	4	2	3
4	3	-	4	4	3
5	5	-	3	lost in 2nd round	

Blastocyst no. 15 (N1) total 66 nuclei

Diploid (XX)	49	nuclei
Tetraploid	10	nuclei
Triploid	2	nuclei
Octaploid	1	nuclei
Chaotic	4	nuclei as follow:

Chaotic nuclei no.	X	Y	18	13	21
1	1	-	2	4	2
2	4	-	2	4	2
3	5	-	3	5	6
4	3	-	5	5	2

Blastocyst no. 16 (N2) total 77 nuclei

Diploid (XX)	55	nuclei
Tetraploid	20	nuclei
Octaploid	1	nucleus
Chaotic	1	nucleus as follows:

Chaotic nuclei no.	X	Y	18	13	21
1	4	-	2	1	1

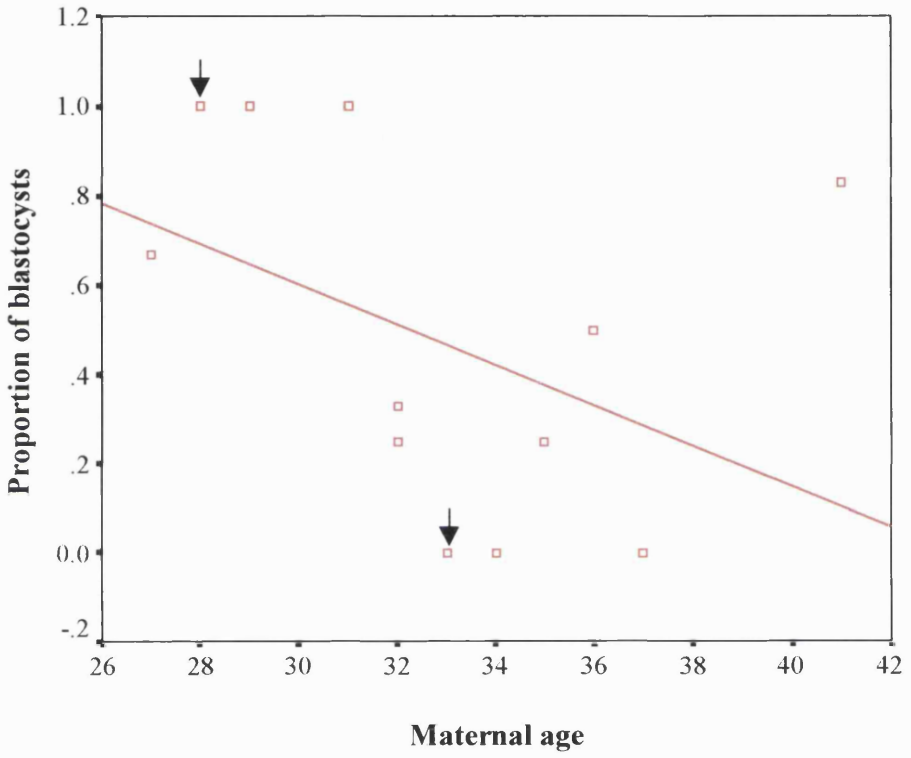
5.3.2.4 Maternal age effect

There was no significant correlation between paternal age and the formation of blastocysts (Spearman's rho correlation coefficient = -0.038, P = 0.901). In addition, there was no difference in the proportion of the cause of infertility (female and male factors) between the arrested and the blastocyst groups (χ^2-test, P = 0.369). For this reason, cases with a male factor were not excluded when the maternal age effect was studied. Figure 5.7 shows the scatter plots between maternal age and proportion of blastocyst formation. The data are from Table 5.7. A total fit line shows that the younger the mother, the higher the proportion of blastocysts. These results reflect that the embryos from younger patients had a better developmental potential than those from older patients.

5.3.2.5 Embryonic sex

Nine of the 21 arrested embryos and 9 of the 19 blastocysts were male. By χ^2-test, there was no difference in the proportion of male and female embryos between arrested and blastocyst groups (P = 0.78).

Figure 5.7 Correlation between proportion of blastocyst formation and maternal age



There was a negative correlation between maternal age and blastocyst formation (Spearman's rho correlation coefficient = -0.568, P = 0.034). Twelve points from 14 patients are seen because there were two pairs of patients with the same age and the same blastocyst proportion and two pairs of points are overlapping (black arrows).

5.4 DISCUSSION

5.4.1 FISH WORK UP

This study aimed to determine chromosome mosaicism in both arrested and blastocyst stage embryos. FISH was used so that information from almost all nuclei from an embryo could be obtained as this procedure can be performed on interphase as well as metaphase nuclei. However, FISH has the disadvantage that only a limited number of chromosomes can be studied at a time because of the limited number of fluorescent colours available. Also, it appears that the more chromosomes studied, the less the FISH efficiency (Ruangvutilert *et al.*, 2000). For the purpose of this study, the chromosomes of interest were sex chromosomes and autosomes whose abnormality are likely to have an impact on reproduction, *e.g.*, resulting in a reproductive failure or a livebirth with disabilities. Thus the main target chromosomes were sex chromosomes and chromosomes 13, 16, 18 and 21.

Recently, five-fluorophore directly-labelled probes are available commercially and allow for analysis of 5 chromosomes in a single FISH. MultiVysion™ PGT consists of probes for chromosomes X (blue), Y (gold), 13 (red), 18 (aqua) and 21 (green). However, in the present study, it was found that probe efficiency was low. This might be due to the difficulty in establishing the optimum conditions according to the protocol with the equipment available at the time as the protocol had to be modified. Obtaining all five signals clearly would be even more difficult in case of polyploid blastomeres which were found to be frequent findings in this study. In addition, in this probe mixture, human placental DNA labelled in aqua was employed for the purpose of nuclei location. DAPI cannot be used as it will interfere with the blue signal from the X chromosome. Using an aqua filter to locate the nuclei, however, was found to be

difficult. Since information from the same 5 chromosome pairs could be obtained from two-round FISH (in the current study) which was less problematic and the cost was lower, FISH using MultiVysion™ PGT was discontinued.

Another way to increase the number of chromosomes to be studied by FISH is to do a repeated analysis. Liu *et al.* (1998) performed three FISH procedures for chromosomes X, Y, 11, 13, 18 and 21 in the same blastomeres and lymphocytes. For blastomeres, the percentages of nuclei with signals out of the initial number of nuclei were 92, 87 and 78% after the first, second and third FISH. For lymphocytes, these percentages were 91, 86 and 81%. Based on the initial number of nuclei, the percentages of nuclei lost were 3, 7 and 13% after the first, second and third FISH respectively for blastomeres and 6, 10 and 14% for lymphocytes. Three-round FISH was not employed in the present study because it was thought to be less efficient. Two-round FISH was considered for the present study, initially for 6 chromosomes: X, Y and 18 in the first round, and 16, 13 and 21 in the second round. For the first round, a commercial probe mixture was readily available. For the second round, a commercial probe mixture was available for chromosomes 13 (green) and 21 (red) locus specific probes. An inclusion of the non-commercial α-satellite probe for chromosome 16 was attempted. The probe was labelled in green and red and ratio mixing of probes was performed to achieve a third colour for the second round FISH. However, poor signals were obtained, probably due to the incompatibility of the commercial locus specific and non-commercial α-satellite probe, resulting in a reduced efficiency of the latter. Inclusion of this α-satellite probe into the first round which contains other α-satellite probes, however, was not attempted as it was thought that if more probes were added

into a probe set, it would result in a lower FISH efficiency and would cause difficulty in scoring, especially in polyploid nuclei. Subsequently, it was decided to remove the probe for chromosome 16 from the study.

Two-round FISH for 5 chromosomes was found to work successfully on fresh embryos. Information of the five chromosomes were achieved from 98.6, 96.8 and 95.8% of the initial nuclei number of male lymphocytes **(Table 5.5)**, arrested embryos and blastocysts **(Table 5.8)** respectively. However, when this strategy was attempted on frozen-thawed embryos, the results were disappointing. This was unlikely to be caused by freezing-thawing *per se* as FISH results from such embryos have been reported (Iwarsson *et al.*, 1999). It is of note that the slides were old at the time of the FISH experiment. They might also have been affected by the transportation conditions. In addition, the spreading was performed by people who were still learning the technique. Thus, it was decided to base the main study on two-round FISH for 5 chromosomes on fresh embryos.

5.4.2 RESULTS FROM THE STUDY

Results were available for chromosomes X, Y, 18, 13 and 21 from 20 arrested embryos and 19 blastocysts. The results show that a high prevalence of mosaicism is found in both groups; 80% (16/20) in the arrested embryos and 89.5% (17/19) in the blastocysts. High levels of mosaicism in blastocysts has also been found by karyotyping (Clouston *et al.*, 1997) and FISH (Benkhalifa *et al.*, 1993; Evsikov and Verlinsky, 1998; Veiga *et al.*, 1999). Before these studies, Angell *et al.* (1987) found an expanded blastocyst to have 10% of the total 107 cells being tetraploid by radioactive *in situ* hybridisation with a Y-chromosome specific probe. They proposed that polyploidy at the blastocyst stage may be a normal developmental feature. Using thymidine for cell division synchronisation to reduce the exposure time to colcimid, Clouston *et al.* (1997) were able to obtain analysable metaphase spreads for karyotyping from 6- to 8-day-old human blastocysts. Of the 73 blastocysts, 17 (23.3%) were diploid/tetraploid mosaic, 7 (10%) were polyploid (mainly tetraploid), and 4 (5.5%) were diploid/aneuploid mosaics. These results were based on 1-6 cells analysed per blastocyst. FISH has an advantage that it can be used to analyse almost all the cells in an embryo as it can be performed on interphase as well as metaphase nuclei. Using single colour FISH for either chromosome X, Y or 18, with the definition of polyploid signals as more than 3 signals for chromosomes X or 18 or more than 2 signals for chromosome Y, to exclude trisomy 18 or common sex chromosome aneuploidies, Benkhalifa *et al.* (1993) estimated that 29% of blastocysts contained more than five polyploid cells. Using multicolour FISH, Evsikov and Verlinsky (1998) found that, in 47 blastocysts where the inner cell mass (ICM) was analysed, 20 had aneuploid cells and 2 had a few tetraploid cells in their ICM. In their 74 cases of euploid blastocysts, only 15 cases contained no

aneuploid cells, and polyploid cells (mostly tetraploid) constituted 4.9% of the total number of cells. Mosaicism was also found in 7/8 blastocysts and 5/8 arrested embryos in the series of Veiga *et al.* (1999).

Mechanism of mosaicism

All types of non-diploid nuclei in mosaic embryos found in this study have been reported in cleavage stage embryos. Aneuploid mosaics can be explained by postzygotic mitotic errors. The error may occur early as evident in blastocyst no. 6 (C3). Nevertheless, blastocyst no. 12 (J3) had just one pair of nuclei, each with monosomy 18 and trisomy 18, and one pair of nuclei, each with XXY and XO. In addition, 2 nuclei with the same sex chromosome anomalies in the same blastocyst were observed in blastocysts no. 3 and 19 (B2 and N5) and one nucleus with XYY was observed in blastocyst no. 17 (N3). In these cases, FISH artefacts could not be definitely ruled out but are unlikely when compared with the corresponding controls. A recent mitotic error is a possible explanation.

In a diploid/tetraploid mosaic, a tetraploid cell may arise from failure of cytokinesis after the chromosomes divide (Harper *et al.*, 1995), or from cell fusion (Benkhalifa *et al.*, 1993). Octaploid nuclei were also found in the present study. They have been observed in a previous study on blastocyst stage embryos (Benkhalifa *et al.*, 1993). They may arise from further failure of cytokinesis of a tetraploid cell. Cell fusion of an octaploid and a tetraploid cell may explain the 12n nucleus found in blastocyst no. 18.

The origin of diploid/triploid mosaicism is not clear. The presence of an extra haploid set may imply an incorporation of another gamete or its genome into one of the

daughter cells derived after the first mitotic division or later. This extra gamete might be a second sperm (in a dispermy event where the second sperm remains unincorporated into the formation of the zygote) or a polar body (Tuerlings *et al.*, 1993). Niebuhr (1974) mentioned another possible mechanism of fertilisation of the first polar body and ovum by separate sperms with the suppression of one second polar body.

The presence of a haploid cell line in a mosaic embryo is also difficult to explain. It may be associated with binucleate cell production with a meiotic type of segregation (Delhanty *et al.*, 1997) or may be an incorporation of a polar body into the embryo (Staessen *et al.*, 1999). Haploid and triploid cells, however, seem less viable or less actively dividing than tetraploid cells. They were present in only a few embryos, with only one or two cells in each. They were seen more often in cleavage stage embryos (Delhanty *et al.*, 1997). Nevertheless, in some instances, triploid cells may persist until later in development as cases of mosaic diploid/triploid have been reported postnatally (Edwards *et al.*, 1994).

Chaotic embryos have been described in the cleavage stage and may be patient related (Harper *et al.*, 1995; Delhanty *et al.*, 1997). It may result from the absence of cell cycle checkpoints leading to chaotic segregation of chromosomes (Harper *et al.*, 1995; Delhanty and Handyside, 1995). In the blastocyst stage, Evsikov and Verlinsky (1998) found 3 such embryos out of 91 blastocysts in their series. In the present series, chaotic nuclei were found with diploid nuclei in 2 arrested embryos and 3 blastocysts, some of which may have originated from uncontrolled division of a tetraploid cell line.

Role of cells with an abnormal chromosome complement

The results in the present study also suggest that embryos with a low proportion of the diploid cell line (*i.e.*, a high degree of mosaicism) have a low developmental potential. This is shown by the fact that 7 out of 8 embryos with less than 70% diploid nuclei were arrested. A selection mechanism operating via the elimination of embryos with a high degree of mosaicism prior to blastocyst formation has been suggested (Evsikov and Verlinsky, 1998). This selection may not operate perfectly as some embryos in the present series were exceptional. For example, some embryos with 100% diploid cells arrested whereas blastocyst no. 6, with only 22.2% diploid cells, was able to develop to the blastocyst stage. However, in this study, "diploid cells" were classified as such based on five chromosomes only. In addition, normal development may depend on other factors as well as the chromosome constitution. This may explain why some embryos arrested despite being uniformly diploid. As for blastocyst no. 6, mosaicism is likely to originate from non-disjunction with clonal expansion of both complementary cell lines. The genetic imbalance of the whole embryo might still be in the range that could be tolerated and the embryo could develop to the blastocyst stage.

In the blastocyst group, more than half of mosaic embryos were diploid/tetraploid or diploid/tetraploid with one or more other cell lines. The formation of polyploid cells may be caused or increased by the unnatural culture conditions. In other mammals, although polyploid cells have been found *in vivo*, a higher rate of mixoploidy has been observed *in vitro* (Murray *et al.*, 1986; Viuff *et al.*, 1999). In humans, the presence of polyploid cells is so frequent in a morphologically normal blastocyst that it has been speculated to be a normal feature and probably these cells are the precursors of some trophoblast cells (Angell *et al.*, 1987; Benkhalifa *et al.*, 1993).

Although cases of mosaic diploid/triploid or diploid/tetraploid have been reported postnatally (Edwards *et al.*, 1994), they are very rare considering their high frequency in the preimplantation period. There are several possible explanations for the absence or disappearance of polyploid cells (and probably including other abnormal cells) from an embryo/fetus proper such as:-

1. Selective diversion of abnormal cells from the embryo proper. From experiments using created tetraploid/diploid mouse embryos, the tetraploid cell line was not or rarely found in the resulting fetuses (reviewed in James *et al.*, 1995). Non-random distribution of tetraploid cells to the extraembryonic cell lineages in mouse embryos have been demonstrated (Nagy *et al.*, 1990; James *et al.*, 1995). James *et al.* (1995) suggested that, from their experiments where no excessive loss of the created chimaeric embryos was found, the selective mechanism was unlikely to involve the death of this kind of chimaeras. The restrictive distribution of the tetraploid cell line in ectoderm should arise from either loss of the tetraploid cells in ectoderm or preferential allocation of the tetraploid cells to the trophectoderm and the primitive endoderm. Later, in created mouse chimaera, the tetraploid cells were shown to be preferentially allocated to the mural trophectoderm at the time of aggregation (Everett and West, 1996). They suggested that the selective allocation of abnormal cells to the trophectoderm might be relevant to some cases of confined placental mosaicism seen in the human. Most of the cases where mosaicism is found in CVS are associated with a non-mosaic diploid fetus (Simoni and Sirchia, 1994; Kalousek and Vekemans, 1996; Wolstenholme, 1996; Hahnemann and Vejerslev, 1997).

However, some selection against trisomic cells in the trophoblast-derived lineages may occur. In mosaic trisomy 8 arising in a diploid zygote, high levels of

trisomic cells have been observed in the extraembryonic mesoderm while the levels in the fetal blood/tissue, amniotic fluid and the cytotrophoblast are low (Webb *et al.*, 1998).

2. Selection against embryos with a high degree of mosaicism. It is not certain if the diversion of abnormal cells to trophectoderm mechanism occurs in the human. Based on the presence of trisomic fetuses that are originated from postzygotic mitotic error (Delhanty and Handyside, 1995), it appears that aneuploid cells are not necessarily diverted to the TE. Evsikov and Verlinsky (1998) suggested that there is probably no selection for euploid ICM. They found that the average degree of aneuploidy in the ICM was similar to the overall blastocyst mosaicism and found a few tetraploid cells in 2 out of 47 ICM samples analysed. They proposed that the selection mechanism operates via the elimination of embryos with a high degree of mosaicism prior to blastocyst formation. The present study also illustrates that, despite the high frequency of blastocysts with mosaicism, the degree of mosaicism was lower than 30% (with the exception of blastocyst no. 6) while most of the embryos with a high degree of mosaicism were arrested. This selection against embryos with a high degree of mosaicism may not be perfectly operating as blastocyst no. 6 in the present study was not arrested. Also, abnormal embryos do develop to blastocysts and implant *in vivo*, giving rise to abnormal fetuses.

3. Elimination of abnormal cells from the embryo or fetus proper. With apoptosis or cell death beginning to operate in embryos at around this stage (Hardy *et al.*, 1989; Brill *et al.*, 1999), abnormal cells may be selectively eliminated. Nagy *et al.* (1993) suggested that the tetraploid component is selected against in all lineages whilst diploid cells differentiate normally and take over the embryo proper. They suggested

that this also resulted in relegation of the tetraploid component to the extraembryonic membranes. Some chaotic nuclei in the present study appeared to originate from a tetraploid cell line. These nuclei are unlikely to develop further as they are highly abnormal and may be subjected to apoptosis. This may lead to the disappearance of the tetraploid nuclei in later development.

All three mechanisms may exist and exert their effect concomitantly. Also, they may not operate with high efficiency. Some results from the present study provide evidence for the second and the third mechanisms. At the same time, it is interesting to observe that there was apparently no selection against the clonal expansion of the monosomic and trisomic cells in blastocyst no. 6 following an early mitotic non-disjunction event. The present study does not give information about the preferential allocation of abnormal cells to the TE. Further work needs to be carried out by studying the ICM and the TE separately. To date, there has been only one such study on human blastocysts (Evsikov and Verlinsky, 1998), whose results negate the selective allocation of abnormal cells to the TE.

Maternal age effect

In this study, maternal age appeared to affect the developmental potential. The proportion of blastocysts out of the total embryos was higher in younger patients. This is in agreement with the findings of Janny and Ménézo (1996) that the percentage of embryos reaching the blastocyst stage was significantly reduced for patients above age 30. In their series, the incidence of embryo blockage at the morula stage was 19% for patients younger than 30, but for patients of 30-34, 35-39 and older than 39 were 27,

28% and 28% respectively. They suggested that the lower blastocyst formation rate in older patients could be explained by an accelerated turnover of maternal ribonucleic acid (RNA) in aged oocytes. Maternal RNA has been shown to affect blastocyst formation in the mouse (Renard *et al.*, 1994). No paternal or male factor effect was found in the present study while previously, a strong paternal effect from sperm quality on blastocyst formation has been demonstrated (Janny and Ménézo, 1994; Shoukir *et al.*, 1998). A small number of cases could mask this effect in the present study. These conflicting results of parental factors on blastocyst formation need further studies.

Embryonic sex

No difference in the embryonic sex ratio between the arrested and the blastocyst groups was observed in this series. In the blastocyst group, there were nine males and ten females. From a recent study, more male than female infants were born after blastocyst transfer (Ménézo *et al.*, 1999). This may reflect more viability of male embryos after implantation.

Clinical implication

With the improvement in extended culture to the blastocyst stage, blastocyst culture has been proposed as a natural biological screen for enhancing the selection of chromosomally normal embryos for transfer (Janny and Ménézo, 1996; Ménézo *et al.*, 1997; Gardner and Lane, 1997). Results from this study and those of Clouston *et al* (1997), Evsikov and Verlinsky (1998) and Veiga *et al* (1999) show that it is not always the case that embryos which manage to reach the blastocyst stage are chromosomally normal. PGD at this stage may become a possible option. This may be performed by

trophectoderm biopsy (Veiga *et al.*, 1997) or day 3 biopsy followed by further culture of embryos to the blastocyst stage and blastocyst transfer (Veiga *et al.*, 1999). The latter strategy also allows more time for analysis. Recently, a group performed embryo biopsy on day 3 for the analysis of β-thalassaemia major and embryos were further cultured in sequential media with embryo transfers performed after the analysis (day 4, 5 or 6). Two healthy babies have been born and two other confirmed unaffected pregnancies are on-going (Kanavakis *et al.*, 1999). However, one should always be aware of the continuing high prevalence of mosaicism at the blastocyst stage as this could lead to misdiagnosis particularly in trophectoderm biopsy since the cells contributing to the inner cell mass will not be tested.

5.4.3 PROBLEMS ENCOUNTERED IN THE STUDY

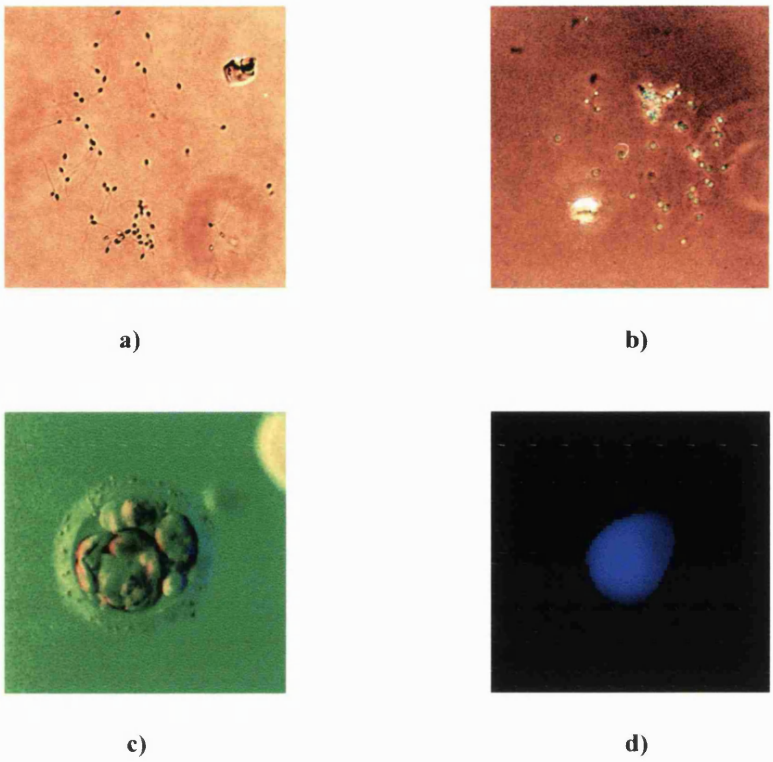
5.4.3.1 Technical problems and precautions

Contamination by sperm **(Figure 5.8)** and cumulus cells **(Figure 5.9)** may cause a misinterpretation of the results. Sperm, however, caused no problems because, with tightly condensed DNA, they yielded no probe access and gave no signals. The sperm contour was also recognisable **(Figure 5.8d)**. Cumulus cells can be removed during the spreading by gentle pipetting several times in PBS **(see also 2.3.1.3)**.

Any remaining cytoplasm will prevent good access of probes to the DNA. The correct spreading technique **(section 2.3.1.3)** and digestion step in the FISH procedure are crucial.

Due to the 3-dimensional nature of nuclei, several layers of DNA are situated on top of one another. Attention needs to be paid to the signals in different planes **(Figure 5.10)** to reduce errors in signal scoring.

Figure 5.8 Possible contamination by sperm

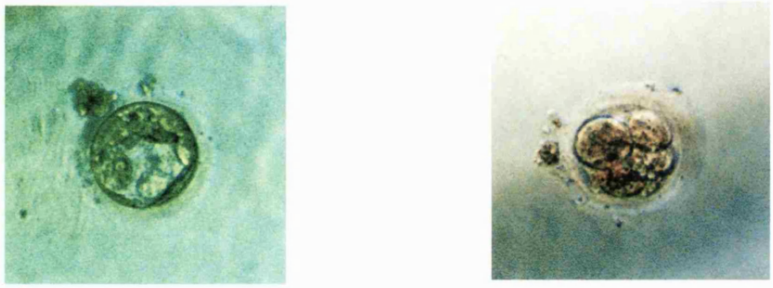


a) b)

c) d)

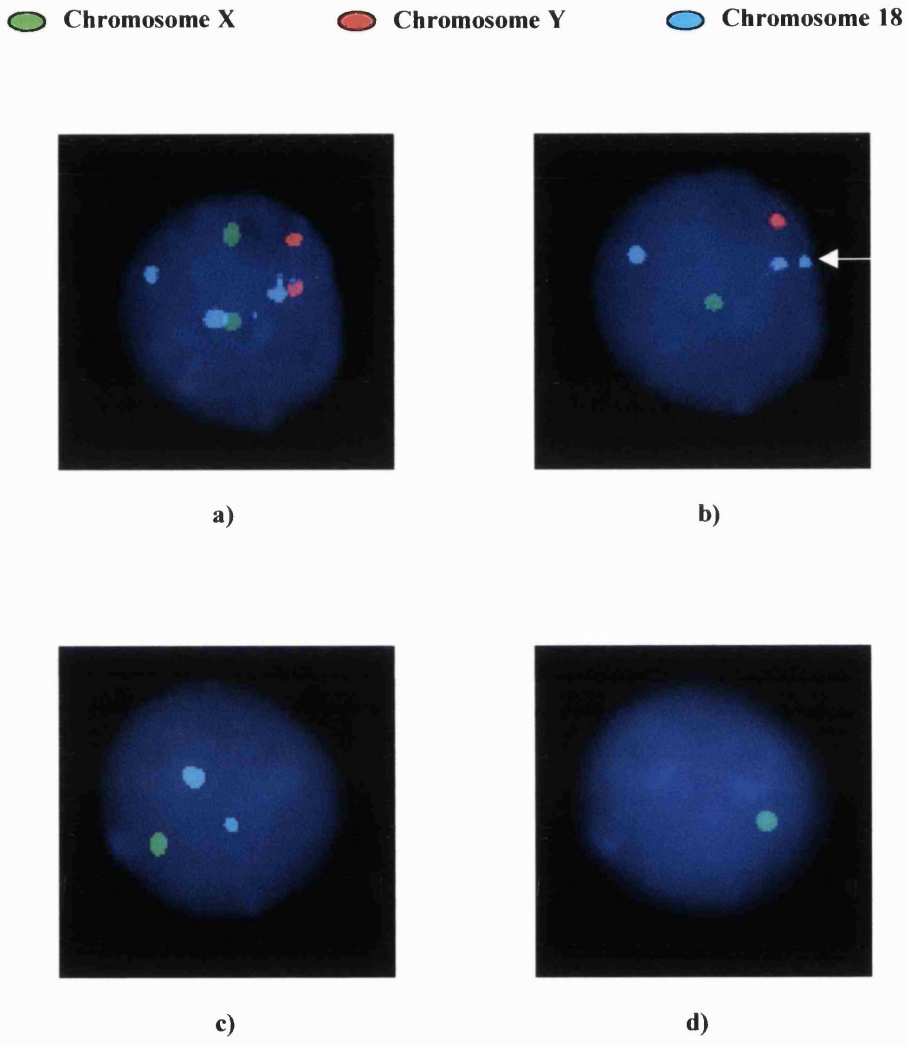
a) and b) Contaminating sperm on a slide. c) an arrested embryo with sperm attached to its zona pellucida. d) A sperm after the FISH procedure. The condensed DNA stained blue without any FISH signals. The contour was a tear-drop shape, with a fading staining at the junction between the neck and the body. The body and tail were digested by pepsin and was not seen after FISH.

Figure 5.9 Possible contamination by cumulus cells



A blastocyst and an arrested embryo with cumulus cells attached to their zonae pellucida.

Figure 5.10 Examples of the first round FISH signals in different planes



a) - b) show the same tetraploid male nucleus (embryo ID J4). In a), two copies each of chromosomes X and Y can be seen while only three copies of chromosomes 18 are visible. In b), the fourth copy of chromosomes 18 can be seen at the far right (arrow) while some chromosomes that can be seen from a) are also visible.

c) - d) show the same diploid female nucleus (embryo ID C3). In c), only one copy of chromosomes X can be seen. Another copy can be seen in d).

5.4.3.2 Interpretation of the data

Some points must be kept in mind when looking at the data. Firstly, the available embryos were surplus from transfer and cryopreservation. Their morphological quality might be lower than those that had been transferred or cryopreserved. However, in total they comprised one-third of the starting fertilised oocytes (FISH results available from spare 39 embryos from the initial 117 fertilised oocytes). As for the quality, 19 of the total 40 surplus embryos that were cultured further reached the blastocyst stage. Therefore, this set of data may be representative for embryos in general. The second issue is that the data obtained represent *in vitro* embryos which may be affected by the culture conditions. Although they may help in further understanding of human preimplantation genetics, *in vivo* embryos may be different. A study of human day-5 embryos *in vivo*, however, is difficult and unethical. Nevertheless, the present results may be applied to *in vitro* conditions and may be useful for IVF and PGD in the future.

5.4.4 FUTURE ASPECTS

Consideration for PGD

With the improvements in blastocyst culture, PGD may be performed on 1-2 cells biopsied from day-3 embryos and the embryos cultured to the blastocyst stage before transfer (Kanavakis *et al.*, 1999). Alternatively, trophectoderm biopsy may be performed on embryos at the blastocyst stage (Veiga *et al.*, 1997). For both of these, an error in diagnosis can be caused by the high prevalence of mosaicism. If the biopsy is performed at the cleavage stage, results are available from 1-2 nuclei and this may miss some important results such as in embryos with reciprocal non-disjunction. Also, some mitotic errors may develop after day 3 since some blastocysts in this series showed a possible recent mitotic error. With regard to trophectoderm biopsy, it may be less likely to have a normal diploid chromosome complement in every nucleus obtained from the procedure because of the high prevalence of mosaicism. Moreover, abnormal cells may be diverted into the trophectoderm since it has been shown that several types of trisomic cells can be found in the placenta while the fetuses are diploid (Wolstenholme, 1996; Hahnemann and Vejerslev, 1997), and confined placental mosaicism has been described (Simoni and Sirchia, 1994; Kalousek and Vekemans, 1996). As a result, trophectoderm biopsy may unnecessarily reduce the number of blastocysts suitable for transfer if all mosaic results are regarded as abnormal. Mosaicism in the TE should be studied further to determine its implication for PGD and its association to the ICM. There has been one study to date where mosaicism in the ICM was determined (Evsikov and Verlinsky, 1998). More studies should be carried out regarding the TE and the ICM separately.

Information from other chromosomes

Using FISH, information from this study was available from a limited number of chromosomes. It would be interesting to look at more chromosomes that are also relevant to embryonic death or fetal loss such as chromosome 16. Also, structural aberrations cannot be detected by FISH. In studies of cleavage stage embryos, several techniques have been attempted in order to obtain information of every chromosome from interphase single cells especially biopsied blastomeres. These techniques include interphase conversion and comparative genomic hybridisation (CGH) (reviewed in Harper and Wells, 1999).

Interphase conversion results in metaphase chromosomes available for a complete karyotyping from single interphase nuclei. It involves transferring an interphase cell, such as a blastomere, into a recipient cell such as a metaphase II bovine oocyte (Willadsen *et al.*, 1999) or an enucleated or intact mouse zygote (Verlinsky and Evsikov, 1999). The metaphase-inducing factors in the recipient cell force the transferred nucleus into metaphase that can be karyotyped either conventionally or by new technologies such as Multiplex or multifluor FISH (M-FISH) and SKY (spectral karyotyping). These techniques have been developed as advanced FISH-based methods that allow for the identification of the 24 chromosomes in a single hybridisation experiment (Speicher *et al.*, 1996; Schröck *et al.*, 1996). They have been shown to improve the detection and defining of some subtle or complex abnormalities which are sometimes missed or difficult to diagnose by a conventional method (Ning *et al.*, 1999; Uhrig *et al.*, 1999).

Like interphase conversion, CGH can determine a karyotype from interphase cells. The principle of CGH is the competition of hybridisation between a reference and

a test DNA labelled in different colours to normal metaphases on a slide. The ratio of hybridisation between the two DNA samples on the metaphase chromosomes is determined with the aid of computer software by the different fluorochromes used to label the reference and the test DNAs. The deviation of this ratio at any location on the metaphase chromosomes suggests a gain or loss of the test DNA in that region. Also, unbalanced structural abnormalities can be detected by CGH. These abnormalities can be missed by a conventional FISH. Studies using CGH on blastomeres from cleavage stage embryos have been reported and various abnormalities involving chromosomes other than those commonly tested by FISH have been observed (Wells and Delhanty, 1999a; Wells *et al.*, 1999b; Voullaire *et al.*, 2000). This emphasises the importance of the study for other chromosomes in blastocysts.

Although ideally information should be available from every cell in a blastocyst, doing so using interphase conversion or CGH can be labour intensive and difficult. Interphase conversion will need insertion of each blastomere to a recipient cell and CGH will need single cell PCR for every cell. In addition, CGH cannot identify ploidy abnormalities because the detection of abnormalities is based on a change in the relative binding ratio of the two genomes (reference and test DNAs) from one locus to another (Piper *et al.*, 1995). A loss or a gain of the whole genome will have a constant relative binding ratio and would not be detected.

Due to the limitation of interphase conversion in a study of a large number of cells and some limitations in CGH especially regarding polyploidy which have been frequently found in the present study, chromosome enumeration by FISH in interphase nuclei is still necessary in blastocysts. In the future, advanced FISH based technologies may be applicable to interphase cells. A study has applied spectral imaging on

interphase lymphocytes and was able to achieve signals from 7 chromosome pairs simultaneously (Fung *et al.*, 1998). However, the expected signals were obtained from 70% of interphase nuclei and various problems needed to be solved. In the meantime, in a study of blastocyst, CGH could be an option in situations where FISH cannot provide the desired information.

Study of the TE and the ICM

As already mentioned, the TE and the ICM in blastocysts should be studied separately. There are several techniques to achieve this. A technique of differential labelling of the TE and the ICM in human blastocysts has been described (Hardy *et al.*, 1989). Antibody-mediated complement lysis of the TE cells is induced and this allows labelling of the TE nuclei with the fluorochrome propidium iodide while the nuclei of the ICM are not labelled because the cells are intact. The whole embryo is rapidly fixed and both the TE and ICM nuclei labelled by a second fluorochrome such as bisbenzimide. The labelled embryo is mounted on a slide. Under a fluorescent microscope with appropriate filter sets, the ICM nuclei appear green from bisbenzimide and the TE nuclei appear orange from the combination of bisbenzimide and propidium iodide. This differential labelling technique can be followed by FISH as demonstrated by a study in which propidium iodide and Hoechst 33342 were used for nuclei labelling (Evsikov and Verlinsky, 1998). A possible alternative to separate the TE and the ICM in a blastocyst is mechanical dissection. This has been carried out in mouse blastocysts to culture the inner cell mass and the trophectoderm separately (Papaioannou, 1988).

More data obtained from blastocysts with separate results for the ICM and the TE may also explain the low fecundity of humans and how to rectify the problem. It may improve the outcome of IVF and may be a guideline for future PGD.

CHAPTER 6

DISCUSSION AND CONCLUSION

CHAPTER 6
DISCUSSION AND CONCLUSION

The results in Chapter 3 confirm the presence of fetal cells in cervical mucus during the first trimester of pregnancy which is potentially useful for early prenatal diagnosis. However, there are yet some obstacles to be overcome. Firstly, the inconsistency in finding fetal cells which could be caused by the true absence of fetal cells or the inadequacy of collection or analysis. It is also not certain if fetal cells are present in cervical mucus of all pregnant women as a physiological event. A larger scale of study should be carried out to address this possibility and at the same time, to improve the collection and analysis techniques. If it is found to be a physiological phenomenon that occurs in every pregnancy, cervical mucus can be a promising sample for non-invasive prenatal diagnosis. In addition, it would be interesting to determine if this fetal cell shedding has any physiological implications in pregnancy. Furthermore, prior to use in a clinical setting, additional studies should be performed to ensure the safety and the efficiency of TCC collection and analysis techniques in ongoing pregnancies. To date, there has been one study which systematically investigated the safety of collecting transcervical samples from ongoing pregnancies (Rodeck *et al.*, 1995). Secondly, the identification of fetal cells for a subsequent genetic analysis. This has to be accomplished from the more abundant maternal cells. Several monoclonal antibodies have been studied with a considerable variation in reactivities of these antibodies including cross reactivity with maternal cells (Rodeck *et al.*, 1995; Bulmer *et al.*, 1995; Miller *et al.*, 1999). In some instances, pure fetal cells are desired for genetic analysis such as in a single gene disorder diagnosis. Isolating clumps of pure

trophoblastic cells by micromanipulation has been successful in a number of cases but some clumps also contain maternal cells or are purely maternal (Tutschek *et al.*, 1995; Adinolfi *et al.*, 1997; Sherlock *et al.*, 1997; Adinolfi and Cirigliano, 2000). Thirdly, there is a possibility of more than one fetal cell line such as in twin pregnancies, mosaicism or chimaerism. Chimaerism originates from more than one zygote, commonly found in twin-twin transfusion. Ultrasonography may help in the diagnosis of twin or chimaerism. Mosaicism originates from one zygote with a postzygotic mitotic error. The error creates a new cell line and, in some originally abnormal embryos, produces a normal cell line which may alleviate the severity of the syndrome or the disease caused by the abnormal cell line. Without a proper identification of fetal cells in a FISH study, a mosaic trisomic female fetus may be misdiagnosed as full trisomic because a normal fetal cell line in a female pregnancy cannot be distinguished from maternal. On the contrary, if the fetus is a male with a normal and an abnormal cell lines, the finding of a normal male cell line can lead to misdiagnosis if one is not aware of the possibility of a presence of another cell line and stops looking further.

In addition to causing misdiagnosis in the current or potential future prenatal diagnoses, mosaicism has some other effects that must be considered. It may alter the phenotypes of the individual from non-mosaic status. An individual with some subtle manifestations of genetic disorders may in fact be a mosaic with various proportions of an abnormal cell line in the lymphocyte or skin fibroblast cultures or specifically in some tissues. Also, some chromosomally abnormal individuals may possess a proportion of normal diploid cells in lymphocytes, skin fibroblasts or some tissues. This may decrease the severity of the phenotypes that are expected in the full form of the abnormality. Thus, mosaicism in prenatal diagnosis may change the prognosis of the

fetus. However, the results in the study of fetal tissues for trisomies 13, 18 and 21 in Chapter 4 do not support the hypothesis that mosaicism with a normal cell line will alleviate the phenotypes and perhaps is a cause of the survival of trisomic fetuses to term. Moreover, a case of confined placental mosaicism was found without a favourable effect to the fetus. However, a separation of cytotrophoblast and syncytiotrophoblast cells was not carried out to define which type of CPM it was. With the respect of mosaicism and the outcome of the fetus, more cases should be studied to obtain a firm conclusion.

Mosaicism is nevertheless an important issue in human reproduction. Its high prevalence in cleavage stage embryos has been appreciated and, along with other chromosome abnormalities, is thought to be responsible for the low fecundity in humans (Delhanty and Handyside, 1995). The prevalence in the early fetal period or later is lower than that found in the cleavage stage, therefore, it is possible that the majority of conceptuses with mosaicism fail to implant. This may be a mechanism of natural selection. It is questionable that embryos with mosaicism are selected against at the cleavage, morula, or blastocyst stage. If they are selected against very early, screening an embryo for transfer may be carried out using prolonged culture and invasive procedures such as embryo biopsy can be avoided. However, the results from Chapter 5 show that a high prevalence of mosaicism persists to the blastocyst stage. This may have two implications. Firstly, the fact that an embryo can develop to the blastocyst stage does not necessarily mean that the embryo is chromosomally normal. Secondly, with some reports that blastocyst stage transfer can increase the implantation or pregnancy rate, a mosaic embryo may have a high survival potential. In this case the proportion of a normal cell line may play a role as shown from Chapter 5 that the

embryos with a better development had a higher percentage of a normal cell line than those that were arrested. Nevertheless, there was an exceptional case where the embryo managed to develop to a nice blastocyst despite being mosaic with all three cell lines from an earlier nondisjunction event. Moreover, a number of mosaic individuals survive to adulthood. This selection therefore does not operate in all cases and blastocyst stage transfer cannot ensure that an embryo is chromosomally normal.

Although mosaicism is prevalent in both prenatal and preimplantation diagnoses, there is a difference between the two periods. Mosaicism with polyploid cell lines has been frequently found in cleavage stage and blastocyst stage embryos. However, it appears that mosaicism with aueuploid cell lines has a better implantation or survival to the later developmental period as the majority of mosaicism found at early prenatal diagnosis by CVS, CPM, is aneuploid/diploid mosaicism.

In conclusion, the presence of fetal cells in TCC samples was confirmed but not in all instances. Problems arise from inconsistency and difficulties in finding and identification of fetal cells. In addition, mosaicism can be a potential problem for this non-invasive technique. It is indeed a problem with conventional prenatal diagnosis and PGD since its presence can lead to misdiagnosis. Its prevalence is also high in preimplantation embryos from the cleavage to the blastocyst stage. In many cases it is not possible to predict the phenotype of the affected embryo or fetus and this renders counselling and decision making difficult.

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PUBLICATIONS ARISING FROM THE THESIS

1. Ruangvutilert, P., Halder, A., Jauniaux, E., Arienzo, M., Cirigliano, V., and Sherlock, J. (1998) A minimally invasive prenatal diagnosis technique for the collection of transcervical cells. *Prenat. Neonat. Med.,* **3:**294-296.

2. Ruangvutilert, P., Delhanty, J.D.A., Rodeck, C.H., and Harper, J.C. (2000) Relative efficiency of FISH on metaphase and interphase nuclei from non-mosaic trisomic or triploid fibroblast cultures. *Prenat. Diag.,* **20:** 159-162.

3. Moore, G.E., Ruangvutilert, P., Chatzimeletiou, K., Bell, G., Chen, C.K., Johnson, P and Harper, J.C. (2000) Examination of trisomy 13, 18 and 21 foetal tissues at different gestational ages using FISH. *Eur. J. Hum. Genet.,* **8:**223-228.

4. Ruangvutilert, P., Delhanty, J.D.A., Serhal, P., Simopoulou, M., Rodeck, C.H., and Harper, J.C. (2000) FISH analysis on day 5 post-insemination of human arrested and blastocyst stage embryos. *Prenat. Diag.,* **20:** 552-560.