# Minimally invasive prenatal diagnosis of inherited disorders employing trophoblastic cells shed into the endocervical canal.

By

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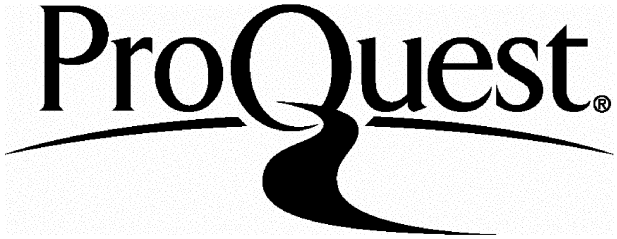


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**To Mum, Dad and Bob**

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# Abstract

Fetal (trophoblastic) cellular elements have been detected in transcervical cell (TCC) samples collected from the uterine cavity and cervical canal of pregnant women between 6 and 15 weeks of fetal gestation. These cells, present in a background of maternal material, have been identified utilising various molecular techniques. Chromosome Y specific DNA sequences have been detected using both fluorescent in situ hybridisation (FISH) and polymerase chain reaction (PCR) assays. Fetal specific short tandem repeat (STR) allele sizes have been identified in TCC samples indicating the presence of fetal DNA. Trophoblast cells have been identified in TCC samples using monoclonal antibodies specific for trophoblast antigens. Using these methods various TCC sampling procedures have been compared for their efficiency of fetal cell retrieval. The safety of TCC sampling has also been assessed by the collection of samples from ongoing pregnancies prior to CVS, and comparison with a control group.

The fetal Rh(d) type has been successfully diagnosed from TCC samples collected from Rh(d) negative mothers. Fetal genetic errors have been identified including trisomy 18, trisomy 21, XYY and triploidy. In attempts to identify recessive fetal disorders and X linked diseases, the isolation of trophoblastic elements has been attempted. Numerous methods have been employed to this end including micromanipulation, trophoblast specific mRNA detection, and magnetic activated cell sorting using trophoblast specific antibodies. Multiplex quantitative fluorescent PCR techniques have been developed to test these isolated cell clumps and individual cells for various inherited disorders. These include the detection of sex chromosome complement, chromosome aneuploidies, the delta-F 508 three base pair deletion causing cystic fibrosis, the single base change causing sickle cell anaemia, and a single base change and two deletions causing beta-thalassaemia. To test separated trophoblast cells, and with a view to potential preimplantation diagnosis, large numbers of isolated single cells were tested with these methods and the results assessed.

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# Abbreviations used in this thesis

| | |
|---|---|
| α-hCG | free alpha subunit of human chorionic gonadotrophin |
| β-hCG | free beta subunit of human chorionic gonadotrophin |
| ADO | allele drop out |
| AFP | alpha-fetoprotein |
| ALF | Automated Laser Fluorescence (Genetic analyser; Pharmacia) |
| ARMS | Amplification Refractory Mutation System |
| bp | base pairs (nucleotides) |
| C/S | caesarean section |
| CVS | chorionic villous sampling |
| DNA | deoxy ribonucleic acid |
| FACS | Fluorescent Activated Cell Sorting |
| FISH | Fluorescent *In Situ* Hybridisation |
| HbF | fetal haemoglobin |
| HbS | sickle cell anaemia |
| ICM | inner cell mass |
| MACS | Magnetic Activated Cell Sorting |
| McAb | monoclonal Antibody |
| min | minutes |
| MOM | multiples of the median |
| mRNA | messenger Ribonucleic acid |
| nRBC | nucleated erythrocytes (red blood cells) |
| NTD | neural tube defects |
| PA | Preferential amplification |
| PAPP-A | pregnancy associated protein A |
| PBS | phosphate buffered saline |
| PCR | Polymerase Chain Reaction |
| PGD | Preimplantation genetic diagnosis |
| QF-PCR | quantitative fluorescent PCR |
| RNA | Ribonucleic acid |
| SDS | sodium dodecyl sulphate |
| sec | seconds |
| STR | short/small tandem repeat |
| Taq | Thermostable DNA polymerase derived from Thermophilus aquaticus |
| TCC | Transcervical Cell |
| TOP | Termination of Pregnancy |
| YAC | Yeast Artificial Chromosome |

# Chapter 1

# Introduction

# Introduction

## Part 1. Prenatal Diagnosis

In recent times medical science has begun to unravel the genetic basis to numerous human disorders (McKusick, 1990). It has become apparent that many defects and disorders result from errors in the genetic makeup of an individual. These changes can be attributed to alterations in the sequence of deoxyribonucleic acid bases (DNA) that are found in the nucleus of every cell (Watson and Crick, 1953). This understanding has enabled the genetic status of an as yet unborn child to be assessed through prenatal diagnosis. Serious birth defects, many of them genetic, complicate and threaten the lives of 3-5% of new-born infants (McKusick VA. 1990; Shepard 1986) and about 0.7% of all live-born infants have a congenital abnormality associated with a chromosomal defect (Thompson et al., 1991). Such disorders account for 20% of deaths during the newborn period and an even higher percentage of serious morbidity in infancy and childhood (Contribution of birth defects to infant mortality; 1989). Other pregnancies are at risk of other serious inherited conditions, from deleterious single or numerous genes passed from one generation to the next. The fiscal and social cost of neonatal intensive care and rehabilitation programs for the severely handicapped is high, but higher still are the immeasurable emotional costs afflicting an affected family. With current social trends toward smaller families and delays in childbearing, coupled with the growing scientific knowledge regarding the basis of genetic disease, prenatal diagnosis has an important role in the management of many pregnancies. Prenatal diagnosis provides not only the option to parents of terminating a severely affected pregnancy, but in many situations the potential for the prevention of the clinical manifestations of a gene defect, such as with selected metabolic disorders by dietary restrictions soon after birth. Knowledge of fetal genetic errors is also paramount in the potential new field of genetic intervention, or gene therapy.

Prenatal diagnosis is possible as, apart from in unusual cases of chimeras and mosaics, every nucleated cell in an individual contains the same DNA. Thus retrieval and analysis of any fetal material, without disruption of the embryo proper, should enable the assessment of genetic health.

## 1.1 Invasive Prenatal Diagnosis Methods

### 1.1.1. Amniocentesis

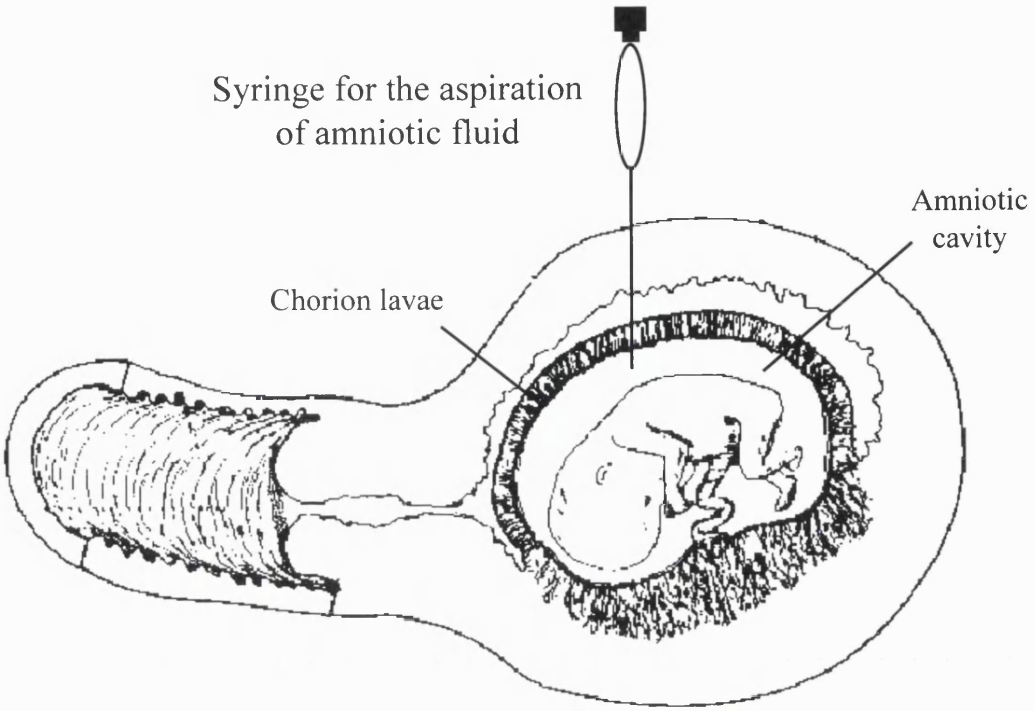
#### 1.1.1.1 Background

Amniocentesis is a technique enabling the collection of amniotic fluid from the amniotic cavity by puncture with a needle (Fig 1.1a). This fluid contains fetal cells originating from a variety of different tissues. Amniocentesis was first proposed in the nineteenth century (Prochownik 1877) in the management of polyhydramnios. However, it was not until the middle of the twentieth century that the technique was applied to prenatal diagnosis of Rhesus isoimmunisation (Bevis 1953). As techniques of analysing sex chromatin became available using quinacrine mustard dye (Pearson et al., 1970), the prenatal diagnosis of X-linked recessive disorders such as haemophilia and muscular dystrophy became possible (Riis and Fuchs 1960; Serv and Margolis 1964). Certain metabolic disorders could also be diagnosed prenatally using amniocentesis (Jeffcoate et al. 1965; Nadler 1968; Dancis 1968). A major advance was made when the amniotic cells were cultured and karyotyped (Steele and Breg 1966). This initially allowed the diagnosis of Down Syndrome (Valenti et al. 1968) and now enables many other inherited disorders to be identified including all common chromosome aneuploidies (trisomies 13 and 18, Turner and Klinefelter Syndromes, triploidy, tetraploidy, triple X females and XYY males), unbalanced translocations, major deletions (such as crit du chat; 5p-, and Wolf Syndrome; 4p-) and the identification of marker chromosomes (UCL Cytogenetics department, personal communication).

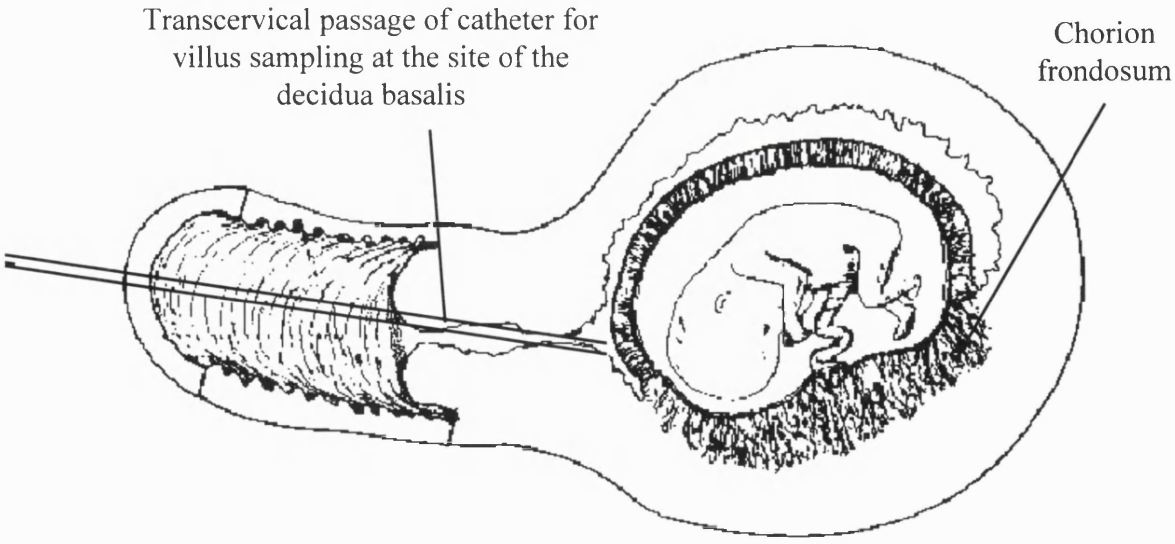
Amniocentesis is now widely accepted and is the most common invasive prenatal diagnostic procedure. With few exceptions it is usually carried out in the second trimester, between 15 and 18 weeks of gestation, at which time approximately 20ml of fluid is removed. Not only can cells from this fluid be cultured and karyotyped, but fetal proteins can also be detected and their levels estimated e.g. alpha fetoprotein, and bilirubin. The results of testing cultured cells can be obtained within 2-3 weeks and are accurate and reliable, with the ability to pick up the loss or gain of a single band on any chromosome (Guidelines for Clinical Cytogenetics, 1994). All cells in the amniotic fluid are fetal derived, and maternal cell contamination in subsequent

cultures caused by the passage of the catheter are rare (0.3% to 0.5%, Lippman et al., 1992; MRC, 1991).

## Figure 1.1a Amniocentesis




## Figure 1.1b Chorionic villus sampling



### 1.1.1.2. Risks & Drawbacks

The inherent risks to the mother from this procedure are negligible although maternal bacterial infection (amnionitis) may occur in 1:8000 procedures (Crane 1983) and has lead to at least one maternal death (Milunsky 1979). As an invasive technique (one which involves transversing fetal membranes), there are potential risks to the fetus.

#### 1.1.1.2.a. Procedure related pregnancy loss

This is defined as the amount of induced fetal loss before 28 completed weeks of gestation above background pregnancy loss rate. This figure is hard to derive because it necessitates the calculation of the background pregnancy loss rate. It is known that a high proportion of all conceptions spontaneously abort (Simpson & Bombard, 1987), 90% of which do so in the first trimester (Boue et al., 1985). The rate of background pregnancy loss is highly dependant on the gestational age. French and Bierman (1962) estimated the natural loss of pregnancy to be 11.8% between the $8^{th}$ and $11^{th}$ week, 16.7 between the $8^{th}$ and the $16^{th}$ week, 17.6 between the $8^{th}$ and the $20^{th}$ week and 4.4%-4.9% between the $12^{th}$ and $16^{th}$ weeks. The spontaneous fetal loss rate before 16 weeks, in pregnancies viable at the start of the $12^{th}$ week of gestation, probably lies between 1 and 9% (Lippman at al., 1984). If prenatal diagnosis is performed in the 8-$9^{th}$ week period, one would expect the background rate to be at the higher end of this range (e.g. 5-9%) while if is performed at 10-11 weeks the background may be lower (e.g. 1 to 5%) (Simpson & Bombard 1987). Any diagnosis performed thereafter would have a lower base spontaneous abortion rate. Procedure-induced miscarriages due to mid-trimester amniocentesis are estimated to be from 0.5% to 1% above the normal pregnancy loss at the same gestation age (Tabor et al., 1986; NICHHD, 1976).

#### 1.1.1.2.b. Additional Fetal risks

There have been isolated reports of fetal injury associated with amniocentesis including scarring (Karp and Hayden, 1977; Eply et al., 1979; Raimer and Raimer, 1984), limb or peripheral nerve injuries (Lamb, 1975; Epley, 1979; Holmes, 1997) and some more serious complications such as bowel injuries (Rickwood, 1977; Swift et al.,

1979), ocular injuries (Merlin and Beyth, 1980; Isenberg and Heckenlively, 1985; Admoni and BenEzra, 1988) and even brain injuries (Youroukos et al., 1980). Another possible procedure induced complication is fetal respiratory stress syndrome caused by a decrease in the number of alveoli. Leakage of amniotic fluid into the vagina occurring during the amniocentesis procedure can account for these problems. Almost all these complications can be attributed to operator error, and when amniocentesis is performed competently they should not occur.

### 1.1.1.2.c. Time of the procedure

Although amniocentesis is now considered a safe and accurate method of prenatal diagnosis, it has the disadvantage that the test is performed no earlier than 15 weeks of gestation. With the additional time required for cell cultures (between 2-3 weeks), a termination of pregnancy in the case of a genetically abnormal fetus can only be performed after 18-20 weeks- in the 2nd trimester. TOP at this late stage has many associated problems: The method of prostaglandin therapeutic abortion is a traumatic and economically costly procedure associated with an increased maternal morbidity compared to first trimester suction curettage, available up to 13 weeks (Abarmsky and Rodeck, 1991). There are also larger emotional problems for the parents caused by increased bonding to the unborn child (fetal movement may have been felt), and increased trauma and social or religious problems after the procedure (Burke & Kolker, 1993; Lawson et al., 1994). Even with the introduction of more rapid methods of genetic defect detection, such as a 24-hour diagnostic test for Down Syndrome using molecular techniques (Pertl et al., 1994), and fluorescent in situ hybridisation (Davies et al., 1994), results can not usually be obtained before 16 weeks of gestation.

### 1.1.1.3. Early amniocentesis

Ideally prenatal diagnosis, and eventual termination if necessary, should be carried out in the first trimester of pregnancy. Heckerling and colleagues (1994) found that a preference for early versus late prenatal diagnosis was the main independent predictor of the choice of CVS over amniocentesis and that test-related miscarriage rates, levels of karyotypic error leading to pregnancy termination, or maternal morbidity from therapeutic abortion did not significantly influence the choice of test. Investigators sought techniques that would maintain the accuracy of amniocentesis

with the benefits of a first trimester procedure. This led to attempts of performing amniocentesis as early as 10-14 weeks of fetal gestation. This procedure theoretically has most of the practical advantages of late amniocentesis (Hanson et al., 1987). However amniotic biochemical analyses before 12 weeks is limited because the corresponding fetal organs are not always functionally mature at this stage (Crandall & Chau, 1995).

The amniocentesis procedure performed prior to 14 weeks also has additional risks. The amniotic space is far narrower in the first trimester and so the risk of fetal injury, particularly with inexperienced operators, is increased. The definitive placenta is only formed from 10 weeks of gestation, and prior to this stage of development the vascularised villi of the chorion frondosum surround the whole gestational sac making the transplacental entry of the needle into the amniotic cavity inevitable. This increases the risk of intra-amniotic and/or feto-maternal haemorrhage. During the first 3 months of fetal development the placenta is separated from the amniotic cavity by the exocoelomic cavity (Section 1.1.5.). Amniocentesis at this time will cause the puncture of the amniotic membrane and possibly allow partial fusion of coelomic and amniotic fluids. The coelomic fluid, which during normal pregnancy does not come into contact with the fetus, shows considerable biochemical differences compared to the amniotic fluid, in particular it is more acidic (pH 7.18vs pH 7.42) (Jauniaux et al., 1994a). The mixing of these two fluids may have a deleterious effect on the developing fetus. Complications may also arise due to the percentage of fluid removed. Removal of 20ml amniotic fluid at 16 weeks gestation constitutes a loss of 5% of overall volume. Removal of 10ml of fluid at 12 weeks constitutes a loss of up to 35% of overall amniotic fluid volume (Silverman et al., 1992). The damage this may induce to fetal organs, such as the developing lungs, may be less reversible than later in pregnancy.

Opinions vary as to the safety of early amniocentesis. It does seem apparent however, that the earlier the procedure is performed, the higher the risk of subsequent miscarriage. Fetal loss rates are difficult to evaluate as the data presented in published studies are still from small sample numbers. In addition, the rate of spontaneous abortion is high prior to 14 weeks gestation and is directly influenced by maternal age. Assessment of fetal loss rates from procedures undertaken on older mothers, at a gestation stage prior to 14 weeks, are thus difficult to evaluate. It is generally accepted that early amniocentesis is associated with a higher procedure related fetal loss rate of up to 5%, (Hanson et al., 1987). One partially randomised trial has demonstrated that early amniocentesis is associated with 3% less chance of successful pregnancy

outcome that CVS performed at the same gestational age (Nicolaides et al., 1994; Table 1.1). These findings are supported by Saura et al., (1994) and Vandenbussche et al., (1994) who in their randomised studies showed fetal loss after early amniocentesis to be significantly higher than after CVS (6.7% in 120 early amniocentesis versus 0% in 64 CVS). In a semi-randomised trial with long-term follow-up involving 212 women, Nagal et al., (1998) also found problems associated with amniocentesis performed prior to 14 weeks gestation compared to CVS at the same stage. Mosaic karyotypes were found in 5.4% of the early amniotic samples compared to none with CVS and with 6.2% fetal loss associated with the former procedure. No pregnancy loss was observed with the group who underwent CVS and there were no incidences of talipes equinovarus compared to 3.1% with early amniocentesis. These findings were corroborated by the Canadian Early and Mid-Trimester Amniocentesis Trial Group (CEMAT, 1998) who in a muticenter random trial showed a significant difference in total fetal losses for early amniocentesis compared with midtrimester amniocentesis (7.6% *vs* 5.9%). In addition, there was a significant increase in talipes equinovarus in the early amniocentesis group (1.3% *vs* 0.1%) and a significant difference in post procedural amniotic-fluid leakage (early amniocentesis 3.5% *vs* midtrimester amniocentesis 1.7%). However, Eiben et al., (1994) studied 1800 pregnancies, each of which had undergone early amniocentesis procedures removing 5-8ml of fluid. They reported only 1% spontaneous abortion up to week 24 and concluded that in their hands the procedure is a safe one- a feeling shared by other groups (Bombard et al., 1994; Tavares et al., 1998). Greenough et al., (1997b) however, concluded that early amniocentesis may be associated with an increased risk of the baby being admitted into neonatal intensive care units.

**Table 1.1 Procedure related pregnancy loss associated with Invasive Prenatal Diagnosis**

| References | CVS | | | Amniocentesis | | |
|---|---|---|---|---|---|---|
| | No. of cases | GA (weeks) | PPL% | No. of cases | GA (weeks) | PPL% |
| MRC, 1991[R] | 1609 | 9-12 (TC) | 9 | 1592 | 15-17 (TA) | 6 |
| Smidt-Jensen et al., 1991[R] | 1175 | 9-11 (TC) | 10.1 | 1041 | 15-16 (TA) | 6.6 |
| | 1191 | 9-11 (TA) | 6.2 | | | |
| Lippman et al., 1993[R] | 1191 | 9-12 (TC) | 7.6 | 1200 | 15-17 (TA) | 7.1 |
| Ammala et al., 119[R] | 400 | 9-11 (TC) | 7.8 | 400 | 16 (TA) | 8.3 |
| Shalev et al., 1994[R] | 356 | 9-12 (TC) | 2.9 | 356 | 14-21 (TA) | 0.9 |
| | | | | 356 | 1012 (TV) | 3.2 |
| Palo et al., 1994 | 821 | 10-15 (TA) | 6.7 | 771 | 13-17 (TA) | 4.4 |
| Shulman et al., 1994 | 250 | 9-13 (TA) | 2.1 | 250 | 9-15 (TA) | 3.8 |
| Nicolaides et a., 1994* | 250 | 10-13 (TA) | 1.2 | 238 | 10-13 (TA) | 5.9 |
| | | | mean=5.95 | | | mean=5.13 |

GA, gestational age; PPL total post-procedure loss; TA transabdominal; TC, transcervical; TV, transvaginal; [R], randomised data; *, only randomised data presented.

These figures have not taken into account the background pregnancy loss rate.

From Jauniaux E and Rodeck C. 1995.

Short and medium term complications of early amniocentesis such as intrauterine bleeding, amniotic fluid leakage and amnionitis are thought to be similar to those reported for late amniocentesis (Baird et al., 1994; Brambati, 1993). Before 9 weeks of gestation however, amniotic fluid samples contain fewer cells and, compared to very early chorionic villous samples, they require longer culture harvest times of up to 40 days more. In addition, their *in vitro* culture success rate for some groups is only about 50% (Hackett et al., 1991), but for others is comparable to CVS (Sundenberg et al,. 1997).

### 1.1.1.4. Filter amniocentesis

A method of performing early amniocentesis without the removal of a large fraction of amniotic fluid, is that of amniocenteses with filtration (Sundberg et al., 1995). This procedure returns aspirated amniotic fluid to the amniotic cavity having removed its cellular content by filtration. Cells obtained in this way can be cultured and karyotyped. In a study of 1160 pregnant women undergoing prenatal diagnosis at 10-13

weeks, who were randomly assigned to either early filter amniocentesis or transabdominal CVS, Sundenberg and co-workers (1997) found there to be no significant difference in fetal loss rates between the two procedures. However, the trial was halted early as a significant increase in the occurrence of talipes equinovarus in the early amniocentesis group was observed (1.7%), associated with sampling at the earliest gestation ages and with the temporary leakage of amniotic fluid after sampling. Interestingly, in the study of early amniocentesis by Nicholaides et al., (1994), a similar frequency of talipes equinovarus was found (1.6%).

### 1.1.2. Chorionic Villus Sampling

#### 1.1.2.1. Background

An alternative method of prenatal diagnosis, possible in the first trimester, was first proposed by Mohr in 1968. In the first 12 weeks of pregnancy the gestation sac is enveloped in a coat of chorionic villi, the total volume of which exceeds the total volume of the fetus. By the analysis of these trophoblast villi, removed by endoscopic biopsy, prenatal diagnosis could be achieved: Chorionic Villus Sampling (CVS). Chorionic villi are mitotic derivatives of the zygote and afford the advantage that most will disappear naturally by about 14 weeks leaving only a portion to become the definitive placenta. It was proposed that removal of some of these actively growing villi will not compromise subsequent fetal development. Mohr obtained samples of extra embryonic tissue from first and second trimester pregnancies (8-20 weeks). Villi were pulled into a hole in the side of a transcervically inserted 'hysteroscope' barrel using vacuum and cut from the chorion by a knife inside the tube.

The first blind transcervical vacuum biopsies were reported from China in 1975. These were carried out at 6-14 weeks' gestation, and 4% induced miscarriage. Successful sex diagnosis was achieved in 94% of continuing pregnancies. Ultrasound guidance was first utilised by Kazy in 1982, and the procedure was adapted to that of today by Old et al., (1982) who used a 1.5mm transcervical catheter under real-time ultrasound guidance and suction to aspirate villi. A 90% sampling success rate was achieved on samples from pregnant women between 7-14 weeks' gestation. By 1983 transcervical CVS had been used to obtain fetal material for the correct diagnosis of Tay-Sachs disease, sickle cell anaemia (HbS), Duchenne muscular dystrophy and argininosuccinicacidura in the first trimester of pregnancy (Ward et al,. 1983; Rodeck

et al., 1983; Simoni et al., 1983; Silverman et al., 1992). Between 9-12 weeks of fetal gestation the uterine cavity is not completely filled by the amniotic sac. This is the ideal time for the passage of the catheter transcervically into the developing placenta.

Chorionic villi can also be obtained using a transabdominal ultrasonically-guided needle (Smidt-Jensen et al., 1984). This method is preferred for women with genital herpes, cervical polyps, or a markedly retroverted uterus. A needle is passed through the mother's abdomen and uterine wall to gain access to the chorionic villi. Chorionic villi are removed by a suction biopsy with a single or two-needle (one within the other) approach. This transabdominal procedure causes patients no subsequent bleeding, but more cramping than with the transcervical approach. It is now more commonly used than the transcervical method.

Chorionic Villus Sampling, either transcervical or transabdominal, is now routinely carried out as a prenatal diagnostic procedure between 10 and 13 weeks of fetal gestation. The procedure has been found to have no adverse perinatal effects (Williams et al., 1987). Direct metaphase preparations are also possible allowing a preliminary aneuploidy assessment after 24 hours. Even allowing for lengthy cell cultures and karyotyping, any ensuing TOP can be completed relatively early during pregnancy. Large amounts of DNA can be obtained and chorionic villi are better than amniocytes for fluorescent in situ hybridisation (FISH) analysis (Evans et al., 1992). With the advent of high resolution ultrasound CVS can theoretically be performed routinely transabdominally from 6 weeks gestation using the freehand ultrasound-guided single needle aspiration technique (Brambati et al., 1988). Several refinements have also recently been proposed including the use of biopsy forceps (Dumez et al., 1984) ~~a double-needle system (Maxwell et al., 1986)~~ and an automatic puncturing apparatus (Popp and Ghirardini, 1990). CVS has now become an established procedure throughout the world providing material for fetal karyotyping and DNA analysis. CVS cannot however be used in assays for which amniotic fluid is essential, such as measurement of the alpha-fetoprotein concentration. CVS is also the ideal technique for first-trimester prenatal diagnosis in multiple pregnancies, sampling for each individual placenta. In such cases, there is no greater risk of pregnancy loss than that associated with singleton pregnancies (Pergament et al., 1992). Problems however may arise if the same placenta is erroneously sampled twice.

### 1.1.2.2. Risks

#### 1.1.2.2.a. Procedure Related Abortions

Randomised trials have demonstrated that a woman assigned to undergo first trimester chorionic villus sampling has a 0.5 to 4.6% lower chance of a successful pregnancy outcome than a woman assigned to a second -trimester amniocentesis (MRC, 1991; Smidt-Jensen et al., 1991; Lippman et al., 1992. Table 1.1). A trial conducted by the National Institute of Child Health and Human Development estimated the procedure-related rate of fetal loss following CVS exceeded that for amniocentesis by 0.8% (Rhoads et al., 1989). It was found that the risks of spontaneous abortion is however significantly increased (up to 10.8%) among women in whom 3 or 4 attempts are made to place the transcervical catheter. These estimates comparing fetal loss due to CVS with fetal loss due to amniocentesis give a fair indication of the relative increase in risk to the fetus of the CVS technique. Due to the higher average age of women undergoing CVS compared to the general population, the calculated CVS procedure related pregnancy loss may be artificially high when compared to the background pregnancy loss rate at the same stage of gestation (Jahoda et al., 1991).

#### 1.1.2.2.b. Bleeding

Using the transabdominal procedure bleeding occurs in less than 1% of patients. With the transcervical method, however, vaginal bleeding occurs in 15-25% of women (Silverman et al., 1992; Rhoads et al., 1989). Subchorionic haematoma formation immediately after transcervical CVS has been shown to occur in 4% of patients (Brambati et al., 1987), but this is only rarely associated with adverse outcome and usually disappears after 16 weeks. Direct vascular injury of small branches of the utero-placental or unbilico-placental circulations may also lead to a retro-placental haemotoma and /or subchorionic haemorrhage and subsequently to a miscarriage. Most bleeding problems occur due to accidental placement of the catheter tip into the vascular decidua basalis underlying the chorion frondosom and is related to operator experience and in particular to the number of attempts needed to obtain a sufficient villous sample (Rhoads et al., 1989; Silverman et al., 1992).

### 1.1.2.2.c. Infection

Intrauterine infection and chronic amniotic fluid leakage are two main medium-term complications of CVS, occurring between a few days and 3 weeks after the procedure. It has been found that 30% of catheters used for CVS have bacteria on them following the operation (Scialli et al., 1985). This has raised concern about the possibility of inducing vaginal flora into the uterus by the transcervical passage of the catheter, resulting in chorioamnionitis. However no association between these bacteria and chorioamnionitis has been found (Garden et al., 1985). Intrauterine infection after CVS is a rare (<0.1%) but serious complication which can lead to maternal septic shock (Barela et al., 1986) and immediate evacuation of the uterine contents (Fisk and Anderson, 1987).

### 1.1.2.2.d. Rupture of membranes

Rupture of the amniotic membranes due to CVS is a very rare occurrence. However, delayed rupture, due to injury of the chorion allowing it to become exposed and damaged, or low grade chorioamnionitis occurs in 0.3% of cases (Hogge et al., 1986).

### 1.1.2.2.e. Rhesus Sensitisation

After CVS an acute rise in maternal serum alphafetoprotein levels has consistently been reported (Schulman et al., 1990) indicating the occurrence of fetal-maternal bleeding. This bleeding in most cases is asymptomatic, and serum levels return to normal ranges by 16-18 weeks. Fetal-maternal bleeding only becomes important in Rh (D) negative women, where a volume as low as 0.1ml of Rh-positive fetal blood in the maternal circulation can cause Rh sensitisation (Zipursky & Israels, 1967). This potential complication can be avoided if all Rh-negative mothers, undergoing CVS, receive Rh(D) immune globulin subsequent to the procedure.

### 1.1.2.2.f. Placental Mosaicism

Most cells cultured from the CVS technique are fibroblasts originating from the placenta. A complete genetic identity between the fetal and extraembryonic tissues

cannot however always be assumed. The inner cell mass of a developing pregnancy is represented by approximately 16 cells of a 64 cell blastocyst. Confined placental mosaicism, in which the placenta is karyotypically different from the embryo, may arise as a consequence of mitotic non-disjunction in one of the other 48 extraembryonic cells early in development (Simoni and Sirchia 1994). This situation may also be exaggerated by the preferential allocation of abnormal cells to the trophoectoderm as seen in studies of mouse chimeras (James and West, 1994). Confined placental mosaicism is thought to occur in approximately 1% of human pregnancies (Silverman et al., 1992) resulting in both false negative and false positive diagnosis. Of 62865 karyotyped chorionic villus samples that were reported to EUCROMIC between 1986-1992, 98.5% showed either a true normal karyotype (94.8%) or a true non-mosaic chromosomal aberration (3.7%) (Hahnemann & Vejerslev, 1997). True fetal mosaicism was diagnosed in about 0.15% and confined placental mosaicism in 1.0%. False positive non-mosaic aberrations were observed in 0.15% and false negative CVS results in 0.03%. Only 0.15% were unclassifiable. Ledbetter et al., (1990) reported the frequency of pseudomosaicism (artefact mosaicism in CVS culture metaphases, not present in the fetus or placenta) to be 1.8%, and indicated that the results of the direct method of chromosome preparation might be less representative of the fetal status than those from the full culture method. It has been shown that between 1-10% of chorionic villus samples need follow-up amniocentesis for an unambiguous diagnosis (Ledbetter et al., 1992; Lippman et al., 1992; Canadian Collaborative CVS-Amniocentesis Trial Group, 1989).

Amniotic cells are derived in the main from the developing fetus. Confined placental mosaicism is therefore not a problem when assessing amniocentesis samples. True fetal mosaicism, reflected in amniocenteses cultures is rare (0.25%) (Hsu and Perlis, 1984). Smidt-Jensen et al., (1993) confirmed that maternal tissue contamination and chromosomal mosaicism/pseudomosaicism occur in 1 and 0.4% respectively and early amniocentesis samples are altered by inherent mosaicism problems 10 times less often.

### 1.1.2.2.g. Maternal contamination

Contamination of CVS samples with maternal cells is a potential problem, particularly with transcervical CVS. Maternal-cell contamination is uncommon however in experienced cytogenetic laboratories, where villus specimens are

meticulously separated from blood and decidual cells before cytogenetic analysis. In such situations maternal contamination is seen to occur at a frequency between 1.9-3.8% (Ledbetter et al., 1990; 1992; Lippman et al., 1992).

### 1.1.2.2.h. Fetal Abnormalities

In 1991 Firth et al., reported an increased incidence of severe limb abnormalities in babies whose mothers had undergone transabdominal CVS prior to 9 completed weeks of gestation (56-66 days). The abnormalities were rare congenital disorders and included varying degrees of limb deficiency, hypoglossia-hypodactylia syndrome and cavernous hemangiomas. Occurring in the normal population at a frequency of one incidence per 17500 live births, (Froster-Iskenius et al., 1988), these abnormalities occurred in approximately 1.5% of offspring from women undergoing early CVS. The babies concerned were found to have normal karyotypes. The deformities sustained were consistent with incomplete morphogenesis and could be due to some kind of vascular insult, disrupting the normal development of the fetus. These observations were confirmed in subsequent studies (Burton et al., 1993; Gruppo Italiano Diagnosi Embrio-Fetali, 1993; Firth, 1994), including cases of transcervical CVS beyond the tenth week of gestation.

Some doubt however has been cast on these findings when further review showed that four of the cases reported by Firth and co-workers were not limb reduction defects (one case each of club foot or finger deformities, and two cases of annular constrictions without limb reduction defects); there had also been one duplication of cases published independently by different groups (Froster and Jackson, 1996). In their assessment of 138996 CVS follow-ups, Froster and Jackson concluded that there was no difference from the background population in the overall frequency or pattern distribution of limb deficiencies, with no correlation between gestational age at CVS. The majority of cases in this study however were collected after 10 weeks of gestation (mean 71.5 days), and complete absence of one or more limbs was observed in 4 cases, all collected between 53 and 63 days. The authors agreed that CVS should be avoided before 60 days gestation. This report was also questioned with regard to sample bias, either by failure of centres to report cases not felt to fit the spectrum of limb defects related to CVS or by the exclusion of cases with limb defects in patterns of multiple malformations owing to pregnancy termination or inadequate description (Evans and Hamerton, 1996).

Most authors now agree that very early CVS is associated with an overall 10-fold increase in the incidence of limb reduction effects and oromandibular-limb hypogenesis. This pattern and a much higher (2-3 times) incidence in limb reduction deformities before 9 weeks compared with sampling after 9.5 weeks suggest a causal relationship (Brambati et al., 1992; Rodeck 1993). CVS is now only carried out after 8-9 weeks' gestation, subsequent to the period of fetal organogenesis, apart from exceptional cases.

### 1.1.2.3. Transcervical CVS versus Transabdominal CVS.

Transabdominal CVS is associated with less secondary bleeding than transcervical CVS. In cases of a posterior placenta or in obese patients, the transcervical approach is probably safer and should be considered for CVS. Conversely, if the ~~amniocentesis is planned and the and the~~ placenta is anterior and obstructing, a transabdominal CVS would be more appropriate and probably safer (Jauniaux and Rodeck 1995). Transcervical CVS has the added advantage that there is no need to enter the peritoneal cavity or transverse sensitive structures. In practised hands both the transabdominal and transcervical techniques are equally safe (Jackson et al., 1992a; Jahoda et al., 1991).

### 1.1.3. Fetal cord blood sampling

Fetal blood can be obtained from approximately 18 weeks of gestation with a 20-22 gauge needle inserted into the umbilical cord under ultrasound guidance (Daffos et al., 1985). This can be achieved at the placental or fetal insertion site, free loop or from the intrahepatic umbilical vein. Such percutaneous umbilical blood sampling allows prenatal diagnosis of many fetal haematologic abnormalities, including isoimmunisation, haemoglobinopathies thrombocytopenia, and coagulation-factor abnormalities, (Shulman and Elias, 1990) and the assessment of viral, bacterial and parasitic infections.

Pregnancy loss post procedure is approximately 6% (Heckerling et al., 1994). However, most fetuses in this group have severe congenital malformations, and thus the background loss rate is high in comparison with that for the population undergoing amniocentesis or chorionic-villus sampling. The rate of fetal loss after percutaneous umbilical blood sampling is therefore about 2% more than the background risk to the

particular fetus undergoing the procedure (Daffos et al., 1985; Shulman and Elias, 1990). Because fetal blood sampling entails a substantially greater risk of pregnancy loss than amniocentesis, it is reserved for situations in which rapid diagnosis is essential, as a fetal karyotype can be obtained from cultured fetal lymphocytes in 3 days, for patients who book late in pregnancies, or for situations in which diagnostic information cannot be obtained by safer means.

### 1.1.4. Fetal biopsy

Fetal biopsy, initially performed by fetoscopy but latterly with ultrasound guidance, has been used for a few very specific cases of prenatal diagnoses. Certain genetic skin disorders, such as epidermolysis bullosa, that could not be diagnosed by DNA analysis required fetal-skin sampling (Rodeck et al., 1980). Fetal-liver biopsy has been used to diagnose ornithine transcarbamylase deficiency (Rodeck et al., 1982), and fetal muscle biopsy has been used to diagnose Duchenne muscular dystrophy in a family in which DNA studies were uninformative (Evans et al., 1991). It is difficult to assess the safely and accuracy of fetal biopsy because experience with these procedures is limited, however the invasiveness of the procedures and necessary trauma to the fetus suggests that procedure related fetal loss would be a serious concern. Rapid advances in DNA technology can be expected to elucidate the molecular basis of many diseases that now require fetal biopsy. As such knowledge is accumulated, the need for these procedures will decline as molecular tests can be performed on amniotic or CVS samples (Dunhill et al., 1995; Christiano et al., 1996a; 1996b; Christiano et al., 1997; Rozen et al., 1985; Nussbaum et al., 1986; Qu et al., 1996; Wong, 1996; Danpure et al., 1994).

### 1.1.5. Coelocentesis

An alternative method of prenatal diagnosis, that of coelocentesis, was proposed in 1991 (Jauniaux et al., 1991). The extra embryonic coelom is a space between the amniotic and chorionic membranes and is lined by an epithelium termed mesothelium. The coelom is found first outside the embryo and a similar cavity develops within the embryo (the intra embryonic body cavity). The fluid within the extra-embryonic coelomic cavity contains cells derived from the fetal extraembryonic mesoderm. Under continuous transvaginal ultrasound monitoring a 20 gauge needle is

introduced transvaginally into the coelomic cavity and the fluid aspirated. Utilising this procedure, successful fetal sexing was achieved using FISH and polymerase chain reactions (PCR) (Jurkovic et al., 1993) and it was proposed that this may present a new method of first trimester prenatal diagnosis. Failure to achieve reliable fetal sexing was however reported by one study where, by examining the coelomic fluid from 35 patients prior to TOP, 2 cases of male pregnancies were not identified and there was also 1 false positive by Y-sequence-specific PCR (Lau et al., 1998).

Diagnosis of fetuses at risk of haemoglobinopathies has been achieved. Coelomic fluid was collected at 7-10 weeks' gestation from 58 women undergoing TOP for social reasons. PCR was used to amplify a 364bp fragment of the human beta-globin gene spanning the site of the single base change causing sickle cell anaemia. The restriction endonuclease DdeI was used to detect the mutation, as the base change abolishes its restriction site. Five women were carriers of the HbS mutation, and the PCR result in 3 of these indicated that the fetus was also a heterozygous carrier. In the remaining two cases, the fetus was diagnosed as normal. Three further coelomic fluid samples were found to be heterozygous for the HbS mutation. In these instances the maternal haemoglobin genotype was normal, indicating paternal transmission of the sickle gene (Jurkovic et al., 1995). A further study was able to use PCR for multiple genetic diagnoses (sexing and single-gene diagnosis) from coelomic cells together with simultaneous DNA fingerprinting to determine that contamination had not occurred (Findlay et al., 1996). Coelomic fluid was also used for the PCR identification of VNTRs and beta thalassaemia status of fetuses from four women undergoing termination of pregnancy at 7-9 weeks (Makrydimas et al., 1997a). All four women and their partners were known carriers of beta-thalassaemia, and there was concordance in the results obtained from the chorionic villi and coelomic cells. Turner's Syndrome has also been identified by coelocentesis (Cruger et al., 1997), and coelomic fluid cells have also been successfully cultured and karyotyped (Crüger et al., 1996).

It has been shown that the fetal heart rate is not affected by coelocentesis which suggests that the procedure does not have a major effect on the fetal cardiovascular system (Makrydimas et al., 1997a; 1997b). Additionally by measuring the maternal serum concentration of alpha fetoprotein (AFP) before and after the procedure, it appears that coelocentesis is not associated with significant feto-maternal haemorrhage (Makrydimas et al., 1997b). Sadly however, coelocentesis has been shown to be associated with a procedure related pregnancy loss rate of 25% (Ross et al., 1997) and so is now considered unsuitable for early prenatal diagnosis.

It has been proposed that coelocentesis may however offer a new opportunity to induce tolerance to foreign grafts or establish chimaerism in human fetuses to avert genetic disease from 28 days post-fertilisation (Edwards et al., 1995). The coelomic cavity appears to be closely associated with the fetal haemopoietic system. By performing two successive coelocentesis, cells can be extracted from the cavity for the genetic typing of embryos in early pregnancy, and then donor cells replaced. The optimal age to inject stem cells designed to produce chimaerism may be at 8-10 weeks embryonic age, and these grafted cells may induce tolerance later in gestation. If tolerance and chimaerism can be established, repeated tissue grafts could be carried out during fetal life and after birth, so that disorders caused by single or multiple gene defects in the haemopoietic system and other organs may be corrected.

## 1.2. Existing Non-invasive Prenatal Diagnostic methods

As described, amniocentesis and CVS are now well established procedures but have the main drawbacks of high procedure related pregnancy loss and late diagnoses respectively. All first-trimester invasive procedures also appear to be associated with an excess of congenital anomalies (Greenough et al., 1997a). Ideally a first trimester prenatal diagnosis technique would be available which is less traumatic than CVS, simpler, cheaper and with less associated risks to mother and fetus.

### 1.2.1. Ultrasound scanning

Ultrasonography is an important aid in the assessment of gestational age, the monitoring of fetal growth, the confirmation of the placental site, the detection of multiple gestation, and the diagnosis of major fetal anomalies. Some teratogens and infections produce only structural abnormalities, which are potentially detectable with ultrasound but not with the other prenatal diagnostic approaches. Such anomalies may even be familial, but no chromosomal or molecular defect is known. Visualisation of the fetal anatomy is essential in diagnosing anatomical defects inherited in polygenic or multifactorial fashion. Individual centres report impressive achievements in the ultrasound diagnoses of renal and bladder anomalies, hydrocephaly, and neural tube and ventral-wall defects (D'Alton & DeCherney, 1993). Ultrasound examination is also useful in mendelian disorders characterised by certain anatomical defects such as skeletal dysplasias.

A four-chamber view of the fetal heart in obstetrical sonography has resulted in a substantial increase in referrals for fetal echocardiography, and the majority of the cardiac anomalies now diagnosed are in this subgroup of referred cases. The sensitivity of the four-chamber view for the detection of congenital heart defects was reported to be 92% in a high risk referred population (Copel et al., 1986).

Ultrasound can also be used for indications of selected fetal aneuploidy. Fetal anomalies characteristic of Trisomy 21 fetuses include duodenal atresia, tracheo-esophageal fistula, esophageal atresia, hydramnios, atrioventricular canal defects, ventricular septal defects, atrial septal defects and hypoplasia. Those for Trisomy 18 include intrauterine growth retardation, hydramnios, clenched hands with overlapping digits, club feet, rocker-bottom feet, ventricular septal defects, omphalocele, diaphragmatic hernia, and choroid-plexus cysts. Those for trisomy 13 include holoprosencephaly, cleft lip and palate, ventricular septal defects, polydactly, omphalocele, and polycystyic kidneys (D'Alton & DeCherney 1993).

In particular, Down Syndrome has been seen to be associated with increasing nuchal translucency, arising from fluid accumulation at the back of the fetal neck. This is an observation that can be detected in the first trimester of pregnancy. In one study using nuchal translucency, a cut-off of more than or equal to 3mm yielded a detection rate of 84% with a false positive rate of 4.5% (Nicolaides et al., 1994). These encouraging results have been supported by findings from other groups (Szabo and Gellen, 1990; Schulte-Vallen and Schindler, 1992; Soothill and Kyle, 1997), but disputed by some (Brambati et al., 1994) with occasional extreme thickening being associated with uneventful pregnancies (Cha'ban et al., 1996).

Ultrasound scanning has the drawback of being primarily a second trimester technique, although some centres are now experimenting with first trimester transcervical ultrasound, (Achiron et al., 1991). Although increasingly useful as an indication of embryonic mal-development, diagnosis with ultrasound is inconclusive, and a positive result leads only to referral for an invasive procedure.

### 1.2.2. Biochemical screening

Non-invasive biochemical screening between 15-22 weeks of pregnancy can provide indications for numerical chromosome anomalies (Wald et al., 1988). This method can detect an estimated 72% of Down Syndrome affected pregnancies with a false-positive rate of 5% (Wald et al., 1994) if gestational age is based on ultrasound

scan examination. The assessment is based on levels of substances detectable in the maternal serum which have been found to be associated with an increase or decrease in affected pregnancies- alpha-fetoprotein (AFP), unconjugated oestriol, free alpha subunit of human chorionic gonadotrophin (α-hCG), and free beta subunit of human chorionic gonadotrophin (β-hCG)- together with maternal age and weight. Any referral for full invasive prenatal diagnosis cannot however be made until the second trimester.

In 1972 Brock and Sutcliffe reported a high level of alpha-fetoprotein (AFP) in the amniotic fluid of pregnancies with fetuses affected by the neural tube defects (NTD) anencephaly, spina bifida or hydrocephalus. AFP is small enough to cross the placental barrier into the maternal circulation and can be measured as maternal serum alpha-fetoprotein (MSAFP) (Adinolfi 1978). About 90% of open NTD cases are associated with MSAFP levels at more than twice the normal at 16-18 weeks of gestation (UK collaborative study, 1977). With cut off levels of MSAFP at 2.5 times the normal level, 90-95% of anencephaly and 75% of open spina bifida can be detected, with a 5% false positive rate. Clearly this screening technique is invaluable for the identification of fetuses suffering from NTDs. Again however, due to the timing of the procedure, any referral for full invasive prenatal diagnosis cannot be made until the second trimester of pregnancy.

Recent advances suggest that screening for Down Syndrome may be effective from 10-12 weeks of pregnancy (Wald et al., 1995). A number of markers have been explored and the levels of some in the maternal serum found to differ with Down Syndrome pregnancies; pregnancy associated protein A (PAPP-A), β-hCG, unconjugated oestriol, AFP, serum hCG, α-hCG, CA-125, SP1, Dimeric inhibin A, Progesterone, Placental alkaline phosphatase, and Placental protein 14 (for review see Wald et al., 1995). The most useful markers have been shown to be PAPP-A, β-hCG, unconjugated oestriol AFP. Expressed in multiples of the median (MOM) these are found in Down Syndrome pregnancies to be at 0.38 MOM, 1.73 MOM, 0.70 MOM and 0.78 MOM respectively (Wald et al,. 1995). When using PAPP-A and β-hCG together with maternal age a detection rate of 62% with a 5% false-positive rate can be achieved (Wald et al., 1995). If nuchal translucency and the maternal serum markers prove to be independent indicators of Down Syndrome, the detection rate would become 80% with a 1.2 % positive rate. Combining these parameters, notable detection rates of 70-85% have been achieved (Sebire et al., 1996; Scott et al., 1996).

Maternal serum biochemical analysis has good potential for non-invasive first and second trimester screening. However the range of this screening program is limited to

Down Syndrome and NTDs, and the diagnosis cannot be certain, only provide an indication of fetal status. Should the screening results suggest an affected fetus, the course of action is for referral to an invasive process with all the inherent risks involved therein. It is important to remember that biochemical and ultrasound techniques are prenatal screening tools, and cannot offer definitive prenatal diagnosis.

To perform non/semi-invasive prenatal diagnosis using alternative methods to the existing invasive procedures, fetal cells must be obtained by another manner. For the success of this proposition, the fetus and fetal cellular material must not be totally self contained within fetal membranes; there must be some kind of 'leakage' of fetal cells. It is known that there are two possible sites of fetal cell migration during pregnancy; through the maternal/fetal placental barrier at the decidua basalis into the maternal blood circulation; or through the decidual capsularis into the uterine cavity, the aspect of study in this research project.

# Part 2. Development of the Placenta

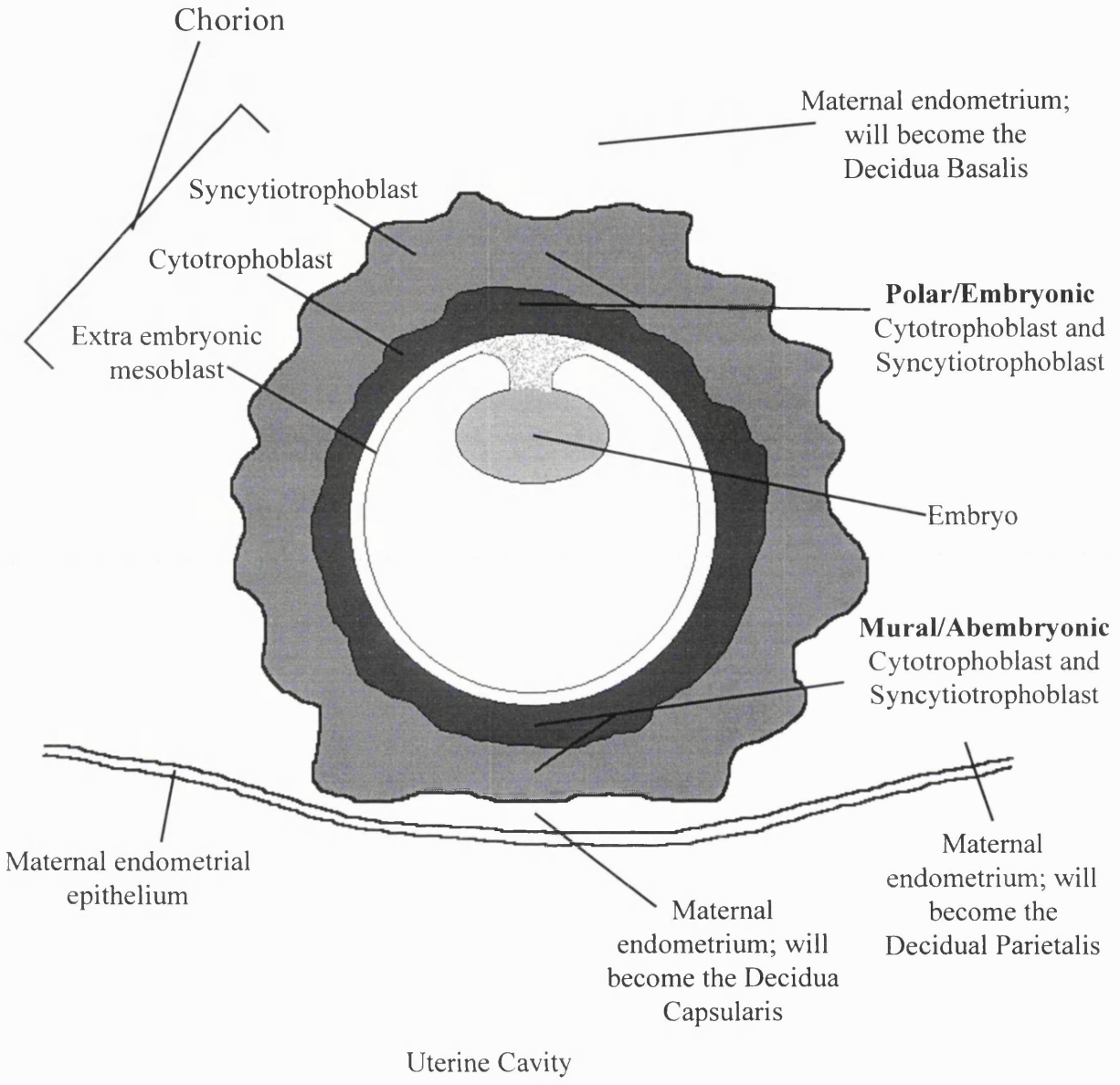
## 1.3. Implantation.

Four days after fertilisation, a zygote divides into 16-32 cells, and a cavity in this mass starts to develop. By this stage in a normal pregnancy the zygote, now termed a blastocyst, will have migrated down the fallopian tube and be approaching the uterine lumen. By day 5 post-conception, the once totipotent cells begin to become differentiated into the inner cell mass (ICM) surrounded by the trophoectoderm (Fig 1.2). The blastocyst, still encased by the zona pellucida, develops a polarity. Cells in the ICM facing the blastocystic cavity begin to form the ventral surface, which is lined ventrally by a basement membrane. By day 6 post-conception the blastocyst consists of approximately 100 cells, and implantation begins. During early implantation two main classes of trophoblast begin to evolve, primitive syncytiotrophoblast and primitive cytotrophoblast. The trophoblast cells adjacent to the ICM, termed the polar trophoblast, form the first contact with the epithelium of the maternal endometrium at the site of implantation. Cells on the opposite side of the embryo to the ICM are termed mural trophoblasts. During implantation the blastocyst hatches from the zona pellucida and thus consists of an outer wall of single layer uni-nucleate trophoblast cells, surrounding the blastocoel and ICM. During later stages of development, this layer remains unicellular and acts as a stem cell population. Their rapid division and subsequent fusion with the syncytium leads to the continual expansion of this mantle (O'Rahilly & Muller 1996).

Implantation is initiated by the intrusion of trophoblastic extensions which penetrate between apparently intact endometrial cells (Enders & Schlafke, 1969). This begins by the attachment of trophoblast and endometrium by their apical cell membranes (Denker, 1974). This is unusual as the apically situated cell membrane of an epithelial cell is not adhesive, in contrast to the basally and laterally located membrane. During implantation the uterine epithelium partly loses its epithelial character of apico-basal polarity, although it does not switch completely to a mesenchymal phenotype (Denker, 1974). This epithelial layer underlying the conceptus becomes eroded. Trophoectodermal processes seem to flow between adjacent epithelial cells, isolating and then dissolving and digesting them. Cytotrophoblast cells on the edge of blastocyst proliferation begin to fuse forming syncytiotrophoblast: a specialised mass of multinucleated cytoplasm with its free surface characterised by numerous microvilli. The polar syncytiotrophoblast causes erosion of maternal tissues, and the cytotrophoblast contributes to further penetration.

Once the epithelium is penetrated, typical mature junctional complexes are often observed to be shared between the uterine epithelium and the invading trophoblast (Enders et al., 1969). Implantation in humans is 'interstitial', with total submergence of the blastomere in the maternal stroma. The blastomere becomes engulfed by an enlarging mass of maternal decidual tissue and by day 7 post-conception is almost completely embedded in the uterine epithelium and endometrial stroma. By day 12 post-conception the conceptus is totally embedded within the uterine wall, with the maternal endometrial epithelium having re-grown over the implantation site. The syncytiotrophoblastic mass expands over the entire blastocystic surface, producing hundreds of branching finger like villi which radiate outwards in all directions (Heap, 1981). Syncytiotrophoblastic villi trees totally surround the conceptus but are thicker beneath the embryonic pole (Fig 1.3).

**Figure 1.2 Blastocyst after Implantation**




Adapted from O'Rahilly and Muller 1996

## 1.4. The uterus

The lining of the uterus can be divided into three layers from interior to exterior: the perimetrium (peritoneal covering), the myometrium (muscular coat) and the endometrium (mucosa). If pregnancy does not occur and there is no blastocyst implantation, the endometrium is shed at the end of the menstrual cycle. If implantation does occur the endometrium remains intact and undergoes cellular changes known as decidualisation. This is caused by the release of progesterone from the corpus luteum during implantation, and the altered endothelium becomes known as decidua. The endometrial surface consists of a simple columnar ciliated epithelium that is continuous. Its stroma is a highly cellular connective tissue with an amorphous extracellular matrix containing relatively few connective tissue fibres. During decidualisation the endometrial stromal cells enlarge, accumulate glycogen and become epithelioid in appearance on cross section (Tabanelli et al., 1992). As blastomere development ensues, the decidua becomes arranged at three topographical sites; the decidua basalis, situated at the deepest pole of the conceptus; the decidua parietalis lines the uterine cavity except at the site of implantation; the decidua capsularis is reflected over the rest of the chorionic sac (Fig 1.3).

## 1.5. The Placenta

During pregnancy, maternally derived mesenchyme first appears from days 7-12 post-conception. The first indication of this is the production of extra-embryonic mesoblast from a caudally situated area of proliferation. The combination of embryonic derived trophoblast and extra-embryonic mesoblast is know as the placenta. Although venerated by the early Egyptians, the placenta was first defined as the organ responsible for fetal nutrition by the Greek physician Diogenes of Apollonia (480 B.C.) (DeWitt., 1958). The term placenta derives from the Latin for flat 'cake' and was first used by Realdus Columbus (1516-1559). Aristotle (384-322 BC) first realised that the fetus is fully enclosed by membranes which he termed chorion. During early pregnancy trophoblast is the most abundant tissue of fetal origin. Trophoblast development depends on imprinted genes that are expressed from only the paternally derived allele (Mochizuki et al., 1996; Franklin et al., 1996). The initial day 5 single layer of cytotrophoblast differentiate into syncytiotrophoblast, villous trophoblast or extravillous (intermediate) trophoblast.

### 1.5.1. Chorionic villi

From day 6 post-conception, groups of cytotrophoblast cells begin to aggregate just below the layer of syncytiotrophoblast. These clumps begin to project into the syncytiotrophoblast and are termed primary villi. These villi begin to acquire cores of mesoblasts (mesoblastic crests) and are termed secondary villi. Approximately 2 weeks post-conception the mesenchymal core then becomes vascularised resulting in tertiary (or chorionic) villi, consisting of the vascularised mesenchymal core covered by cytotrophoblast and syncytiotrophoblast. The first chorionic villi are not free processes but villous stems, and are covered by two layers of trophoblast; the inner layer of cytotrophoblast, the so called 'Langhans' cells', and an outer layer of syncytiotrophoblast. Although both layers persist to maturity, the inner layer becomes discontinuous with advancing gestation. New production of syncytium from the Langhans layer continues throughout pregnancy, although it slows down in later gestation. Villi branch extensively and are vascularised, but contain no blood vessels in their mesoblastic core before 19 days post-conception when the first fetal capillaries are observed (a complete fetoplacental circulation is established around the beginning of the fifth week of gestation). They entirely surround the implanted blastomere, stemming from both the polar and mural regions, and begin to form villous trees.

The development of a villous tree starts by the formation of side branches on the trabeculae. The earliest of these are composed of syncytiotrophoblast alone and so are termed syncytial sprouts. The syncytium represents one large, continuous sheet of cytoplasm in which the nuclei freely float. Frequently the nuclei bunch up irregularly, producing 'knots' or 'buds'. In early gestation these are seen arising in an apparently random pattern from the surfaces of mesenchymal and immature intermediate villi, and at all stages of gestation a proportion of these sprouts break away (Castellucci et al., 1989). Mesenchymal invasion into the proximal end of the non-shed true sprout is soon followed by the formation of capillaries, so rendering conversion into a tertiary villus complete. Up until the fifth week post-conception, mesenchymal villi formed in this way progress to become primitive stem villi. However, after this point some mesenchymal villi differentiate into immature intermediate villi (Castellucci et al., 1989) The latter continue to produce many new syncytial sprouts until they themselves are transformed into stem villi. Hence, growth of the more structural elements of the villous tree is very rapid in the first trimester. Full villous trees consist of stem villi, mature intermediate villi, terminal

villi, immature intermediate villi and mesenchymal villi (Fig 1.4). Up until the eighth week of gestation these trees cover the entire chorion (Fig 1.3).

Early in the embryonic period, some chorionic villi develop dense masses of trophoblastic cells at their tips. These 'cytotrophoblastic cell columns' make contact with the eroded endometrium and spread out as part of the lining of the intervillous space. In the basal region they form the cytotrophoblastic shell or early basal plate. Later the villous cores extend through the cell columns to become anchored to the decidua (anchoring villi). Anchoring villi are in direct contact with the decidua. Most villi, however, retain free tips in the intervillous space; floating villi.

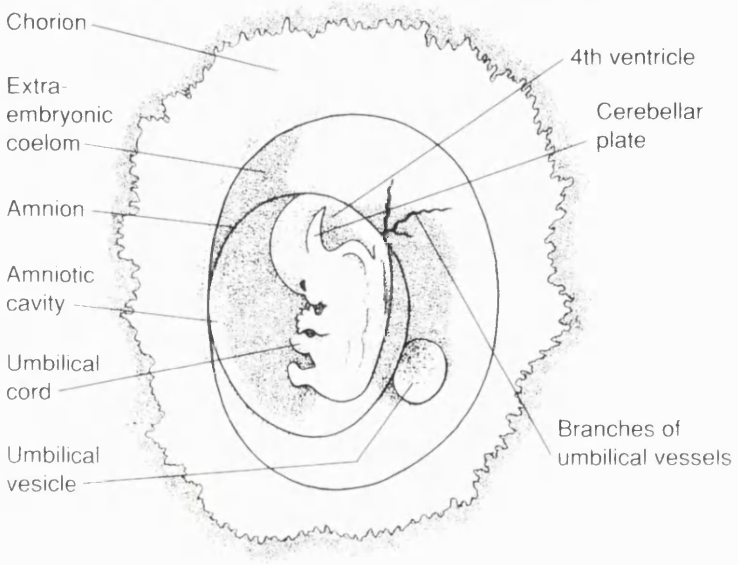
### 1.5.2. Cell columns and cell islands

Cell columns are the trophoblast connections of larger anchoring villi to the basal plate. These are segments of the villous trees that persist in the primary villus stage, because mesenchymal invasion during formation of secondary villi does not reach the most basal segments of the anchoring villi. Because of continuous cytotrophoblast proliferation at the stromal-trophoblastic interface the cell columns serve as segments of longitudinal growth of the anchoring villi. From their distal ends, cytotrophoblasts may invade the basal plate, thus contributing to the growth of the latter. Because of this, cell columns serve as one of the richest sources for the so-called extravillous cytotrophoblast. Fibrinoid deposition at the surface of the cell columns slowly buries them into the basal plate. As soon as they are completely incorporated into the plate, the cytotrophoblastic proliferation slows down. After partial degeneration of the cells and complete disintegration of their structure, cell columns largely disappear in the course of the last trimester and can only rarely be observed in term placenta.

Cell islands are largely comparable structures. They too are formed from villous tips that have not been opened up by connective tissue during the transition from primary to tertiary villi. The only difference is that these villous tips are not connected to the basal plate, as are anchoring villi. Also , the cytotrophoblast of the cell islands proliferates and later becomes largely transformed into fibrinoid, which surround clusters and strings of surviving extravillous cytotrophoblast. Sometimes central degeneration and liquefaction causes the development of fluid-filled cysts inside the cell islands. Cell biology studies concerning the proliferative behaviour of villous cytotrophoblast, the expression of growth factor receptors and of oncogene protein products, and the interactions with extracellular matrix have not revealed any differences between cell islands and cell

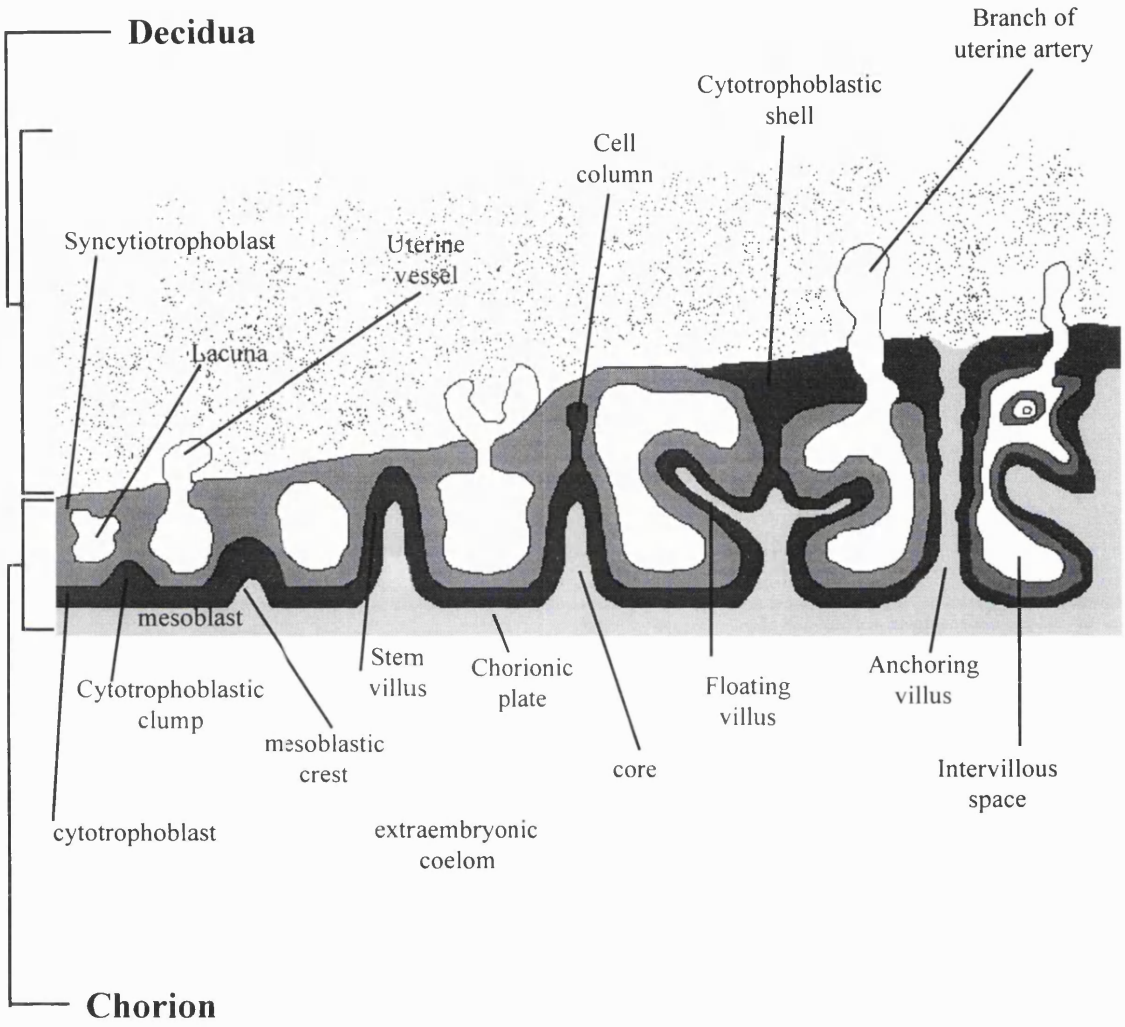
columns (Castellucci et al., 1989; 1991a; 1991b).

**Figure 1.3 Human embryo at 7 weeks of gestation**




From O'Rahilly R, & Muller F., 1996

**Figure 1.4** **The development of chorionic villi**




Simplified scheme showing the development of chorionic villi to form the mature placenta. Cytotrophoblast gives rise to syncytiotrophoblast, lacunae appear, the chorion is formed and chorionic villi develop.

From O'Rahilly R, & Muller F., 1996.

### 1.5.3. Intervillous space

The anchoring villi are attached to the decidua basalis by solid cytotrophoblastic cell columns. The cytotrophoblast cell columns function primarily as growth zones for the cytotrophoblast cells that proliferate and subsequently migrate in either a chorionic direction (anchoring villi) or a basal direction (basal plate), where they differentiate into specialised trophoblast cell types constituting the extravillous cytotrophoblasts, either 'endovascular type' or 'intermediate type' also called the 'X-cells'. The intermediate type constitute single cells found to be loosely intermingling with maternal decidua cells. Extensive modulation of the placental 'floor' and of the lateral edges of the placenta take place, the villi develop into complex structures, and gradually the final shape of the placenta is formed. The extravillous intermediate cell type also exist in the 'cell islands' of the subchorionic sheet of trophoblast, and in the 'giant cells' (multinucleated cells) of the basal plate.

The second type of extravillous cytotrophoblast, the endovascular type, start invading the maternal vasculature on about day 9 or 10 post conception. Their main function is believed to be the remodelling of uteroplacental arteries in order to meet the need for increased blood supply to the feto-placental unit during the second and third trimesters of pregnancy.

### 1.5.4. Uteroplacental circulation

The uteroplacental arteries are branches or direct continuation of myometrial arteries. As soon as they enter the decidua, where they run parallel to the basal plate, these end-arteries are called 'spiral arteries' because of their spiral course. Each radial artery terminates as two or three spiral arteries.

Spiral arteries undergo marked physiological changes during normal pregnancy. Single cytotrophoblasts (endovascular type) migrate against the pressure gradient, deep into the maternal spiral arteries where they replace the endothelium of the vessel, and subsequently into the tunica media where they replace in part the contractile smooth muscular layer (Hamilton and Boyd 1966). It is thought that this vascular phenomenon occurs in two stages, the conversion of the decidual segments of the spiral arteries by a wave of endovascular trophoblast migration in the first trimester and the myometrial segments by a subsequent wave in the second trimester (Pijnenborg et al., 1983). The physiological changes are achieved in the upper decidua by the action of endovascular

and perivascular trophoblasts, whereas in the deeper decidua endovascular trophoblast cells are principally involved (Pijenenborg; et al., 1980). During the first trimester the lumen of the spiral arteries are sometimes completely blocked by plugs of endovascular cytotrophoblast (Brosens, 1977; Jaffe et al., 1997).

In sections of first trimester placenta, one customarily finds very active growth of both cytotrophoblast and syncytium; into the intervillous space, with only tenuous connections to the stromal base of the villi. As pregnancy progresses, this pattern of outward growth (sprouting) diminishes, until at term the syncytiotrophoblast appears to be stretched rather thinly over the surface of the villi.

During implantation endometrial stromal cells enlarge and become decidual cells. As the syncytium advances into the decidua it engulfs endometrial capillaries and produces disintegration of the vessel walls. These are believed to be the future lacunae which coalesce to form the intervillous space. These lacunae are usually said to contain blood, but it has been shown that during early embryogenesis the intervillous space lacks blood flow so these actually contain an acellular fluid, mainly plasma (O'Rahilly & Muller, 1996). Penetration of the maternal blood vessels is accompanied by proliferation of endovascular cytotrophoblast within the arterioles. The trophoblast is separated from the underlying stroma by a basement membrane. The stroma of the villi contains collagenous fibrils, reticular cells, fibroblasts, and macrophages.

### 1.5.5. Chorionic plate

At day 14 post-conception, the primary chorionic plate consists of three layers: syncytiotrophoblast, cytotrophoblast, and extraembryonic mesenchyme. They separate the intervillous space from the blastocystic cavity. Trophoblastic proliferation with subsequent degeneration and fibrinoid transformation (Langhans' fibrinoid) causes continuous growth of the primary chorionic plate. Around the fourth and fifth week of pregnancy, allantoic blood vessels reach the primary chorionic plate through the connecting stalk and start protruding into the stem villi, which branch off from the chorionic plate. As soon as the expanding amnionic sac comes into close contact with the mesenchymal surface of the chorionic plate (eighth to tenth week post conception), the definitive chorionic plate is formed. It is composed of the following layers: Langhans' fibrinoid lining the intervillous space and largely replacing the original syncytiotrophoblast; one or several layers of cytotrophoblast; chorionic mesenchyme; a

spongy layer with many clefts, which indicate the border between chorion and amnion; amnionic mesoderm; and amnionic epithelium, which lines the amnionic cavity (Benirschke K, and Kaufmann P, 1990).

### 1.5.6. Trophoblasts at the decidual basalis

#### 1.5.6.1. Syncytial knots

These are characterised by a multi-layered group of aggregated nuclei that bulge only slightly on the trophoblastic surface. When seen in histology sections a proportion are likely to be only representations of tangential sections; (Cantle et al., 1987) Their numbers increase with advancing gestation. A few are characterised by densely packed nuclei that show a more condensed chromatin and sometimes severe pyknosis.

#### 1.5.6.2. Syncytial sprouts

The large aggregates of syncitial nuclei protruding into the intervillous space in a mushroom- like fashion are usually called syncytial sprouts (Boyd and Hamilton 1970). This name is inappropriate as it implies that these structures are correlates of the villous sprouting which occurs in earl pregnancy, where syncytial sprouts project into the intervillous space, and their villous attachment is often being restricted in a stalk-like manner. These sprouts usually possess a central concentration of clumped syncytial nuclei, and appear to be identical in structure and nature. Most of these projections represent no more than the initial phase in the development of a new terminal villus as a vascularised mesenchymal core, also containing a layer of cytotrophoblast cells, is later formed.

The second variety of syncytial sprouts initially have the same structure as villous sprouts and seem to arise in the same fashion. Their fate, however, is quite different, for unlike incipient villi, the sprouts of this second type acquire neither cytotrophoblast nor stromal components. The stalk of attachment joining them to the syncytium becomes increasingly attenuated, and may eventually rupture, so that the sprout itself becomes a small spherical or ovoid mass of syncytium, varying in size, and floating freely in the intervillous space. Such free syncytial sprouts can be found regularly in endometrial and myometrial veins at all subsequent gestation stages. Histologically isolated masses of syncytium are regularly found, lying freely in the early intervillous

space, after the 13th or 14th day of development (Boyd and Hamilton, 1970). In addition these may also be groups of cytotrophoblasts derived from the cytotrophoblastic shell rather than syncitial sprouts. Sprouts, whether free in the intervillous space or attached to the villi often show signs of degeneration, especially pyknosis. Often, however, they are well preserved and, in electron micrographs, show extremely well developed brush borders (Hamilton and Boyd, 1966). The possible reasons for their formation include local hyperplasia of the syncytium, trophoblastic degeneration, structural expression of ischamenia or hypoxia, hypertension or fetal malperfusion of placental villi.

### 1.5.7. Trophoblasts at the decidual capsularis

During implantation and embryonic development villi develop over the entire surface of the chorionic sac although they are more profuse over the embryonic pole. The villi over the abembryonic pole interact with the endometrium that has overgrown the implanting blastocyst termed the decidua capsularis. As the conceptus enlarges, the decidua capsularis becomes progressively stretched and the placental villi in this region are compressed. By the end of week 8 the implantation site bulges into the cavity of the uterus, but the decidua capsularis has yet to fuse with the opposite wall of the uterus, the decidua parietals. As a result the villi over the abembryonic pole may receive a relatively poor supply of nutrients, which could account for the fact that beginning around the ninth week post-conception they begin to degenerate. This is accompanied by a disappearance of the associated intervillous space, and by a fusion of the primary chorionic plate with the trophoblast shell. The chorionic sac becomes secondarily smooth in this region and is referred to as the chorion laeve. The first patches of the smooth chorion appear opposite to the implantation pole at the so-called abembryonic or anti-implantation pole. From there, they spread over about 70% of the surface of the chorionic sac by the fourth month of gestation. By the second trimester of pregnancy, almost all the chorion surface in contact with the decidua capsularis is smooth and villi only remain at the embryonic pole (chorion frondosum), where they constitute the definitive discoidal placenta. The decidua capsularis eventually comes into permanent contact with the decidual parietalis and as pregnancy progresses undergoes marked atrophy and later disappears, so that the decidua parietalis comes into contact with the chorion laeve. Between the $15^{th}$ and $20^{th}$ week post conception, both decidual layers fuse with each other thus obliterating the uterine cavity. From this point onward, the smooth chorion has contact over nearly its entire surface with the decidual surface of the uterine wall and may function as a paraplacental exchange

organ. Because of the deficiency of a fetal vascularisation of both the smooth chorion and the amnion, all paraplacental exchange between fetal membranes and fetus has to pass the amniotic fluid. The mean thickness of the fetal membranes at term, after separation from the uterine wall, is about 200-300μm. The membranes show some similarity with the structure of the chorionic plate (Bourne, 1962). The innermost layer, the amnionic epithelium, encloses the amnionic fluid. The epithelium itself rests on a thin layer of amnionic mesoderm. Near the placental margin, persisting ghost villi, embedded in fibrinoid, split the cytotrophoblast into two layers. These continue into the placenta and become confluent with the chorionic and basal plates, receptively. Attached to the outer surface of the cytotrophoblast is a decidual layer. The latter indicates that separation of the membranes, as in the placenta, does not take place along the materno-fetal interface but, instead, it cleaves somewhat deeper.

## Part 3. Fetal Cells in the Maternal Peripheral Blood Circulation

During pregnancy, mother and fetus are obviously in physical contact and the exchange of chemicals and nutrients continually occurs. However, the potential passage of cells between the two is a phenomenon that is still largely unresolved. By the time intervillous and fetal circulation is established in the human placenta, fetal and maternal blood are in close proximity to each other with the two blood streams separated by the placental barrier, also called the 'placental membrane'. This membrane is composed of trophoblast, basement membranes, possibly villous stroma, fetal capillary walls and fetal capillary endothelium. The thickness of the placental membrane gradually decreases with advancing gestation and as the villous tree enlarges; the terminal villi become smaller while the relative contribution of the fetal capillaries increases (Jackson et al., 1992b). As the membrane thins fetal blood flow and blood pressure increases (Sutton et al., 1990). Where the fetal capillaries and sinusoids bulge beneath the villous surface, the cytotrophoblast is either extremely thin or absent (Hamilton and Boyd, 1966). However at no time during normal pregnancy is there any physical breakdown of this barrier preventing large scale cellular exchange.

Initial physiological interest in feto-maternal cellular exchange lay in the possible migration of maternal cells into the fetal circulation. If maternal lymphocyte cells were to reach the fetal circulation in large numbers, they could induce a graft-versus host reaction and eventually abortion. Although isolated cases have been reported (O'Reilly et al., 1973), this is clearly not usually the case. This suggests that on the whole maternal cells, which would be foreign to the fetus, did not pass through the placenta. This observation was supported by studying the pregnancies of mothers with leukaemia (Gramblett at al., 1958; Bernard et al., 1964). Any self replicating metastatic white blood cell that entered the fetal blood stream would proliferate unchecked and cause the fetus to suffer from the same condition. This was shown not to be the case as mothers suffering from leukaemia were shown to have normal offspring in almost all cases. Loewenstein et al., (1971) demonstrated that in the mouse less than 100 leukaemia cells of the strain used in the investigation transferred to the circulation of a developing embryo would be sufficient to induce neonate leukaemia. When pregnant female mice were inoculated with leukaemia cells of the same strain in no case was this conferred to the developing fetus.

If there is passage of maternal cells into the fetal circulation, and these cells undergo mitosis, there is the potential for fetal micro-chimaerism; the existence of two

blood cell lines within the fetus. In a study by Schroder et al., (1974) 27000 lymphocyte mitoses from 10 male infants were examined, and one child was found to have maternal 46XX cells at a frequency of 0.7%. However, large scale lymphocyte karyotyping and genetic investigations over the last 20 years have failed to show micro-chimaerism to be a common occurrence. This would also suggest that if maternal stem cells do reach the fetus through the placenta, it is a very rare event and not a part of normal gestation.

It has recently been proposed that the persistence of fetal stem cells in some mothers may cause long term microchimerism and have pathological effects. It has been shown that a large proportion of women with systemic sclerosis, a disease of as yet unknown origin which often occurs in women after their childbearing years, have fetal cells persisting in their circulation or tissues (Artlett et al., 1998). This study demonstrated the presence of Y-chromosome specific sequences using PCR in the peripheral blood of 32 out of 69 affected women (46%) compared to 4% in controls. Nucleated cells containing Y chromosomes were detected in skin-biopsy specimens by FISH in 11 of 19 women with systemic sclerosis (58%), with 9 of the 11 known to have carried male fetuses. The disease has many clinical and histopathologcal similarities to chronic graft-versus host disease with instances having been seen to occur from bone marrow transplants (Bell et al., 1996) and is three to eight times more common in women than men (Steen and Medsger, 1990). It is proposed that in these rare cases fetal T-cells circulate in the mother with the host immunologically unaware of their presence. These cells are not therefore immunologically eliminated as would have been expected and in time they may mediate a graft-versus-host reaction, resulting in autoimmune disease.

Before prenatal diagnosis was proposed utilising fetal cells that had migrated into the peripheral maternal blood circulation, there was physiological interest as to whether such cell migration was indeed a natural occurrence. It had been observed that in some cases mothers developed antibodies against fetal histocompatability antigens; i.e. the formation of anti-paternal cytotoxic antibodies (Ferrone et al., 1976). Of particular interest was rhesus immunisation in Rh(D) negative mothers which caused pregnancy complications due to the rejection of Rh(D) fetuses by the action of the maternal immune system (Zipursky and Israels, 1967). This demonstrated that fetal blood had indeed come into contact with the maternal immune system to illicit the response. However, Rh(D) sensitisation is not as common as one would expect if blood exchange were a normal occurrence, and is rare in primigravida. When Rh(D)

sensitisation does occur, it is most often late in pregnancy (after 24 weeks) showing that fetal red blood cells may not routinely enter the maternal circulation in the first two trimesters of pregnancy (Bowman & Pollock, 1987; Mollison et al., 1987). Materno-fetal blood exchange may only occur during the mechanical rigours of childbirth. At the time of delivery FISH studies have shown that maternal cells constitute between 0.04-1% of cord blood from male gestations (Hall et al., 1995), whereas PCR based assays reveal maternal cells in 42% of fetal cord blood samples and fetal cells in 51% of maternal blood samples (Lo et al., 1996).

### 1.6. Studies on whole blood.

Following the introduction of the PCR method for the amplification and identification of specific DNA sequences, studies on maternal peripheral blood using PCR for the amplification of Y-chromosome DNA sequences have demonstrated the presence of nucleated male cells in the blood of women carrying male fetuses. These techniques are unable to identify the type of fetal cell detected, merely indicate their presence. Estimates of the number of fetal cells present in maternal blood samples vary. Based on DNA studies by PCR amplification or Southern blot hybridisation, the ratio of fetal to maternal nucleated cells was estimated to be more than 1 in $10^6$ at 8-12 weeks of gestation (Kao et al., 1992), less than 1 in $2.5x\ 10^4$ at 14-16 weeks of gestation (Nakagome et al., 1991), less than 1 in $10^5$ at 16 weeks of gestation (Schwinger et al., 1989), 1 in $5x10^5$ at 16 weeks of gestation (Bianchi et al., 1990), 1 in $10^7$ to 1 in $10^8$ in the first and second trimesters (Price et al., 1991), less than 1 in $7x10^4$ at 24-36 (Adinolfi et al., 1989) and less than 1 in $5x10^3$ throughout the entire gestational period of 8-40 weeks (Ganshirt-Ahlert et al., 1990). Using an avidin-biotin based immunoaffinity system, Hall and Williams (1992) estimated the frequency of fetal cells to be $1:4.75x10^6$ to $1.6x10^7$ of the nucleated cell fraction of maternal blood.

Lo et al., (1989) collected blood from 19 pregnant women, of whom 12 were found to have male fetuses. Using a PCR specific for the Y chromosome, the sex of the fetus was predicted in all cases, with no false positives or negatives. It was claimed that this technique could detect a male cell in a female population at a dilution of $1:10^5$. However, it was later shown by Nakagome et al., (1991) that the particular Y chromosome sequence employed has autosomal homologies and in their next study Lo et al., (1990) experienced two false positives and four false negatives. In a more recent publication Lo and co-workers developed a nested PCR assay able to detect the

equivalent of a single male cell in $3x10^5$ female cells (Lo et al., 1993a). Using this procedure sex prediction was attempted for the fetuses of 75 women through all three trimesters of pregnancy. This was successfully achieved in 86%, 67% and 87% of women in the first, second and third trimesters respectively. Thomas et al., (1994) used Y-specific PCR following the pregnancies of 5 women who became pregnant by *in vitro* fertilisation. In the 2 women found to be carrying male fetuses, Y-specific signals were detected at 33 and 40 days of gestation, and continued to be detectable to term, ceasing to be seen from 8 weeks post-partum. However, Liou et al., (1993; 1994) used Y-PCR to detect fetal cells from male pregnancies in blood samples collected from 19 women with male fetuses, and could not detect Y sequences until 6-12 weeks' gestation. Hamanda et al., (1993) again used Y-PCR but could not consistently detect a Y-specific product in women with male fetuses prior to 15 weeks' gestation, although the proportion of fetal to maternal cells increased with progressing gestation; $0.27x10^{-5}$ fetal to maternal cells in the first trimester; $3.52x10^{-5}$ in the second trimester; and $8.56x10^{-5}$ in the third trimester.

The question of how much fetal blood would need to be present in the maternal circulation for it to be detected was addressed by Fewings and Adinolfi (Published in Adinolfi 1991). They injected male donor blood into non-pregnant female volunteers then, having allowed the blood to disperse in the whole circulation, took a sample of volunteer blood and using Y-PCR attempted to detect Y DNA. The PCR technique could identify Y-specific DNA in a female DNA background at a dilution of 1 to $7x10^4$. It was concluded that 0.2ml of male blood was required to detect the Y chromosome. Bowman and Pollock (1987) and Mollison et al., (1987) estimated that there may be up to 0.2ml of fetal blood in the maternal circulation in most cases, and in 0.21% of pregnancies between 28-30 weeks' gestation, there may be up to 1ml. However, Hamanda et al, (1993) using an approximate quantitative Y-PCR method, estimated that only 0.2µl fetal blood is present in a 20ml maternal sample which extrapolates to only 50µl fetal blood in the total maternal circulation.

In 1990, Bianchi et al., used quantitative PCR employing radioactive nucleotides, to amplify a region on the Y chromosome. The number of fetal cells present in each sample was estimated by comparing the intensity of the radio-labelled band with that obtained using known aliquots of male cells. Experiments were carried out on 20ml of blood collected from women with male fetuses, and it was estimated that in this volume there were on average 16 Y-bearing cells. The same tests performed on 20ml of blood collected from women with a female fetus indicated there were 1.45

Y containing cells- thought to be either false positive results or residual cells from previous male pregnancies. From these results it was estimated that each 20ml sample contained 0.04% fetal blood, i.e. 10μl. In a later paper blood samples were collected from women with normal euploid pregnancies (n=199) mostly prior to any invasive procedure (190/9), and from women carrying male aneuploid pregnancies, mostly after an invasive test (27/31) (Bianchi et al., 1997). Although no conclusive proof can be derived from these data due to the almost exclusive collection of one group of samples subsequent to an invasive procedure, the mean of detectable cells appeared to increase when the fetus was aneuploid. The mean number of male cells detected from 16ml of blood collected from women with normal male pregnancies was 19; range 0-91 (compared with 2 from normal female gestations [range 0-24]). The mean number of male cells detected from 16ml of blood collected from women with 47 XY+21 pregnancies was 110 [range 0-650].

Diagnosis of fetal disorders has also been achieved using unsorted, whole blood. Camaschella et al., (1990) obtained maternal blood from 3 pregnant women at risk for fetal beta-thalassaemia/haemoglobin $Lepore_{Boston}$. Haemoglobin $Lepore_{Boston}$ is a haemoglobinopathy caused by a 7-kilobase deletion in the beta-globin cluster. Using PCR to amplify haemoglobin $Lepore_{Boston}$ fragments from the maternal blood of normal women whose partners carried the deleted gene, the condition was correctly identified in two fetuses, and confirmed absent in the third. Lo et al., (1993b) used whole blood in an attempt to diagnose the Rh(D) status of fetuses carried by Rh(D) negative mothers. Without any enrichment for fetal cells, the blood from 21 Rh(D) negative pregnant women was collected and subjected to PCR for Rh(D) sequences. Marginal success was achieved; however there were 4 false positives and 2 false negatives. Greifman-Holtzman et al., (1994) also looked to identify the Rh(D) status of an embryo by examining the peripheral blood of pregnant women. Blood was collected from 9 Rh(D) negative women at 10-22 weeks gestation, prior to amniocentesis or CVS. Rh(D) positive cells were identified in 7 of the 9 samples, all of which were found to come from women with Rh(D) negative fetuses. The remaining two women gave birth to Rh(D) negative infants (Rh(D) negative twins in one case) confirming this diagnosis.

All the aforementioned studies were performed on whole blood from the maternal peripheral circulation. For any realistic future diagnosis, fetal cells would have to be identified or isolated. Isolation or enrichment techniques would have to be directed toward a specific cell type. The human placenta has a villous haemochorial

structure. Unlike other forms of placenta, the haemochorial placenta is characterised by the absence of maternal endothelial, stromal and epithelial layers, resulting in direct contact of the maternal blood with the fetal syncytiotrophoblasts. Exposure of syncytiotrophoblast to the maternal blood flow may result in trophoblast cells being shed into the maternal circulation. In addition, the reduced barrier between the fetal and maternal circulations may allow passage of fetal leucocytes and erythrocytes into the maternal blood stream (Adinolfi 1992b; 1992c). If any of these nucleated cells could be isolated, non-invasive prenatal diagnosis of fetal genetic disorders would be possible.

## 1.7. Trophoblasts at the site of the decidua basalis

The first report of trophoblast deportation came in 1893 from a German pathologist named Schmorl. He discovered large multinucleate trophoblasts trapped in lung capillaries from autopsy sections of 14/17 (82%) women dying from eclampsia whereas none were found in similar sections from 4 women dying from other causes (Schmorl 1893). This provided indirect evidence of trophoblast cell deportation in human pregnancy. Since trophoblastic lung embolism at first seemed to be confined to cases of eclampsia he suggested that the deportation of trophoblast cells may be the cause of this condition. Soon after his first report however, Schmorl recognised it to be a phenomenon not confined to eclampsia.

Bardawil and Toy (1959) and Attwood and Park (1961) reported more detailed observations from a larger study. Attwood and Park (1961) studied lung sections from 220 women dying in pregnancy, during delivery or in the pueperium, and found evidence of syncytiotrophoblast in the lung sections from 96 (44%) of these. They confirmed that trophoblast cell deportation is increased in pre-eclampsia; trophoblasts were detected in 81% of women dying of pre-eclampsia or eclampsia compared with 48% in the control group. Amniotic fluid embolism and deportation of entire chorionic villi was diagnosed in only 2 cases, which is the same number that was found amongst the 109 cases studied by Bardwil and Toy (Bardawil and Toy 1959). The number of syncytio trophoblast found in lung tissue appeared to be related to uterine activity as it increased with later gestation and the onset of labour, with the highest levels seen in women who died either during labour or within the first 24 hours afterwards. No syncytio-trophoblast was found later than 15 days post-partum, suggesting that the cells are quickly destroyed. Indeed, shrinkage of the trophoblast emboli, with increased pyknosis of nuclei were observed and

was related to the number of days post-partum. Placental infarction was suggested as a possible cause of trophoblast cell deportation.

The findings of Bardawil and Toy supported the contention of Schmorl that trophoblastic embolism appears to be a regular event, especially in eclampsia. In terms of recorded pathological diagnoses, pulmonary embolic syncytium occurred frequently in eclampsia (13/16), pre-eclampsia (2/3), and toxaemia (3/3). It was also common in miscarried pregnancies (5/6), and in cases of massive haemorrhage (2/3).

The low incidence of lung embolism by entire chorionic villi observed in the two studies suggests that whole placental villi are only deported when there has been what Attwood and Park called 'placental commotion'- for example, it has been found in association with amniotic embolism (Attwood and Park 1961). The detection of chorionic villi in the peripheral blood of healthy pregnant women was however reported (Luz et al., 1966). Boyd and Hamilton also claimed to have occasionally observed villi in the uterine veins remote from the intervillous space, although they thought it was an artefact (Boyd and Hamilton 1970). However, in comparison with the transport of syncytial sprouts, villous deportation or deportation of decidual tissue fragments appears to be rare.

To determine whether trophoblast can pass through the lungs and enter the peripheral circulation, Douglas et al., (1959) carried out a study which provided for the first time *in vivo* confirmation of trophoblast in the maternal circulation. Blood samples were obtained from pregnant women at various stages of gestation from a placental side broad ligament vein at caesarean section (C/S), the inferior vena cava (by placing a catheter through the femoral vein), and the cubital vein (Douglas et al., 1959; Thomas et al., 1959). Typical syncytial fragments were demonstrated in 8/13 (62%) broad ligament vein samples from as early as 18 weeks gestation with higher numbers at 18-22 weeks of pregnancy than at term, but only in 3/33 (9%) of the samples obtained from the inferior vena cava, of which one was from a patient suffering from pre-eclampsia. In the sample obtained at 18 weeks of gestation syncytiotrophoblast was detected at a frequency of approximately 1 per ml of blood. However, no trophoblast cells were seen in any of the 80 peripheral blood samples collected.

Olivelli and Palo did not find syncytial sprouts in the blood from the peripheral circulation in 60 pregnant women, whereas 4/6 samples drawn from the uterine vein, contained syncytial sprouts (Olivelli and de Palo, 1964). In a similar study, an inflow rate of approximately 100000-150000 syncytial fragments per day was suggested, based on the detection of 3-10 syncytial fragments per ml of blood (Ikle, 1964). He also reported a

case where blood was obtained from a broad ligament vein at only 8 weeks' gestation. The specimen was drawn prior to manipulation and contained numerous trophoblastic cells.

Jaameri et al., (1965) were the first to specifically search for trophoblast cells in the circulation of pre-eclamptic patients. They prepared buffy coat slides from blood drawn at the time of C/S from both the right and left uterine vein and scrutinised these for trophoblast in 10 women with pre-eclampsia and 10 normal controls sectioned for other reasons. Trophoblast cells were detected in 9/10 women with pre-eclampsia and in 7/10 controls. An average of 3.02 cells/ml of blood were detected in women with pre-eclampsia and 0.15 cells/ml in the controls; the difference being statistically significant. Placental lesions did not appear to have any influences on the occurrence of syncytial fragments, neither was there any correlation between the time of delivery and the amount of detected trophoblast.

In a similar study Chua et al., (1991) used a monoclonal antibody staining technique (anti-cytokeratin) to identify trophoblast cells on cell-smears. Uterine vein blood from ten women with proteinuric pre-eclampsia requiring C/S and ten women requiring C/S for other reasons were examined. The number of trophoblast cells detected per ml of blood was found to be increased in the pre-eclampsia group. Cells were detected in 10/10 pre-eclamptic pregnancies (an average of 10.9 cells/ml) compared with the normal control group where trophoblast was only detected in 2/10 (0.1 cells/ml). Trophoblast cells (mononuclear cells only) were detected in the peripheral blood of only 1/5 women with pre-eclampsia, confirming that most trophoblast cells, especially the larger syncytial fragments, are trapped in the lungs. There was no correlation with the severity of the disease (degree of hypertension or proteinuria), but an acute maternal or fetal event leading to emergency delivery was associated with increased trophoblast deportation.

Conflicting reports exist as to whether manipulation of the uterus affects the release of trophoblast. During the procedure of tubal ligation and pregnancy termination at 20 weeks of pregnancy, Ikle catheterised the uterine vein and collected blood samples, during and after the procedure (Ikle, 1961). Before uterine manipulation a trophoblast concentration of 2 per ml of uterine vein blood was detected. During uterine palpation or operative procedure only 3 syncytial fragments per 5ml of blood were detected, and at the end of the operation no cells were detected, suggesting that surgical manipulation of the uterus did not cause increased efflux of trophoblast cells from the placenta. However, it was later convincingly demonstrated that trophoblast cell desquamation may be an

episodic event dependent on some degree of uterine manipulation (Wagner, 1968). Wagner catheterised the inferior vena cava and sampled before, during and after surgical procedures, and during simple palpation of the uterus in (a) 16 women during normal ongoing pregnancy (2-4 months gestation), b) 14 women at the time of curettage for incomplete abortion, c) 2 cases of missed abortion, d) 2 cases of hydatiform mole, and e) three cases of tubal pregnancy. Syncytiotrophoblast were detected in 12/14 (75%) cases of normal pregnancy, but only during palpation of the uterus. Cells were detected in only 2/14 (14%) cases of incomplete abortion, despite the fact that copious amounts of placental tissue was obtained during curettage. In both cases of missed abortion cells were detected during curettage. In none of the three cases of tubal pregnancy were any syncytial fragments detected. Interestingly, in only one of all the cases studied (an hydatiform mole) were syncytiotrophoblast detected prior to intervention. In the second case of hydatiform mole no cells were detected prior to intervention, but cellular transport increased considerably during uterine curettage. It was concluded that the numbers of trophoblast cells released relates to the size of placental attachment.

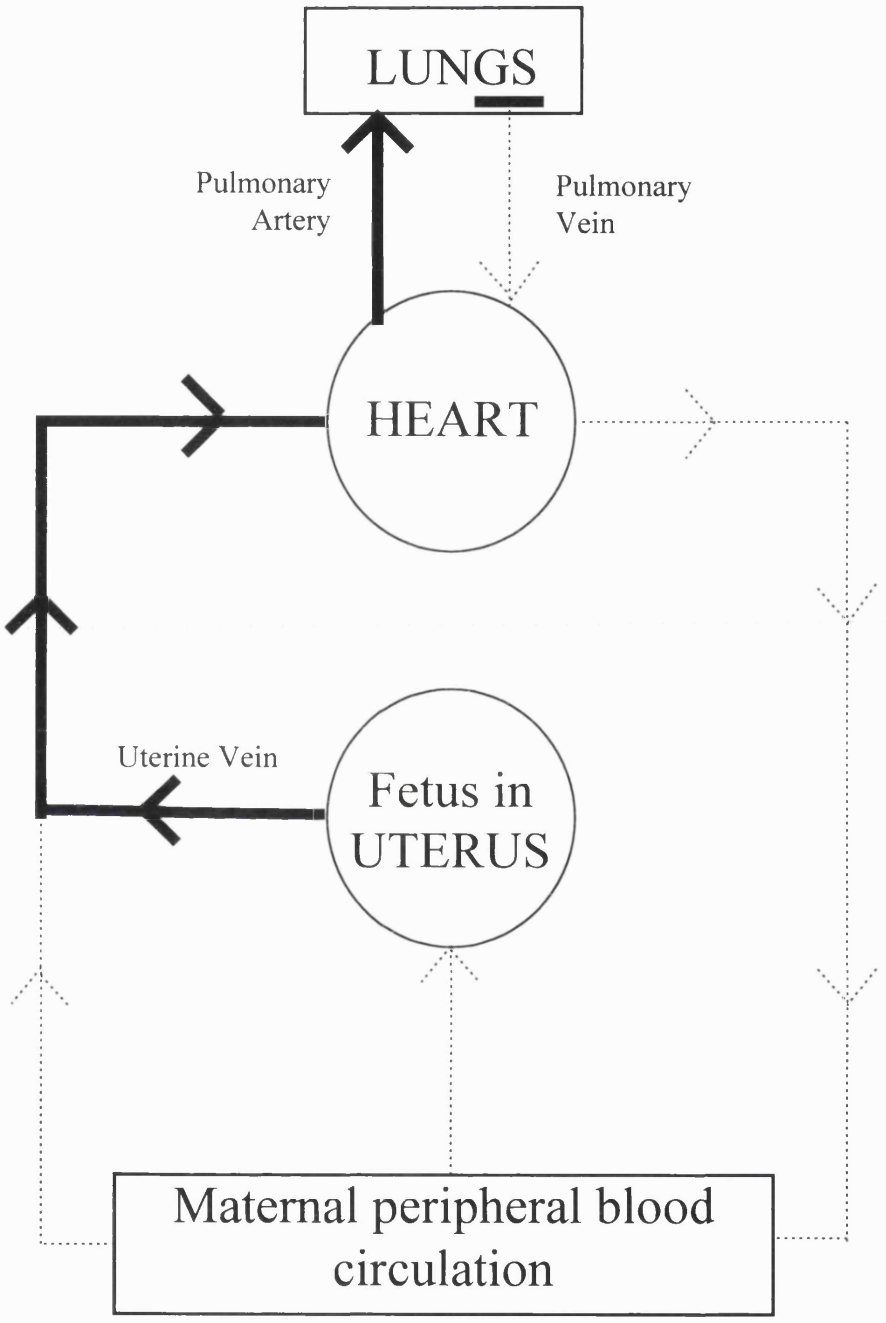
It is not usual for severe cases of pre-eclampsia to be monitored for changes in central venous blood pressure by pulmonary artery catheterisation. Lee et al (1986) studied three pulmonary artery blood samples collected (1) during first stage, (2) immediately after placental removal, and (3) 4 hours post-partum from 14 women suffering from severe pre-eclampsia. Cell smears were examined for the presence of squamous epithelial cells as well as trophoblast, mucus threads and fat. Squamous cells or trophoblast were demonstrated in 3/4 (21%) samples. Two of the three women experienced eclamptic seizures before sampling and in these cases squamous cells were detected in samples obtained within four hours of delivery. The third patient was noted to have trophoblast cells in the pulmonary arterial blood collected during labour, but not in the subsequent peripartum sample. No numbers were recorded and no morphologic description of the detected cells was given.

Goodfellow and Taylor in 1982 were able to identify cells with trophoblast morphologically after enrichment by gradient centrifugation of whole blood however definitive proof that these cell clumps were fetal in origin was lacking. The production of a monoclonal antibody (McAb) (H315) raised against chorionic villi cellular trophoblast which was not reactive against maternal white blood cells offered a new method of identifying the fetal trophoblasts (Johnson et al., 1981). Covone et al., (1984a; 1984b) used a flow sorting technique to select cells that expressed placental alkaline phosphatase and found that a small number of cellular elements reacted with

H315. It is possible however, that these reactive cellular elements were of maternal origin and had absorbed the H315 antigen released from the fetus. In 1988 Covone et al., stained H315 positive cellular elements from maternal blood samples enriched for trophoblasts with a FISH probe specific for the Y chromosome. A small number of cells appeared to demonstrate the presence of a Y-chromosome, although only from a small proportion of H315 positive cells. Although there has been some limited success using McAb raised against trophoblast, the lack of any truly specific marker has hampered success. In 1990 Mueller et al., claimed to have had notable success at identifying fetal trophoblasts in the maternal peripheral blood circulation using a new McAb and PCR for the Y-chromosome. The McAb used in this experiment however was never made generally available, and this work has never been substantiated.

A new approach of negative selection was employed by Sargent et al., (1994a; 1994b) and Johansen et al., (1995) .Using magnetic activated cell sorting (MACS) with a McAb specific to leukocytes (CD45), followed by the hypotonic selective lysis of red blood cells, trophoblasts can be enriched up to 32 fold with a 78% recovery rate in artificial mixtures (Johansen et a., 1995). However fetal trophoblasts have been isolated from the peripheral maternal blood circulation of only 10% of normal pregnant women (Johanson 1998). Trophoblasts are more consistently recovered from women with pre-eclampsia or from the uterine vein of non-eclampsic women.

**Figure 1.5 Trophoblasts in maternal peripheral blood**




Blood circulation not containing trophoblast

Blood circulation containing shed trophoblast

Adapted from Sargent et al., 1994

The lack of expression of the class I and class II HLA-antigens by syncytiotrophoblast (Sargent 1993; Redman 1983) provides an explanation as to how trophoblast can lodge in maternal pulmonary capillaries and persist for some length of time without provoking an inflammatory or immunological rejection response. Long before it was shown that syncytiotrophoblast do not express either classical or non-classical class I or II major histocompatability complex (MHC) antigens, it was suggested that they could somehow antigenically 'present' the fetus to the mother and thus prevent it's immunological rejection (Thomas et al., 1959). It is however difficult to ascribe any active biological role for deported trophoblast in the protection of pregnancy, as trophoblast migration has so far only been shown in humans and in the chinchilla (Billington 1970; Billington and Weir 1967) and not in any other primate or in rodents.

The diagnosis of fetal 47XYY aneuploidy by Y chromosome specific FISH performed on trophoblasts isolated from the maternal peripheral blood was reported by Cacheux et al., (1992). The authors attempted to isolate trophoblast cells by combining immunomagnetic removal of maternal lymphocytes and flow cytometry sorting using anti-trophoblast monoclonal antibodies. Examining 1387 sorted nuclei, 59 (4.25%) were shown to contain one or two Y-positive signals. Although this figure is comparable to the false positive rates in many laboratories, the authors were confident that the cells identified were trophoblastic in origin.

The possibility of villous trophoblast and fetal blood cells entering the maternal circulation as a normal occurring phenomenon in early pregnancy is highly dependant on the establishment of an intervillous circulation. Until this time it is difficult to imagine how fetal cells can enter maternal circulation, except for endovascular cytotrophoblast which are in direct contact with maternal blood in the spiral arteries.

After establishment of an intervillous circulation however, the excessive 'sprouting' taking place in the growing placenta offers plenty of opportunity for syncytial fragments to break off and enter maternal blood leaving villous cytotrophoblast and possibly fetal capillaries exposed to the maternal blood flow. Due to their size, however, the majority of syncytiotrophoblast are likely to get trapped in the capillary bed of the lungs (Fig 1.5), where they have a very limited life-span as emboli, cause no local inflammatory reaction and can be seen by radiography. Syncytiotrophoblast does not undergo mitosis (Rhine & Milunsky, 1979) so proliferation of deported syncytiotrophoblast once at the lung would not occur.

## 1.8. Fetal leukocytes in the maternal peripheral blood circulation

### 1.8.1. Lymphocytes

#### 1.8.1.1. Cultures

In 1969 Walknowska et al., cultured lymphocytes from the peripheral blood of women pregnant with male fetuses. This study was performed prior to chromosome banding technology, and so the presence of 5 acrocentric chromosomes was assumed to indicate 46XY metaphase and therefore to be a fetal cell. It was claimed from this work that between 0.1-1.5% of the cultured lymphocytes were fetal. Despite the fact that, based on a maternal blood volume of 4.5 litres and a 1% proportion of fetal to maternal cells, this would indicate the presence of 45ml of fetal blood in the maternal circulation, these findings were corroborated in other studies (De Grouchy & Trebuchet, 1971; Schindler et al., 1972; Takahara et al., 1972) although Y-containing metaphases were also observed in samples collected from mothers who later delivered female babies. However other researchers, using the same approach, were unable to repeat these findings (Angell & Adinolfi 1969; Jacobs and Smith, 1969; Adinolfi, 1975). Zilliacus et al., (1975) studied a total of 112000 cells in mitosis, cultured from 10 maternal blood samples collected from pregnant women. No Y-containing metaphases could be observed. This negative result was confirmed by Schroder and co-workers in 1977 who again failed to detect 46XY nuclei. A more recent publication by Selypes & Lorenz (1988) however, claimed that using an 'air culturing technique' up to 30% of cells in metaphase were 46XY and therefore fetal. This conclusion was refuted by Youssef et al., (1990) who, using the same technique, could not repeat these findings.

#### 1.8.1.2. Quinacrine mustard staining

An alternative method of identifying male cells was developed in 1970, highlighting Y chromatin DNA with quinacrine mustard dye (Pearson , 1970). Although this methodology is sometimes unreliable, staining regions of chromatin on autosomes (Polani & Mutton 1971; Thomsen & Niebuhr 1986), successful fetal sex prediction was achieved using maternal blood by some researchers (Schroder & de la Chapelle 1972; de-Grosset et al; 1974; Schroder et al., 1974; Zilliacus et al., 1975;

Schroder et al., 1977, Kirsch-Volders et al., 1980) but not by others (Zimmerman & Schmickel 1971, Adinolfi & Gorvette 1974).

### 1.8.1.3. Paternal HLA selection

Herzenberg and colleagues attempted to isolate fetal lymphocytes from selected mothers who were HLA-A2 negative, and whose partner was HLA-A2 positive; the fetus in these cases was thus expected to be HLA-A2 positive (Herzenberg et al., 1979; Iverson et al., 1981). Leukocytes were first separated from whole blood using a Ficoll-Hypaque gradient and then subjected to fluorescence activated cell sorting (FACS) for HLA- A2 positive cells employing a rabbit antiserum. In the first study, 12 mothers who had previously undergone amniocentesis and were known to be carrying male fetuses, were selected and sorted cells stained with quinacrine mustard for evidence of the Y-chromosome. In five of these cases a small proportion of sorted cells were shown to have a bright fluorescent spot (range 0.3%-1.6%)- at levels comparable to false positive results (Thomsen & Niebuhr, 1986). The second study included 30 women known to have a male fetus, and demonstrated only a tiny fraction of HLA-2A positively sorted cells to have a bright quinacrine mustard Y-chromosome spot. Despite PHA stimulation, none of the sorted cells could be induced to divide and so no metaphase spreads were analysed. Karyotypes were achieved in two reports by Tarapel et al., (1989; 1993) using the same HLA sorting method but all were shown to be of maternal origin. Paternal HLA antigens, absent in the mother, were again employed to try and detect fetal lymphocytes in maternal blood using FACS (Covone et al., 1984b). However, the metaphase spreads derived from stimulated sorted cells were all shown to have a 46,XX chromosome complement irrespective of whether the fetus was male or female, showing the cells to be maternal in origin.

Yeoh et al., (1990) again used FACS to select for HLA-2A positive cells in the peripheral blood of pregnant HLA-2A negative mother. There was a further antigen difference between the mother and fetus; the mother was DR4 negative and the fetus positive. PCR and southern blotting was employed to successfully detect the DR4 DNA sequence in the sorted cells, demonstrating the presence of fetal DNA. This result however, could not be repeated on unsorted whole blood. Preliminary experiments had shown the assay to have the sensitivity of detecting one DR4 positive cell in a $10^5$ DR4 negative cell mix. It was therefore concluded that there was less than 1 fetal cell in $10^5$ maternal lymphocytes. This ratio extrapolates to approximately 50µl of fetal blood in

the entire maternal circulation. Sargent et al., (1994a) used the same approach to sort HLA-2A positive cells from HLA-2A negative mothers and then used FISH for the Y-chromosome, attempting to correlate these results with the sex of the fetus. However, the mean percentage of Y-containing cells in the 3 samples from mothers with HLA-2A positive male fetuses was 1.06%, a figure not significantly different from that obtained from the female fetus controls.

### 1.8.2. Granulocytes

One report by Wessman et al., in 1992 claimed to have detected fetal granulocytes in the peripheral blood of pregnant women. Gradient centrifugation was employed to isolate the leukocyte fraction from whole blood, and a granulocyte specific monoclonal antibody (McAb) used to identify the cells followed by FISH for the Y-chromosome. Y-specific cells were recovered from eight pregnant women, seven of whom gave birth to male infants. Together with the false positive result, the specificity of the monoclonal McAb used in this study is questionable and the surprisingly high number of fetal cells said to be present, prior to any fetal cell enrichment technique casts doubt on this report. The presence of granulocytes in the maternal circulation is subject to the same theoretical objections as that of lymphocytes and these findings have yet to be substantiated.

### 1.8.3. Leukocyte Summary

Despite the early optimism it would seem that leukocytes, if present at all in the maternal circulation, are there at extremely low numbers. Studies aiming to isolate cells displaying paternally derived antigens or fetal markers often erroneously assume these sorted cells to be fetal without further corroboration. For example Kuloxik & Pawlowitski (1982) used FACS with a McAb specific for alpha fetoprotein and assumed all positive cells to be fetal. It was later shown however that these cells were in fact maternal (Adinolfi, 1982). In a study by Mowbray et al., in 1985, five women with recurrent miscarriages had their partners lymphocytes injected directly into their periphery circulation as part of 'leukocyte immunisation' therapy. The HLA type of both individuals was known, and whole blood, extracted at various times after the inoculation was sorted for the partner's antigens. The sorted cells were exposed to a sensitive Y-specific PCR assay to demonstrate the presence of male blood cells.

Samples from all 5 women were all Y-positive immediately after injection, only 3 were positive after 1 hour, and only 1 after a 24 hour period. After one day, no Y-sequence DNA was detectable as the invading cells appeared to have been destroyed by the maternal immune system. It may be that fetal 'semi-allogenic' cells may act differently, but it would seem that should any fetal leukocyte cells enter the maternal circulation, they are unlikely to survive as they express paternal major histocompatability complex antigens and would therefore be destroyed in a short period of time. The presence of leukocyte cells within the maternal blood is probably due to sporadic fetomaternal haemorrhage and not a regular or normal occurrence in pregnancy. Most studies successfully detecting these cells, have been carried out after amniocentesis or CVS, procedures now known to induce leakage of fetal blood (Los et al., 1989;1996; Smidt-Jensen et al., 1993; 1994; Rodeck et al., 1993).

Should leukocytes enter the maternal circulation and somehow survive, it is proposed that these cell may well persist (Simpson and Elias, 1994a). Indeed one study, Bianchi (1994b) flow sorted for antibodies that bind cell surface antigens present on B cells (CD19 and CD23), T lymphocytes (CD3, CD4, CD5) and haematopoietic stem cells (CD34 and CD38). Eight women, who had delivered male infants between six months and 27 years earlier, were studied. Y-specific PCR was employed on the sorted cells. Male cells were detected in none of the sorted B cells, from one of the sorted T-lymphocyte cells and in 6 of the 8 sorted stem cell fractions, one from the blood of a woman who had give birth to a male offspring 27 years previously. This would suggest that most post-partum women are in fact low grade chimeras with fetal stem cells. However, Hsieh et al., in 1993 followed 28 women who had delivered a male fetus. One week after gestation 26 of the 28 women showed Y-specific DNA sequences in their blood. By 4 months, only 11 of the 28, and by 8 months only 2 of 23 were positive suggesting that micro-chimaerism is a short-lived phenomenon. Thomas et al., (1995) were able to detect Y-specific DNA in the blood samples tested from 2 mothers with male fetuses from 7 weeks' gestation until delivery. They confirmed the absence of long term micro-chimaerism by being unable to continue to find this sequence in the blood of any of these women 8 weeks after birth. In some rare cases however, the passage of fetal stem cells into the circulation of the mother occur with these cells evading the maternal immune system and establishing microchimerism.

## 1.9. Nucleated erythrocytes (red blood cells), nRBC

Bianchi et al., (1990) were the first to report the presence of fetal nucleated erythrocytes (nRBCs) in maternal peripheral blood. FACS was performed to positively select for the transferrin receptor (CD71); an antigen expressed on early erythrocytes (but also on monocytes and certain activated lymphocytes). FACS uses the LDS-751 nuclear stain to flow sort nucleated cells which also removes platelets, anucleated cells and debris from the whole blood mix, negating the need for gradient centrifugation. PCR for the Y chromosome was performed, and the group identified male (fetal) sequences in sorted cells from 6 samples collected from women with male fetuses, but also from 2 with female fetuses (false positives).

Other groups began to flow sort for CD71, and an additional nRBC marker, Glycoforin-A; the major sialoglycoprotein of the erythroid cell membrane (Price et al., 1991) which is present in the erythrocyte lineage and absent in leukocytes, as well as for cell size and granularity (Wachtel et al., 1991; Price et al., 1991; Simpson and Elias, 1993; 1994a; 1994b). Employing nested primers for the Y chromosome on sorted cells, Wachtel et al., (1991) identified 12/12 male fetuses and 5/6 females (one false positive). In addition to FACS, magnetic activated cell sorting MACS has also been employed in attempts to isolate nucleated red blood cells (Holzgreve et al., 1992; Bianchi, 1994a; 1994b; Bianchi et al., 1994a). As it began to be recognised that despite complex positive FACS sorting nRBCs were still in the vast minority of sorted cells, other ways of enriching them were attempted. Negative selection (the removal of antibody specific cell types) is now widely used, particularly for selection against cells displaying the antigen CD45. This is a surface antigen specific to most leukocytes, including lymphocytes and monocytes. MACS is now most commonly used for this negative selection process, either with standard MACS sorting equipment, or on antibody coated flasks (Simpson et al., 1995). This enrichment step is a preliminary stage designed to remove maternal leukocyte cells prior to positive FACS selection of nRBC cells. Additional antibodies for negative selection have also been assessed including CD15 and CD14, again leukocyte specific antigens, but without as much success (Simpson et al., 1995) and CD32 (Zheng et al., 1993). Additional markers specific for nRBCs have also been assessed including CD36 a thrombospondin receptor (Bianchi et al., 1993) but without notable success.

Even after anti-CD45 maternal leukocyte depletion, CD71 and Glycophorin A positive FACS separation, nRBCs still remain mixed among a great majority of

maternal cells (Simpson and Elias, 1994b). Further identification with fetal specific markers must therefore be employed. One such marker is fetal haemoglobin (HbF), first used by Zheng et al., (1993). HbF, constituting 90% of the haemoglobin in a 12-32 week fetus is intracytoplasmic and has a high affinity for oxygen, enabling the absorption of oxygen by the fetal circulation from the maternal blood circulation. HbF is made up of two alpha and two gamma haemoglobin chains whereas haemoglobin in mature erythrocytes consists of two alpha and beta chains. HbF begins to be expressed from 6 weeks of fetal gestation, when the erythropoiesis centre shifts from the yolk sac to the fetal liver. A monoclonal antibody, specific for the gamma chain would therefore be specific for nRBCs. In their study, Zheng et al., depleted leukocytes from whole maternal blood using MACS with antibodies CD45 and CD32. Sorted cells were then assessed with simultaneous immunophenotyping with the mouse anti-HbF antibody, UCH gamma, and FISH analysis using chromosome X and Y specific DNA probes. Male fetal nRBCs were successfully identified, however due to the fact that the fetal haemoglobin is a cytoplasmic marker and not present on the surface membrane of cells, the fetal cells had to be fixed onto a slide. Due to this procedure, after identification, possible applications of the fetal cell are restricted.

Nucleated red blood cells are known to constitute about 10% of the red blood cells in an 11-week fetus and are present in the peripheral blood of normal non-pregnant adults in extremely low numbers. It was therefore erroneously presumed that any nRBC present in the blood of pregnant women would be fetal. Many early reports concentrated merely on identifying nRBC & assumed them to be fetal. However it has been shown that as maternal blood volume increases with pregnancy, it appears that the erythroid progenitor expansion to meet this demand results in proliferation of erythroblast & other nucleated erythroid cells (Pembrey and Weatherall 1971; Pembery et al., 1973). The number of maternal blood cells increases by 25% and all new cells will be nucleated and express markers such as CD 71, glycophorin A and HbF, the very markers used to isolate 'fetal' cells. It has also been shown that nRBCs with HbF are present in non-pregnant people (Zago et al., 1979) especially when suffering from hypoxia anaemia or thalassaemia. Early optimism regarding the numbers of fetal nRBCs in the maternal peripheral circulation has thus become muted (Adinolfi, 1992b; 1992c).

Estimations in the literature of the frequency of fetal nRBCs in maternal blood vary. Busch et al., (1994) and Zheng et al., (1993) claimed the frequency of fetal nucleated red blood cells in maternal nucleated cells is between $10^{-7}$ and $10^{-6}$, whilst

Sohda et al., (1997) reported that the frequency of fetal nRBCs varies from 8.1 x $10^{-5}$ to 1.6 x $10^{-5}$. Hamada et al., (1993) estimated the frequency of fetal nRBC to maternal cells to be 1 in $10^5$ in the first trimester but to increase to 1 in $10^4$ at term. Reading et al., (1995) claimed their technique enabled 70% cell recovery and were able to retrieve on average 3 cells from 15ml of maternal blood- this would suggest a ratio of no more than 1 cell in every 3ml of unsorted maternal blood (less than 1500 nRBCs in the entire circulation). Bianchi et al., (1995) used MACS negative selection of CD45 positive cells, then FACS positive selection for CD71 positive cells. Between 230 and 685000 cells were sorted from each 20ml blood sample, and FISH for the Y chromosome showed this to contain on average 21 fetal cells. Using CD45 depletion and then positive selection for HbF fewer cells were sorted (48-240) with similar numbers of fetal cells. Many sorted samples from mothers with male embryos did not yield any Y-positive sorted cells. In the same year Simpson et al., (1995) also used negative MACS depletion for CD45 positive leukocytes and positive FACS sorting for CD71 positive cells. Using FISH for the Y chromosome and scoring 20-3000 sorted nuclei, they were able to detect male (fetal) cells in 11 of 19 male pregnancies using 20ml blood samples at a frequency of 0-7 cells per sample. Using the same negative depletion technique, but positive selection for HbF, male cells were seen in 12 of 14 male pregnancy blood samples at a frequency of 0-8 cells per sample scoring 40-3000 nuclei.

An alternative method for further identifying fetal cells from a positively sorted fraction is to use micromanipulation to remove single nRBCs from sorted cells on a slide (Takabayshi et al., 1994). This methodology was utilised in three studies by Sekizawa et al., where maternal blood samples were enriched for nucleated erythrocytes by density-gradient centrifugation which were then individually transferred into a PCR tube under light microscopy with the use of a micromanipulator. In selected cases the authors claimed that subsequent PCR on these single cells enabled the diagnosis of fetal muscular dystrophy (Sekisawa et al., 1996a), fetal Rh(d) type (Sekisawa et al., 1996b) and whole genome amplification employing primer extension preamplification (PEP) followed by fetal HLA typing (Sekisawa et al., 1998).

Micromanipulation was also employed by Eggeling et al., (1997) to obtain pure fetal DNA. Single nucleated erythrocytes were separated and enriched from maternal blood by a triple density gradient and the monoclonal antibody CD71 in combination with a magnetic activated cell sorter. Single cells were then microscopically examined and individually collected. Using polymorphic STR markers and the amelogenin region

of the sex chromosomes, fetal cells could be identified. It was concluded that 30% of the sorted nucleated erythrocytes were of fetal origin.

Valerio et al., (1997) collected blood samples after invasive procedures or prior to termination of pregnancy (TOP) from 7 women who had fetuses with chromosome aneuploidy. Using MACS they positively selected cells for erythroid progenitor factors; CFU-E (colony forming unit-erythroid) and M-BFU-E (mature burst forming unit-erythroid). The positively sorted cells were cultured and assessed with FISH for the appropriate aneuploid chromosome and confirmed to be fetal. However these cells may well have entered the maternal circulation as a direct result of the prenatal diagnostic procedures. In an attempt to emulate this research Chen et al., (1998) cultured erythroblasts from 16 maternal blood samples, both with and without prior enrichment by MACS. The resulting colonies were tested for evidence of fetal DNA by PCR and FISH for the Y chromosome, and the PCR amplification of polymorphic STRs. No evidence of fetal DNA could be detected from any of the tested samples and the authors conclude that this method is not reliable for the selection of fetal erythroblasts.

As with fetal leukocytes, fetal nRBCs cells will express paternally inherited surface antigens that are not necessarily common to the mother. It could be assumed that the maternal immune system would attack any fetal nRBCs expressing incompatible ABO or Rh(D) antigens destroying any migrating fetal nRBC. This has been shown to be the case in mice (Bonney and Matzinger, 1997). However it has been recorded that human fetal nRBCs contain fewer reactive A or B sites than adult cells (6000 to 7000 per fetal cell versus 26000 per adult cell), and such sites are also farther apart (Voak & Williams, 1971). It has also been suggested that fetal RBC antigens are not as strong and also possibly not as specific as adult antigens, offering additional protection against interactions with maternal antibodies (Tovey, 1952). Should fetal nRBCs enter the maternal blood stream, and survive any maternal immune response, it is unlikely that any will survive longer than 90 days (Simpson & Elias, 1994a; 1994b) as fetal cells in the erythroid lineage have a very short life-span. There is therefore no chance of any persisting long after pregnancy.

Utilising these enrichment techniques, the diagnosis of fetal chromosome aneuploidies has been achieved. In 1991 Price et al., reported the detection of trisomies 21 and 18 from fetal nRBCs in maternal blood. Fetal nucleated erythrocytes were flow sorted on the basis of four parameters: cell size, cell granularity, CD71 and Glycophorin-A. FISH with probes specific for chromosome 21 identified a small

number of trisomic cells in maternal blood taken from a mother known to be carrying a Down Syndrome fetus one week after she had undergone chorionic villus sampling. A small number of fetal trisomy 18 cells were also identified using FISH from blood collected immediately before chorionic villus sampling. Fetal trisomy 21 nRBCs were also detected in samples collected from women after amniocentesis by Elias et al., (1992) and Bianchi and Klinger (1992) although none of these studies were performed blind, and it has been established that invasive prenatal diagnosis procedures cause materno-fetal transfusions (Los et al., 1996). Ganshirt-Ahlert et al., in 1993 used a combination of triple density gradient and MACS for CD71 positive cells for the enrichment of fetal nRBCs followed by FISH for chromosomes known to be aneuploid in the fetus. They claimed to have identified 15 cases of fetal trisomy (five cases with a trisomy 18 and ten cases with a trisomy 21) although cells with three signals were seen in all normal control samples. Their diagnosis was based on the fact that in all of the aneuploid pregnancies that the percentage of cells with three hybridization signals did not overlap with those of normal controls. Many of these samples were collected subsequent to an invasive procedure, and the FISH tests were not performed blind.

Using micromanipulation, diagnosis of fetal sickle cell anaemia and beta thalassaemia was achieved utilising fetal nRBCs enriched from maternal blood (Cheung et al., 1996). Density gradient cell separation was used to isolate mononuclear cells, followed by positive FACS for CD71. Fetal nRBCs were further identified by using an anti-fetal haemoglobin stain. Individual HbF stained cells were identified under a microscope and isolated by micro-dissection from the slide to be tested by PCR. In this manner, up to 20 fetal cells were identified from 18ml of maternal blood, and the diagnosis of two normal fetuses from two pregnancies at risk of sickle cell and beta thalassaemia was achieved (the cell did not display the mutant form of DNA carried by either mother or father). One sample had however been collected subsequent to CVS.

The large proportion of cells sorted from the 47 XX+21 patient (Elias et al., 1991) that were fetal (74% ) raises the hypothesis that the placenta, in some aneuploid pregnancies, allows relatively more fetal erythrocyte cells into the maternal circulation than is true for euploid pregnancies. It appears that in cases of fetal chromosomal abnormalities, especially Down Syndrome, the number of fetal nucleated red blood cells in the maternal circulation may indeed increase (Ganshirt-Ahlert et al., 1993), perhaps reflective of underlying placental pathology (Prof. P. Polani,. personal communication). This suggestion that physico-mechanical properties of the intervillous

blood flow are abnormal corresponds with reports of increased transfer of fetal cells in women suffering from pre-eclampsia, where uteroplacental perfusion is thought to be abnormal. This hypothesis is supported by Bianchi et al., (1997) who found up to 650 fetal nRBCs in 20ml of sorted maternal blood from mothers with trisomy 21 fetuses; with an average 5.8 fold increase over normal pregnancies. There are however no specific findings that characterise a placenta with trisomy 21 and which may explain increased leakage across the placental membrane. However, many abnormalities have been found sporadically such as an increased incidence of single umbilical artery (SUA), increased syncytial knotting and cellularity of the villous stroma, and unusually small placentas and vascular abnormalities in trisomy 18 (Arizawa and Nakayama 1992). In trisomy 13, the placenta is also frequently found to be smaller and more often has SUA and dysmature villi. No characteristic pathological placental changes have yet been found in trisomy 21.

Wachtel et al., (1996) employed charge flow separation as a novel method of nRBC enrichment. This employs a horizontal crossflow fluid gradient opposing an electric field in a separation chamber. Cells flow vertically, perpendicular to the gradient and the electric field. According to their characteristic surface charge densities, the different cell types are separated from each other and focused in different compartments where they can be directed into waiting collection tubes. It was found that nRBCs separated into the same channels as some leukocytes. When the sorted cells from blood samples corresponding to male fetuses were tested by Y-FISH there were false positives and negatives, false results that 'vanished' after two weeks, and when male cells were detected they were at a frequency of less than 1:600 contaminating cells. It was estimated that fetal nRBCs were among maternal nucleated cells at a frequency of $10^{-5}$ to $10^{-4}$ , and constituted between 0.16-0.5% of the sorted fraction. It was also concluded that more than 65% of nRBCs were maternal in origin. The diagnosis of fetal trisomies 18 and 21 were achieved from samples collected after invasive procedures. As the erythrocytes subsequent to charge flow separation were still viable, attempts were made to culture erythroid stem cells. Maternal erythrocytes were lysed with exposure to $NH_4Cl$ and $NH_4HCO_3$ (Boyer et al,. 1976). Of the 75 clones derived from the sorted cells of male fetuses, 5 were found to be male with Y-FISH. A further study, incorporating these data, claimed to verify these findings (Wachtel et al., 1998). A 15-16ml sample of peripheral blood was collected from 225 pregnant women, mostly between 10-18 weeks gestation, Nucleated red blood cells were identified in 67% of these. Dual FISH for the X and Y chromosomes was

performed on a small number of selected samples (17). From the assessment of a limited number of nuclei it was observed that ~30% of nRBCs were fetal in origin, although within this small number of tests a false negative, a false positive, one case of contamination and one failure of the FISH technique were also recorded. Extrapolating these results, the authors claim that two thirds of pregnant women have an average of 1800-2200 fetal nRBCs in 15-16ml of their peripheral blood. Although these reports offer some optimism, they have yet to be substantiated.

An interesting early report by Lo et al., (1997) found that from blood samples collected prior to amniocentesis or delivery from 30 women with males fetuses, 80% [24/30] had detectable Y-specific DNA in 10μl of their blood plasma by PCR and 70% [21/30] had Y-specific DNA in 10μl of their blood serum, with no false positive results. The PCR assay they employed was found to be able to detect one male genome equivalent in $10^5$ female genome equivalents of DNA. When the same assay was carried out on whole blood from the same women, Y-positive results were seen in only 17% [5/30].

A further report by the same group (Lo et al., 1998) used real time quantitative fluorescent PCR to amplify Y specific fetal DNA and a sequence of the β-globin gene as a maternal marker. It was shown that the concentration of maternal DNA was significantly higher in serum than plasma and increased significantly during pregnancy. Fetal DNA was detected in both serum and plasma and the concentration was seen to increase with gestation. Fetal DNA levels were higher in serum and could be detected from as early as 7 weeks' fetal gestation. In early pregnancy the fractional concentration of fetal DNA totalled 3.4% and 0.13% maternal DNA in plasma and serum, increasing to 6.2% and 1.0% respectively. Calculations of the density of fetal DNA revealed that there was the equivalent of one fetal genome in ~39μl maternal plasma in early pregnancy, increasing to one in ~3μl by the third trimester. The Y-specific amplification products correctly predicted fetal sex in all cases (125 male and 88 female).

Although these reports are yet to be substantiated, the findings support the theory that should any fetal cell (leukocyte or erythrocyte) migrate into the maternal circulation it would be lysed by maternal immunological actions releasing the DNA into solution. Should fetal DNA be confirmed to circulate in the maternal blood plasma, potential prenatal diagnostic applications would be limited to detecting genes present in the father and absent in the mother.

## 1.10. Fetal Cells in Maternal Blood- Summary

It is now thought that with existing FACS, MACS and other enrichment technology, male fetal nRBCs can only be detected in 36% of samples collected from women with a male fetus, and then at an average frequency of 8 cells per 20ml sample, with male cells being present in 6% of female pregnancies (Professor Laird Jackson, personal communication). It seems that due to an increase in maternal erythrogenesis during pregnancy, fetal nRBCs only constitute up to a third of all nucleated erythrocytes (Slunga-Tallberg et al., 1995). Although there may be more fetal cells in samples collected from mothers with aneuploid fetuses, the limitations and technical difficulties associated with this line of research for potential non-invasive prenatal diagnosis suggest that any potential clinical application is a long way off. It would appear that should any fetal cells cross the placental barrier they are most likely destroyed by the maternal immune system, and may well be there due to a sporadic haemorrhage rather than a ubiquitous process. Even bimanual pelvic examination (squeezing the uterus prior to blood sampling) may cause transient passage of fetal cells in maternal blood. Under normal conditions a pressure gradient exists between the fetal capillaries and the intervillous space which favours cell-traffic in the direction of the maternal intervillous blood space. If the integrity of the chorionic epithelium is defective however, it is possible that such a gradient could be momentarily upset, such as when the mother changes from the lying to the standing position or during abnormal uterine contractions.

The degree of contact between maternal and fetal circulations would appear to be more than adequate to allow generous amounts of cells, subcellular fragments or free molecules to enter the maternal circulation if the placental barrier is accidentally disrupted. The total villous surface of the placenta is enormous. Any trauma that disrupts the integrity of the chorionic epithelium will leave the underlying villous stroma with the fetal capillaries exposed, allowing leakage in either direction. It is common in histological sections of the placenta to find areas with defects in the cellular continuity of the decidual basalis (Dr. Kaufmann, personal communication). These areas are then usually covered by small patches of fibrin or fibrinoid material and these lesions are found to accumulate with advancing gestation. Such lesions are sometimes referred to as 'Kline lesions' and are thought to be sites of possible fetal bleeding and coagulation. Kline inferred that the coagulation in capillaries and the fibrin deposits were the primary lesions (Kline 1951). However, they have also been suggested to arise from the exposure of the naked villous

basal membrane to the maternal intervillous blood, after trophoblast detachment (Benirschke, 1994).

Intervillous thrombi occur much more commonly in placentas within which a disturbed intervillous circulation can be inferred, such as in erythroblastosis fetalis, hydrops fetalis, pre-eclampsia and in some other conditions (Benirschke and Kaufmann, 1990).When the composition of such thrombi was assessed, fetal haemoglobin-containing cells were detected in quantities well in excess of the numbers seen in maternal blood (Kaplan et al., 1982), suggesting that an intervillous thrombus may start near a ruptured fetal vessel with subsequent addition of maternal blood and clot formation. Hence, this may be one point of entry of fetal cells into the maternal circulation. Devi et al., (1968a; 1968b) found that the Kline-lesions, as well as operative delivery, correlated with fetal bleeding. The more frequently the lesions were present, the greater was the size of the transplacental haemorrhage. Trauma , such as car accidents, external fetal version, and amniocentesis, may enhance or initiate such fetal bleeding. Often, however, fetomaternal haemorrhage occurs without known antecedent disruption of the fetal tissue (Benirschke and Kaufmann, 1990).

Studies detecting fetal cells in the maternal circulation subsequent to a prenatal diagnostic invasive procedure (CVS or amniocentesis) should be considered with caution as feto-maternal transfusion is well known to occur in association with these techniques (Los et al., 1989;1996; Smidt-Jensen et al., 1993; 1994; Rodeck et al., 1993).

# Part 4. Fetal Cells in the Uterine Cavity

## 1.11. Transcervical Cell (TCC) History

In 1971 Shettles reported that chorionic cells were shed into the cervical mucous plug of pregnant women. He used a cotton swab to obtain mucus smear samples from the mid cervix of 30 women in all three trimesters of pregnancy and applied the quinacrine hydrochloride fluorescein dye test to reveal Y chromosome containing cells. He detected Y bearing nuclei in 18 of the samples and thus predicted the sex of fetus in these cases to be male. At the time of reporting from his ongoing study 10 pregnancies had come to term, with the sex of all deliveries in concordance with his findings; 4 female and 6 male. These results could not be repeated by Bobrow and Lewis (1971) who examined midcervical smears and cervical mucus from 9 pregnant women, 6 carrying a male fetus and 3 a female. Using the same quinacrine dye they found the proportion of apparent Y positive cells from female pregnancies averaged 2.52% and from male pregnancies averaged 3.04%. Both these percentages were within the known range of false positives in female control material; where fluorescing clumps of condensed chromatin and other artefacts may be mistaken for the 'Y' fluorescent spot.

In 1972 Warren et al reported to have obtained fetal cells from endocervical mucus. In a blind study, the sex of the fetus was correctly predicted in 50 women in all stages of gestation. These claims were disputed by two studies the following year (Tusk & Sasaki, 1973; Goldstein et al., 1973). Tusk and Sasaki studied 16 pregnant women from 7-12 weeks after the first day of the last menstrual period, prior to elective termination. Smears were obtained from midcervical mucus with a cotton swab and visualisation of Y chromosome containing nuclei attempted using quinacrine mustard. No Y containing cells were identified in any sample, despite there being 12 pregnancies with male fetuses (confirmed by examination of aborted material). However, lavage of the uterine cavity with sterile saline carried out on 3 patients, did reveal Y containing clumps of chorionic cells in the 1 case of a male pregnancy. Goldstein et al., (1973) studied mid-cervical smears of 38 patients at various stages of gestation, but were unable to identify any Y containing cells using quinacrine mustard, despite examining at least 100 nuclei from each sample and there being at least 7 male fetuses.

In 1975, Manuel et al. obtained mid-cervical smears from 20 women in the second trimester of pregnancy prior to elective abortion and again used quinacrine mustard staining in an attempt to detect Y containing cells. No relationship between fluorescent spotting and sex of the fetus was observed, however the presence of multiple spots in many nuclei highlighted the unreliability of the staining technique.

Positive results were obtained by Rhine et al., in 1975, who used quinacrine staining to detect male cells in endocervical smears collected by cotton swabs from 36 pregnant women during all 3 trimesters. Between 100-300 nuclei were scored from each case, and samples displaying more than 3% of cells with an apparent Y chromosome signal were deemed to be trophoblast shed from a developing male fetus. The sex of the fetus was correctly predicted in 31 of the 36 cases with no false positive results (erroneous male predictions). The percentage of cells containing a Y fluorescent body ranged from 4.0% to 22.7% in samples from male fetus pregnancies (mean 8.9%), and 0.0% to 3.0% in female fetus pregnancies (mean 0.8%). The 5 incorrect results were false negatives (males born, females predicted) and it was suggested that they were due to the collection of smears from a cervical region below the area of fetal cell accumulation.

In 1977 Rhine et al. published data from further studies where efforts were made to collect samples from the area of the internal os, not the mid cervix of pregnant women in the first trimester, using a device entitled the Antenatal Cell Extractor (ACE). (The internal os, or isthmus, is the restricted region between the corpus and cervix). This enabled lavage of the uterine cavity, using less than 5ml sterile saline. Tissue collected with this method was successfully cultured producing cells with trophoblast morphology in 6 of the 32 cases in the first study, and 18 of the 21 samples in the second. These cells were karyotyped and polymorphic differences from maternal karyotypes were observed in 17 of 36 successful cultures, although no Y chromosome was detected due to the low quality of the metaphase spreads achieved. In the third stage of the study 13 pregnant women had two sequential lavage samples retrieved. Eight samples were examined histologically and seen to contain tertiary villi. The presence of human chorionic gonadotrophin was assayed from the culture media of a further 3 duplicate samples by radioimmunoassay and the levels found to be significantly en excess of control cultures from skin fibroblasts. In all cases cell sampling was carried out 8 days prior to termination of pregnancy. During this eight day period no untoward effects of the sampling procedure were seen to affect either the mother or fetus.

In 1977 Varner et al., also reported successful results with endocervical samples obtained from abortion patients between the 6th and 12th weeks of pregnancy. They searched their slides for multinucleated syncitial fragments derived from the syncytiotrophoblast of the developing membranes. These were compared to the syncytium from the abortus tissue for the presence of Y bodies. Of the 106 smears examined, 60 had more than 100 syncytial nuclei present. Y-body sex prediction analyses from these 60 patients were in 95% agreement with the Y-body analysis of the abortus trophoblast.

In 1978 Amankwah et al. obtained cotton wool swab midcervical smears from below the mucous plug in 71 pregnant women in all 3 trimesters. Thirty three of these were shown to be carrying male fetuses and 38 female. Atabrine staining was carried out for Y chromosome detection, and between 100-300 nuclei scored in each cases. Fetal sex was correctly predicted in only 55% of cases with extensive false positive and negative results. The following year, Rhine and Milunsky published a further report sampling 53 first trimester patients prior to abortion (Rhine and Milunsky, 1979). They were able to successfully culture 37samples (70%). Fetal chromosomes were demonstrated to be present in 26 (49%), including a 46,XY male karyotype from an endocervical specimen obtained at 9 weeks of gestation. Attempted culture of samples obtained from second trimester pregnancies were less successful.

In 1980 Goldberg et al., attempted endocervical lavage using 5ml of saline on 30 pregnant women in the first trimester of pregnancy (8-13 weeks) using the ACE apparatus prior to elective termination. Twelve of these terminations were found to be of male fetuses and the lavage samples of these cases were cultured and examined for the presence of XY metaphases. Of the 12 cases, 9 produced successful cultures and were karyotyped. Despite observing cytotrophoblastic elements in the original samples, all cultures contained female karyotypes indicating them to be of maternal origin.

Coleman in 1982 reported the results of an preliminary study collecting intrauterine lavage samples from 12 women in the 1st trimester of pregnancy prior to elective TOP. All samples were seen to contain decidua as well as villus fragments. When cultured however, all metaphases were found to be maternal as adjudged by the absence of XY karyotypes and banding polymorphisms. It was proposed that since intact trophoblast tissue grows slowly in culture because of the inhibiting effect of the trophoectodermal layers, contaminating maternal cells had 'overgrown' the fetal ones.

With the evolution of relatively safe transcervical chorionic villus sampling, research in this area of prenatal diagnosis halted until 1992 when Griffith-Jones et al.,

utilised the technology of the Polymerase Chain Reaction (PCR) to amplify Y chromosome specific DNA sequences in transcervical samples. Samples were collected from 33 women prior to termination at 9-13 weeks' gestational age. Samples were obtained with a cotton wool swab from the vagina, cervix, and transcervically 1cm beyond the internal os in 26 women. In the remaining 7 women, samples were obtained by flushing the lower uterine cavity with 5ml saline from an adapted catheter. Fetal sexing, using PCR to amplify a Y-specific sequence, was performed blind (without prior knowledge of the sex of the fetus) and was correct in 25 out of the 26 initial subjects investigated (9 male) with one false negative. It was concluded that for consistently accurate results, samples needed to be collected from the uterine cavity, beyond the internal os. Collection of cells from the mid-cervix and vagina produced unreliable results by PCR analysis, indicating the region to be unsuitable for shed fetal trophoblast cell accumulation and more prone to spermatozoa contamination. Of the seven samples obtained by intrauterine lavage, all were found to contain syncitial fragments displayed by morphological studies and staining with placenta specific monoclonal antibodies.

Unfortunately these results could not be repeated by Morris & Williamson (1992) who found a Y chromosome-specific sequence using PCR in only 4 of 13 male pregnancies by testing cervical smears, and 7 of the 13 by testing the mucus plug biopsy. They also misdiagnosed some female pregnancies blaming this discrepancy on the presence of sperm from a partner after intercourse. The sampling technique used in this study however did not collect cells from beyond the internal os, and patients were not asked to abstain from sexual intercourse. The authors also highlighted the potential risk of infection of the fetus induced by the procedure.

In 1993 Fluorescent In Situ Hybridisation (FISH) was utilised to highlight the presence of fetal cells in endocervical flushes (Adinolfi et al., 1993). Intrauterine lavage was performed on a mother found to be carrying a fetus with trisomy 18 by conventional CVS at 10 weeks and 2 days gestation. The lavage procedure was carried out 8 days after the CVS. FISH was performed on the transcervical sample with a chromosome 18 specific centromeric probe. Of the cells examined, 25% were found to contain three chromosomes 18 indicating them to be of fetal origin- the remaining cells containing 2 signals (maternal origin). Trisomy 18 of the fetus was confirmed by performing the same FISH procedure on placental samples following termination of the pregnancy. Unfortunately due to the transcervical sample being obtained subsequent to a CVS biopsy, the possibility that the placental cells were shed into the cervix as a

result of the procedure itself could not be dispelled (Gaudoin., 1993). In the same study, endocervical lavage was also performed on a further 11 mothers prior to termination. Primed in situ labelling (PRINS) was performed with a Y specific DNA sequence. Male cells were observed in all 7 pregnancies with male fetuses (2%-33%) with two false positive results in the 4 female fetus cases. Morphological studies were also carried out which showed syncytium-like clumps, some of which reacted to immunohistochemical staining with monoclonal antibodies raised against trophoblastic antigens.

## Part 5. Thesis Aims

This thesis aimed to assess the efficiency of various TCC collection techniques using numerous methods for the identification of fetal cells and the calculation of their abundance. Diagnosis of fetal conditions using a minority fetal cell population in the presence of a abundance of maternal cells was attempted. The possibility of isolating fetal cells using various methods was then addressed, and potential assays that could be applied to these cells explored. (Table 1.2).

**Table 1.2 Thesis Aims**

| Method of fetal cell detection | Methods attempted for fetal cell isolation | Potential prenatal diagnostic assays |
|---|---|---|
| PCR for the X and Y chromosomes | MACS | Sexing |
| FISH for the X and Y chromosomes | Trophoblast specific mRNA identification | Cystic Fibrosis |
| Fetal Rh(D) blood type | Micromanipulation | Sickle Cell Anaemia |
| Paternally inherited fetal STR allele | | Beta Thalassaemia |
| Trophoblast specific Monoclonal Antibody | | Chromosome aneuploidy (PCR) |
| Fetal chromosome aneuploidy detection (FISH) | | |

## 1.12. Fetal cell detection

### 1.12.1. PCR for the Y chromosome

As with Y chromosome specific PCR performed on the peripheral blood of pregnant women (Lo et al.,1989; 1990; 1994c), this fetal cell detection method is only informative for samples collected from women with male fetuses. In these cases, evidence of the Y chromosome indicates the presence of fetal DNA in the TCC sample. The same assay performed on TCC samples collected from women with female pregnancies gives an indication of the accuracy of the procedure, highlighting the number of false positive results.

### 1.12.2. FISH for the Y chromosome

As with maternal blood studies (Hamanda et al., 1993), this method can only be useful for detecting cells from male fetuses. Dual FISH was utilised, minimising the chances of false positive results. The same assay performed on TCC samples collected from women with female pregnancies gave an indication of the accuracy of the procedure, highlighting the incidence of false positive results.

### 1.12.3. Paternal polymorphism detection

A further method of detecting the presence of fetal cells in TCC samples, is to detect a genetic polymorphism, where a mother does not contain a sequence of DNA present in the fetus through paternal inheritance. Detecting this sequence of DNA in the TCC sample would indicate the presence of fetal cells.

#### 1.12.3.1. Rh(D)

The fetal Rh(D) blood type when born to a Rhesus negative mother is an example of one such polymorphism. Additionally, determining the Rhesus blood type of a developing embryo has important clinical implications. The RhD-negative blood group is a moderately rare polymorphism in the general population with approximately 15% of people displaying the Rh(D) negative phenotype (Mourant et al. 1976). Thus any Rh(D) negative mother is likely to have an Rh(D) partner. Depending on whether

he is heterozygote or homozygote positive, she will have a 100% or 50% chance of having an Rh(D)-positive baby (the fetus having paternally inherited the Rh(D)-positive allele). Fetal blood cell antigens are expressed in the first trimester of gestation (Lilford, 1990). This can cause problems in some pregnancies due to fetomaternal haemorrhaging and the subsequent sensitisation of the mother against fetal antigens; i.e. her formation of anti-Rh(D) antibodies (Zipursky and Israels, 1967; Bowman and Pollock 1987; Mollison et al. 1987). Future pregnancies of the sensitised mother are also at risk. The problem can be avoided by giving the mother anti-D prophylaxis, however it is still advantageous to determine the Rh(D) type of the fetus during pregnancy. Obtaining a sample of fetal DNA by chorionic villus sampling will enable the determination of the fetal Rh(D) type, but the sampling process itself, aside from its own inherent risks, may induce sensitisation. This is indicated by the rise in alpha-fetoprotein in the maternal peripheral blood circulation (due to fetomaternal haemorrhaging) immediately after the procedure (Warren et al. 1985). Thus detecting the Rh(D) type of an unborn fetus using TCC samples has major clinical implications. Rh(D) type was first prenatally diagnosed using PCR by Bennet et al,. (1993). If, by using the same assay on TCC cells, the fetal Rh(D) could be assessed this would have significant clinical applications.

### 1.12.3.2. Short tandem repeats (STR)

The DNA of many species contains short sequences of nucleotides that are reiterated from a few to several thousand time, ultimately forming sequences of various length (Litt and Luty,1989; Edwards et al.,1991; Sutherland et al.,1994). The terminology used to describe these tandem repeats still differs, but one particular group is often referred to as microsatellite and consists of repeated DNA units formed by 2 to 6 base pairs (bp), while the term minisatellite is reserved for repeats up to 100 bp. A common type of tandem repeat is formed by two bp, with the bases AC (adenosine and cytosine) on one DNA strand and TG (thymidine guanine) on the other. These dinucleotide repeats are a frequent source of human genetic DNA variations and are readily detectable with polymerase chain reaction (PCR) assays. Since the number of copies of a repeated unit at one locus often differs in the two homologous chromosomes - thus producing heterozygosity - these microsatellites are frequently used as markers of choice for the construction of genetic linkage maps. Some triplet

repeat sequences - CTG (CAG), CGG (GCC) and GAA (CTT) -are associated with specific genetic disorders and fragile sites.

Since 1993, the less frequent tetranucleotide repeats, here referred to as small (or short) tandem repeats (STRs), have been used to investigate specific chromosome disorders (Mansfield,1993; Pertl et al.1994,1996;1997; Adinolfi et al.,1994) in view of their polymorphism and stability during the life of an individual. They are also claimed to be more suitable for multiplex quantitative assays due to their minimal production of stutter bands during PCR tests.

Before the introduction of QF-PCR, PCR amplification of tandem repeats was performed using non fluorescent primers to detect aneuploidies and to investigate the origin of the non-disjunction in patients with Down Syndrome (DS) (Mutter and Pomponio,1991; Eggeling et al.,1993). In a study of 87 families with an affected patient, the paternal origin of the additional chromosome 21 was determined in 68 cases (78%) using two loci (Petersen et al.,1991). In a subsequent investigation the paternal origin of this chromosomal abnormality could be established in 93% of cases by using seven different highly polymorphic markers (Petersen et al.,1992). These results were comparable to the rate of success observed in two large collaborative studies in which most PCR products were analysed by southern blot analysis (Antonorakis et al.,1991; Sherman et al.,1991). In a subgroup of families, where cytogenetic and DNA polymorphic markers were compared, discrepant cytogenetic determinations were observed in 3 out of 31 cases (Antonorakis et al.,1991) and in 2 out of 25 cases (Sherman et al.,1991), thus documenting the more accurate diagnostic value of PCR tests with microsatellites.

Due to the highly polymorphic nature of STRs they can be utilised for the investigation of the origin of DNA. By assessing the fetal and maternal STR allele sizes, identification of the paternal STR in TCC samples was used to indicate the presence of fetal DNA.

### 1.12.4. Monoclonal antibodies (McAb)

If a monoclonal antibody could be generated that was specific for trophoblast cells (Mueller et al., 1987; 1990; Bulmer et al., 1995; Sargent et al., 1994a; Martin et al., 1997) this could be used for the identification of fetal cells.

### 1.12.5. Fetal chromosome aneuploidy detection with FISH

Any de-nova fetal chromosomal aneuploidy will obviously be absent in the mother. Therefore detecting cells displaying this aneuploidy will highlight fetal cells. This can be achieved even if fetal cells are present in overwhelming maternal cell background by using the FISH technique with chromosome specific probes.

## 1.13. Fetal cell isolation

All the aforementioned techniques aim to identify a sequence of DNA absent in the mother but present in the fetus from paternal inheritance. The diagnostic applications of such approaches are obviously limited. For meaningful prenatal diagnosis fetal material will have to be isolated in pure form.

### 1.13.1. Magnetically Activated Cell Sorting (MACS)

If a monoclonal antibody that was specific to trophoblast cells could be generated, it may be possible to use magnetic activated cell sorting (MACS) to enrich fetal cells from TCC samples.

### 1.13.2. Identification of trophoblast specific mRNA

As there are no cell surface antigens which are exclusively trophoblast specific, it may be possible to identify an intracytoplasmic marker found only in trophoblast cells. An ideal candidate is the beta subunit of human chorionic gonadotrophin (β-hCG).

Human chorionic gonadotrophin is a glycoprotein derived from placental trophoblast and is composed of two subunits, α and β, which are subsequently joined non-covalently (Laphorn et al., 1994). β-hCG is encoded by a closely linked multigene family (Talmadge et al., 1984). The polypeptide is passed from the developing placenta into the maternal blood circulation, and is first immunologically detectable in maternal serum at around the time of blastocyst implantation (Ahmed and Klopper, 1983). It is extremely abundant in the terminally differentiated syncytiotrophoblastic layer of the villus but absent in proliferating mononuclear cytotrophoblasts (Bonduelle et al., 1988). Maternal decidual expression of β-hCG mRNA has been shown to be below

detection level in Northern blot analysis (Brizot et al., 1995), and the cDNA encoding this polypeptide had been sequenced (Fiddes & Goodman, 1980). Identification of this cytoplasmic transcript will thus unequivocally highlight a syncytial trophoblast.

A new technique for the detection of RNA *in situ* based on sequence-dependent annealing of unlabeled specific oligonucleotide primers to intracellular RNA and subsequent chain elongation catalysed by reverse transcriptase was presented by Mogensen et al., (1991). They showed that biotin-labelled nucleotides can be incorporated and the cDNA synthesised *in situ* can thus be detected using fluorescein-conjugated avidin. A further advance was proposed by Heniford et al., (1993) using an *in situ* assay that coupled reverse transcriptase with the polymerase chain reaction (*in situ* RT-PCR) thus producing numerous transcripts of a desired, cell specific, sequence. This method has been used for the localisation of bicoid transcripts in the *Drosophila* egg and analysis of *Drosophila* embryos, sea urchins, amphibians, teleost fish and rodents (for review see Rosen & Beddington, 1993). Should this method be applicable to TCC samples, it could be possible to identify fetal syncytial clumps and use them for prenatal diagnosis.

### 1.13.3. Micromanipulation

The principle of removing desired cellular material for separate analysis from a contaminating population has been applied for the isolation of fetal nucleated erythrocytes from whole maternal blood (Takabayshi et al., 1994; 1995). If trophoblast cells or clumps of cells were microscopically identifiable, then they too could be removed from the whole TCC sample and examined.

## 1.14. Potential prenatal diagnostic assays

If isolation of pure fetal material does prove possible by one of the aforementioned methods, then the amount of material will be exceedingly small. Should any prenatal diagnostic techniques be applied, they would have to be designed for use on a minute amount of DNA.

### 1.14.1. Aneuploidy detection using PCR

The great majority of chromosomal abnormalities (95%) are due to numerical variations of chromosomes 21, 18, 13, X and Y (Cuckle and Wald,1990). Their diagnosis is usually performed using *in vitro* cultures of nucleated cells (lymphocyte, amniocytes or chorionic cellular elements), followed by the analysis of metaphases stained by conventional procedures (eg. Giemsa) or fluorescent *in situ* hybridization (FISH) (Adinolfi and Crolla,1994). While these procedures allow accurate diagnoses, they are time consuming and require great technical expertise. Their main disadvantage is that definitive results can be obtained only after several days of *in vitro* cultures, thus delaying the time for therapeutic interventions, if required. The proposition of culturing fetal cells from TCC samples is also hampered by the fact that syncytiotrophoblast do not undergo mitosis.

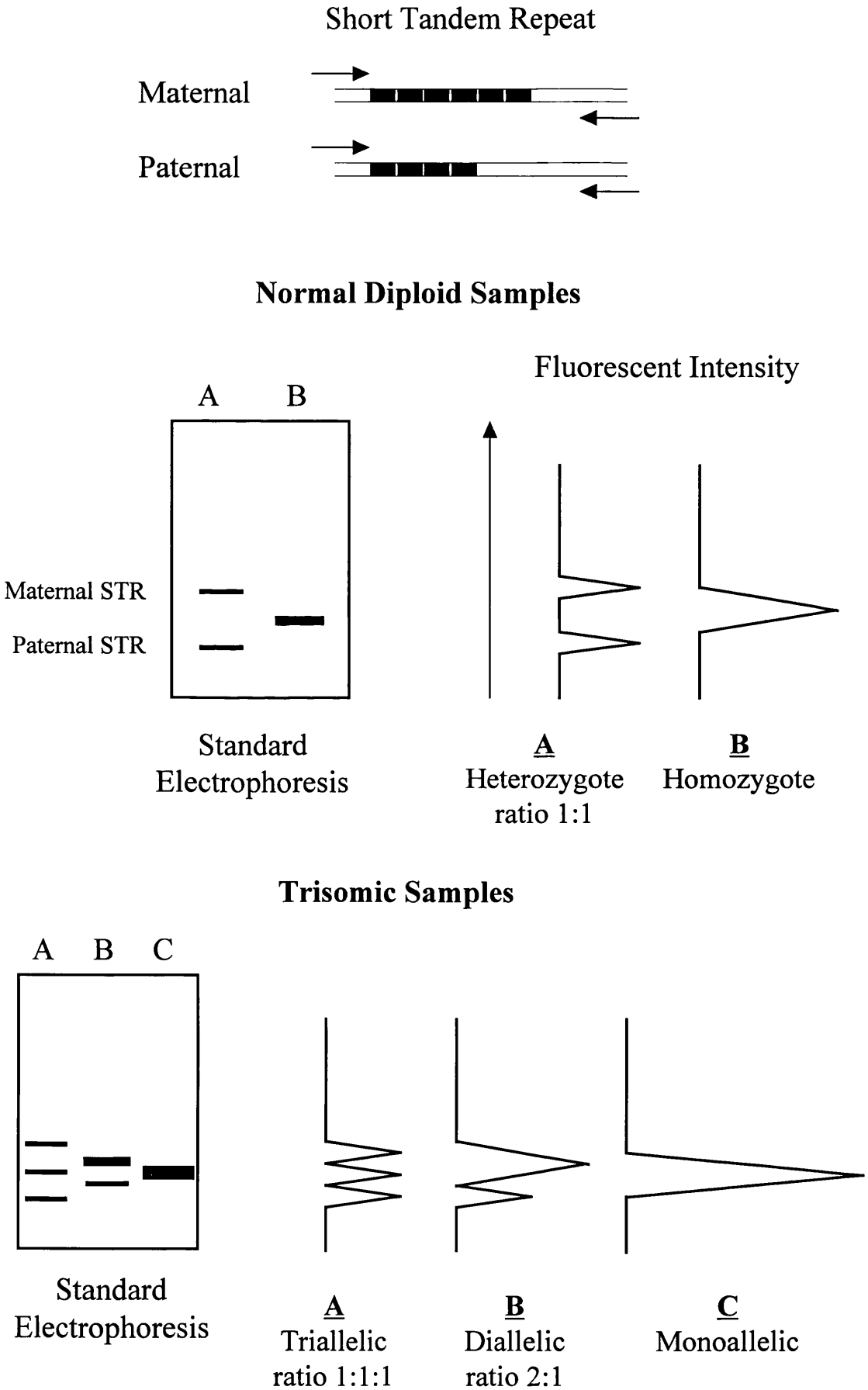
As an alternative, cells in interphase may be investigated using single or multiple colour FISH (Kuo et al.,1991; Davies et al.1994; Adinolfi and Crolla,1994). Tests by FISH on nuclei in interphase are limited to a restricted set of chromosomes and consequently to major abnormalities, even if several chromosome-specific probes are employed. Furthermore amniotic cells seem less suitable for interphase FISH, particularly when tested using probes for chromosome 21 (Davies et al.,1994).

#### 1.14.1.1. STR quantitation

In 1991, Lubin et al. described a precise gene dosage assay using a quantitative PCR strategy in which a chromosome X specific marker was coamplified together with a reference marker and the ratio of the amplification products compared between controls and unknown samples. They tried to apply this strategy to detect trisomies 21 and 18; however several comparisons were required to develop statistically solid results. On the base of these pilot studies, Mansfield (1993) developed a chromosome dosage strategy with fluorescent primers to detect trisomies 21 and 18. The great majority of samples (9 out of 13) from karyotypically proven DS patients produced trisomic triallelic patterns; two showed two peaks with ratios 2:1, while one sample had only one peak. For the detection of trisomies 18, Mansfield (1993) employed two STR markers close to the 5'end of the myelyn basic protein (MBP). All cases of trisomy 18 were clearly diagnosed, but, occasionally, the marker at locus A produced lower trisomic diallelic peak ratios than those expected.

To evaluate the possibility of using quantitative fluorescent PCR (QF-PCR) as a means of aneuploidy detection DNA from uncultured amniotic fluid fetal blood and tissue samples was amplified with the small tandem repeat (STR) marker D21S11. This STR marker has 12 alleles and a heterozygosity of 90% (Sharama and Litt, 1991). Quantitative analysis of the fluorescent intensity of chromosome -21-STR amplification products was expected to show two peaks with a ratio of 1:1 in most normal individuals. Only homozygous subjects would show a single peak. In such cases, the ratio between the internal reference marker and STR fluorescent peaks was expected to be 1:1, thus allowing distinction between homozygous normal and homozygous trisomic samples (Fig 1.6). Trisomy 21 samples were expected to fall into two major groups; those with three STR peaks of similar intensity (1:1:1) (i.e. three different STR alleles) or two peaks with a ratio of 2:1 (two copies of an identical STR allele and one of a different allele). Only a few samples would be expected to have identical STR markers on all three chromosomes 21, and thus to show a single peak of fluorescent activity that could be correctly interpreted by comparison with the area of the internal control peak.

**Figure 1.6 The use of short tandem repeats for aneuploidy detection**



### 1.14.2. PCR assays refined for use on small cell numbers

Having established the reliability of this technique, it was assessed for its reliability for use on a small number of cells, such as would be isolated from a TCC sample. Additional PCR assays were also refined for use on small groups of cells and their reliability assessed. These included the detection of the amelogenin region of the sex chromosomes (Nakagome et al., 1991; Nakahori et al., 1986; 1991b), the 3bp deletion delta-F 508 causing cystic fibrosis, (Rommens et al., 1990), use of the amplification refraction mutation system (ARMS) for the detection of the single base changes causing sickle cell anaemia (Newton et al., 1989) and thalassaemia (Baysal et al., 1992; Athanassiadou et al., 1995).These QF-PCR assays were combined into multiplex QF-PCRs for the simultaneous assessment of numerous loci and chromosome aneuploidy detection, which were applied to trophoblast cell clumps isolated from TCC samples.

### 1.14.3. PCR assays assessed for use on single cells

Should these tests need to be performed on a single isolated fetal cell, there are the potential problems of allele-drop-out (ADO) and preferential amplification (PA) of one allele (Findlay et al., 1995a; 1995b; Ray et al., 1996). Distortion of the starting ratio of DNA sequences may occur when the starting template for PCR is the single genome present in one cell. Single cells were thus tested with all the aforementioned assays and multiplexes to assess the accuracy of single QF-PCR.

## 1.15. Safety of TCC sampling

The entire basis of this research is that should prenatal diagnosis prove to be feasible from TCC samples, this method of fetal cell retrieval is safer than existing invasive procedures. The safety of TCC sample collection was thus assessed on continuing pregnancies.

The ultimate aim of this project is to establish a method to consistently retrieve and isolate fetal trophoblasts from the endocervical canal of pregnant women by a non- or minimally invasive- procedure, and successfully utilise these cells for first trimester prenatal diagnosis.

# Chapter 2

# Materials and Methods

# 2. Materials and Methods

## 2.1. Materials

### 2.1.1. Chemicals

Unless listed below, all chemicals used in this study including those used for the preparation of buffers were supplied by BDH Chemicals, Pool, Dorset and were of AnalaR grade. Additional companies that supplied chemicals are listed below.
Biorad: ammonium persulphate (AMPS), NNN'N'tetramethylenediamide (TEMED).
Fluka Paraformaldehyde
National diagnostics; UltraPure Sequigel, Histopaque
Sigma Chemical Company; ethidium bromide, standard grade agarose, Tween 20 detergent (polyoxyethlene sorbitan monolaurate), phosphate buffered saline (PBS) tablets,
Sodium dodecyl sulphate (SDS)
Vector Laboratories, USA: avidin-FITC Vectashield antifading medium [containing 0.1 μg/ml 4',6-diamidino-2-phenylindole (DAPI) as counterstain]
All water used was distilled and de-ionised.

### 2.1.2. Enzymes

Desiccated Proteinase K (Arthobacter leteus) was provided by Sigma chemicals, as was collagen. Thermostable *Thermus aquaticus* (Taq) DNA polymerase was initially supplied by Super Taq and Promega, UK. In later experiments the DNA polymerase 'Supertaq-Gold' was used, supplied by P.E.- Applied Biosystems, UK. DNA polymerase was also supplied as a component of the Boehringer Mannheim nick translation kit and the Gibco BRL Bio nick kit. These kits also included DNAase I. All enzymes were stored at -20°C. Thermostable rTth Reverse Transcriptase was supplied in kit form from Perkin Elmer-Applied Biosystems. This enzyme acts as a reverse transcriptase or DNA polymerase in the same buffer conditions, depending only on the temperature. Ribonuclease inhibitor was supplied by MMBI Fermentas, Italy. RNAse was supplied by Sigma Chemical Company, as was Heparinase.

### 2.1.3. Nucleic Acids

Deoxynucleoside triphosphates (dATP, dGTP, dCTP, dTTP) for PCR were supplied by Promega, UK. Deoxynucleoside triphosphates used for reverse transcription were provided in kit form from PE-ABI. DNA size standards (100bp ladder, 1Kb ladder) were supplied by Gibco BRL and stored at -20°C. Oligonucleotide primers for PCR were obtained from Oswel DNA services or P.E.- Applied Biosystems and were diluted to 10pM/µl working concentration, aliquoted and stored at -20°C (Table 2.1). Primers for fluorescent PCR and analysis on the A.L.F (Section 2.2.5.3.2.a.) were provided by Oswell DNA Services and 5' end labelled with fluorescein. Primers for fluorescent PCR and analysis on the Prism™ 310 (Section 2.2.5.3.2.c.) were supplied by PE-ABI and 5' end labelled with FAM [6-carboxyflluorescein] (blue), TET [4, 7, 2', 7'-Tetrachloro-6-carboxyfluorescein] (green), or HEX [4, 7, 2', 4', 5', 7'-Hexachloro-6-carboxyfluorescein] (yellow). Salmon/herring sperm DNA was supplied by Sigma Chemical Company at a concentration of 10mg/ml and was kept at 4°C.*Cot* 1 DNA was proved by (BRL Life Technologies).

The centromeric alpha satellite probe specific for chromosome 18 employed for fluorescent in situ hybridisation was purchased from Oncor, UK.

### 2.1.4. Solutions and Buffers

All solutions and buffers were made using distilled de-ionised water. To increase shelf life solutions were sterilised by autoclaving at 15lbs psi (per square inch), 121°C for 30 minutes. Unless otherwise stated storage was at room temperature (15-25°C).

**AD buffer**

0.2M NaOH, 1% SDS

**Anti-fade medium**

9 parts 2% 1,4-diaza-bicyclo-(2,2,2)-octane (DABCO); 1 part 0.2M Tris-HCl, pH7.5, 0.02%$NaN_3$; 0.5µg/ml 4,6-diamidino phenylindole (DAPI). Stored at -20°C and protected from light.

**Bacterial lysis buffer**

50mM glucose, 10mM EDTA, 25mM Tris, pH8

**Red Blood Cell Lysis Buffer** (Method 1)

0.32M sucrose, 10mM tris-CL pH7.5, 5mM $MgCl_2$ 1% v/v triton X-100, autoclaved and stored at 4°C

**Cosmid broth**

1.2%w/v tryptone, 2.4% w/v yeast extract, 0.5% v/v glycerol, 0.072M $K_2HPO_4$, and 0.028M $KH_2PO_4$

**CVS buffer**

150 mM NaCl, 25 mM EDTA in sterile $H_2O$, pH7, stored at 4°C

**Heparinase buffer**

20mM Tris, 4mM $CaCl_2$, 0.01% Albumin, 50mM NaCl, pH7.5.

**Hybridisation mix**

50% deionised formamide; 20% w/v dextran sulphate; 2X SSC; 0.1mM EDTA, pH8; 0.2mM Tris-HCl, pH7.6

**Loading buffer** (agarose gels) (10X)

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

**Loading buffer** (for ALF)

10% dextran blue; 90% formamide

**MACS buffer**

PBS with 1% BSA

**Paraformaldehyde buffer**

1% Paraformaldehyde (Fluka); 1% w/v $MgCl_2$

**PBS** (phosphate-buffered saline)

0.0027M KCl, 0.137M NaCl, 0.01M $Na_2HPO_4$, 0.01M $KH_2PO_4$, pH7

**Polyacrylamide** (6%; 8M Urea)

210g Urea, 75ml 40% acrylamide, de-ionized with duolite (Sigma, UK) made up to 500ml with $H_2O$.

**Proteinase K buffer**

20mM Tris-HCl, pH7.5; 2mM calcium chloride ($CaCl_2$)

**SET (1X)**

10 mM Tris-CL pH7.5, 10 mM NaCl, 1 mM EDTA

**SSC (20X)**

75.3g NaCl, 88.2g Sodium Citrate in 1 litre of $H_2O$, pH7 (adjust with NaOH)

**SSCM**

4X SSC with 5% Marvel™ non-fat dried milk

**SSCT**

4X SSC with 0.05% Tween 20

**Super-Taq Polymerase Buffer**

PE-ABI, UK (10X)

**T 0.1E**

100mM Tris-HCl, 1mM EDTA in sterile $H_2O$, pH7

**Taq Polymerase Buffer**, [in house] (10X)

16.6mM $(NH4)_2$ $SO_4$; 67mM Tris-HCl, pH8.3; 1.5mM $MgCl_2$; 10mM Beta-mercaptoethanol and 170 μM bovine serum albumin (BSA)

**Taq Polymerase Buffer**,

Promega, UK (10X)

**Taq Polymerase Buffer**

Supertaq, UK (10X)

**TBE**

90mM Tris-HCl, pH8; 90mM; boric acid; 2mM EDTA- made up as a 10X concentrate.

**TE**

10mM Tris-HCL, pH8; 0.1M EDTA pH8

**TKM1**

0.24g TRIS, 0.15g KCL, 0.41g $MgCl_2.6H_2O$, 0.15g EDTA in 200ml $H_2O$, pH7.6

**TKM2**

100ml TKM1, 2.34g NaCl

**TN**

0.1mM Tris-HCL, pH7.5; 0.15M NaCl. Made as a 10X stock

**TNB**

As TN but with the addition of 0.5% blocking agent (Boeringer Mannheim), dissolved after incubation for three hours at 60°C, filtered through 1mm filter paper (Whatman) and stored at -20°C.

**TNE**

0.2M NaCl; 10mM Tris-HCl, pH8.0; 1mM EDTA

### 2.1.5. Kits

| | |
|---|---|
| Qiagen, Uk: | QIAamp® Tissue DNA extraction kit |
| Boehringer Mannheim: | Nick translation kit |
| Gibco BRL: | Bio-nick kit |
| PE-ABI: | GeneAmp® EZ rTth RNA PCR Kit |

### 2.1.6. Cell culture media

Cell culture flasks (50ml and 200ml) were supplied by Nuclon$^{TM}$. RPMI 1640 medium, non-essential amino acids, phytohaemaglutinin (PHA) and the antibiotics glutamine, penicillin, and streptomycin were supplied by Gibco. Iscoves modified DMEM medium was obtained from Imperial, while fetal calf serum was purchased from Globepharm Ltd. Storage was at -20°C except for RPMI, Iscoves , and non-essential amino acids which were stored at 4°C.

**Iscoves medium**: For the culture of 1ml of blood: 17ml of Iscoves medium; 2ml GPS (glutamine, penicillin, and streptomycin); 2ml fetal calf serum; 200µl of phytohaemaglutinin (PHA).

**RPMI medium**: For 100ml: 76ml sterile distilled water; 9ml 10X RPMI; 10ml fetal calf serum; 1ml GPS (glutamine, penicillin and streptomycin); 1ml non-essential amino acids; 3ml 5.3% sodium hydrogen carbonate ($NaHCO_3$); 0.5-1.5ml NaOH. Stored at 4°C.

### 2.1.7. Growth of Bacteria containing FISH probe inserts

Casamino acids, Bacto-agar, and Bacto-yeast nitrogen base (minus amino acids) required as components of S.D. media were supplied by Difco, while β-mercaptoethanol, ampicillin, adenine, and tyrosine were all provided by Sigma Chemical Company. Glycerol, sucrose and glucose were supplied by BDH.

### 2.1.8. FISH materials and solutions

Nick translation reagents were supplied in kit form by Boehringer Mannheim and Gibco BRL. Sigma Chemical Company supplied the mouse anti-digoxin (MαD), rhodamine conjugated rabbit anti-mouse (RαM), and rhodamine digoxigenin labelled probes. Detection of biotinylated probes required fluorescein isothiocyanate (FITC) conjugated avidin, and biotinylated anti-avidin from Vector. All components of the immunocytochemical detection were stored at -20°C. Propidium iodide and diamidiophenylindole (DAPI) counterstains were supplied by Sigma Chemical Company and stored at 4°C, the anti-fade mounting medium was supplied by Vector Labs.

### 2.1.9. FISH Probes

The following probes were used during this study;

**pSV2X5** (Pertl et al., 1994).

Repetitive sequence, specific to the human X chromosome centromere, labelled with digoxigenin.

**PBam X5** (Willard et al., 1983)

An alphoid probe of 2Kb insert size, specific for the centromeric region of the human X chromosome, directly labelled with fluorescein-12-2'deoxouridine-5' triphosphate (FITC-12-dUTP, Fluorogreen, Amersham, UK 2121)

**cY98** (Courtesy of Dr. Johnathon Wolfe).

An alphoid probe recognising a 3.4Kb repeat on the long arm of the human Y chromosome long arm, directly labelled with rhodamine-4-deoxuridine-5'-triphosphate (TRITC, Fluorored, Amersham, UK RPN 2122)

**Chromosome 18 centromeric alpha satellite** probe was purchase from Oncor, UK

**YAC 896** (Human Genome Mapping Project Research centre; Hinxton Hall, Cambridge UK)

This YAC probe of 800kb mapped specifically to human chromosome 13. To enable 3 colour FISH to be employed one aliquot of this probe was labelled with fluorescein (green) and the other with rhodamine (red). Equal amounts of these aliquots were then mixed producing a yellow signal, distinguishable from pure green or red.

**Y190** (Courtesy of Dr. Eric Muller).

Repetitive sequence, specific to the human Y chromosome centromere, labelled with biotin.

**cCMP21.2/6** (Zheng, 1992).

Single copy cosmid contig mapping proximal to human Chromosome 21q22, labelled with biotin.

**242c** (Davies et al., 1994).

Single copy cosmid contig mapping to human Chromosome 21q22.3, labelled with digoxigenin.

Alpha satellite centromeric repeat probes for chromosomes 18, 13 and 1 were initially provided by Oncor and latterly grown in transfected *E.coli* following standard protocols.

## 2.2. Methods

### 2.2.1. Isolation extraction and purification of DNA

#### 2.2.1.1. DNA extraction from blood

#### 2.2.1.1. a. **Method 1** (Standard method)

All laboratory procedures were carried out under sterile conditions. Fresh blood (2-5ml) was obtained from the peripheral circulation in sterile EDTA (ethylenediaminetetra-acetic acid) tubes. The blood was transferred into a sterile centrifuge tube, to which 10ml of Red Blood Cell Lysis Buffer (Section 2.1.4.) was added. The centrifuge tube was spun at 4°C, 600g for 20min, and the supernatant poured off and discarded. Using a Pasteur pipette, the pellet was re-suspended in 4.5ml 1xSET (Section 2.1.4.), 250ml 10% SDS (10g SDS in 100ml autoclaved distilled water), and 100µl Proteinase K (at 10mg/ml $H_2O$). This suspension was incubated overnight at 37°C.

After incubation, 2.5ml of phenol (TE saturated) was added to the solution, and the tube placed on a roller for 10min, followed by centrifugation at 1600g for 5min at room temperature. The aqueous (top) layer was then removed using a 1000µl hand pipette (Gilson) and a pipette tip which had been cut to make a wider bore, thus reducing the area exposed to suction and allowing delicate aspiration. This aqueous layer was added to 5ml phenol/isoamyl-alcohol/chloroform (25:1:24), then set aside. To the organic (bottom) layer, 2.5ml of 1X SET was added and the tube placed on a roller for 10min, spun at 1600g for 5min. The aqueous layer was removed as before, and added to the phenol/ isoamyl-alcohol/ chloroform mix previously set aside. This was placed on a roller for 10min, and centrifuged at 1600g for 5min. The aqueous layer was once more removed and added to 5ml of isoamyl-alcohol/chloroform (1:24). This was rolled and centrifuged as before. The aqueous layer was then removed, added to 5ml of isoamyl-alcohol/chloroform (1:24), rolled and centrifuged again. The aqueous layer was finally placed into a tube, to which was added 1/10 volume 3M Sodium acetate, and 2 volumes of cold 100% ethanol to precipitate the DNA. This tube was centrifuged for 10min at 700g and the liquid poured off the white DNA pellet. The pellet was either freeze dried for 15min or left to dry by evaporation at room

temperature for 2 hours. The DNA pellet was suspended in 200ml T0.1E (Section 2.1.4.).

### 2.2.1.1. b. Method 2 (Lahiri et al., 1991)

This method of DNA extraction was adopted during the later stages of this project, as the protocol was far quicker, the solutions used non-hazardous and the yield of DNA was found to be equivalent to that achieved by the standard method. The 2-5ml of blood, collected in an EDTA anti-coagulant tube, was transferred into a centrifuge tube containing 5ml of TKM1 (Section 2.1.4.), and 125µl of nonidet p40 (BDH, UK). These were well mixed by inversion and shaking. The tube was then centrifuged at 850g for 10min and the supernatant carefully decanted. The pellet was then re-suspended in 5ml TKM1 and 125µl nonidet p40 and spun as before. These washes and spins were repeated until the redness of the pellet was no longer apparent. The pellet was then thoroughly re-suspended in one drop of TKM1. To this cell suspension, 0.8ml of TKM2 (Section 2.1.4.) and 50µl of 10% SDS were added and mixed thoroughly by pipetting up and down. The centrifuge tube was then incubated for 30-120min at 55°C until all visible lumps dissolved. To this solution, 300µl of 6M NaCl was added and mixed well. This mixture was then transferred into a 1.5ml microcentrifuge tube and centrifuged at 13000g for 5min. The supernatant was then removed and placed into a new microcentrifuge tube. Two volumes of 100% ethanol were added to the supernatant at room temperature, and the tube inverted until the DNA was seen to precipitate. The DNA was spun down at 13000g for 10min, the supernatant carefully poured off and the pellet washed in 70% ethanol at -20°C before being re-suspended in 200µl T 0.1E.

### 2.2.1.1. c. Method 3 (Qiagen QIAamp® Blood kit)

Two hundred microlitres of blood from an EDTA collection tube were transferred to a clean 1.5ml microcentrifuge tube and extraction performed according to manufacturer's instructions.

#### 2.2.1.1.1. Heparinase Treatment

Any DNA extracted from blood that had been collected in Heparin tubes had to be treated with heparinase prior to PCR. The DNA pellet was dissolved in Heparinase buffer (Section 2.1.4.) to a concentration of 0.5μg/μl DNA. To this sollution10μl of Heparinase (0.1U/μl stock) was added prior to an incubation at 25°C for 2 hours.

### 2.2.1.2. DNA extraction from Placental Tissue

#### 2.2.1.2.a. Method 1 (Standard method)

Approximately 0.25g of villus placenta was isolated from aborted material in a sterile hood using dissection scissors and forceps. This material was extensively broken up with the scissors and suspended in 500ml CVS buffer (Section 2.1.4.) in a 1.5ml microcentrifuge tube. To this tube, 10μl 10% SDS and 10μl Proteinase K (10mg/ml) were added and mixed. The tube was then incubated overnight at 37°C.

Following incubation, 500μl phenol/isoamyl-alcohol/chloroform (25:1:24) was added to the tube and mixed well by inverting for 2-3min. The tube was then spun at 13000g for 2min and the aqueous layer transferred to a fresh tube using a hand pipette (Gilson) and cut tip as with blood extraction **Method 1**, taking care not to transfer any of the interface. Then, 500μl of phenol/isoamyl-alcohol/chloroform was again added and mixed prior to centrifugation as before. The aqueous layer was transferred to a 1.5ml tube containing 500μl isoamyl-alcohol/chloroform (1:24) and mixed well. The tube was centrifuged as before and the aqueous layer transferred to another 1.5ml tube containing 500μl isoamyl-alcohol/chloroform. After spinning again at 13000g for 2min the aqueous layer was then transferred to a clean 1.5ml tube to which 2 volumes of 100% ethanol and 1/10 volume 4M NaCl were added and mixed by inversion until the DNA was precipitated. The tube was then centrifuged at 13000g for 10min at 4°C and the supernatant removed by aspiration with a hand pipette. The DNA pellet was then washed with 70% ethanol before being re-suspended in 150μl T.01E.

#### 2.2.1.2.b. Method 2 (Qiagen QIAamp® Blood kit)

Approximately 0.1g of villus placenta was isolated as before and meticulously macerated with sterile scissors. The placental tissue was then suspended in 200μl PBS

(Section 2.1.4.) and substituted for whole blood with the Qiagen extraction procedure (Blood DNA extraction **Method 3**).

### 2.2.1.3. DNA extraction from whole Transcervical Cell Samples (TCC)

If excess mucus was present in any TCC sample it was treated with acetyl cystine prior to any DNA extraction procedure (Section 2.2.7.2.a.).

#### 2.2.1.3.a. **Method 1** (Standard method)

Samples were transferred to a 1.5ml microcentrifuge tube and centrifuged at 13000g for 5min. The supernatant was removed and the cell pellet re-suspended in 100µl of CVS buffer, to which 2-5µl of proteinase K (10mg/ml) (according to pellet size) and 5µl of 10% SDS were added. The tube was then vigorously mixed by vortexing and incubated at 37°C overnight. Standard DNA extraction with Phenol/ isoamyl-alcohol/ chloroform then proceeded exactly as if for placental samples (**Method 1**) except 1/5 of all reagent volumes were used.

#### 2.2.1.3.b. **Method 2** (Qiagen QIAamp® Blood kits)

The whole TCC sample was transferred to a 1.5ml tube and centrifuged at 13000g for 5min. The supernatant was removed and the cell pellet re-suspended in 200µl of PBS (Section 2.1.4.). The TCC cells in suspension were substituted for 200µl of whole blood in the Qiagen kit and the same protocol followed with the exception of the final step: Due to the smaller number of cells in the starting 200µl solution, the bound DNA was finally eluted with 50µl of $H_2O$ to increase the concentration of DNA.

### 2.2.2. Single cell and clump isolation

### 2.2.2.1. Cell preparation

Due to the possibility of contamination, all isolation procedures were carried out in a class II/Laminar flow hood. Cell clumps and single cells were isolated using

micromanipulation with plug tips (Anachem, UK) under an inverted microscope (Tutschek et al., 1995).

Cells were obtained from transcervical cell samples, cultured fibroblasts, buccal mouth washes, whole blood and cultured chorionic villus samples (CVS). Fibroblast cell cultures were washed twice with Hanks balanced salt solution (Gibco, UK), then incubated at 37°C for 2min in 5ml of 20:1 Versene (2g EDTA in 1 litre Hanks): 2.5% trypsin (Gibco UK). Three drops of fetal calf serum were then added to halt the enzymatic action. The cell suspension was spun at 180g for 5min, the supernatant removed, and the cells re-suspended in sterile phosphate buffered saline (PBS) to achieve a suitable cell density for micromanipulation.

Buccal mouth washes were spun at 180g for 5min and the supernatant replaced by sterile PBS. This was repeated and the cells re-suspended in a suitable volume for micromanipulation.

Whole blood (5-10ml) was added to an equal volume of Histopaque (Sigma Diagnostics, UK) and spun at 700g for 20min. The cell layer containing lymphocytes was removed and diluted with sterile PBS to a suitable cell density for single cell isolation.

CVS cultures were performed according to standard cytogenetic protocols (Heaten et al., 1984), [kindly provided by UCL cytogenetics department] and exposed to trypsin treatment as with fibroblasts. The cell suspension was then spun at 180g for 5min and the supernatant replaced by sterile PBS. This was repeated and the cells re-suspended in a suitable volume of sterile PBS for micromanipulation.

### 2.2.2.2. Cell isolation

Cell clumps with the morphology of trophoblast intended for diagnosis were collected directly in 2µl from TCC samples. If excess mucus prevented easy pipetting, the TCC sample was treated with acetyl cystine (Section 2.2.7.2.a.). Positive control placental cell clumps were obtained by shaking placenta derived from TOP in sterile PBS and collecting free placental villi in 2µl of solution with a p2 Gilsen hand pipette while viewing under an inverted microscope. Control 'clumps' from all other sources consisted of 10-50 cells collected in 2µl of solution using inverted microscopy (Olympus).

To isolate single cells, approximately 50µl of cell suspension was pipetted onto a 5cm diameter sterile petri dish (Steralin, UK). Approximately five 50µl droplets of

sterile water were placed around the cell suspension. While viewing under the inverted microscope (Olympus) one cell was aspirated from the solution using a p2 Gilsen hand pipette reserved for this purpose alone. This cell was then pipetted into a surrounding water droplet to confirm that only a single cell had been collected. This process was repeated with at least one further droplet prior to the final transfer of the single cell, in a 2µl volume, into individual 0.5ml microcentrifuge tubes ready for cell lysis and PCR.

The method used to manipulate the single cell into the PCR mix had the shortfall of not being able to see the cell entering the tube. At each washing step it was certain that only one cell was present in the final drop of medium, however, due to the opaque nature of the PCR tube, the cell could not be seen entering, or leaving the tip at the final expulsion. It was noticed that occasionally cells would adhere to the inside of tips and would not be expelled. The purpose of this part of the study was to assess quantitative fluorescent PCR (QF-PCR) amplification from a single genome, not the efficiency of single cell collection. It was thus considered that the methodology used was acceptable.

### 2.2.2.3. DNA extraction/preparation from cell clumps and single cells.

Single cells, or cell clumps ready for DNA preparation were in 2µl sterile PBS/medium/water in a 0.5ml microcentrifuge tube. The extraction procedure was identical for both clumps and single cells, with the exception of the concentration of Proteinase K. To each tube 1µl of 17µmol/l sodium dodecyl sulphate (SDS), 2µl of Proteinase K (125mg/ml for single cells; 400mg/ml for cell clumps) and 1 drop of paraffin oil (Sigma) were added prior to incubation at 37°C for 30-60min and then at 99°C for 15min. The tube was pulse centrifuged for 5sec and the single cells, now in a 5µl volume, were used for PCR within 1 hour, or stored at -20°C for up to 3 months. For each single cell experiment a negative control consisting of 2µl of cell media, underwent the same DNA preparation and subsequent PCR reaction. An additional negative control of 5µl water in place of DNA was used to test every PCR mix. If either of the negative controls showed the presence of contaminating DNA after the PCR assay, the results of the entire set of tests were discarded.

### 2.2.3. DNA extraction from Fibroblast, Lymphoid, and CVS cultures

The Qiagen® DNA extraction kits (Section 2.2.1.1.c.) were also used for the extraction of DNA from fibroblast, lymphoid and CVS cultures. The volume of culture available for DNA extraction varied greatly from the 200µl necessary for use with the Qiagen kit. If the volume was greater than 200µl, the culture was spun in a centrifuge tube at 400g and sufficient supernatant removed to leave 200µl. If the volume was insufficient, PBS (Section 2.1.4.) was added up to the 200µl mark. The cultured cells, now in a volume of 200µl, were substituted for 200µl of whole blood in the Qiagen kit and the same protocol followed.

### 2.2.4. Fluorometry

The concentration of extracted DNA was determined using a Hoefer Scientific Instruments TKO 100 Fluorometer according to the manufacturer's instructions.

### 2.2.5. The Polymerase Chain Reaction (PCR)

Polymerase chain reactions were all performed in a total volume of 25µl. Three different PCR machines were employed; Omnigene (Hybaid), Touchdown (Hybaid) and PCR cycler 2400 (PE-ABI). The PCR conditions desired were accurately reproduced on all three machines as described below.

#### 2.2.5.1. Oligonucleotides

Numerous oligonucleotides were utilised through the duration of this study in varying combinations (Table 2.1). All primers were replicas of published oligonucleotides (Sharma and Litt, 1992; Utah marker development group, 1995; Polymeropoulos 1992; Boylan 1990; Edwards, 1992) with the exception of the fluorescent amelogenin primers which were specifically designed from the published sequence of this region (Nakahori et al., 1991a;1991b). For fluorescent analysis of PCR products the forward primer from each set was 5'-end labelled with a fluorescent dye (Section 2.2.5.3.2.). For analysis on the Pharmacia Automated Laser Fluorescence (ALF) 5'-fluorescein was utilised (Oswell, UK). For analysis on the PE-ABI Genescan 373 primers were 5'-end-labelled with either 2',7'-dimethyloxy-4', 5'-dichloro-6-

carboxy-fluorescein (green) or 5-carboxy-fluorescein (blue) (PE-ABI). For analysis on the ABI Prism™ 310 dyes 6-FAM, [6-carboxyfluorescein] (blue), TET [4, 7, 2', 7'-Tetrachloro-6-carboxyfluorescein] (green), or HEX [4, 7, 2', 4', 5', 7'-Hexachloro-6-carboxyfluorescein] (yellow) with filter set C were used. When a primer set was used individually, each primer was included at a concentration of 5pM in the 25μl final reaction volume. When combined in multiplex reactions, primer concentrations were altered to adjust for more and less efficient individual reactions so producing equivalent final PCR product amounts (Table 2.1). This was achieved through the comparison of PCR product yields generated from single primer set reactions performed on the same concentration of DNA.

**Table 2.1** Primers employed for PCR assays, their chromosomal location, label, concentration (conc.) within a multiplex, heterozygosity where appropriate and product size.

| Marker Name (F= forward) (R= reverse) | Sequence of Primers | Chromosome Location | Heterozygosity | Size (bp) | Conc. (pM) | Label | Reference |
|---|---|---|---|---|---|---|---|
| D21S11 (F)<br>D21S11 (R) | 5'-tat gtg agt caa ttc ccc aag tga-3'<br>5'-gtt gta tta gtc aat gtt ctc cag-3' | 21q21 | 0.93 | 172-264 | 20<br>20 | 6-FAM (blue)<br>- | Sharma and Litt (1992) |
| D21S1414 (F)<br>D21S1414 (R) | 5'-aaa tta gtg tct ggc acc cag ta-3'<br>5'-caa ttc ccc aag tga att gcc ttc-3' | 21q21 | 0.88 | 330-380 | 2.5<br>2.5 | 6-FAM (blue)<br>- | Genome data base |
| D21S1411 (F)<br>D21S1411 (R) | 5'-atg atg aat gca tag atg gat g-3'<br>5'-aat gtg tgt cct tcc agg c-3' | 21q22.3 | 0.93 | ≥239 | 17.5<br>17.5 | HEX<br>- | Utah marker development group (1995) |
| D21S1412 (F)<br>D21S1412 (R) | 5'-cgg agg ttg cag tga gtt-3'<br>5'-ggg aag gct atg gag gag a-3' | 21 | 0.80 | ≥305 | 5<br>5 | 6-FAM<br>- | Utah marker development group (1995) |
| D18S535 (F)<br>D18S535 (R) | 5'-tca tgt gac aaa agc cac ac-3'<br>5'-aga cag aaa tat aga tga gaa tgc a-3' | 18q12.2-12.3 | 0.92 | ≥138 | 5<br>5 | TET<br>- | Genome data base |
| MBP (F)<br>MBP (R) | 5'-gga cct cgt gaa tta caa tc-3'<br>5'-att tac cta cct gtt cat cc-3' | 18q23-q ter | Locus A- 0.80<br>Locus B- 0.79 | A- 102-124<br>B- 208-232 | 20<br>20 | 6-FAM<br>- | Polymeropoulos (1992); Boylan (1990) |
| D13S634(F)<br>D13S634(R) | 5`- tcc aga tag gca gat gat tca at-3`<br>5`-cct tct tct tcc cat tga ta-3` | 13q14.3-22 | 0.81 | ≥375 | 20<br>20 | HEX<br>- | Genome data base |
| D13S631(F)<br>D13S631(R) | 5`-ggc aac aag agc aaa act ct-3`<br>5`-tag ccc tca cca tga ttg g-3` | 13q31-32 | 0.94 | ≥209 | 2.5<br>2.5 | HEX<br>- | Genome data base |
| XPHRT(F)<br>XPHRT(R) | 5'-atg cca cag ata ata cac atc ccc-3'<br>5'-ctc tcc aga ata gtt aga tgt agg tat-3' | Xq26.1 | 0.73 | 260-302 | 5<br>5 | FAM<br>- | Edwards et al., 1991 |
| HbS (F)<br>HbS (R) normal<br>HbS (R) mutant | 5'-acc tca ccc tgt gga gcc ac-3'<br>3'-tcg tct tca gac ggc aat gac ggg aca c-5'<br>3'-acg tct tca gac ggc aat gac ggg a-5' | 11 | - | norm- 203<br>mut- 199 | 10<br>5<br>5 | 6-FAM<br>none<br>none | Modified after Newton et al., 1989 |
| IVS1-110 (F)<br>IVS1-110 (R) normal<br>IVS1-110 (R) mutant | 5'-acc tca ccc tgt gga gcc ac-3'<br>5'-acc agc agc cta agg gtg gga aaa tac acc-3'<br>5'-acc agc agc cta agg gtg gga aaa tag agt-3' | 11 | - | 389 | 20<br>10<br>10 | none<br>6-FAM<br>HEX | Modified after Newton et al., 1989 |
| CF (F)<br>CF (R) | 5'-gtt ttc ctg gat tat gcc tgg cac-3'<br>5'-gtt ggc atg ctt tga tga cgc ttc-3' | 7 | - | norm- 100<br>mut- 97 | 10<br>10 | 6-FAM<br>- | Courtesy of Dr. Boris Tutschek |
| AMXY (F)<br>AMXY (R) | 5'-ctg atg gtt ggc ctc aag cct gtg-3'<br>5'-taa aga gat tca tta act tga ctg-3' | Xp22.1-22.3<br>Yp11 | - | X- 1000<br>Y- 823 | 30<br>30 | unlabelled | Nakahori et al., (1991b) |
| AMXY (F)<br>AMXY (R) | 5'-ctg atg gtt ggc ctc aag cct-3'<br>5'-atg agg aaa cca ggg ttc ca-3' | Xp22.1-22.3<br>Yp11 | - | X- 432<br>Y- 252 | 10<br>10 | HEX<br>- | Sherlock et al., 1998 |

### 2.2.5.2. Amplification

PCR reactions performed on extracted DNA were set up on a clean bench using sterile pipette tips. All PCR reaction mixtures for single cells and cell clumps were set up in a laminar flow cabinet using dedicated Gilson pipettes and sterile, DNA free filter plug pipette tips (Anachem).

Amplification of the sex chromosome amelogenin region, with standard agarose electrophoresis and ethidium bromide PCR product identification was performed in a 25μl reaction volume as follows: 5μl of sample or control DNA (at ~25ng/μl) was added to a 15μl PCR reaction kit containing 0.25μl of both forward and reverse primers (10pM stock), (after Nakahori et al. 1991b); 1μl of dNTP (5mM), 2.5μl of enzyme reaction buffer (Super Taq), and made up to 25μl volume with sterile $H_2O$. The following Hot Start PCR (Chou et al., 1992) was then performed:

| | | | |
|---|---|---|---|
| Step 1: | 94°C | 15min. | |
| | | | |
| Step 2: | 94°C | 1min. | |
| | 65°C | 1min. | |
| | 72°C | 2min. | Step 2 for 35 cycles |
| | | | |
| Step 3: | 72°C | 5min. | |

One unit of taq polymerase (Super Taq) in T.01E was then added to the PCR mix under the mineral oil.

PCR products were stored at 4°C until electrophoretic assessment (Section 2.2.5.3.1.).

All fluorescent PCR reactions, whether employed for the PCR of extracted genomic DNA, cell clumps, or single cells were identical as regards the reaction temperatures, Hot Start protocol and PCR reagent concentrations in the PCR mix. Only the number of PCR cycles varied; 19 for 400ng of extracted DNA; 24 for 50ng of extracted DNA; 30 for a clump of 10-50 cells; and 35 for a single cell.

PCR mastermixes were prepared containing varying numbers and amounts of fluorescent primers (Table 2.1), and final concentrations/volumes of the following; 0.2mM of each 2'-deoxynucleoside 5'-triphosphate (dATP, dGTP, dCTP, dTTP), 2.5µl of 10X enzyme reaction buffer (16.6mM $(NH_4)_2 SO_4$; 67mM Tris-HCl, pH8.3; 1.5mM $MgCl_2$; 10mM Beta-mercaptoethanol and 170µM bovine serum albumin; [latterly supplied by Promega]), (1.5µl of $MgCl_2$ (from 25mM stock, Promega, UK) if Promega ready made $MgCl_2$ free buffer was employed. In a 0.5ml microcentrifuge tube 15µl of this mix was added to either 5µl DNA, or 5µl solution containing a prepared single cell or cell clump and overlaid with approximately 50µl mineral oil (Sigma). The 20µl volume within the PCR tube was then heated to 94°C for at least 5min prior to the addition of 5µl T0.1E containing 1.5 units of taq (Promega, UK) under the oil in the Hot Start PCR method (Chou et al., 1992). The following PCR cycling was performed:

| | | | |
|---|---|---|---|
| Step 1: | 94°C | up to 15min (Hot start). | |
| Step 2: | 93°C | 48sec | |
| | 60°C | 48sec | |
| | 72°C | 1min | Step 2 for 19-35 cycles |
| Step 3: | 72°C | 5min | |

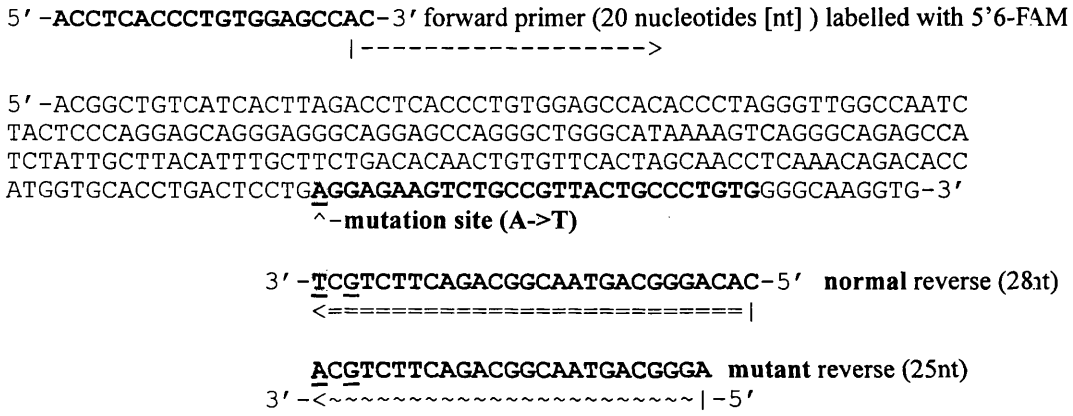
When Super-Taq-Gold (PE-ABI) was used with single cells in the later stages of the project, the need for Hot Start PCR was removed. 1.5 units of the Super-Taq - Gold enzyme was added directly into the PCR mastermix and 20µl added to the 5µl solution containing the single cell. In addition, the initial 15min step at 94°C was increased to 15min at 96°C as this higher temperature was reported to reduce allele drop out (Ray et al., 1996). If no PCR products were seen after amplification, the microcentrifuge tube was placed into the block for a further 5 cycles and reanalysed.

### 2.2.5.2.1. Sickle Cell Anaemia (HbS) Detection

Three primers were used simultaneously to detect the sickle cell (HbS) single base pair substitution (A to T) in the beta globin gene (Table 2.1) in an adapted Amplification Refractory Mutation System (ARMS) procedure (Newton et al., 1989). The forward primer was 5'labelled with fluorescein (Section 2.2.5.3.2.a.). The 'normal'

allele-specific reverse primer, was designed with its 3' terminal nucleotide complementary to the normal base at the mutation site. The 'mutant' reverse primer was three base pairs (bp) shorter at the 5' end, and at the 3' terminus matched the mutant nucleotide. To make the reverse primers more discriminating, an additional mismatch two nucleotides 5' of the mutation site was introduced in both. The products of PCR thus differed by three base pairs in length (Fig 2.1.).

**Figure 2.1** Primers used for the analysis of the HbS single base pair mutation.




### 2.2.5.2.2. Beta Thalassaemia IVS1-110 Detection

For the detection of the point mutation IVS1-110 causing beta-thalassaemia (Baysal et al., 1992), three primers were again used in an adapted ARMS reaction (Table 2.1); one common forward primer and two reverse primers, each specific to either the normal or the mutant nucleotide. The forward primer was unlabelled while the normal reverse primer, specific at its 3' terminus to the wild-type nucleotide, was labelled with a fluorochrome (6-FAM; Perkin Elmer ABI; Section 2.2.5.3.2.c.). The mutant reverse primer, specific at its 3' end to the mutated nucleotide, was labelled with a different fluorochrome (HEX; Perkin Elmer ABI; Section 2.2.5.3.2.c.). Despite their identical size, the PCR products could be identified due to the differing fluorochromes annealed to each of the reverse primers.

For the detection of two deletions in the haemoglobin gene causing beta thalassaemia (one 4 base pairs, and one 619 base pairs) the sequences for two primer sets used for the routine prenatal diagnosis of the same mutations, were provided by Dr. Mary Petrou, UCL Perinatal Centre. These were used in a duplex reaction with the primer set flanking the 4bp deletion site fluorescently labelled and the resulting PCR products assessed by removal of 1μl PCR product from the microcentrifuge tube in the PCR block after 29 cycles using the ABI Prism™ 310. The tube was then replaced onto the PCR block and put through a further 21 cycles for ethidium bromide assessment of the other deletion site.

#### 2.2.5.2.3. Cystic fibrosis (CF) delta F-508 Detection

The delta F-508 three base pair deletion which is responsible for most cases of cystic fibrosis in Caucasian populations (Rommens et al., 1990) was identified using primers flanking the region of the deletion site (Table 2.1), and the forward primer labelled with 6-FAM (Perkin Elmer ABI). The PCR product lengths were 95bp for the mutant allele and 98bp for the wild-type.

#### 2.2.5.2.4. Sex chromosome detection.

The amelogenin primers were designed to co-amplify products of 432bp from the X chromosome and 252bp from the Y chromosome (Nakahori et al., 1991a; 1991b; Sherlock et al., 1997).

#### 2.2.5.2.5. Aneuploidy detection.

Eight STR loci have been utilised from 4 different chromosomes, individually and in various combinations (Table 2.1). In each case the forward primer from the set was labelled with a fluorescent dye. Initially, a single marker (D21S11) was investigated for use in determining chromosome 21 aneuploidies. Two further investigations were then undertaken, combining this STR with the analysis of further repeats in multiplex PCR reactions. In the first, DNA from 54 normal sources (23 blood samples; 31 amniotic fluid samples) and 22 aneuploid samples (10 trisomy 21 fetal blood and tissues; 10 trisomy 21 amniotic fluids; 1 trisomy 18 fetal tissue; 1 triploid fetal tissue) were assessed using multiplex PCR.

Information from the STR D21S11 was confirmed by the use of a primer set annealing at different locations surrounding the same repeat sequence (D21S1414). A single primer set was also included which resulted in the amplification of two separate, but closely linked, tetranucleotide repeats within the myelin basic protein (MBP) gene on chromosome 18 (Polymeropoulos et al,. 1992). Primers for the amelogenin region of the sex chromosomes were also included to determine the presence of the X and Y chromosomes. This research was performed in collaboration with Dr. Barbara Pertl, Graz Austria.

A second study employed two multiplex PCR assays combining information from eight STR loci on four chromosomes. The first set consisted of primers for the loci D21S1414, MBP, D13S631, D13S634 and the amelogenin region of the sex chromosomes. The second set included primers for the loci XHPRT, D18S535, D21S1412, D21S1411 and D21S11. Normal DNA from 60 sources (28 blood; 32 amniotic fluid) and aneuploid DNA from 25 sources (20 trisomy 21; 2 triploid; 3 trisomy 18; 1 trisomy 13) were assessed using these reactions. This research was performed in collaboration with Dr. Barbara Pertl, Graz Austria.

### 2.2.5.3. PCR product detection

#### 2.2.5.3.1. Agarose gel electrophoresis

PCR products were run on a 1% agarose gel containing ethidium bromide (0.3μg/ml) prepared by the microwave heating of 0.5g agarose in 50ml of 1X TBE until all the agarose had been dissolved (~2min at full power). The ethidium bromide was added to the molten agarose which was mixed and then poured into minigel moulds (8mm x 100mm, Cambridge Electrophoresis) with 8 or 16 well gel-slot-formers and left to set at room temperature. Gels were then immersed in 50ml of 1X TBE. Eight microlitres of each sample were mixed with a one tenth volume of loading buffer and pipetted into well-slots. Electrophoresis proceeded for 30-60min alongside a 100bp or Kilobase size marker ladder. Gels were viewed under ultra violet trans-illumination. Amplification of the Y chromosome produced a product of 823 base pairs where as amplification of the X chromosome produced a product of 1kb.

### 2.2.5.3.2. Fluorescent PCR Product Detection

Fluorescent PCR products were separated and analysed using one of three automated laser DNA analysers; Automated Laser Fluorescence [ALF] from Pharmacia; Genescan 373a Sequencer or the ABI Prism™ 310 from Perkin Elmer-Applied Biosystems. Each device had its own provided software (ALF Fragment Manager V1.1, Genescan 672 and Genescan version 2.0.2 respectively). The specific PCR products were sized and the amount of each PCR product evaluated by the extent of fluorescent activity, equal to the area of the fluorescent peak generated.

#### 2.2.5.3.2.a. Pharmacia Automated Laser Fluorescence (ALF)

Two microlitres of fluorescent PCR product were mixed with 4μl of loading buffer (Pharmacia) and 0.5μl of relevant size standards (100bp, 150bp, 300bp, 50-500bp ladder, Pharmacia) and were run on a 0.35mm 6% polyacrilamide, 8M urea gel (Ultrapure Sequigel; National Diagnostics, UK) at 1900V, 55W, 42mA, 42°C with a sampling interval of 0.84sec.

#### 2.2.5.3.2.b. Genescan 373a Sequencer

Two microlitres of PCR products were mixed with 3μl loading buffer (10% dextran blue 90% de-ionised formamide) and 0.5μl Genescan-2500 Rox (PE-ABI) containing reference molecular size standards. Electrophoretic analysis was carried out through a 6% denaturing polyacrilamide gel (8M urea) at 2500 volts, 40 miliAmps, 45 watts at 45°C.

#### 2.2.5.3.2.c. Prism™ 310

When using the Prism™ 310, 1μl of each fluorescent PCR product, mixed with 12μl of de-ionised formamide and 0.5μl of size standards (Genescan 500-TAMRA) were run through the capillary (15kVolts, 24min at 60°C).

### 2.2.6. Tissue Culture

#### 2.2.6.1. CVS cultures

CVS cultures, kindly provided by University College London Cytogenetics unit were set up with standard protocols (Heaten et al., 1984).

#### 2.2.6.2. Fibroblast cultures

Fibroblast cell lines in fetal calf serum (FCS) plus 5% dimethyl sulfoxide (DMSO) were stored in cryotubes under liquid nitrogen (-196°C). When required the cell samples were thawed at 37°C, added to 15ml of RPMI medium in a 25ml tissue culture flask and placed in a 5% $CO_2$ incubator at 37°C. Cell culture medium was renewed every 2-3 days by carefully decanting off approximately 10ml of starved medium and adding a similar volume of fresh medium.

#### 2.2.6.3. Lymphocyte cultures

One millilitre of blood, collected in a heparin tube was transferred into a 25ml culture flask, to which the following were added: 17ml of Iscoves modified DMEM medium (Imperial) with 1% GPS (glutamine, penicillin, streptomycin), 2ml of FCS (fetal calf serum), and 0.2ml PHA (phytohaemaglutinin). The flask was then agitated by inversion, and incubated for 72 hours at 37°C. Over this incubation period, the flask was agitated at 24 hour intervals.

#### 2.2.6.4. Cell culture harvest

Cell cultures for DNA extraction or single cell/cell clump PCR analysis were treated as previously mentioned. Lymphocyte cells for FISH slide preparation, after incubation had 100µl of colchicine added to the flask and the cell suspension agitated. The flask was then left for a further 60min at 37°C. The contents of the flask were then transferred to 2 conical centrifuge tubes which were centrifuged at 250g for 5min. The supernatant was decanted leaving the pellet at the base of the tube. Hypotonic KCl (0.075M) was then added a drop at a time whilst, by flicking the tube, the pellet was re-suspended. The tubes were left at room temperature for 15min, then centrifuged at

250g for 5min. The supernatant was once again discarded, and the pellets re-suspended in freshly made fixative. The tubes were then centrifuged again at 250g and re-suspended in fixative. This step was repeated until the pellets were white and the supernatant clear.

### 2.2.7. Side preparation

#### 2.2.7.1. Slides for morphological, histological and immunohistochemical studies

TCC samples were washed with PBS, and spun at 180g for 5min in a 10ml centrifuge tube. The supernatant was removed with a disposable pipette and the remaining cells re-suspended in 2-5ml fresh PBS according to cell-pellet size. Samples with a large number of cells were thoroughly mixed using repeated suction and expulsion from a disposable pipette, and then re-spun, the supernatant removed, and the cells re-suspended.

For direct light microscopic analysis, approximately 200µl of the TCC sample was spread on a slide for direct visualisation using phase contrast microscopy. This had to be performed rapidly (~5min) before the solution on the slide dried.

For immunohistochemical analysis a proportion of the TCC sample was re-spun and the supernatant removed. The cells were re-suspended in RPMI 1640 medium (Gibco, UK) containing 10% fetal calf serum. The TCC cells were then spread over glass slides and left to air-dry overnight. The TCC cells were then fixed by exposure to acetone at room temperature for 5-10min. Slides were kept at 4°C for conventional or immunohistochemical staining.

For morphological examination, slides were stained with Leishman's stain (Burr's, BDH, UK) for analysis under phase contrast microscopy.

Immunoperoxidase and immunofluorescent staining were performed using monoclonal antibodies (McAb) FT141.1, H315 (from Professor Peter Johnson, University of Liverpool) and IO3 (from Dr. F. Carr, Glasgow) raised against trophoblast antigens. Slides were rehydrated in 0.05M Tris, 0.15M saline, pH7.6 (TBS) and incubated for 10min with 1:10 normal rabbit serum in TBS to block non-specific binding sites. Excess serum was removed and the smears were then incubated for 30min with the primary monoclonal antibody, appropriately diluted in TBS. After two

10min washes in TBS, the smears were overlain for 30min with biotinylated rabbit anti-mouse immunoglobulins (Dako, High Wycombe, UK) diluted 1:500 in TBS. After a further two TBS washes, the slides were incubated for 30min with streptavidin conjugated with peroxidase or fluorescein (Dakopatts, Denmark) was employed as second antiserum. The peroxidase was developed with 3,3'-diaminobenzidine (DAB) (Sigma chemicals) containing 0.02% hydrogen peroxide. The reaction was stopped after 5-10min by washing in excess water.

### 2.2.7.2. Slides for FISH

#### 2.2.7.2.a. TCC samples

TCC samples were transferred to a 10ml centrifuge tube. If excessive mucus was present in a TCC sample, 400µl acetyl-cystine was added and the sample incubated at 37°C and shaken regularly until the mucus dissolved. The TCC cells were then pelleted by centrifugation at 180g for 5min and the supernatant removed. Cells were re-suspended in PBS and once more spun at 180g for 5min before the supernatant was removed. The cell pellet was then re-suspended in 2ml PBS, 2ml KCl (100mM) preheated to 37°C, and the tube incubated at 37°C for 15-20min. After incubation, 1ml of fix (methanol:acetic acid, 3:1) was added and mixed prior to further centrifugation (180g, 5min). The supernatant was removed and the cells re-suspend in 5ml fix and re-centrifuged (180g for 5min). The supernatant was then removed and the cells re-suspended in 5ml fix. Centrifugation (180g, 5min) followed, the supernatant was again removed and the cells re-suspended in ~1ml fix to achieve the desired concentration. Fixed cells in suspension were stored at -20°C.

#### 2.2.7.2.b. Placenta

Approximately 0.5cm$^3$ white villus placenta was cut up as fine as possible using sterile scissors and placed in a 10ml centrifuge tube. The placental cells were then suspended in PBS and mixed by rapid suction and expulsion with a disposable pasteur pipette. The cells were then pelleted by centrifugation at 180g for 5min, the supernatant removed and the cells washed by re-suspension in PBS, spun again at 180g for 5min with the supernatant once more removed. Cells were then re-suspended in 2ml PBS and 1ml of collagenase (2mg/ml) prior to an incubation at 37°C, with regular

agitation, until the cells were seen to disagregate. The cells were once more pelleted by centrifugation at 180g for 5min, the supernatant removed, and the cells washed in 2ml PBS then re-spun at 180g for 5min. The supernatant was then removed and the cell pellet once more re-suspended in 2ml PBS. Two millilitres of KCl (100mM stock, preheated to 37°C) was added. The tube was then incubated at 37°C for 15-20min. After incubation, 1ml fix (methanol:acetic acid, 3:1) was added prior to further centrifugation (180g, 5min), supernatant removal and re-suspension in 5ml fix. Centrifugation (180g, 5min), removal of supernatant and re-suspension in 5ml fix was then repeated, followed by centrifugation (180g, 5min), removal of supernatant and re-suspension in ~1ml fix to achieve the desired concentration. Fixed cells were stored in suspension at -20°C.

When slides for FISH were prepared, the TCC, lymphocyte or placental cell suspension was centrifuged again for 5min at 180g and the supernatant discarded. The pellet was then re-suspended in fresh fixative (methanol:acetic acid, 3:1) until a cloudy suspension was achieved. Slides were first cleaned in methanol with a drop of concentrated HCl to remove any grease, then dried by wiping with a lint-free cloth. Approximately 100µl of the cell suspension (from TCC, placental or control lymphocytes) was then dropped from a distance onto the centre of the slide. Two drops of fresh fixative were then added to the slide and left for 3-5sec, adhering the cells firmly to the slide surface. The slide was then immediately shaken dry. FISH was always performed on freshly prepared slides.

### 2.2.7.2.c. Slides for RT-mRNA PCR

For all RT-mRNA experiments, poly-l-lysine coated slides were used to increase fixation of cells onto the slide surface. These were prepared by soaking normal glass slides (BDH, UK) in poly-l-lysine (Sigma) diluted 1:10 with distilled water for 5min, then allowing them to air dry. Once coated, slides were stored at 4°C.

Slides were prepared from TCC, placental, buccal, and lymphocyte and mixed population samples. Buccal cells were obtained by a simple mouth wash using sterile water. Lymphocytes were obtained from whole blood using Ficoll cell separation methods (Section 2.3.5.1.). Trophoblasts were obtained from fresh placental samples obtained after TOP which was cut up as fine as possible using sterile scissors. Cells from all sources were prepared in the same manner: Cell suspensions were washed with

PBS three times, by successive centrifugation (180g, 5min), supernatant removal and re-suspension. Approximately 200μl cell suspensions were spread directly onto poly-l-lysine slides and allowed to air-dry. As the cytoplasm of cells was to be examined, all cells were left as intact as possible, with no pre-treatment with hypotonic sollution. Three slide types were prepared by using mixed and pure suspensions; 100% trophoblast, 100% lymphocyte or buccal cells, and 1:1 trophoblast:buccal/lymphocyte. Cells were fixed to the slides by being submerged in 4% paraformaldehyde (50ml PBS, 1.3ml 37% paraformaldehyde) at room temperature for 10min in a coplin jar. Slides were washed in PBS for 5min and incubated in 100μl of 10ug/ml proteinase K (10μl of 10mg/ml proteinase K in 1ml of PBS) at 37°C for 10min. The slide was then placed on a PCR block and heated to 94°C for 5min to denature the proteinase K, and left to air-dry.

**2.2.7.2.d. Slides for FISH from isolated clumps**

Cell clumps intended to be tested by FISH were isolated in <2μl of solution by micromanipulation and placed into a 0.5ml microcentrifuge tube containing 5μl of collagenase (2mg/ml) which was then incubated for 37°C for 45min. Fifteen microlitres of hypotonic KCl solution (50mM) was then added to the same tube which was further incubated at 37°C for 30min. Without removing any solution , an equal volume of fixative (3:1 methanol:acetic acid) was added and the tube spun for 5min at 180g. A fraction of the supernatant was then removed with care taken not to dislodge any of the invisible cell pellet at the base of the tube. One hundred microlitres of fixative were then added to the tube and the cell pellet resuspended by pipetting up and down. The cells were then re-spun at 180g for 5min, a fraction of the supernatant once more removed and then resuspended in 100μl fix. One final spin was performed, and then a fraction of supernatant removed to leave the cells in approximately 10μl of fix. The cells were carefully resuspended in this volume and pipetted onto a glass slide. The ~10μl volume of cell suspension was then left to air dry before fresh fix was placed over the cells on the slide. This was removed after 10 seconds, and the slide entered into a standard FISH protocol.

## 2.2.8. FISH Probe Preparation

### 2.2.8.1. Bacterial Probe Inserts

Bacteria containing FISH probe inserts were cultured in 10ml cosmid broth (Section 2.1.4.) at 37°C for 24 hours. To this was added 0.2mg of ampicillin per millilitre of broth. After incubation 10ml of culture was centrifuged at 5000g for 10min and the supernatant discarded. The cell pellet was resuspended in 200µl of bacterial lysis buffer (Section 2.1.4.) and the mixture incubated at room temperature for 10min. This preceded two 5min incubations on ice, the first after the addition of 400µl AD buffer (Section 2.1.4.) and the second after 300µl of 3M sodium acetate (pH5.2) had been added. Each step required thorough mixing. Next the mixture was transferred to a 1.5ml microcentrifuge tube and spun for 5min at 10000g. The clear supernatant was then collected into a new microcentrifuge tube containing 600µl isopropanol and the mixture incubated at -70°C for 10min or at -20°C for approximately 16 hours. Precipitated DNA was recovered by centrifugation at 20000g for 5min, after which the supernatant was removed and the pellet air dried. Finally the DNA pellet was dissolved in 200µl of 0.3M sodium acetate (pH 6.5).

An equal volume of equilibrated phenol (Gibco BRL) was then added and the mixture vortexed. Organic and aqueous layers were separated by centrifugation at 5000g for 5min, after which the aqueous layer was transferred into a fresh tube. DNA precipitation was then accomplished by addition of 200µl of isopropanol and incubation at -70°C for 10min. The precipitate produced was spun at 20000g for 5min and the supernatant discarded. The pellet was dried and then resuspended in 200µl of TE (Section 2.1.4.). Next RNA was removed from the solution by incubation for 15min at 37°C after the addition of 10µl of 1mg/ml RNase. The DNA solution was further purified by phenol-chloroform extraction. For this purpose an equal volume of phenol was added to the solution and the two phases mixed together vigorously. Again centrifugation at 5000g for 5min caused formation of discrete organic (phenol) and aqueous layers. The DNA containing aqueous layer was collected into a fresh microcentrifuge tube and the extraction repeated this time using an equal volume of chloroform rather than phenol. Finally DNA was precipitated once more, this was achieved by the addition of a $1/20^{th}$ volume of 4M NaCl and 3 volumes of absolute ethanol, followed by a 10min incubation at -70°C. The precipitated DNA was recovered

by centrifugation , dried, and resuspended in 50µl of TE. Storage was at 4°C for up to 6 months.

### 2.2.8.2. Probe Labelling

FISH probes were initially labelled with biotin-14-dATP or digoxigenin (DIG)-11-dUTP by nick-translation using BioNick Labelling System Kits (BRL Life Technologies, UK) following the manufacturers recommended protocols. Probes were then eluted through a sephadex column (Nick™- Pharmacia), equilibrated with 1X TNE (Section 2.1.4.) to remove un-incorporated nucleotides and collected in a final volume of 400µl which was stored at -20°C.

The repetitive probes pBam X5 and cY98 were directly labelled with fluorescent nucleotides as previously described (Harper and Delhanty, 1996). On ice, the following were mixed in a 1.5ml microcentrifuge tube: 5µl of 10X nick translation buffer (0.5M Tris-HCl, pH7.5, 0.1M $MgSO_4$ 1mM dithiothreitol [DTT, Sigma]), 5µl of 0.1M DTT, 4µl of nucleotide mix (consisting of 0.5mM 2'deoxyguanosine 5'-triphosphate, 0.5mM 2'deoxyadenosine triphosphate, 0.5mM 2'deoxycytidine 5'-triphosphate, 0.1mM 2'deoxythymidine 5'-triphosphate [each of which from 100mM stock, pH7.5, Pharmacia] ), 3µl 1mM label, 1µg probe DNA, 2µl DNA Polymerase I (Promega), 5µl of 1:1000 Dnase (DNAse I [bovine pancreas grade II, Boehringer Mannheim] in 20mM Tris-HCl, pH7.6 50mM NaCl, 1mM DTT, 50% glycerol, diluted 1:1000 with bidistilled water). The volume in the tube was made up to 50µl with sterile water. The tube was then incubated at 15°C for 2 hours. After 1 hour another 5µl of DNAse 1:1000 was added. After the incubation 5µl of 0.5M EDTA was added and the tubes kept on ice. DNA precipitation was achieved by the addition of 5µl herring sperm DNA, 1/10 volume 3M sodium acetate and 1ml ice cold ethanol. The tube was then inverted to mix and incubated at -70°C for 1 hour or -20°C overnight. Tube contents were then centrifuged at 13000g for 15min, the supernatant removed and the pellet left to air dry in the dark. The probe was re-suspended in 20µl hybridization buffer (60% formamide [BDH], 2XSSC) and stored at 4°C away from direct light.

To obtain a yellow coloured FISH probe, an equal amount of the probe labelled green was added to the same probe labelled red. This yellow signal, distinguishable from pure green and red, enabled triple colour FISH to be employed.

### 2.2.8.3. Preparation of Probes

To prevent repetitive sequences in cosmid probes from hybridising to non-target regions of DNA during FISH, repetitive DNA (Cot-1 or salmon sperm) was pre-hybridised to the purified probe. Repetitive probes, at 2ng/μl in 50% formamide, 10% dextran sulphate, 2X SSC, 40mM/l $NaH_2PO_4$,500 μg/ml sheared sonicated salmon sperm DNA (ssDNA) pH7, were denatured by heating to 75°C for 3min and were then cooled rapidly on ice ready for application to a slide. Specific sequence probes were prehybridised with *Cot* 1 DNA (BRL Life Technologies) present at 10-50 fold excess by suspending the DNA probe at 7ng/μl together with the *Cot* 1 DNA in a solution of 50% formamide, 10% dextran sulphate, 2X SSC, pH 7, containing ssDNA at 100 fold excess. The DNA was denatured by heating to 75°C for 3min and incubated at 37°C for 1-3 hours.

### 2.2.9. Preparation of Slides

Unless otherwise stated, all slides were always washed and treated in coplin jars with a solution of 50ml. Slides were either;

a) treated with 100μg/ml RNAse A (Sigma Chemical Co.) in 2X SSC (20X SSC = 175.3g NaCl, 88.2g. Sodium Citrate in 1 litre of $H_2O$, pH 7) at 37°C for 1 hour, or

b) washed in 10mM HCl at 37°C for 5min, then incubated in 10μg/ml pepsin in 10mM HCl for 10min at 37°C.

The latter protocol was utilised in the later stages of the project as it produced equally satisfactory results in a shorter time.

Slides were then washed once in 2X SSC for 5min, once in PBS for 5min and then dehydrated in an ethanol series (70%, 90%, 100%) 5min in each.

### 2.2.10. Fluorescent in Situ Hybridisation

Immediately before application of the FISH probe, chromosomal DNA was denatured by incubation of the slides in 70% formamide, 2X SSC, 40mM/l $NaH_2PO_4$,

pH7, for 2min 30sec at 65°C. After being dehydrated by washing in a 4°C ethanol series (70%, 90%, 100% ethanol, 5min in each), the slides were air dried. The denatured probes were applied to the slides under coverslips which were sealed with rubber solution glue and incubated at 37°C for approximately 16 hours.

### 2.2.10.1. Post Hybridisation Washing and Signal Detection

After probe hybridisation the rubber glue solution from around the coverslip was carefully removed and slides placed into a coplin jar containing 2X SSC.

Slides exposed to repeat probes were incubated in 50% formamide, 0.2X SSC, pH7 at 37°C for 40min, then washed twice in 2X SSC for 5min at room temperature.

Slides exposed to single copy probes were washed three times (5min per wash) in 50% formamide, 2X SSC, pH7 at 45°C, three times (5min per wash) in 0.1X SSC, pH7 at 60°C, and once for 5min in 4X SSC, 0.5% Tween-20, pH7. They were then blocked with normal rabbit serum diluted 1/10 in 4% BSA, 4X SSC, pH7.

Slides with directly labelled probes were then incubated in 60% formamide/2X SSC pre-warmed to 37°C for 5min. They were then washed twice in 4X SSC/0.05% Tween 20 at room temperature for 5min and dehydrated in an ethanol series before being left to air-dry and mounted in Vectashield antifading medium (Vector Laboratories, USA) containing 0.1 μg/ml 4',6-diamidino-2-phenylindole (DAPI) as counterstain.

Slides with biotin labelled probes were incubated with avidin-FITC (Vector Laboratories, USA) (5μg/ml in 0.1% BSA, 4X SSC, pH7) for 20min followed by three washes (5min each) in 4X SSC, 0.5% Tween-20, pH7. They were then incubated for 20min in biotinylated anti-avidin (Vector Laboratories, USA) diluted to 5mg/ml as above, washed three times and finally incubated again in avidin-FITC. After one wash in 4X SSC, 0.5% Tween-20, pH7, and two washes in PBS, the slides were mounted in Vectashield antifading medium (Vector Laboratories, USA) containing 0.1μg/ml 4',6-diamidino-2-phenylindole (DAPI) as counterstain.

Signals produced using digoxigenin labelled probes were developed by incubating the slides in TRITC-sheep anti-digoxigenin (Boehringer Mannheim GmbH, Germany- 10μg/ml in 0.1% BSA, 4X SSC, pH7) for 20min. The slides were then

washed once in 4X SSC, 0.5% Tween-20, pH7, twice in PBS and mounted in Vectashield antifading medium (Vector Laboratories, USA) containing 0.1μg/ml 4',6-diamidino-2-phenylindole (DAPI) as counterstain.

When biotin and digoxigenin labelled probes were used simultaneously, probe hybridisation was visualised by incubating the slides in TRITC-sheep anti-digoxigenin and avidin-FITC (10 and 5μg/ml respectively in 0.1% BSA, 4X SSC, pH7) for 20min followed by three washes (5min) in 4X SSC, 0.5% Tween-20, pH7. They were then treated once with biotinylated anti-avidin and once more with avidin-FITC as above and mounted in Vectashield antifading medium containing 0.1μg/ml DAPI.

Slides were examined by direct visualisation using fluorescent microscopy; a Nikon optiphot with an Omega dual band-pass filter for simultaneous FITC and TRITC detection and a Nikon UV 2a filter for detection of DAPI fluorescence. Images were captured using a Zeiss Axioskop microscope linked to a Kaf 1400 cooled Charged Couple Device (CCD) camera with Smartcapture analysis computer software (Vysis, UK). All FISH experiments were carried out including positive control slides to confirm successful probe hybridisation. When examining TCC slides, as many whole interphase cells within the probe hybridisation zone as possible were scored. On most slides this cell number ranged from 200 to 400 nuclei.

When using the X and Y specific FISH probes on TCC material, nuclei containing one X and one Y signal were deemed to be of male (fetal) origin, and those with two X signals to be female.

When using the dual cosmid contigs specific for chromosome 21, one dual coloured doublet (with the red and green signals in close proximity) was deemed to represent the chromatin of one chromosome 21. Nuclei with broken membranes or clearly visible cytoplasm were not scored.

### 2.2.11. *In situ* RT-mRNA PCR

Primers for reverse transcription of the beta- subunit of human chorionic gonadotrophin mRNA, followed by PCR of the generated amplicon (RT-mRNA PCR) were designed from the published cDNA sequence (Fiddes & Goodman, 1980):

Reverse transcriptase primer: **GCG GAT TGA GAA GCC TTT ATT GTG G**
(and Forward PCR primer)

Complementary (Reverse): **CTG GCT GTG GAG AAG GAG GGC TGC**
primer for PCR

The optimal annealing temperatures for these primers during PCR was calculated as 61°C by use of the following formula:

Annealing Temperature ($T_M$) = 69.3 + (GC x 0.41) - (650 / L)

Where 'GC' is the percentage of nucleotides that are either guanine or cytosine (48 and 66.7 respectively) and 'L' is the number of nucleotides in the primer (25 and 24 respectively).

To each slide under a 22mm diameter coverslip (BDH, UK) 10µl GeneAmp® EZ rTth RNA PCR Kit mix was added containing the following reagents all but the primers provided in the kit: 2.5µl $Mn(OAC)_2$ ,1.5µl PCR forward Primer (10pM/µl stock), 1.5µl Reverse primer (10pM/µl stock), 0.25µl RNAse inhibitor (Sigma), 0.5µl dATP, 0.5µl dGTP, 0.5µl dCTP, 0.5µl dTTP/ dUTP, 2µl 5X reaction mix, 0.2µl EZ rTth enzyme, made up to 10µl with double distilled (dd) $H_2O$.

For reactions attempting visualisation with fluorescently labelled nucleotides, only 0.1µl of the triphosphate dTTP was added, the remainder substituted with 1µl of FITC-labelled dUTP from the Bio-nik kit (Bohringer Mannheim). For reactions attempting visualisation with avidin conjugated FITC, only 0.1µl of the triphosphate dTTP was added, the remainder substituted with 1µl of BIO- dUTP from the Bio-nick kit (Bohringer Mannheim).

The coverslip was sealed with cowgum and the slide placed onto a metal PCR block. The thermostable rTth enzyme used in this kit acts as a reverse transcriptase or

DNA polymerase in the same buffer conditions depending only on the temperature. Test experiments using a slide thermometer (Cytocell, UK) had shown that due to inefficient heating, the temperature of a slide on the PCR block was 2°C lower than the block setting. All temperatures were thus set 2°C higher than required. The block was set to the following temperature steps:

| | | | |
|---|---|---|---|
| Step 1 | 62°C | 30min | ( to enable reverse transcription) |
| Step 2 | 95°C | 2min | denaturation |
| | 63°C | 1min | annealing and extension |
| | | | Step 2 x 30 cycles |
| Step 3 | 63°C | 5min | final extension |

The cowgum & coverslip were then carefully removed and the slides immersed in 4X SSC for 5min. Two 5min washes in 2X SSCT proceeded followed by a 20min incubation in SSCM (100ml 4xSSC, 5g filtered marvel) at room temperature.

If the RT-mRNA PCR had been carried out with fluorescently labelled nucleotides, visualisation was attempted at this stage: Slides were washed in PBS, allowed to air dry, then mounted in Vectashield antifading medium (Vector Laboratories, USA) containing 0.1 μg/ml 4',6-diamidino-2-phenylindole (DAPI) as counterstain.

If the RT-mRNA PCR had been carried out with biotin conjugated nucleotides, the slides were incubated for 20min at room temperature with 100μl avidin-FITC (2.5μl FITC, 500μl SSCM). Slides were then washed for 5min in SSCT, 5min in PBS and air-dried. Slides were mounted in Vectashield antifading medium (Vector Laboratories, USA) containing 0.1 μg/ml 4',6-diamidino-2-phenylindole (DAPI) as counterstain.

Visualisation was attempted using an MRC 600 confocal laser microscope (Biorad) with MRC 500/600 software.

Negative controls consisted of slides with no EZ rTth enzyme added, everything else being constant.

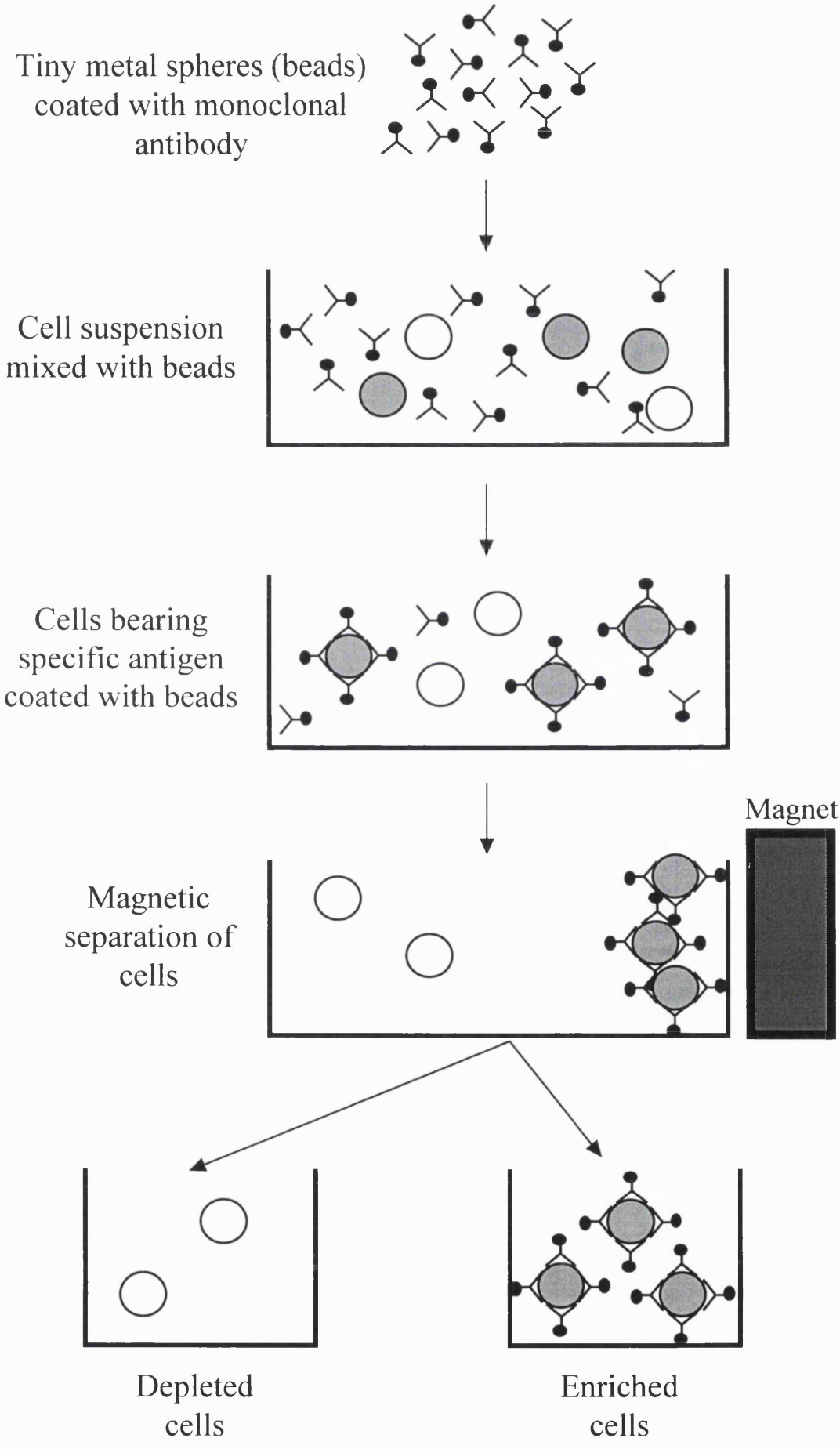
## 2.2.12. Magnetic Activated Cell Sorting (MACS)

MACS was attempted using the monoclonal antibodies (McAb) 340 (Martin et al., 1997) and αEGFR (epidermal growth factor receptor) (Kawagoe et al., 1990) both raised in mice. These were provided courtesy of Lindy Durrant, Nottingham University.

Two types of magnetic beads were compared in this study- Minimac beads (Minimac, UK) and Serotec Ltd (Figure 2.2). Beads, conjugated with goat anti mouse antibodies and sheep anti mouse antibodies respectively. The Minimac beads were ~0.5nm in diameter whereas the Serotec beads were ~3.5μm. The Minimac columns supplied for bead separation had mesh pore sizes of 35-42μm (approximately 4 cell diameters). As these pores would not allow the passage of cell clumps, the Serotec Ltd apparatus was used for bead separation.

Three solutions were made; 100% trophoblasts, 100% lymphocytes/buccal cells, and 1:1 mixes. Solutions were equilibrated to approximately $1x10^6$ cells per 500μl using a haemocytometer (Graticules Ltd, UK) and transferred into a 1.5ml microcentrifuge tube. The tube was centrifuged at 180g for 5min, the supernatant removed and the cells re-suspended in 500μl buffer (PBS with 1% BSA [Section 2.1.4.]) . The primary antibody was added and tubes incubated at 4°C for 12min. Primary antibody consisted of 5.5μl of McAb EGFR (1:100), or 55μl McAb 340 (1:10 from a stock of 2.66μg/ml). After incubation 1ml MACS buffer (PBS with 1% BSA [Section 2.1.4.]) was added to the tube which was spun at 180g for 5min. The supernatant was then removed and the bead/cell pellet re-suspended in 1.5ml MACS buffer. The tube was then spun as before, the supernatant removed and the pellet re-suspended in 500μl MACS buffer. The magnetic beads were then added to this solution at a concentration of 50X cell number (~$5x10^7$ beads) according to the manufacturer's instructions. The tube was then left for 12min at room temperature. The 1.5ml tube was then placed into the separation rack for 5min and the solution (negative fraction) carefully aspirated. The tube was removed from the rack, the beads resuspended in 1ml MACS buffer and mixed. The tube was then replaced in the rack for 5min and the negative fraction removed once more. The beads/cells were finally resuspended in 200μl buffer and smeared onto a slide for microscopic examination. Negative controls consisted of the same solutions with no addition of primary antibody.

**Figure 2.2; Magnetic Activated Cell Sorting (MACS)**



### 2.2.13. TCC collection methods

All TCC sampling was carried out with the aid of real time transabdominal ultrasound scanning. The presence of a viable pregnancy and an estimation of gestational age were established prior to any procedure. With the patient in the dorsal lithotomy position, a posterior vaginal speculum was inserted, and the vagina and cervix cleaned with antiseptic solution.

Initially TCC samples were collected form the cervix of pregnant women using a cotton wool swab. Due to difficulties in retrieving cells from the cotton wool this method of cell collection was abandoned. TCC samples were then collected by three different methods from pregnant women, at 6-14 weeks of fetal gestation (with the exception of a few selected cases up to 17 weeks); aspiration, cytobrush and lavage (Figs 2.3.1; 2.3.2). A further adaptation of the aspiration method was also employed using a Pipelle catheter (Pipelle De Cornier™, Neuilly-en-Thelle, France). This device consists of an internal piston within a narrow exterior catheter. As the piston was withdrawn this generated negative pressure pulling the TCC sample into the catheter. TCC sampling procedures were performed by Professor Charles Rodeck, Dr. Peter Soothill, Dr. John Kingdom, Dr. Boris Tutschek, Mr R.H.T. Ward, Dr. U. Montemagno, and Dr. Pornpimol Ruangvutilert at the University College Hospital Fetal Medical Unit and the Elizabeth Garret Anderson Hospital.

**Figure 2.3.1. Methods of TCC collection**

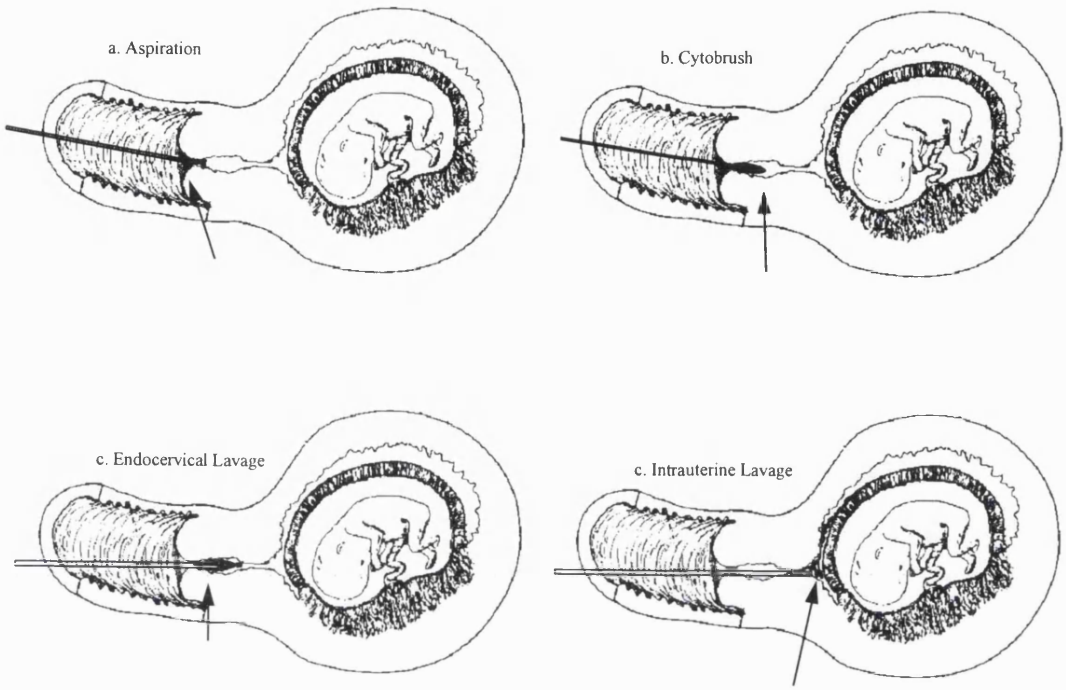
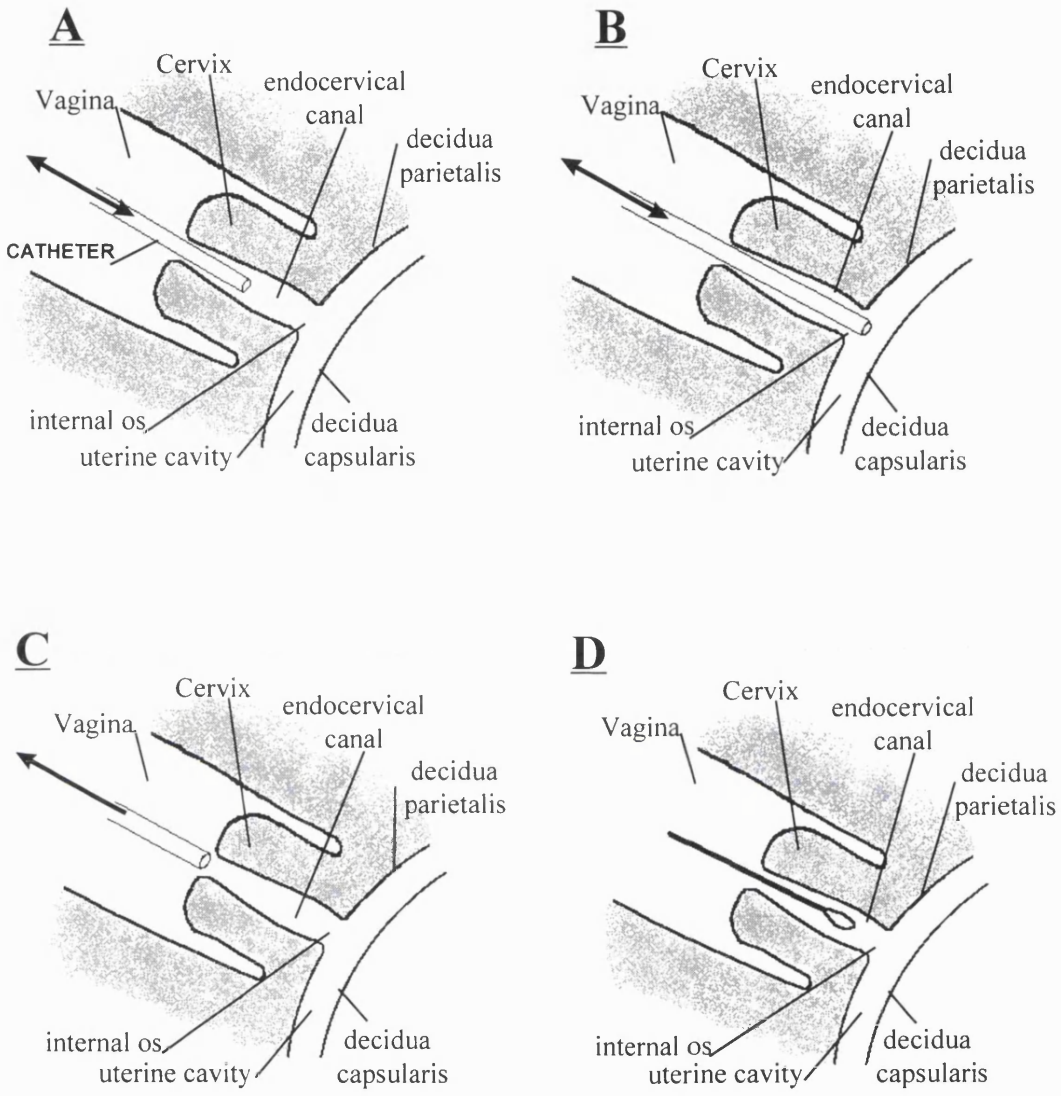


**Figure 2.3.2 TCC collection techniques in detail**




Methods for the collection of TCC samples. **A**. Endocervical lavage; **B**. Intrauterine lavage (arrows indicate instillation and expiration of saline); **C**. Aspiration of cervical mucus; **D**. Cotton swab or cytobrush.

### 2.2.13.1. Mucus Aspiration

The tip of a 3ml catheter was placed in the endocervical canal below the mucus plug. The catheter was attached to a 10ml-50ml syringe (Rocketmedical) containing 3ml of sterile fluid. The mucus from the endocervical canal was then aspirated by suction of the syringe, cervical mucus and transcervical cells being collected directly in the fluid. Initially phosphate-buffered saline (PBS) was used as the collection media. Occasionally, to maintain better cell morphology, MEM media was used. Samples were kept at 4°C prior to subsequent processing.

When using the Pipelle apparatus for TCC aspiration sampling, the contents of the catheter were expelled into a sterile vessel containing 2ml sterile PBS.

### 2.2.13.2. Cytobrush

A cytology brush (Sterling Winthrop, UK) was inserted into the endocervical canal and rotated whilst being withdrawn, to trap any endocervical mucus. The end of the brush was cut off into a sterile vessel containing 10ml sterile PBS. This was then shaken to dislodge the cervical debris into the saline.

### 2.2.13.3. Lavage

Lavage was performed using a flexible plastic tube (Universal filling cannula [UN 888/100], Universal Hospital Supplies Ltd., London, England) connected to a syringe containing 5-10ml of physiologic sterile saline (0.15M NaCl). After insertion of the tube into the endocervical canal, the saline was slowly injected and then immediately drawn back into the tube and syringe with gentle aspiration. Two forms of lavage were performed during the duration of this study differing only in the positioning of the catheter in the endocervical canal. With endocervical lavage, the distal end of the tube was positioned in the mid-endocervical canal, with intrauterine lavage the distal end of the tube was inserted 1cm through the external os, into the uterine cavity.

## 2.2.14. TCC Preparation

Cells obtained from all collection methods were washed with PBS, and spun at 180g for 5min in a centrifuge tube. The supernatant was then removed with a disposable pipette and the remaining cells re-suspended in 2-5ml fresh PBS according to cell-pellet size. Lavage and cytobrush samples with a large number of cells were thoroughly mixed using repeated suction and expulsion from a disposable pipette, and then re-spun, the supernatant removed, and cells re-suspended.

If there was excessive mucus present in a TCC sample, or the mucus was very viscous, 500µl of acetyl-cystein (Parvolex, 200mg/ml, Evan) was added to the sample which was then incubated at 37°C for 30min with intermittent shaking to dissolve the mucus and dislodge cells (Section 2.2.7.2.a.). The solution was then centrifuged at 180g for 5min, the supernatant removed and the cells resuspended in PBS.

### Preliminary Examination

After washing, an aliquot (~200µl) of the TCC sample was spread on a slide or Petri-dish for direct visualisation using phase contrast microscopy. This gave an approximate indication of the quality of the sample and the cell density. In the early part of the study, the material was washed and pipetted, breaking up cell clumps. In the second half of the study, all samples were subjected to light-microscopic evaluation using an inverted microscope whilst the cells were still suspended in the original sampling solution.

## 2.3. TCC Sample Groups

TCC samples were collected from pregnant women between 6 and 13 weeks gestation, in 5 distinct groups. All participants in this study gave their informed consent, and approval from the UCL ethical committee was obtained for all work. Blood was taken from the peripheral circulation of all women participating in this study prior to any TCC sampling. This served as reference maternal material.

### 2.3.1. Group A

This consisted of aspirate (n=33) or lavage (n=21) samples collected from women prior to elective termination of pregnancy (TOP). Following all TOP, villous placenta samples were collected as reference fetal material.

### 2.3.2. Group B

This consisted of TCC samples collected only by aspiration, (n=130) prior to CVS from women intending to continue their pregnancy. All patients in Group B were monitored either through the regular follow-up of the Fetal Medicine Unit, UCL, or by sending a questionnaire to the General Practitioner. This letter, inquired about any procedure-related discomfort or complications to pregnancy outcome. An approximately equal number of pregnant women who underwent CVS procedures without TCC sampling also had their pregnancies monitored. By comparison to this control group the risk of the aspiration procedure was assessed.

### 2.3.3. Group C

This consisted of lavage (n=11) or cytobrush (n=11) TCC samples collected prior to TOP. These samples were all collected by the same operator and used in a blind, comparative study to assess the relative efficiency of the two collection methods.

### 2.3.4. Group D

This consisted of TCC samples collected sequentially by aspiration and lavage (n=22) from each pregnant woman prior to TOP. Following all TOP, villous placenta samples were collected as reference fetal material.

### 2.3.6. Group E

The Pipelle device was employed to retrieve TCC aspiration samples from 56 pregnant women, between 6 and 15 weeks of fetal gestation, seeking TOP for non-medical reasons. Prior to any procedure maternal blood was collected from all mothers and following TOP villous placenta samples were retrieved as reference fetal material. Samples were tested by PCR for the presence of Y chromosome DNA using primers specific for the amelogenin region of the sex chromosomes. In the same multiplex PCR reaction two STR loci (D18S535 and D21S11) were examined for the presence of the paternally inherited allele indicating the presence of fetal DNA.

### 2.3.5. Group F

This consisted of TCC samples collected by mucus aspiration from selected women carrying fetuses at risk of specific genetic defects; cystic fibrosis, sickle cell anaemia, or beta thalassaemia.

### 2.3.5.1. Samples for single cell/cell clump QF-PCR

Samples were also collected for the analysis of single cells and cell clumps. Buccal wash cells and blood samples were collected from numerous normal individuals, carriers of specific mutations and homozygous affected individuals. CVS cultures derived from heterozygous and homozygous affected fetuses were kindly provided by the UCL cytogenetic department. Umbilical cord blood samples from trisomy 21 and 18 fetuses were kindly provided by the UCL and UMDS cytogenetic departments. Sources of single cells used for single cell QF-PCR are listed in Table 2.2.

All assays were first assessed in blind studies performed on extracted control DNA samples, with the results compared to those obtained from independent tests performed by established standard protocols. The tests were then

applied to DNA prepared from a small number of cells (10-50) before single cells. All primers were then combined in multiplex reactions to assess compatibility.

| Cell type | QF-PCR Assay | | | | | |
|---|---|---|---|---|---|---|
| | HbS | CF | IVS1-110 | Amg | STR | |
| | | | | | normal | trisomic |
| **Fibroblasts** | - | 1 hom affected **18**<br>1 het cell line **12**<br>Total **30** | - | - | - | - |
| **Trophoblasts** | 1 hom norm **20**<br>1 het **20**<br><br>Total **40** | - | - | - | - | 2 trisomy 21 **40**<br>1 trisomy 13 **20**<br>1 trisomy 18 **20**<br>Total **80** |
| **Buccal** | 1 hom normal **50**<br>1 hom affect **50**<br>2 het **50**<br>Total **150** | 1 hom normal **16**<br>1 het **17**<br><br>Total **16** | 1 hom normal **25**<br>1 hom affected **25**<br>1 het **24**<br>Total **74** | 1 male **20**<br>1 female **20**<br><br>Total **40** | 1 di-allelic **150** | - |
| **Squamous** | - | 1 het **17** | - | - | - | - |
| **Lymphocytes** | - | 1 hom norm **14** (12) | - | - | 1 di-allelic **30**<br>1 di-allelic **20**<br><br>Total **50** | 1 trisomy 18 **30**<br>1 trisomy 21 **20**<br>1 trisomy 21 **20**<br>Total **70** |

**Table 2.2** Single cells tested by QF-PCR assays. All STR data is from assays performed using a single marker [excluding multiplex results]. Cells tested for the single base changes causing HbS and beta-thalassaemia or the delta F-508 deletion causing CF are either homozygous (hom) normal or affected or heterozygous (het).

#### 2.3.5.1.1. Sickle Cell Anaemia

One hundred and ninety single cells from normal, heterozygote and homozygote affected individuals were tested (Table 2.2.).

#### 2.3.5.1.2. Beta Thalassaemia IVS1-110

Seventy four single buccal cells from normal and affected homozygous individuals and heterozygous carriers of the IVS1-110 mutation were isolated and tested (Table 2.2.).

#### 2.3.5.1.3. Cystic fibrosis delta F-508

Seventy seven single cells were investigated using this assay (Table 2.2.).

#### 2.3.5.1.4. Sex chromosome detection.

This assay was applied to 40 single cells (Table 2.2.).

#### 2.3.5.1.5. Aneuploidy detection by QF-PCR.

Six STR markers were utilised individually and in various combinations. Two hundred normal and 150 individual trisomic cells of known origin were tested using all of six STR markers in single primer set reactions, and the derived PCR product ratio assessed (Table 2.2.). Eighty two cells, from 2 cell lines trisomic for chromosome 21 were then tested with a multiplex QF-PCR containing primers specific for 3 STR repeats on chromosome 21, and one on chromosome 18. Prior to single cell PCR, control DNA from each cell source was prepared and amplified using the same assay. This provided a check of the allele sizes and ratios to be expected.

# Chapter 3

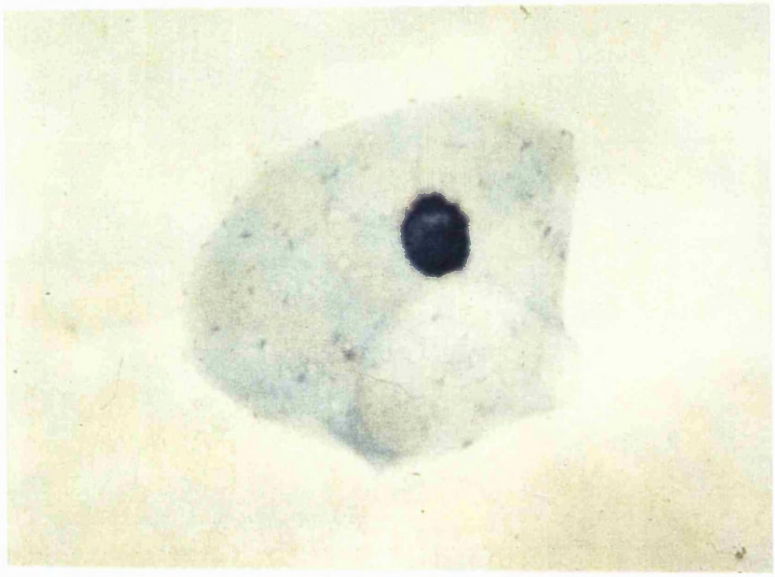
# Results

# Results

Almost all TCC samples collected were examined under light microscopy to determine their quality, and see whether they contained trophoblastic cellular elements. Random samples were further assessed by conventional staining. Following such analysis various different cell types could be observed. Maternal ectocervical squamous cells, and endocervical columnar epithelial cells (Fig 3.1a) were ubiquitously visible. Other maternal cells included leukocytes (macrophages, histocytes, lymphocytes, polymorphs, neutrophils and lymphocytes) as well as erythrocytes and cells of unknown origin (Fig 3.1b). Trophoblast cells could also been seen in some samples as syncitial fragments which could be identified by the presence of sprout formations containing multiple nuclei (Fig 3.2a) and presumed cytotrophoblasts with the characteristics of large, irregular, pleomorphic nuclei and abundant cytoplasm (Fig 3.2b).

## Figure 3.1a Maternal Squamous Cells in a TCC sample

A



B

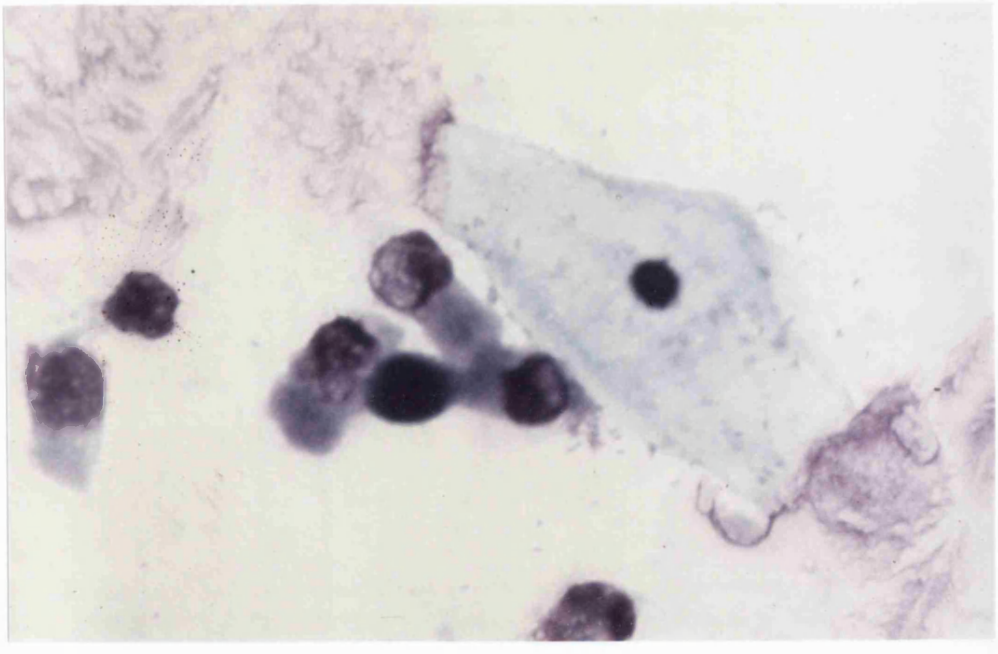
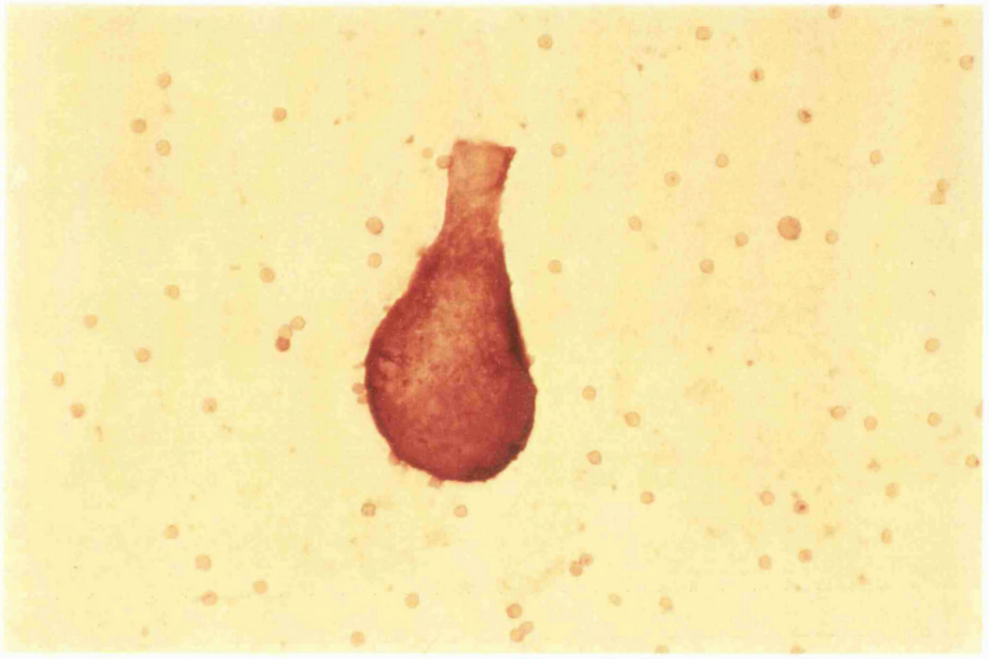


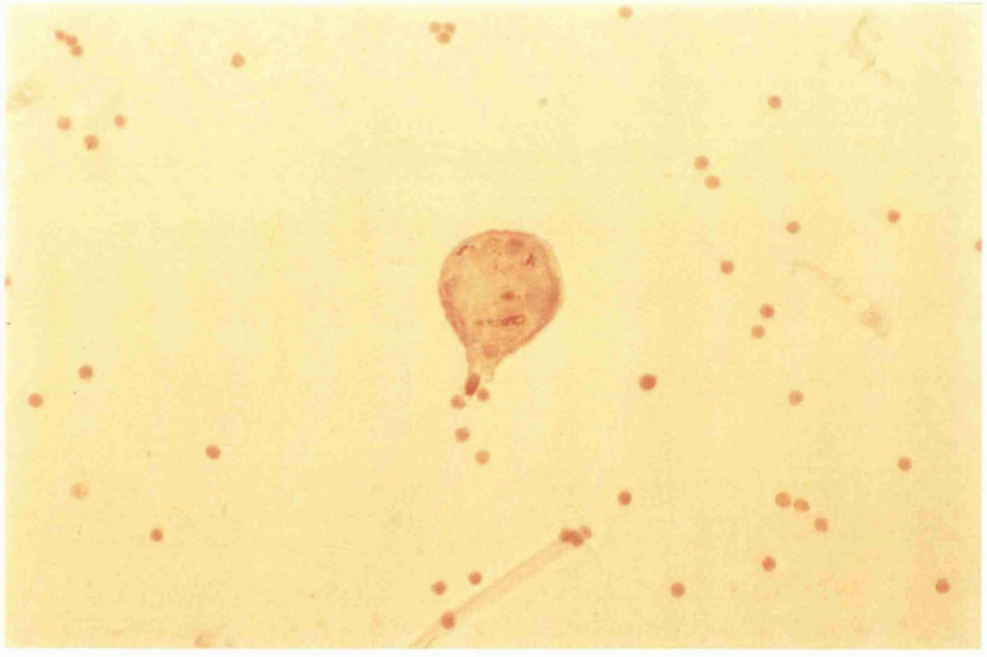
Image A shows an isolated maternal squamous cell identified in an aspiration sample stained with Leishmans. Image B shows a squamous cell surrounded by cells of unknown origin, again stained with Leishmans.

**Figure 3.1b Other cells observed in a TCC sample**

a)



b)



Figures a) and b) shown apparent syncytial trophoblastic buds surrounded by maternal erythrocytes.

**Figure 3.2a Syncytial fragment in TCC sample**

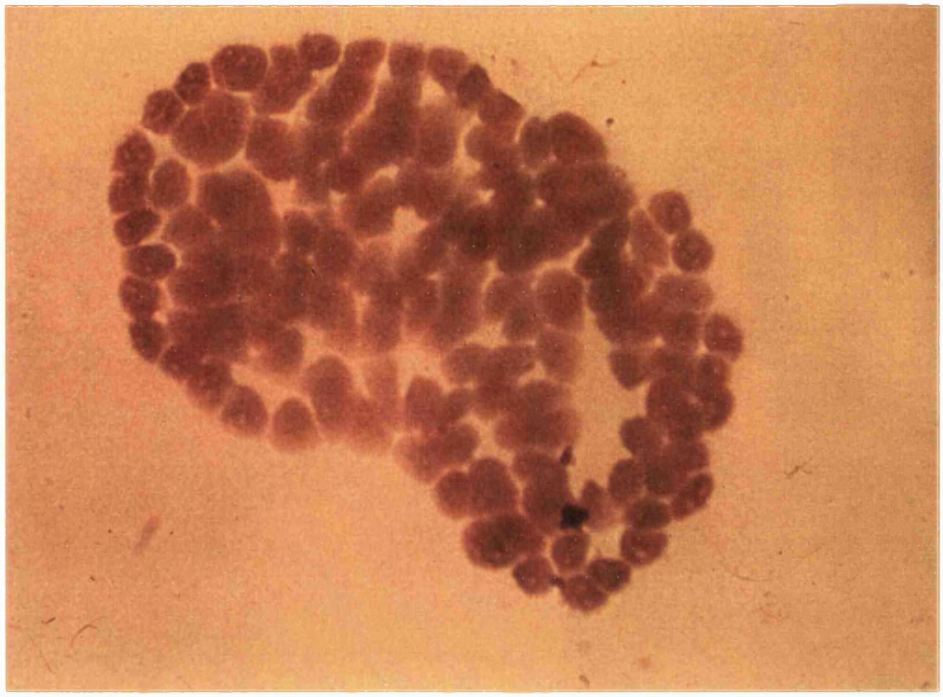
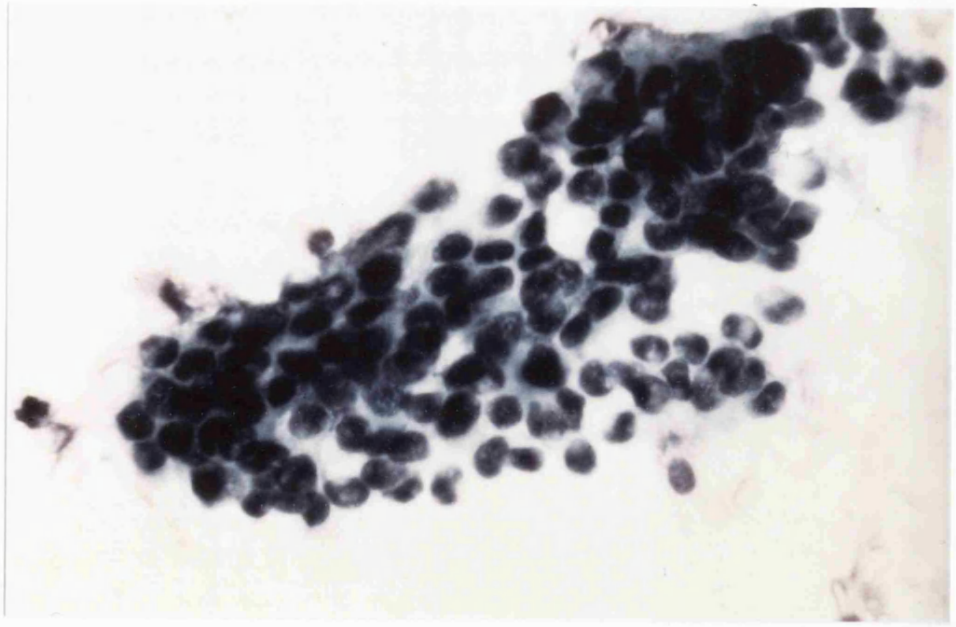


**Figure 3.2b Clump of trophoblast cells in TCC sample**



## 3.1. GROUP A

Selected TCC samples from Group A were tested by various molecular methods in attempts to identify the presence of fetal DNA.

### 3.1.1. Dual FISH for the X and Y Chromosomes

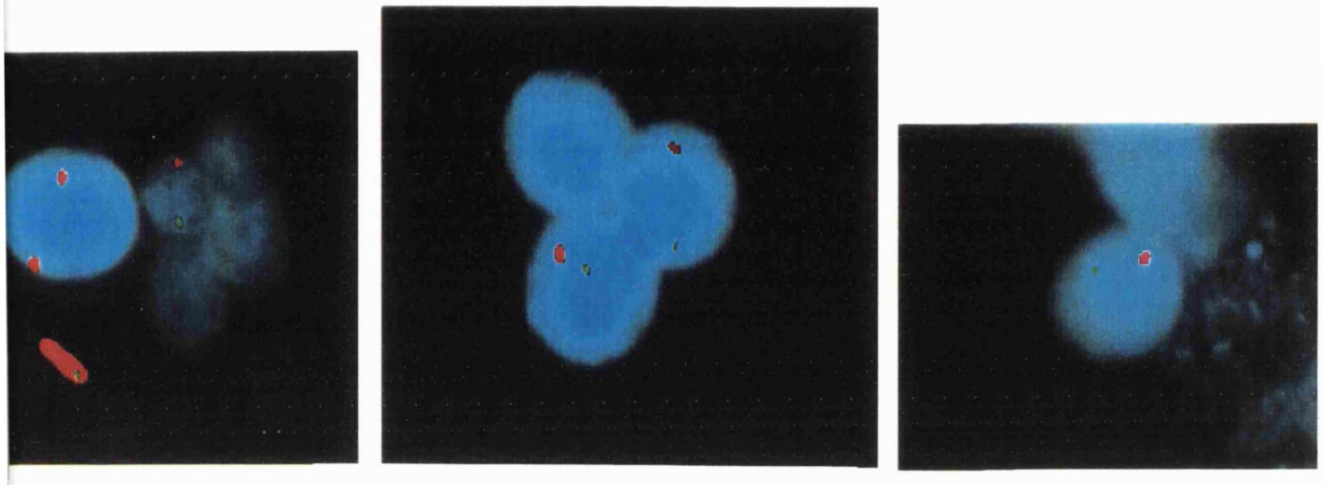
TCC samples were scanned for nuclei containing one X and one Y signal. If such nuclei were observed, the sex of the fetus was predicted to be male (Fig 3.3). In the absence of XY nuclei, the fetus was considered female. These results were compared to the sex of the fetus as detected by subsequent dual XY FISH of placental cells obtained after TOP. In most cases approximately 300 TCC cells were scored (Table 3.1).

The accuracy of this dual FISH method for identifying male fetal cells in a background of female nuclei was assessed by scoring control female lymphocyte preparations with every experiment. An apparent male nucleus was never observed (~100 nuclei scored per slide with over 100 slides examined). This negligible false positive rate allowed, with confidence, any XY bearing nuclei on a TCC slide to be adjudged to be fetal in origin.

**Figure 3.3 XY Nuclei in TCC samples using two colour FISH**

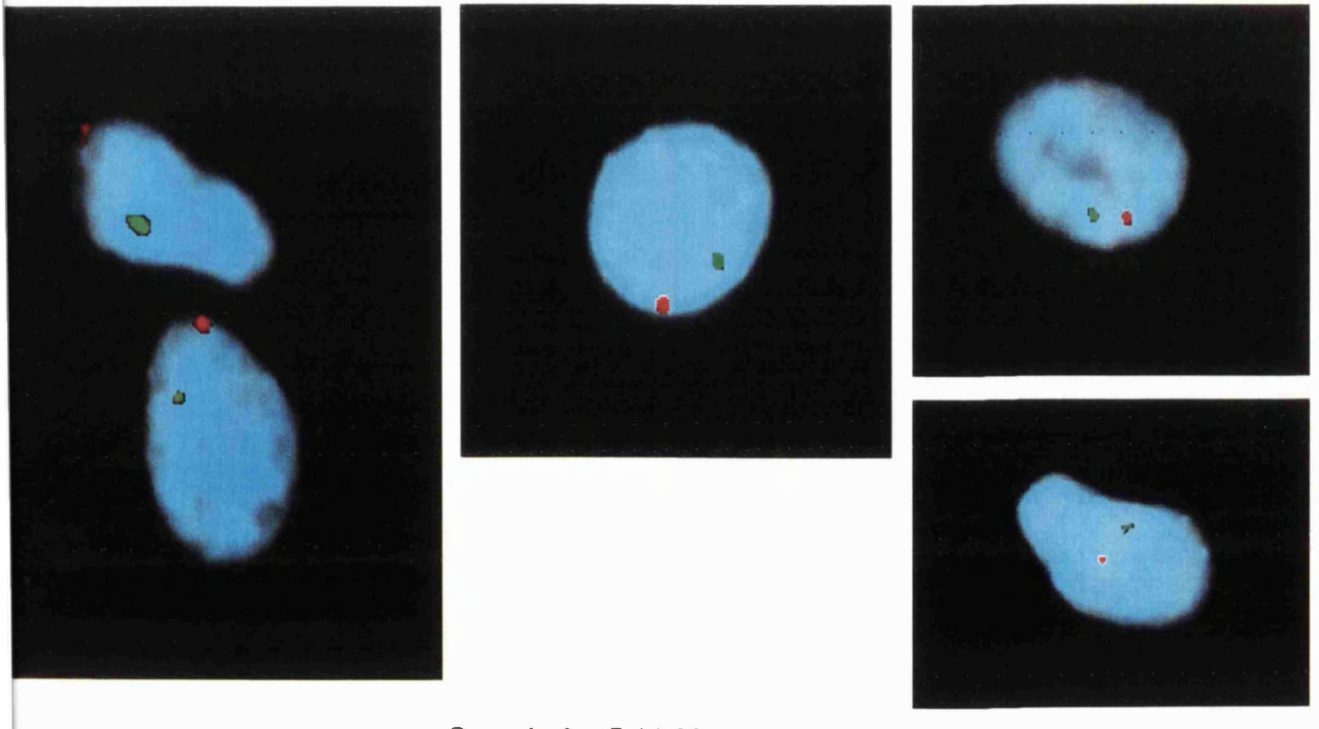
Transcervical Cells having undergone Fluorescent In Situ Hybridisation with :

**Y190** [Chromosome Y specific] labelled green
**PSV2X5** [Chromosome X specific] labelled red



**Sample 1 30.11.93**

NB: The foetus was confirmed to be male by conventional karyotyping.



**Sample 3 5.11.93**

NB: The foetus was confirmed to be male by conventional karyotyping.

**TABLE 3.1 Sexing of TCC samples collected by lavage or aspiration (Group A).**

| SAMPLE | Tested by FISH | | | Tested by PCR | | |
|---|---|---|---|---|---|---|
| | Total | Male* | Female | Total | Male* | Female |
| Aspiration | 16 | 2/5 | 11/11 | 33 | 7/14 | 19/19 |
| Lavage | 2 | 1/1 | 1/1 | 6 | 2/2 | 4/4 |

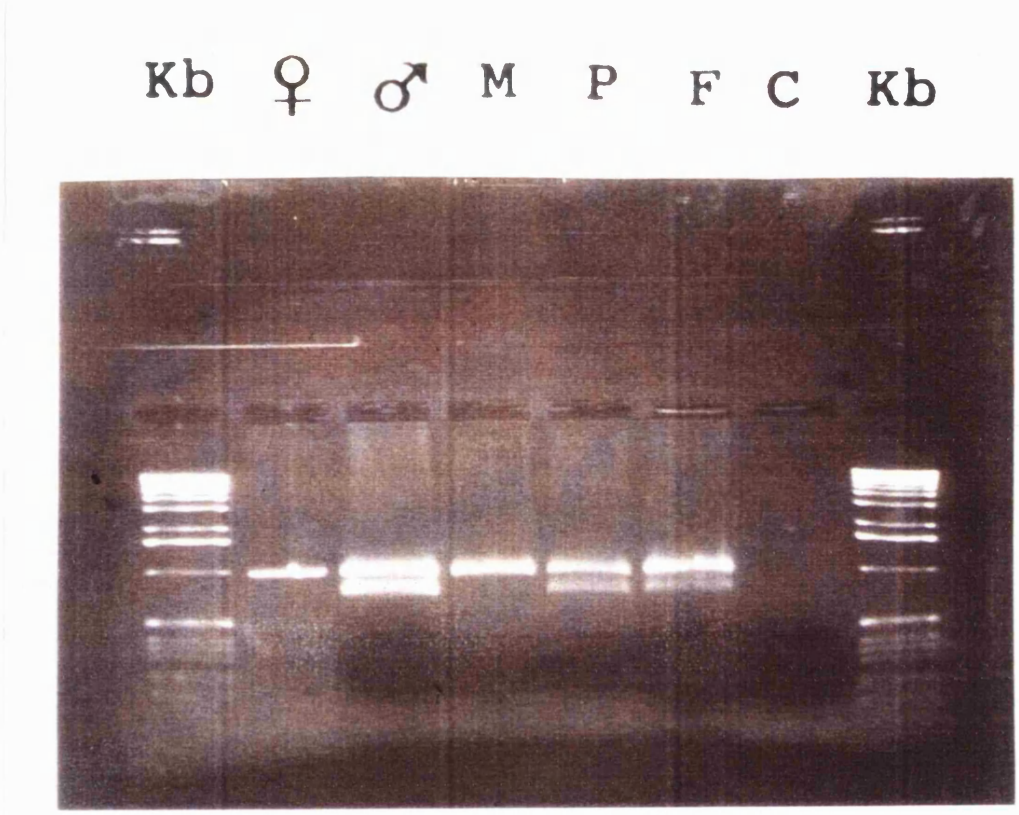
* Number of 'male' sexed TCC samples from total number of male gestations tested.

### 3.1.2. PCR of the Amelogenin Region of the Sex Chromosomes

(Ethidium Bromide PCR product detection)

Amplification of the amylogenin region of the X and Y chromosomes using primers after Nakahori et al., (1991b) produces two PCR products or bands, each specific to one chromosome (X= 1000 bp, Y= 823 bp). The presence of Y chromosome DNA within a sample is thus indicated by the presence of the smaller PCR product (Fig 3.4). As DNA is extracted from (and thus PCR performed upon) whole TCC samples, and not just on a few cells, this technique is potentially more sensitive than dual XY FISH.

**Figure 3.4 XY PCR; ethidium bromide product detection**




PCR amplification of the amelogenin region of the sex chromosomes was performed, and the products of the PCR separated on an agarose gel. For approximate sizing of the PCR products, the outer lanes contain a DNA '1Kb ladder'. This PCR was performed on control female and male DNA, DNA from maternal peripheral blood- **M**, DNA from placental material acquired after TOP- **P**, and on DNA obtained from a TCC lavage, or flush, sample- **F**. The same PCR reaction was also performed on a negative control- **C**. From lane **P** of the electrophoretogram it can be seen that the fetus is male, indicated by the presence of the Y specific band, absent in the maternal blood sample. A band of the same length can also be seen in the lavage sample, **F** showing it to contain DNA specific to the Y chromosome and thus of fetal origin. The Y chromosome specific band is relatively weaker in the lavage sample in comparison to the placental sample due to the presence of maternal cells in TCC samples.

From Group A, 57 TCC samples were examined by one or other of the aforementioned methods. Using PCR 39 samples (33 aspirations, and 6 lavage) were tested, with FISH, 16 aspiration and 2 lavage TCC samples were examined (Table 3.1). It can be seen that a good relationship was observed between the results of sexing TCC lavage samples by FISH and PCR. In aspiration samples examined by FISH, Y fluorescent signals were detected in 2 out of 5 samples from pregnancies with male fetuses, with three false negative results. Eleven out of 11 samples in the absence of any XY cells were correctly diagnosed as being derived from female pregnancies, i.e. no false positives. PCR amplification was carried out in 33 TCC aspiration samples. Correct sexing was achieved in 7 out of 14 TCC samples retrieved from mothers with male fetuses (7 false negatives) in 19 out of 19 samples obtained from pregnancies with female fetuses (no false positives; Table 3.1).

The different methods of TCC sampling showed great variability in the quantity and nature of cells obtained. When examined by FISH, the percentage of male cells detected in positive aspirate samples obtained from women with male fetuses, varied from 0.2-2%. In the Y positive lavage samples, XY cells were present at 0-50% concentration.

### 3.1.3. PCR of the STR D21S11

The DNA extracted from selected samples in Group A (lavage=16, aspirate=29) were also tested by PCR for the presence of the paternally inherited allele of the polymorphic STR, D21S11 (Table 3.2). Maternal allele sizes were determined by the PCR of maternal blood. Fetal allele sizes were determined by the PCR analysis of the placental DNA samples obtained after TOP. In the TCC samples, the presence of the paternal STR allele size inherited by the fetus (present in the placental sample but absent in the maternal) is evidence of fetal DNA. In a minority of cases, the DNA extracted from the placenta displayed the same D21S11 alleles as the maternal blood. In these cases the fetus had inherited a paternal allele of the same size as the non-inherited maternal allele. This meant that both the fetus and mother had identical STR sizes, and the assay was non-informative.

In the PCR amplification of any repeat sequence a minority population of PCR products are generated which are slightly smaller than those expected, differing by the number of DNA bases in the repeat itself. These are termed 'stutter bands' which are created by slippage of the PCR products during their formation, in the elongation stage

of PCR (Walsh et al., 1996; Fig 3.5). In some cases, the paternal allele appeared to be present in the TCC samples, but at a level only comparable to visible stutter bands and background fluorescence. In this situation, the presence of fetal DNA in the TCC sample was inconclusive, and therefore termed 'possible'.

**TABLE 3.2: Results of D21S11 PCR amplification from TCC samples collected by lavage or aspiration (Group A).**

| Collection Method | Total Sample Number | Presence of Paternal allele | | | |
|---|---|---|---|---|---|
| | | Present | Absent | Non-informative[1] | Possible[2] |
| Aspiration | 29 | 4 (13.8%) | 14 (48.3%) | 6 (20.7%) | 5 (17.2%) |
| Lavage | 16 | 4 (25%) | 8 (50%) | 3 (18.8%) | 1 (6.3%) |

1 Placental (fetal) alleles the same size as the maternal alleles

2 The paternal allele was present, but at a level comparable to stutter bands and background fluorescence.

As shown in Table 3.2, a paternally derived STR allele was detected in 4 out of 16 lavage and 4 out of 29 aspirate samples (Fig 3.6). Three lavage and 6 aspirate samples were non-informative.

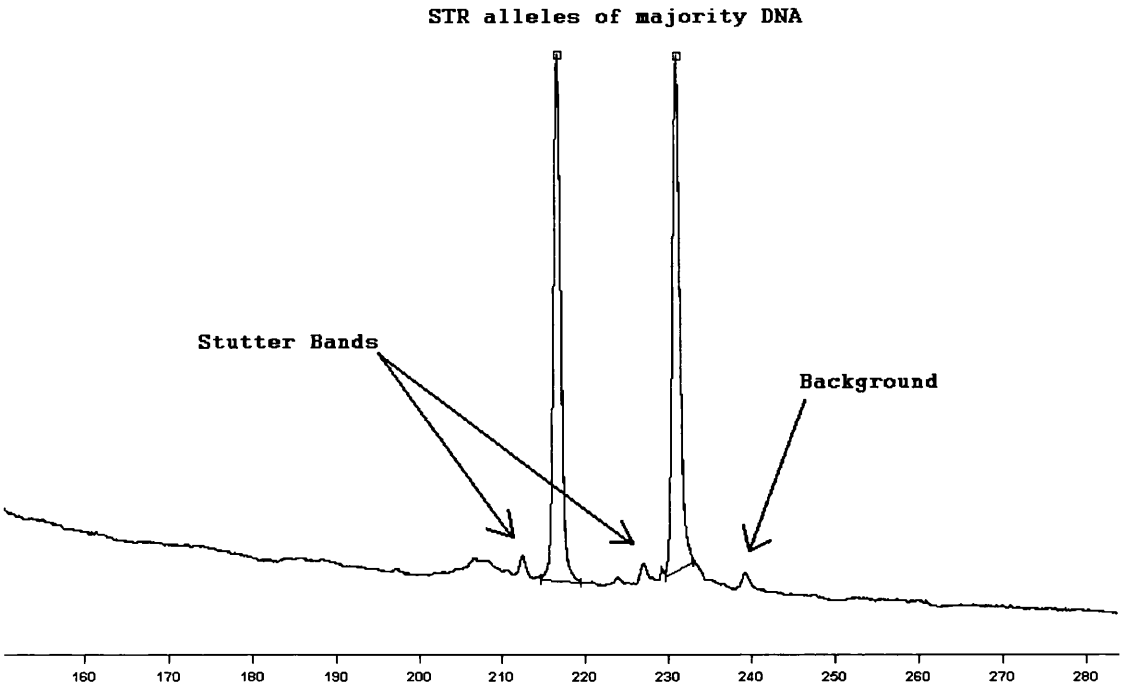
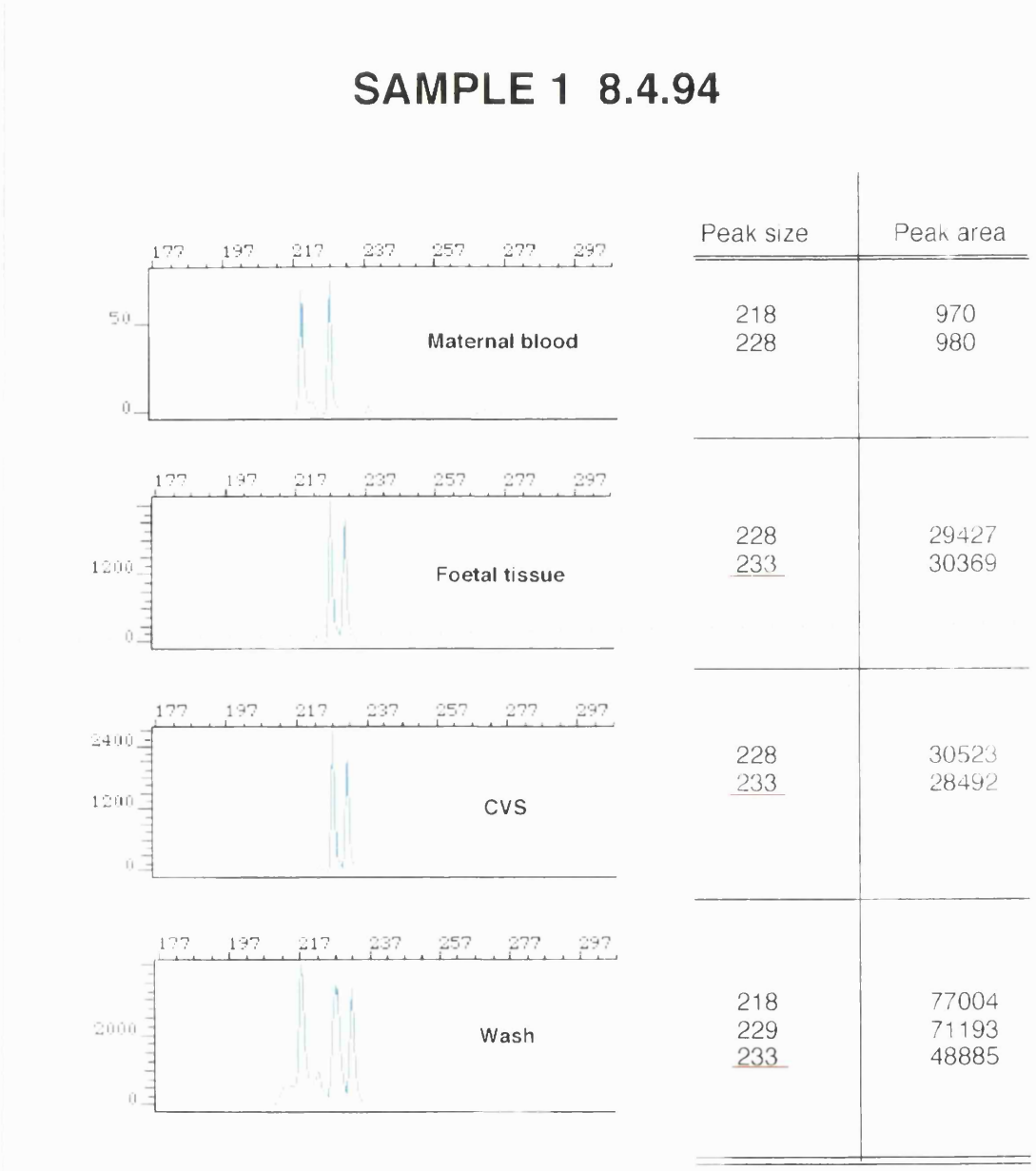



**Figure 3.5** **Difficulties in the detection of trace levels of DNA with fluorescent quantitative PCR due to the presence of 'stutter-bands' and 'background' fluorescence.**

**Figure 3.6 Fetal STR detected in TCC sample**




The fetus in this case has inherited a paternal STR allele of 233bp which is present in the placental and fetal tissue samples but absent in the maternal blood. The presence of this allele in DNA extracted from the lavage, or wash, sample clearly indicates the presence of fetal DNA.

### 3.1.3.1. Sensitivity of Fetal STR allele detection

Using a fluorescent detection system, with every lane of fluorescent DNA products run, there will be a background of detected fluorescence and visible stutter bands (Walsh et al., 1996). For a fluorescent DNA product to be detected without doubt, the peak of fluorescent intensity must exceed the intensity of the stutter bands and background level. An artificial mixture was thus set up whereby two DNA samples of known allele sizes and concentration were mixed, prior to PCR using primers specific for the STR allele D21S11. Subsequent fluorescent detection of the products of PCR was performed with both the Genescan and the ALF apparatus. The concentration of one DNA was progressively decreased, simulating the situation of low level fetal DNA present in a sample containing an overwhelming majority of maternal DNA. The lowest concentration of DNA at which the fluorescent intensity of generated PCR products exceeded both background and stutter band intensities was noted. In two separate experiments it was observed that, to be confidently detected, the percentage of minority DNA would have to exceed 2.5% of the total DNA concentration. For all those samples not displaying a clearly visible paternal peak, the only conclusion that can be drawn is that fetal cells were not present in the TCC samples at a concentration greater than 2.5%.

### 3.1.4. FISH for Trisomy 21

Two of the intrauterine lavage samples collected in Group A prior to elective TOP were from mothers with advanced age (>35). Using routine pre-procedure ultrasound both these fetuses were seen to display increased nucal translucency (4mm and 5mm) so were considered to be at high risk of trisomy 21. Cells were prepared from the TCC samples and exposed to dual FISH with two chromosome 21 contigs; each doublet of red and green fluorescent signals indicating one chromosome 21 (Section 2.2.10.1.). Samples of placenta obtained following TOP were simultaneously cultured and karyotyped for confirmation of the fetal chromosome complement.

Using this methodology, a chromosome 21 is only deemed to be present in a sample if two fluorescent signals (one green and one red) are recorded in close proximity. It is unlikely therefore that a false positive result will occur as non-specific hybridisation of both fluorescent dyes (producing one doublet) would have to transpire, within the same region of the same nuclei. The incidence of false positive results on

control lymphocytes was seen to be less than 1 in over 5000 nuclei scored. The presence of >3 nuclei containing 3 doublets was thus deemed sufficient to formulate a conclusion of fetal trisomy (Davies et al., 1994).

In one sample, no cells containing three 'doublets', and therefore three chromosomes 21, were found in over 500 scored nuclei. The karyotype of this sample was later confirmed to be 46,XX, verifying this result. In the second sample however, four cells were found out of 500 counted (0.8%) which each had 3 chromosomes 21 present in the nuclei (Fig 3.7a). Subsequent karyotype results confirmed the fetus to have trisomy 21.

**Figure 3.7a** **Dual colour FISH for chromosome 21 using cosmid contigs.**

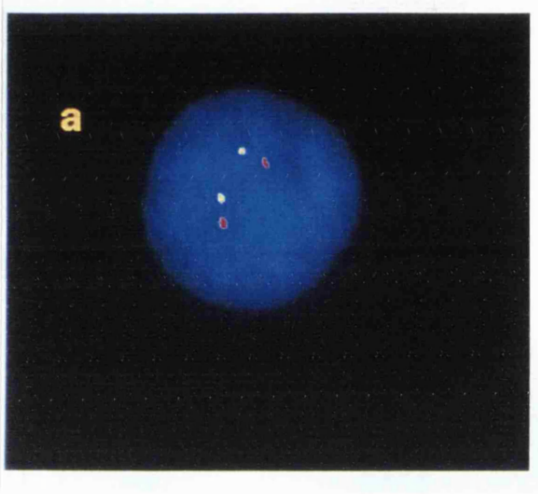



**Image a) shows a nucleus with two doublets and therefore two chromosomes 21.**

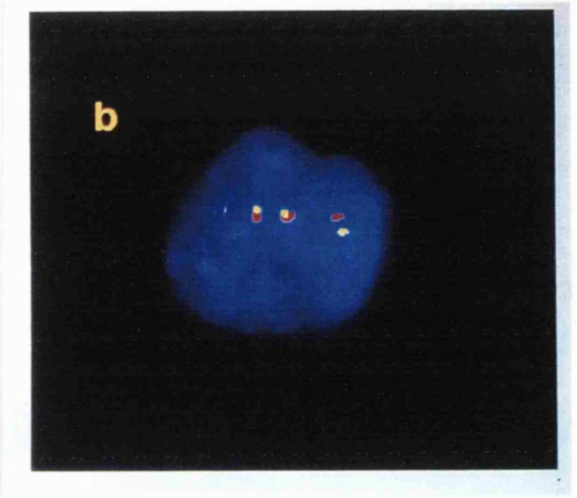
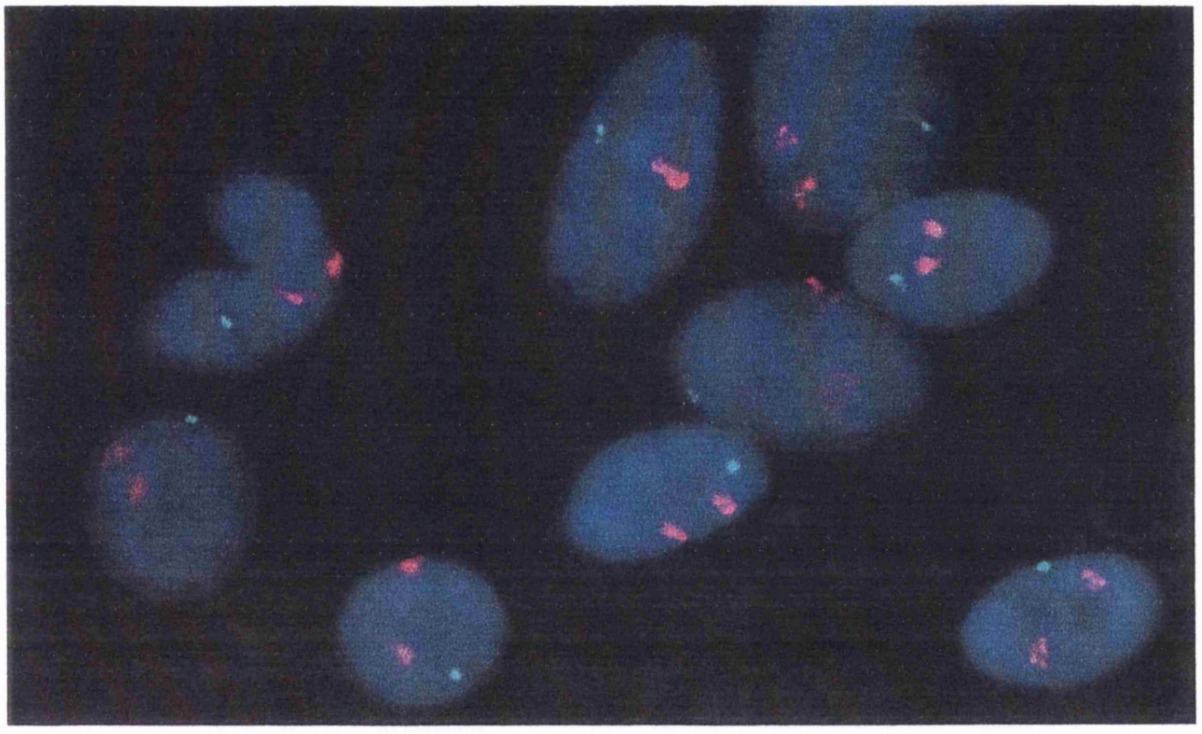



**Image b) shows a nucleus with three doublets and therefore three copies of chromosome 21.**

## Figure 3.7b



FISH for the X and Y chromosomes performed on TCC cells revealing a clump of fetal nuclei with one X (green) chromosome and two Y (red) chromosomes.

## 3.2. GROUP B

Group B consisted of TCC samples, collected by aspiration prior to CVS from 130 women intending to continue with their pregnancy. The outcome of these pregnancies with respect to the health of the baby and mother was monitored to establish the safety of this procedure. The position of the placenta, fetal gestation time (between 6 and 12 weeks) and the name of the operator was noted for each sample. The pregnancies of 145 women who underwent a CVS procedure without prior TCC sampling was followed up as a control group (see Section 3.16).

### 3.2.1. FISH for the X and Y Chromosomes

A random group of 33 of these TCC samples were analysed by FISH (n=22) or PCR (n=11) for the detection of Y specific DNA as previously described. The predicted fetal sex from these results was compared to that obtained from the analysis of chromosomes in metaphases from cultured chorionic tissue (Table 3.3). Fetal sex diagnosis was thus performed blind.

A correct diagnosis of the sex of the fetuses was achieved by FISH tests on all 15 specimens recovered by aspiration from women with female conceptuses (no false positive results), and in 5 out of 7 samples from pregnancies with male fetuses (2 false negative results). The frequency of XY cells in Y-positive samples varied from 0.7% to 3%. The analysis of one aspiration sample collected from a woman with a male fetus which showed no evidence of containing XY cells was limited to only 20 nuclei.

**Table 3.3: Sexing of TCC samples collected by aspiration prior to CVS (Group B).**

| | Tested by FISH | | | Tested by PCR | | |
|---|---|---|---|---|---|---|
| | Total | Male | Female | Total | Male | Female |
| Aspiration | 22 | 5/7 | 15/15 | 11 | 2/2 | 9/9 |

### 3.2.2. PCR of the Amelogenin Region of the Sex Chromosomes

As shown in Table 3.3, 11 samples from Group B were exposed to the PCR sexing reaction as previously described. The sex of the fetus was correctly diagnosed in all samples (male= 2, female= 9) as confirmed by conventional karyotyping.

### 3.2.3. FISH for Trisomy 21

From group B, aspiration samples were collected prior to CVS, from 13 mothers at risk of chromosomal abnormalities. All these women were considered at sufficient risk of having a baby with trisomy 21, due to their age (>37), and were to undergo a CVS biopsy. These aspirate samples were tested with FISH for the number of chromosomes 21, as previously described. At least 300 nuclei were scored from each sample. No cells containing three chromosomes 21 were observed. All samples were examined without prior knowledge of the karyotype of the fetus. The predicted results of normal fetuses were subsequently confirmed in all cases by conventional CVS cell culture and karyotyping.

### 3.2.4. FISH for other Chromosomal Abnormalities

From one patient in Group A, a transcervical CVS, performed 10 weeks 4 days after the last menstrual period, showed a fetal karyotype of 47,XYY. Before TOP at 16 weeks 6 days, a TCC sample was collected by aspiration. FISH with X and Y specific probes performed on placental tissue obtained after TOP showed 95% of the 450 cells scored to be XYY and 5% to be XY. Using an inverted microscope a clump of cells with the morphological characteristics of a syncytiotrophoblastic cellular element was isolated from the TCC aspiration sample (Section 2.2.2). When tested with the same FISH assay, 50 of the 62 cells in this isolated clump were shown to contain one X and two Y chromosomes and thus to be of fetal origin (Fig. 3.7b). The remaining 12 cells had two X chromosomes and were therefore of maternal origin. No fetal cells were found using FISH in the remainder of the TCC sample despite the examination over 400 nuclei; however, PCR using primers specific for the amelogenin region of the sex chromosomes performed on DNA extracted from the whole TCC samples showed the presence of Y specific DNA.

One aspirate sample in Group B was obtained from a pregnant woman prior to elective termination of a fetus in the 15th week of gestation. TCC samples were not normally obtained from women with a pregnancy at this stage, however karyotyping of a previously obtained CVS sample had revealed that the fetus carried the chromosomal abnormality; 48,XYY+13. Probes specific for the Y chromosome, X chromosome, and chromosome 13 were hybridised to this TCC sample using triple colour FISH. The sample was subsequently screened for abnormal (fetal) cells. No abnormal cells were detected in the 900 cells examined in this sample.

## 3.3. GROUP C

A new collection technique, that of cytobrush was assessed in a comparative study with lavage samples (11 cytobrush samples, 11 lavage samples), performed on 22 women undergoing TOP between 6 and 12 weeks of gestation (Group C). These samples were stained with conventional Leishman stain and studied with light microscopy. Cells of fetal origin were also identified using monoclonal antibodies (McAb) specific to placental antigens, stained with fluorescence or peroxidase detection systems. TCC samples were also tested with dual XY FISH, XY PCR and STR PCR techniques, as previously described (Table 3.4).

**Table 3.4 Comparison of cytobrush and lavage for the retrieval of TCC samples**

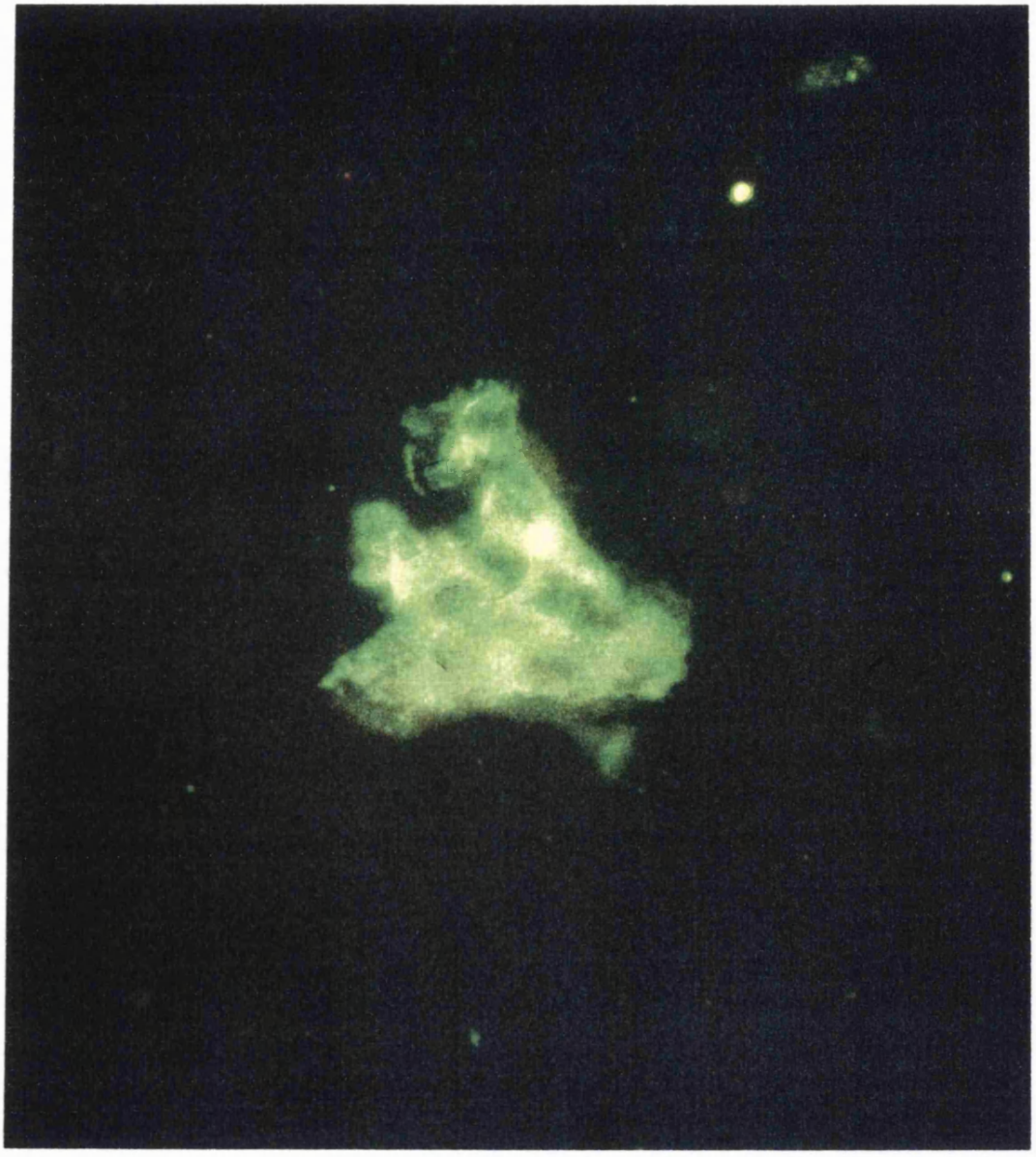
| Sampling technique[1] | Gestational age (weeks) | XY PCR placenta | XY PCR TCC | FISH TCC (%XY) | Fetal STR detected in TCC | Total cells | Syncytio-trophoblast | Cyto-trophoblast[2] |
|---|---|---|---|---|---|---|---|---|
| L1 | 6 | Male | Male | 50 | + | +++ | + | + |
| L2 | 6 | Female | Female | 0 | - | + | - | ++ |
| L3 | 8 | Male | Male | 5 | - | + | - | ++ |
| L4 | 7 | Female | Female | 0 | - | +++ | - | + |
| L5 | 9 | Female | Female | 0 | + | + | - | + |
| L6 | 9 | Male | Male | 40 | + | +++ | + | + |
| L7 | 7 | Male | Male | 0* | - | + | - | ++ |
| L8 | 8 | Female | Female | 0 | - | +++ | + | ++ |
| L9 | 7 | Female | Female | 0 | + | +++ | + | ++ |
| L10 | 6 | Male | Male | 8 | + | + | - | ++ |
| L11 | 8 | Male | Male | 2 | - | + | - | ++ |
| CB1 | 7 | Female | Female | 0 | - | + | - | ++ |
| CB2 | 7 | Female | Female | 0 | - | ++ | - | ++ |
| CB3 | 6 | Female | Female | 0 | - | +++ | - | + |
| CB4 | 7 | Male | Male | 2 | non-inform | + | - | ++ |
| CB5 | 10 | Male | Male | 5 | + | ++ | + | + |
| CB6 | 8 | Female | Female | 0 | non-inform | ++ | - | ++ |
| CB7 | 12 | Male | Male | 0* | - | + | - | ++ |
| CB8 | 7 | Female | Female | 0 | - | + | - | ++ |
| CB9 | 10 | Male | Male | 0* | - | + | - | ++ |
| CB10 | 12 | Male | Male | 2 | - | + | - | ++ |
| CB11 | 10 | Male | Male | 1 | non-inform | ++ | - | ++ |

1 Samples collected by lavage (L) or cytobrush (CB); 2 Cytotrophoblasts detected using McAb IO3; * No XY cells found in >200 nuclei counted.

### 3.3.1. Microscopy and Antibody Staining

Following conventional staining, squamous and endocervical cells, syncytiotrophoblastic cellular elements, neutrophils, lymphocytes and red blood cells could readily be recognised, but the assessment of the number and type of cells was inevitably subjective and could only be expressed in arbitrary values (-, +, ++, +++; Table 3.4). The two methods of sampling showed great variability, but all samples contained squamous cells; syncytiotrophoblastic cellular elements were seen in four of eleven lavage samples, but were detected in only one cytobrush sample. By phase contrast microscopy and conventional staining with Leishmans, cytotrophoblasts could not be readily identified with certainty. Single cytotrophoblasts are sometimes difficult to distinguish from macrophages and groups of cytotrophoblast from inflamed endocervical epithelial cells or metaplastic squamous cells. The presence of cytotrophoblast cells was confirmed in some samples with McAbs FT141.1, H315, and IO3 (Fig 3.8). The morphology of the positive cells varied greatly within each sample; however cellular debris was more commonly seen in samples collected by cytobrush, than in those retrieved by lavage.

**Figure 3.8 TCC cell clump stained with trophoblast specific McAb**



This clump of cells, with the apparent morphology of trophoblast, were exposed to the trophoblast specific McAb 340 (courtesy of Dr. Lindy Durrant) which was conjugated to a green fluorescent molecule (fluorescein) and visualised with microscopy.

### 3.3.2. FISH for the X and Y Chromosomes

Using dual X and Y FISH, high percentages of Y-positive cells were observed in two samples obtained by lavage (L1 and L6); in the other specimens from mothers with male embryos, the incidence varied from 2% to 8% where between 200-300 nuclei were scored in each case (Table 3.4). This was a higher percentage than that observed in the brush samples, where FISH analysis demonstrated a lower number of Y chromosome containing cells in samples from mothers with male embryos (1% to 5%).

### 3.3.3. PCR of the Amelogenin Region of the Sex Chromosomes

The results of analysing the sex of the fetuses, performed by PCR on transcervical cells, were in agreement in every case with those carried out using the same method on samples of villous placenta collected following termination of pregnancy (Table 3.4). There were no false positive results.

A good correlation was observed between the result of testing the transcervical samples with PCR to detect X and Y derived DNA sequences, and those obtained using FISH and X and Y probes in all but three specimens. Cells containing fluorescent Y signals could not be detected in one sample (L7) obtained by lavage, and in two (CB7 and CB8) retrieved using a cytobrush; all three were Y-positive using PCR, and the results were confirmed on villus placental tissue obtained after TOP.

### 3.3.4. PCR of the STR D21S11

In six transcervical samples (5 collected by lavage and one by cytobrush), three STR peaks were observed. One peak, due to the presence of fetal DNA, was detected in the villus placenta DNA extract but was absent in the corresponding maternal sample (Fig 3.6). Three uninformative patterns (CB4, CB6, and CB11) were observed. Samples which did not display the paternal peak either did not contain fetal cells or they were present at a concentration below 2.5% (Fig 3.5).

## 3.4. GROUP D

Two TCC samples were collected sequentially, the first by aspiration and the second by lavage from 22 pregnant women before TOP for non-medical reasons (Group D). The two samples from each woman were collected on the same day with minimal interval between each procedure.

### 3.4.1. FISH for the X and Y Chromosomes

All these TCC samples were tested blind by dual, XY FISH, together with relevant controls. The sex of the fetus was later established by testing nuclei prepared from chorionic villi, obtained after TOP. Accordingly, 13 fetuses were found to be male and 9 to be female (Table 3.5).

When TCC samples collected by aspiration were tested by dual FISH for the presence of X and Y chromosomes, fetal sex prediction was correctly achieved in 8 out of 13 samples obtained from women with male fetuses (Fig 3.9) and 9 out of 9 women with female fetuses. With the TCC samples collected by subsequent lavage from the same pregnant women, fetal sex prediction by FISH was again correctly performed in all 9 samples obtained from mothers with female fetuses, and in 8 out of 13 specimens from pregnant women with male conceptuses. In this group the incidence of nuclei with a Y fluorescent signal in Y-positive samples ranged from 0.23-7.1%. Combined FISH analysis of TCC samples obtained by aspiration and lavage from the 13 pregnancies with male conceptuses showed that sexing was correctly performed in 12; in 4 cases both aspiration and lavage samples contained XY nuclei, and in the remaining cases either the aspiration or lavage TCC sample provided evidence of nuclei with one X and one Y fluorescent signal.

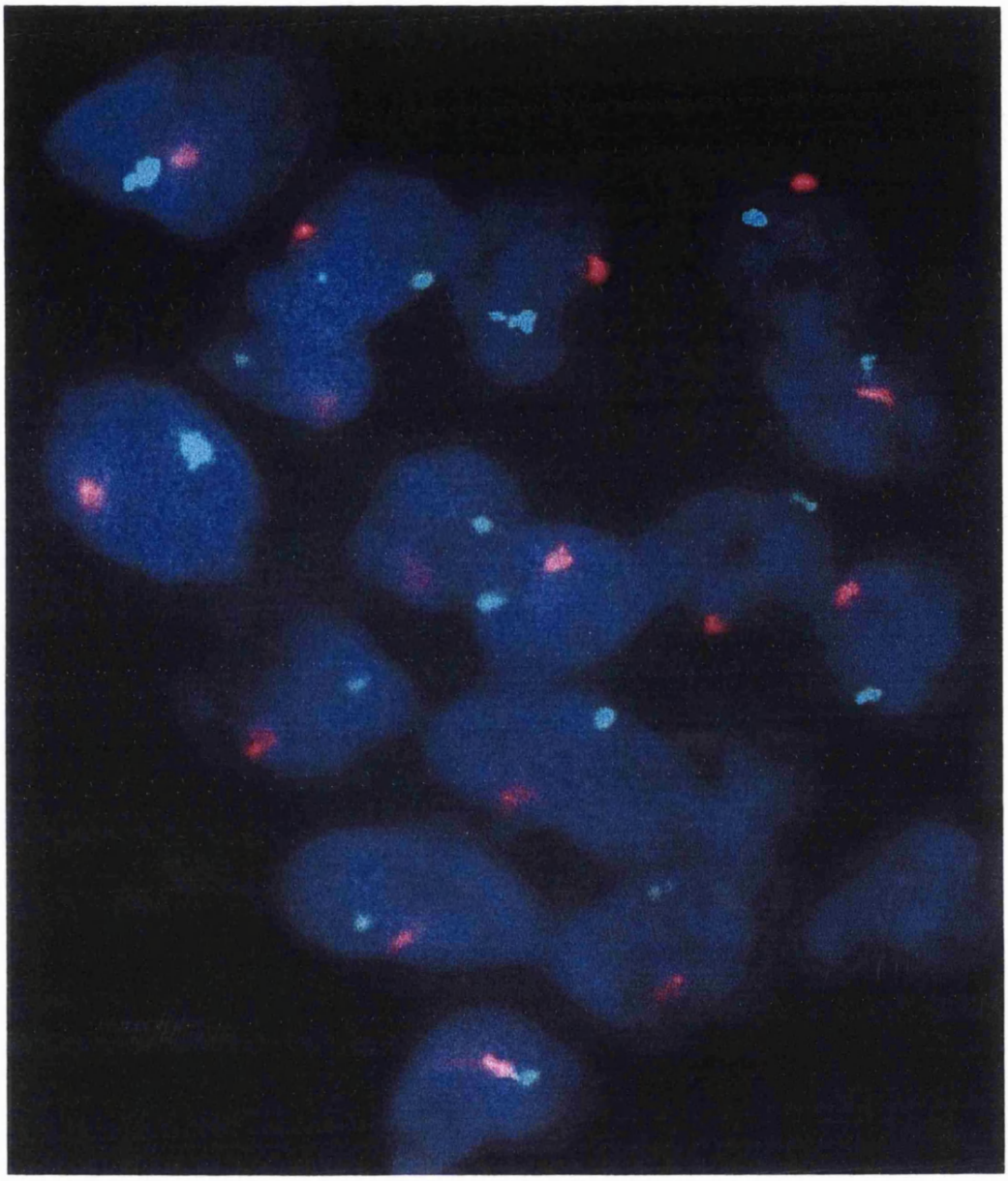
**Table 3.5 FISH sexing of TCC samples collected sequentially by aspiration and lavage (Group D).**

| SAMPLE | Male* | Female |
|---|---|---|
| Aspirate n=22 | 8/13 | 9/9 |
| Lavage n=22 | 8/13 | 9/9 |
| Placenta n=22 | 13 | 9/9 |
| Aspirate or Lavage | 12/13 | 9/9 |

* One fetus was found to be triploid, with two X signals and one Y signal in each cell (see later text) but for this table was treated as 'male'.

Nb. FISH latterly performed by Dr. Ashutosh Halder

**Figure 3.9 Dual XY FISH showing clump of fetal cells in TCC**



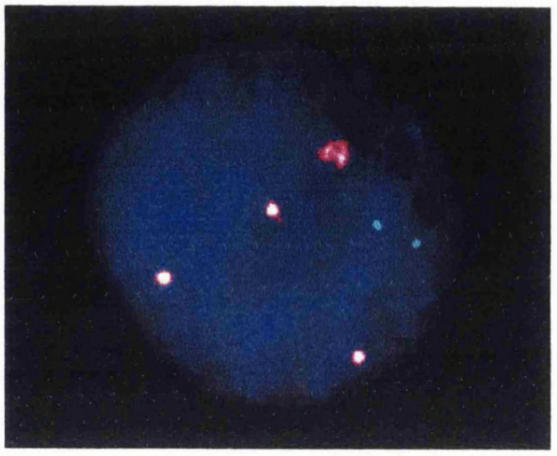
A clump of male (fetal) cells detected in a lavage sample by fluorescent in situ hybridisation. Each nuclei clearly shows two fluorescent signals; one green (X chromosome) and one red (Y chromosome).

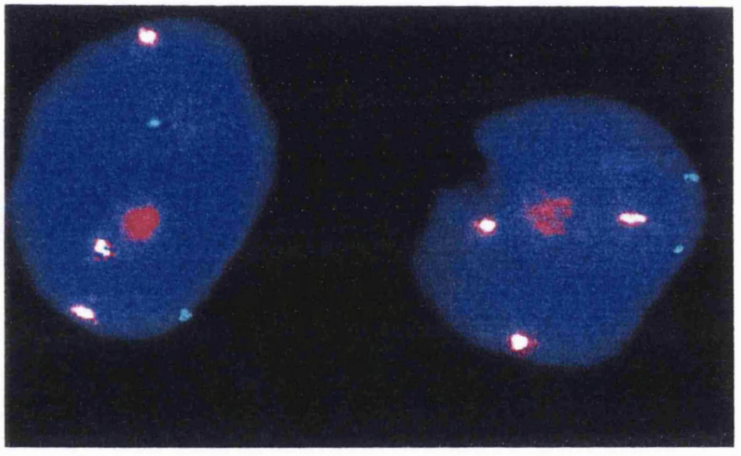
### 3.4.2. Fetal Triploidy

During the course of this comparative study, one placental sample was found to contain cells with one Y and two X chromosome signals suggesting the fetus to be either 47XXY or triploid. This sample was further investigated using a dual labelled chromosome 1 repetitive probe, as well as the X and Y probes, in a triple colour interphase FISH procedure. The placental cells were shown to contain 3 chromosome 1 signals and thus to be almost certainly derived from a triploid fetus (Fig 3.10).

Dual colour XY-FISH was performed on the aspirate and lavage samples obtained from this patient. The results of the FISH tests showed that 6.7% of nuclei (7 out of 104 counted) from the aspirated sample and 2.7% (3 out of 111) nuclei from the lavage sample exhibited two green (XX) and one red (Y) signal and were of fetal origin (Fig 3.11a). Clumps of cells with the morphology of trophoblastic cellular elements were isolated from the remainder of these samples using micromanipulation. Most of these isolated clumps were shown to be exclusively of fetal origin using FISH and PCR(see Section 3.8.1).

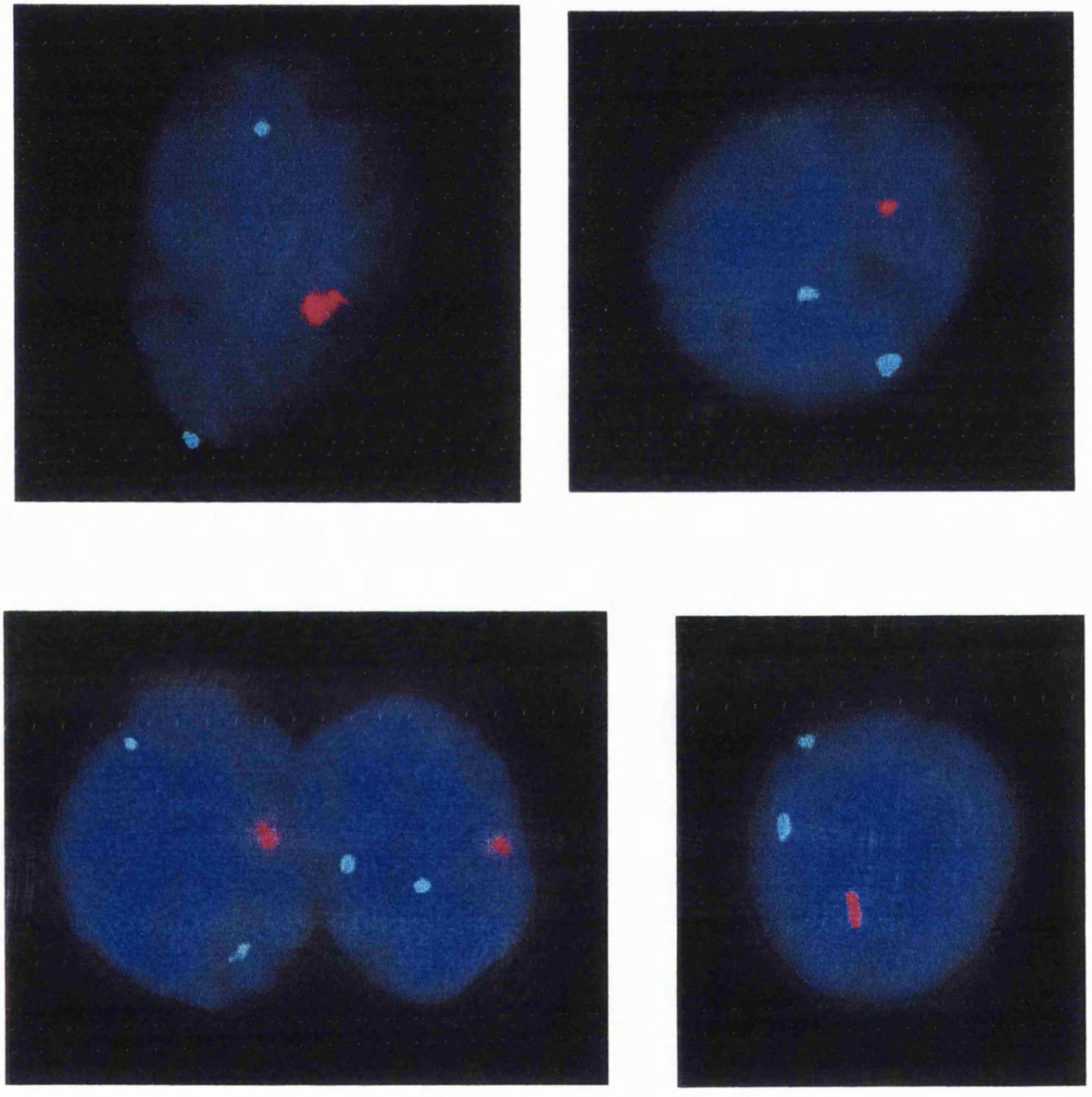
**Figure 3.10**





Three colour FISH performed on triploidy placenta cells. Within each nuclei, the two green fluorescent spots indicate the presence of two X chromosomes, the single red spot highlights a single Y chromosome and the three yellow fluorescent spots reveal the presence of three copies of chromosome 1.

**Figure 3.11**



Fetal triploidy cells detected in a lavage sample by FISH. Cells show two X chromosomes (green) and one Y chromosome (red).

## 3.5. GROUP E

TCC samples collected by Pipelle aspiration were analysed by PCR for the presence of X- and Y- specific DNA sequences and the results compared with those obtained from testing the corresponding placental tissues. As shown in Table 3.6, out of 25 TCC samples collected from mothers with male fetuses, 17 (68%) showed a chromosome Y-derived PCR amplification peak. This was not observed in any of the 31 TCC samples retrieved from mothers with females fetuses.

Another 27 TCC samples were tested by PCR for the presence of STR-derived sequences. STR markers of paternal origin were detected in eight TCC samples, corresponding to a similar fetal marker detected in placental extracts (Fig 3.12). In five cases the STR markers in the TCC samples were similar to those present in maternal blood, with a fetal marker present only in the placental DNA. In 12 cases the STR markers were similar in maternal blood, placenta and TCC, thus the samples were non-informative. In a further two cases, the fetal STR allele was observed in the TCC samples, but only at a level comparable to non-specific PCR products. It could therefore only be concluded that there was a possibility of the presence of fetal DNA. There were no significant differences in the success of detecting fetal DNA with regard to gestational age.

**Table 3.6** The presence of Y-derived and STR sequences in placenta and TCC samples collected by Pipelle aspiration.

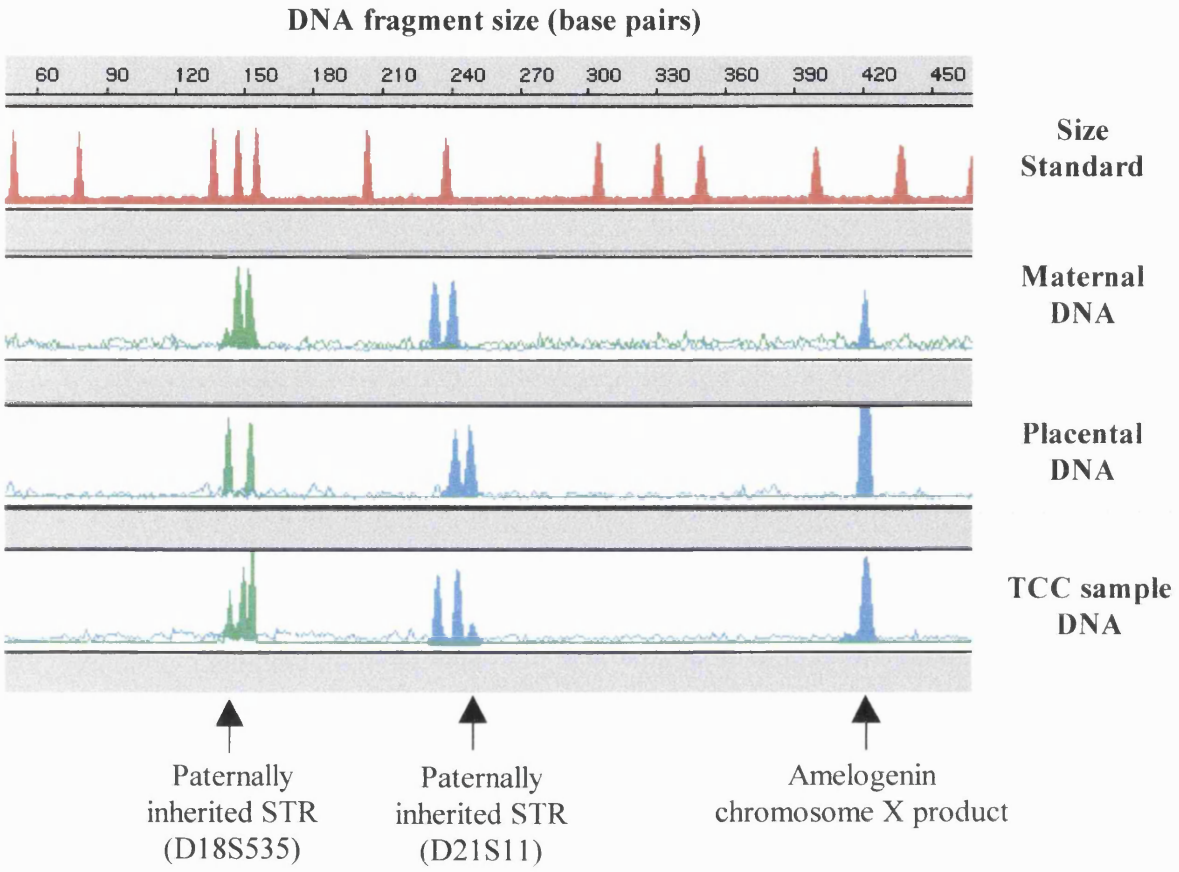
| PCR for X and Y | **Total** | **Placenta XY** | **TCC XY** | **Placenta XX** | **TCC XX*** |
|---|---|---|---|---|---|
| | 56 | 25 | 17 (68%) | 31 | 31 |
| PCR for the STR markers | **Total** | **STR fetal positive**** | **STR fetal negative** | **Possible+** | **Non-informative** |
| | 27 | 8 (29.6%) | 5 | 2 | 12 |

* No TCC sample from female fetuses was found to be Y-positive.

** There was full agreement between the results of testing placenta and TCC samples.

\+ The fetal STR allele was observed in the TCC sample, but only at a level comparable to non-specific PCR products.

**Figure 3.12**




Peaks of fluorescent intensity indicate products of PCR, separated by capillary electrophoresis. DNA from the transcervical cell sample clearly displays the paternally inherited STR from both the D21S11 and the D18S535 markers. The chromosome Y amelogenin PCR product (252 base pairs) is absent from both placental and TCC DNA indicating the fetus to be female.

## 3.6. Fetal Rh(D) typing in Rh(D) Negative Mothers

The DNA from all TCC samples collected from Groups A and B, where the mothers were Rh(D)-negative, was extracted and exposed to a PCR reaction to determine the fetal Rh(D) type (Carritt et al., 1994). Samples were collected by aspiration of cervical mucus from 6 mothers with ongoing pregnancies, and lavage from 6 mothers prior to TOP. Results of PCR typing performed on TCC samples were compared to either blood typing after the resulting birth, or PCR results from samples of placenta (or CVS) respectively (Table 3.7). All mothers with Rh-negative fetuses (n=4) had Rh(D)-negative PCR results from their transcervical samples, i.e. there were no false positives. Of the 7 Rh(D)-positive pregnancies, all but 2 had Rh(D) positive transcervical sample PCR results. The 2 false negative results were assumed to be due to collection of too few fetal cells for the sensitivity of the PCR reaction. The PCR for the Rh(d) type was performed by Dr. Tim Kemp.

**Table 3.7: Rh(D) typing of TCC samples collected from Rh(D)-negative mothers.**

| SAMPLE* | Rh(D) typing by PCR | | | New-born Rh(D) |
|---|---|---|---|---|
| | Mother | TCC | CVS | |
| 1A | - | + | Not Done | + |
| 2A | - | + | Not Done | + |
| 3A | - | - | Not Done | + |
| 4A | Not Done | - | Not Done | - |
| 5A | - | - | - | Not Done |
| 6A | - | - | - | Not Done |
| 7L | - | + | + | Not Done |
| 8L | - | - | - | Not Done |
| 9L | - | + | + | Not Done |
| 10L | - | - | - | Not Done |
| 11L | - | - | + | Not Done |
| 12L | - | + | + | Not Done |

* TCC collection method; A= aspiration, L= lavage.

Shaded cells indicate non-concordance of TCC and fetal tissue tests.

## 3.7. Isolating pure fetal samples

The prenatal diagnosis of single-gene disorders (e.g. autosomal recessive and X-linked conditions) requires the use of pure fetal cells free of maternal contaminants. This may be achieved by the micromanipulation of cellular elements with trophoblast morphology from TCC samples, into sterile chambers. These cells can then be exposed to quantitative fluorescent PCR and FISH tests. This micromanipulation technique was developed and largely performed by Doctor Boris Tutschek.

## 3.8. STR quantitation; Quantitative Fluorescent PCR

One hundred and thirty four samples of DNA were tested by quantitative fluorescent PCR (QF-PCR) for the STR D21S11 to assess the validity of this procedure as a technique for the detection of chromosome aneuploidy. Of the 17 amniotic fluid samples known to be normal from cytogenetic analysis, 16 produced two STR peaks with a mean intensity ratio of 1.07 (Table 3.8); the other samples showed one STR peak (homozygote). Of 91 amniotic fluid samples tested blind, 68 samples produced two STR peaks (mean ratio 1.102); cytogenetic analysis confirmed that these samples had been obtained from normal conceptuses. Seven samples produced only one STR peak and were shown to be from homozygous normal samples.

Eight amniotic fluid samples produced two STR peaks with a mean ratio of 2:1. This demonstrated that one STR allele, with double the amount of PCR product, had been present in duplicate in the starting DNA; trisomic *di-allelic*. These eight fluids were shown to be from Down Syndrome fetuses. Another 8 samples had three peaks (1:1:1; trisomic *tri-allelic*) (Table 3.8); cytogenetic analysis confirmed them to be from fetuses with trisomy 21. Ten of 13 blood samples from normal adults showed two STR peaks (mean ratio 1.034:1). Three samples from fetuses of children with Down Syndrome showed two STR peaks with a mean ratio of 1.9:1; a further 4 blood samples from Down Syndrome fetuses showed three peaks (1:1:1) (Table 3.8). Three samples of tissue from Down Syndrome fetuses had two STR peaks (mean ratio 1.96:1) (Table 3.8); three other samples produced three STR fluorescent peaks.

Only 3 of the 134 samples (2.2%) produced uninformative results. From 1 amniotic sample no DNA could be extracted. A sample from a normal fetus produced three STR peaks (1:1:2). The combined areas of the first two peaks and that of the third peak were identical (1:1). The first two peaks differed in size by four bases (one repeat-

unit) and may have resulted from mosaicism for one STR allele. This result may also have been due to maternal cell contamination in the amniotic sample. The third unsatisfactory result was observed in amniotic fluid from a Down Syndrome fetus; two STR peaks with a ratio of 4:1 were produced. This unexpected pattern my also be due to mosaicism for an STR marker, or maternal cell contamination.

Since the two abnormal STR amplification patterns could readily be identified, their occurrence did not invalidate the diagnostic value of this procedure. It was clearly shown that QF-PCR is a reliable technique for the identification of chromosome copy number. This initial STR work was performed together with Dr. Barbara Pertl.

**Table 3.8 Ratio of QF-PCR product peak areas**

| Sample | Total tested | Zygosity | Relative peak areas of heterozygotes | | |
|---|---|---|---|---|---|
| | | | mean peak ratio | Standard Deviation | Range |
| Normal Samples | | | | | |
| Amniotic cells (known to be normal) | 17 | 16[1] | 1.070 | 0.060 | 1.005-1.224 |
| Amniotic cells (blind study) | 75 | 68[1] | 1.102 | 0.095 | 1.001-1.310 |
| Blood | 13 | 10[1] | 1.034 | 0.041 | 1.005-1.127 |
| Trisomic samples | | | | | |
| Amniotic cells (blind study) | 16[3] | 8[2] | 2.000 | 0.224 | 1.631-2.317 |
| Blood | 7[3] | 3[2] | 1.909 | 0.007 | 1.817-2.180 |
| Fetal tissue | 6[3] | 3[2] | 1.964 | 0.156 | 1.902-1.935 |

1 Normal di-allelic

2 Trisomic di-allelic

3 15 samples were trisomic tri-allelic

The same QF-PCR assay, performed on small numbers of amniotic cells (2µl of fluid) using more cycles of PCR, was shown to be reliable. This demonstrated the feasibility of assessing the origin of cell clumps isolated from TCC samples by this QF-PCR technique.

When STR markers were combined in multiplex QF-PCR reactions (Table 2.1), it was found that each marker was amplified independently, and could be assessed individually for aneuploidy detection. This significantly reduces the chance of a DNA sample having a homozygous allele pattern and therefore being non-informative (Fig 3.13). Trisomic (22) and normal (54) DNAs were assessed using multiplex PCR and primers for the STRs D21S11 and MBP (Table 3.9). The primers for MBP amplified two separate but closely linked repeats within the MBP gene, while amplification of the STR D21S1414 provided confirmation of the D21S11 STR result- hence two informative ratios could be obtained for each chromosome. When tested with D21S11, 46 out of 54 normal samples were heterozygous, showing two STR peaks with a mean fluorescent intensity ratio of 1.06 (standard deviation [SD]=0.065) (Table 3.9). Amplifications using D21S1414 confirmed the presence of 46 heterozygotes with a mean ratio of 1.09 (SD=0.078). PCR assays using MBP primers showed 35 of the 54 samples to be heterozygous at locus A with a mean fluorescent activity ration of 1.14 (SD=0.115) and 45 heterozygous at locus B with a ratio of 1.10 (SD=0.088). Out of the 54 normal amniotic and blood samples, 8 were homozygous for D21S11 and thus for D21S1414, whilst 6 were homozygous for both alleles of the MBP marker.

With the D21S11 marker, 10 out of 20 trisomic samples showed three peaks with a peak ratio of 1:1:1 (tri-alleleic). The remaining 10 samples displayed a di-allelic pattern with two peaks and a mean intensity ratio of 2.07 (SD=0.211) (Table 3.9). This was confirmed with the use of the D21S1414 primers showing the same 10 di-allelic profiles with a mean peak ratio of 1.91 (SD=0.080). Only one sample in this study produced spurious results: One trisomy 21 fetal blood sample showed a trisomic di-allelic pattern when using the D21S11and D21S1414 markers, the ratios for the two peaks (1.99 and 1.97 respectively) clearly indicating the presence of three chromosomes 21. However, when using the MBP-STR primer, a ratio of 1.6 for locus A and a ratio of 1.07 for locus B were observed. Thus, while thee result for locus A is ambiguous, the locus B ratio clearly reflected the diploid chromosome 18 content of the tested DNA.

**Table 3.9**

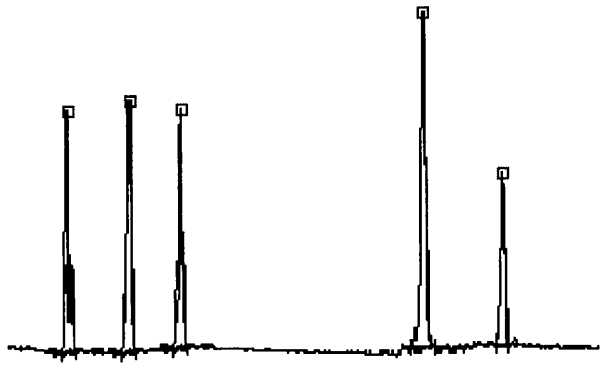
**DNA samples tested by QF-PCR with STR markers D21S11, D21S1414 and MBP**

| Normal Samples | | | | | | |
|---|---|---|---|---|---|---|
| Sample (no: 54) | Marker | Homozygous | Heterozygous | Relative peak areas of the heterozygous samples* Mean | SD | Range |
| Normal Blood (no: 23) | D21S11 | 4 | 19 | 1.06 | 0.069 | 1.00-1.25 |
| | D21S1414 | 4 | 19 | 1.10 | 0.082 | 1.00-1.23 |
| | MBP locus A | 8 | 15 | 1.09 | 0.047 | 1.02-1.15 |
| | MBP locus B | 4 | 19 | 1.09 | 0.077 | 1.01-1.26 |
| Amniotic fluid (no: 31) | D21S11 | 4 | 27 | 1.06 | 0.063 | 1.0-1.25 |
| | D21S1414 | 4 | 27 | 1.09 | 0.076 | 1.0-1.24 |
| | MBP locus A | 11 | 20 | 1.19 | 0.132 | 1.02-1.45 |
| | MBP locus B | 5 | 26 | 1.10 | 0.094 | 1.00-1.29 |

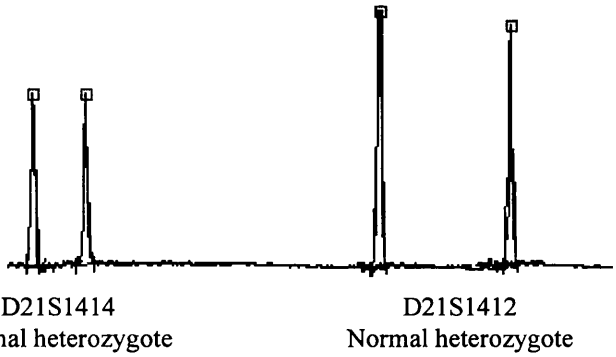
| Trisomic samples | | | | | | | |
|---|---|---|---|---|---|---|---|
| Sample (no: 22) | Marker | Tri-allelic | Di-allelic | Mono-allelic | Relative peak areas* Mean | SD | Range |
| Trisomy 21, Fetal blood and tissue (no: 10) | D21S11 | 4 | 6 | | 1.91 | 0.368 | 1.68-2.37 |
| | D21S1414 | 4 | 6 | | 1.88 | 0.081 | 1.77-1.99 |
| | MBP locus A | 8 heterozygotes[a] | | | 1.11 | 0.119 | 1.00-1.39 |
| | MBP locus B | 9 heterozygotes[b] | | | 1.10 | 0.105 | 1.00-1.34[a] |
| Trisomy 21, amniotic cells (no: 10) | D21S11 | 6 | 4 | | 2.13 | 0.115 | 2.02-2.31 |
| | D21S1414 | 6 | 4 | | 1.96 | 0.043 | 1.89-2.00 |
| | MBP locus A | 7 heterozygotes[d] | | | 1.22 | 0.101 | 1.13-1.42 |
| | MBP locus B | 7 heterozygotes[d] | | | 1.11 | 0.031 | 1.06-1.15 |
| Trisomy 18, Fetal tissue (no: 1) | D21S11 | heterozygous | | | 1.19 | | |
| | D21S1414 | heterozygous | | | 1.02 | | |
| | MBP locus A | | 1 | | 1.71 | | |
| | MBP locus B | | 1 | | 2.03 | | |
| Triploid Fetal tissue (no: 1) | D21S11 | | 1 | | | | |
| | D21S1414 | | 1 | | 1.97 | | |
| | MBP locus A | | 1 | | 1.71 | | |
| | MBP locus B | | 1 | | 2.02 | | |

* The means were calculated by dividing the larger by the smaller area.
a One sample (not included) produced a consistent ratio of 1.60 (see text).
b Two samples were homozygous
c One sample was homozygous
d Three samples were homozygous

**Figure 3.13** **Multiplex QF-PCR amplifying STR markers**




Down Syndrome DNA




Normal DNA

The presence of either a tri-allelic (1:1:1) profile, or a di-allelic profile with the peak areas at a ratio of 2:1 indicate the presence of 3 chromosomes and therefore trisomy. A normal heterozygote DNA will have two peaks of fluorescent activity with an area ratio of 1:1.

In a further study used two multiplex PCR assays to amplify nine STR loci (D21S1414; D21S11; D21S1411; D21S1412; D13S631; D13S634; MBP; D18S535; XHPRT) from 4 chromosomes (21, 13, 18 and X) were employed. Primers specific for the amelogenin region of the sex chromosomes were also included. The DNA from 85 amniotic fluid, fetal tissue and fetal blood samples were analysed (Tables 3.10a &3.10b).

**Table 3.10a The ratio of STR PCR product peak areas from normal DNA samples**

| **Normal Blood Samples (n=28)** | | | | | |
|---|---|---|---|---|---|
| Marker | Heterozygous | Homozygous | Relative peak areas* | | |
| | | | Mean | SD | Range |
| D21S11 | 21 | 7 | 1.04 | 0.025 | 1.00-1.10 |
| D21S1414 | 19 | 9 | 1.04 | 0.042 | 1.00-1.20 |
| D21S1411 | 24 | 4 | 1.06 | 0.066 | 1.00-1.28 |
| D21S1412 | 25 | 3 | 1.08 | 0.058 | 1.00-1.21 |
| D18S535 | 21 | 7 | 1.06 | 0.045 | 1.00-1.15 |
| MBP locus A | 19 | 9 | 1.09 | 0.051 | 1.01-1.21 |
| MBP locus B | 23 | 5 | 1.07 | 0.051 | 1.01-1.20 |
| D13S631 | 22 | 6 | 1.05 | 0.059 | 1.00-1.22 |
| D13S634 | 20 | 8 | 1.11 | 0.065 | 1.01-1.23 |
| Amelogenin | 9 male; 19 female | | | | |
| XHPRT (n=19) | 12 | 7 | 1.12 | 0.086 | 1.00-1.28 |
| **Normal Amniotic fluid samples (n=32)** | | | | | |
| Marker | Heterozygous | Homozygous | Relative peak areas* | | |
| | | | Mean | SD | Range |
| D21S11 | 26 | 6 | 1.09 | 0.077 | 1.00-1.24 |
| D21S1414 | 27 | 5 | 1.09 | 0.062 | 1.00-1.24 |
| D21S1411 | 28 | 4 | 1.08 | 0.070 | 1.00-1.23 |
| D21S1412 | 25 | 7 | 1.10 | 0.078 | 1.00-1.27 |
| D18S535 | 27 | 5 | 1.10 | 0.074 | 1.01-1.28 |
| MBP locus A | 22 | 10 | 1.15 | 0.107 | 1.02-1.43 |
| MBP locus B | 26 | 6 | 1.09 | 0.088 | 1.00-1.44 |
| D13S631 | 23 | 9 | 1.08 | 0.063 | 1.00-1.23 |
| D13S634 | 26 | 6 | 1.12 | 0.087 | 1.00-1.28 |
| Amelogenin | 12 male, 20 female | | | | |
| XHPRT (n=20) | 13 | 7 | 1.19 | 0.063 | 1.00-1.19 |

* The means were calculated by dividing the larger by the smaller area.

**Table 3.10)b The ratio of STR PCR product peak areas from trisomic DNA samples**

| Trisomy 21 samples (n=20) | | | | | | |
|---|---|---|---|---|---|---|
| Marker | Tri-allelic | Di-allelic[a] | Mono-allelic[a] | Relative peak areas* Mean | SD | Range |
| D21S11 | 3 | 11 | 4 | 2.06 | 0.227 | 1.83-2.59 |
| D21S1414 | 6 | 12 | 2 | 2.01 | 0.135 | 1.77-2.29 |
| D21S1411 | 9 | 8 | 3 | 2.02 | 0.233 | 1.80-256 |
| D21S1412 | 11 | 8 | 1 | 1.98 | 0.155 | 1.75-2.21 |
| D18S535 | - | 17 | 3 | 1.11 | 0.055 | 1.01-1.21 |
| MBP locus A | - | 15 | 5 | 1.17 | 0.110 | 1.02-1.44 |
| MBP locus B | - | 19 | 1 | 1.08 | 0.068 | 1.00-1.18 |
| D13S631 | - | 16 | 4 | 1.10 | 0.053 | 1.04-1.21 |
| D13S634 | - | 16 | 4 | 1.12 | 0.103 | 1.00-1.33 |
| Amelogenin | 10 male, 10 female | | | | | |
| XHPRT (n=10) | - | 8 | 2 | 1.04 | 0.028 | 1.00-1.09 |

| Other aneuploid samples (n=20) Mean ratio of Peak intensities. | | | |
|---|---|---|---|
| Marker | Triploid (n=2) | Trisomy 18 (n=2) | Trisomy 13 (n=1) |
| D21S11 | 1.90 (2 di-allelic) | 1.12 (1 het) | 1.13 |
| D21S1414 | 1.96 (2 di-allelic) | 1.21 (1 het) | 1.16 |
| D21S1411 | 2.12 (1 di-allelic[b]) | 1.07 (2 hets) | homoygous |
| D21S1412 | 1.80 (1 di-allelic) | 1.06 (2 hets) | 1.24 |
| D18S535 | 2.01 (2 di-allelic) | 2.01 (2 di-allelic) | 1.12 |
| MBP locus A | 2.40 (2 di-allelic) | 2.00 (2 di-allelic) | 1.01 |
| MBP locus B | 2.10 (2 di-allelic) | 1.82 (2 di-allelic) | 1.08 |
| D13S631 | 2 tri-allelic | 1.08 (1 het) | homozygous |
| D13S634 | 1.75 (1 di-allelic) | 1.13 (2 hets) | tri-allelic |
| Amelogenin and XHPRT | 1 triallelic, 1 XXY or XYY | 1 XY, 1XX (1.29[c]) | XX (1.02) |

* The means were calculated by dividing the larger by the smaller area.

a For STR markers not on chromosome 21, 'di-allelic' should be interpreted as 'heterozygous' and 'mono-allelic' as 'homozygous'.

b Samples not mentioned as di-allelic or heterozygous were tri-allelic or homozygous respectively.

c Ratio of XHPRT peak areas.

Table 3.10a shows the results obtained with DNA samples extracted from 28 normal blood samples and 32 normal amniotic fluid samples together with the frequency of heterozygosity and homozygosity for the various markers, the mean intensity of the ratios of the peak areas, the standard deviations and the ranges. Two

blood samples were homozygous for both chromosome 18 markers, and 7 out of 19 female blood samples were homozygous for the X chromosome marker. All the other blood samples were heterozygous for at least one marker specific for each of the analysed chromosomes. Two amniotic fluid samples were homozygous for both markers specific for chromosome 13, one sample was homozygous for the chromosome 18 specific markers, and 7 out of 20 female samples were homozygous for the X-chromosome marker. When tested with one of the chromosome 13 markers, three amniotic fluid samples (not included in Tables 3.10) were shown to have abnormal results. Two normal samples, tested with D13S631 showed an STR pattern with two peaks and fluorescence intensity ratios of 2.06:1 and 1.84:1. The third sample produced a heterozygous STR pattern with two peaks and a ratio of 1.7:1 when tested with D13S634. These fluorescence intensity ratios, with this chromosome 13 marker, were indicative of the presence of trisomy 13. However, the other chromosome 13 marker, D13S631, produced a heterozygous 1:1 pattern in two samples and a homozygous STR pattern in the third.

The results of testing 20 samples trisomic for chromosome 21 are displayed in Table 3.10b. Most samples showed either a tri-allelic (ratio 1:1:1) or a di-allelic (ratio 2:2) pattern with markers specific for chromosome 21. The markers specific for chromosomes 18, 13 and X showed a normal pattern with either two peaks and a relative fluorescence intensity ratio of 1:1 or a single STR peak. All but three samples were heterozygous for at least one marker specific for each chromosome. One sample was homozygous for both the chromosome 13 markers, and 2 out of 10 female samples were homozygous for the chromosome X marker.

The results of testing samples with trisomies 18, 13, and triploidy are shown in Table 3.10b. Two samples with triploidy were detected: all autosomal markers produced either a tri-allelic pattern with three peaks of similar fluorescence intensities or a diallelic pattern with a ratio of 2:1. The trisomy 18 samples showed a di-allelic pattern with all markers specific for chromosome 18. The trisomy 13 samples showed a tri-allelic pattern with the chromosome 13 marker S13S634 and a homozygous pattern with a single peak when tested with the marker D13S631.

Of the 85 DNA samples in this study only three normal samples produced an apparently false-positive result; when tested with one of the markers specific for chromosome 13. All samples were heterozygous for a least one marker specific for chromosome 21; 3 of the 85 samples were homozygous for all markers specific for

chromosome 18, and another 3 samples were homozygous for the chromosome 13 markers.

### 3.8.1. QF-PCR performed on isolated cell clumps

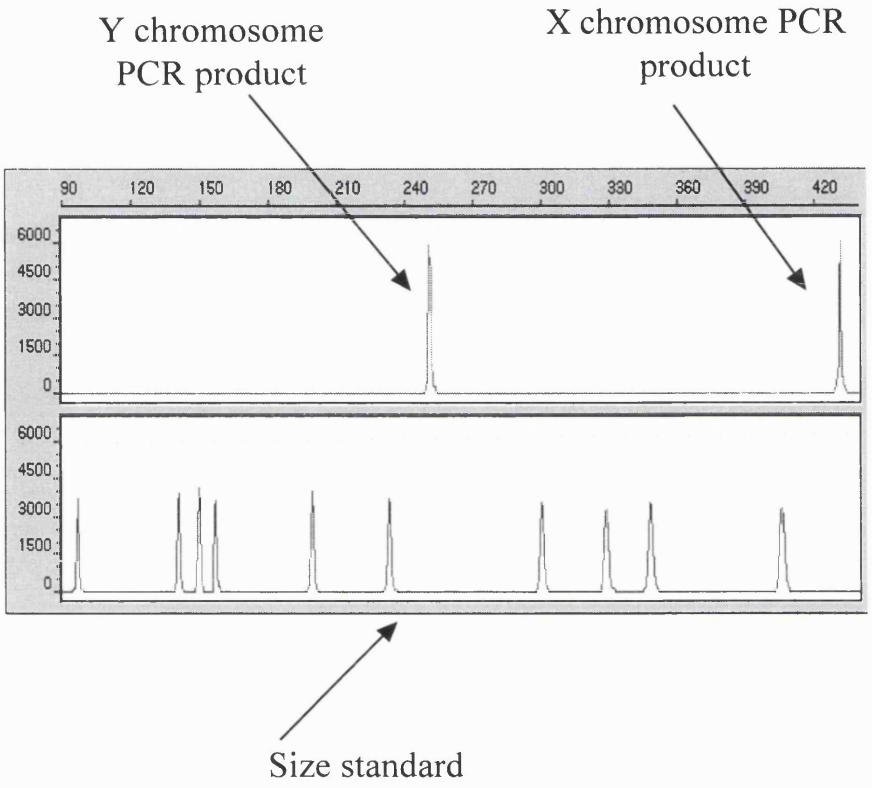
Cell clumps seen to have the morphology of trophoblasts under an inverted microscope were isolated from selected TCC samples collected from Groups A, B and D and tested by PCR for the STR D21S11 alleles. Some cell clumps were also tested simultaneously for the presence of X and Y chromosomes using a multiplex PCR reaction with primers for the amelogenin region of the sex chromosomes. A new primer set was designed for use with fluorescent primers (Table 2.1; Fig 3.14). The fluorescent primer set was first assessed in a preliminary study of 20 DNAs from known normal male and female donors. In every case of amplification from 46XX DNA, only one peak of fluorescent PCR products was produced, as only the X-chromosome amelogenin region was present as a template. Two peaks of DNA, one generated from the X chromosome, and one from the Y were seen in all samples amplified from 46XY DNA. This multiplex technique allows the origin of the isolated issue clump to be ascertained in all informative cases by comparison to maternal and placental results.

Preliminary tests were performed to validate the PCR amplification of D21S11 STR markers from single buccal cells isolated from different individuals ( named **a, b & c**) by micromanipulation (Fig 3.15). After 35 cycles of amplification two cell-free negative controls showed no specific amplification. Three buccal cells from individual **a** showed the same heterozygote peak pattern (PCR products 226 and 238 bases) also seen in the amplification from extracted blood DNA (data not shown). The same concordance was observed in single buccal cells from individual **b** (218 and 226 bases) and **c** (214 and 218 bases). The peak area ratios, representing the amount of PCR products, were all near 1.0 (0.99, 1.07, 1.14; 0.87; 1.04), indicating absence of preferential amplification (PA) or allelic drop-out (ADO). As STR amplification was shown to reliably produce the allele sizes present in the one template of a single cell's nucleus, it was concluded that the DNA from a clump (10-50) of cells could be assessed in the same manner for the identification of allele sizes and thus cell clump origin determination.

From 28 patients undergoing transcervical CVS or termination of pregnancy for non-medical reasons 140 isolated clumps were analysed. The results are reported in

Table 3.11 (mucus aspiration); Table 3.12 (lavage), Table 3.13 (aspiration followed by lavage) and Table 3.14 (summary).

**Figure 3.14** **QF-PCR for the X and Y chromosomes**




The same amelogenin primer set amplifies regions on both the X (432bp) and Y (252) chromosomes producing PCR products that can be differentiated by electrophoresis. The inclusion of fluorescently labelled primers allows PCR products to be analysed by fluorescent sequencing apparatus (in this case the ABI Prism™ 310). Each PCR product is run simultaneously with a DNA ladder provided, labelled with a different colour fluorescence allowing accurate size assessment.

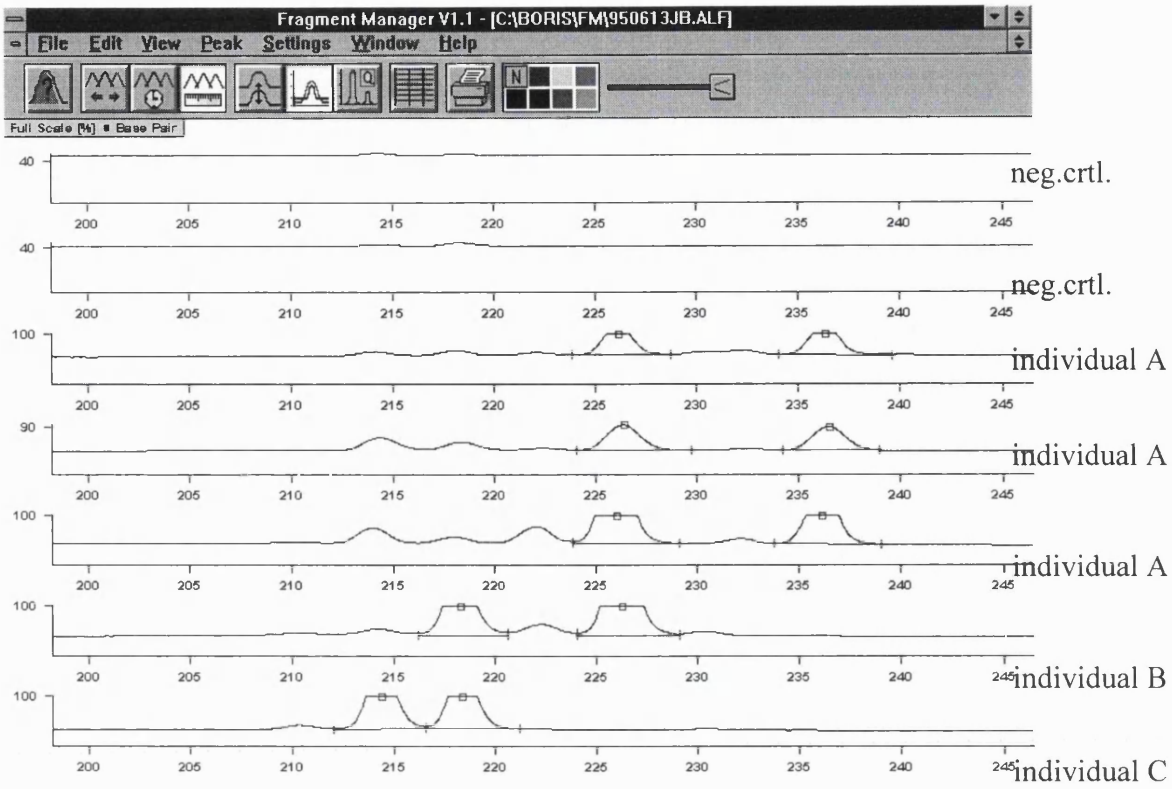



**Figure 3.15** Fluorescent PCR analysis of a polymorphic DNA marker (D21S11) from single buccal cells from different individuals isolated by micromanipulation. Seven PCR results are displayed. The horizontal axis denotes the product length, the vertical axes the product amount. PCR products are represented by peaks with small boxes on top. The three individuals tested are all heterozygote for D21S11. Among them, there are three different alleles.

## 3.9. FISH performed on isolated cell clumps

A small number of cell clumps isolated from TCC samples collected from women with male fetuses were also tested by dual XY FISH to determine whether the isolated cells were of fetal or maternal origin (Table 3.12) (FISH performed by Doctor Ashutosh Halder).

## 3.10 Combined QF-PCR and FISH results TCC cell clumps

Tested cell clumps isolated with micromanipulation from TCC samples were divided into three groups according to the TCC collection method; **Group 1** comprised 22 TCC samples collected by mucus aspiration prior to either TOP or CVS (from TCC Groups A & B; Sections 3.1 and 3.2); **Group 2** comprised three TCC samples collected by lavage prior to TOP (from TCC Group A; Section 3.1); **Group 3** comprised four patients where clumps of cells were isolated from samples collected by aspiration and then subsequent lavage (from TCC Group D; Section 3.4). In all these cases, where TCC samples had been obtained prior to TOP, cell clump STR sizes were compared with those determined in tested placenta and maternal blood. The STR sizes obtained from cell clumps obtained from TCC samples collected prior to CVS were compared with those determined in maternal blood, and from DNA extracted from the remains of the CVS cultures provided after routine karyotype analysis by the UCL cytogenetics department.

In Group 1 three cases produced non-informative STR allele sizes, so precluded the detection of purely fetal cell clumps. Nineteen samples were informative for the STR, from which seven yielded clumps of purely fetal material (Table 3.11). In five of these (patients 4, 8, 12, 19, 22) more than one fetal cell clump was observed. In another three cases both fetal and maternal allele sizes were detected by STR PCR. This confirmed that the isolated clump of cells were of fetal origin, but showed maternal DNA to also be present in the sample.

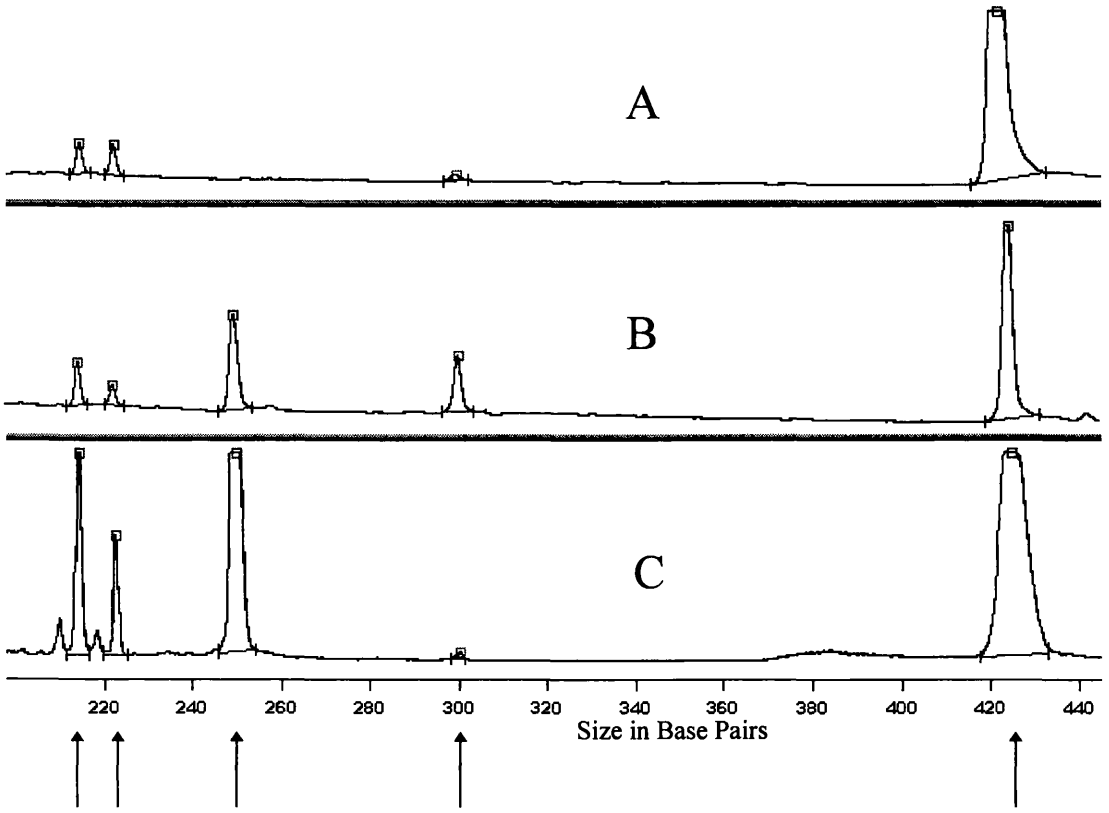
In the second group (lavage) one isolated clump contained exclusively fetal cells (patient 23, Table 3.12.); FISH of the remaining material revealed 4.7% fetal XY cells. In the other two cases, a total of seven isolated clumps were of maternal origin as judged by the results of FISH (patient 24, male fetus) or STR (patients 24 and 25)

analysis. In one of these cases (patient 24) the mucus and lavage showed 5.2% and 7.1% single fetal cells, respectively, after removal of the cell clumps (Table 3.12).

In the third group 15 isolated clumps from three patients were found to be fetal, while one cell clump from another patient was found to be maternal (Table 3.13). The placenta of patient 27 (Table 3.13) was found to be abnormal with one Y and two X chromosomes. In order to distinguish between a karyotype of 47XXY and triploidy, triple colour FISH for chromosomes X, Y and 1 was carried out and showed three chromosomes 1, two X and one Y signal in all cells (Fig 3.10), showing the fetus to be triploid. In 7 out of 7 clumps isolated from mucus and 3 out of 6 from lavage, tested by XY FISH, all nuclei showed XXY signals showing them to be of fetal origin.

In this patient, further investigation was performed by fluorescent duplex PCR on one isolated cell clump from the mucus aspiration and from DNA extracted from maternal blood and placenta (Fig 3.16).

**Figure 3.16 QF-PCR showing triploidy TCC cell clump**




**Quantitative multiplex PCR analysis from an XXY triploid pregnancy.**
DNA was amplified from maternal blood (**A**), placenta (**B**). and a clump of cells isolated from the transcervical mucus aspirate (**C**). Relative abundance of D21S11 alleles and amelogenin PCR products are compared. The two D21S11 alleles (chromosome 21 marker) with a signal intensity ratio of 2:1 in both the placental and isolated cell clump indicate the presence of three copies of chromosome 21. These results combined with both X- and Y- specific PCR products co-amplified at the amelogenin locus, are consistent with the FISH diagnosis of fetal triploidy.

**Table 3.11 Assessment of the origin of cell clumps isolated from cervical mucus aspirations and analysed with the polymorphic STR marker D21S11.**

| Patient | Procedure post-TCC sampling | Gestational age (weeks) | Number of tested clumps | Cell clump result | | | |
|---|---|---|---|---|---|---|---|
| | | | | Maternal[1] | Fetal[2] | Mat/Fet[3] | Failed[4] |
| 1 | TOP | 6 | 6 | 1 | | 1 | 4 |
| 2 | TOP | 10 | 4 | 1 | | 1 | 2 |
| 3 | CVS | 10 | 5 | 3 | 1 | | 1 |
| 4 | CVS | 12 | 3 | | | 2 | 1 |
| 5 | CVS | 10 | 2 Mat/NI[5] | | | | |
| 6 | CVS | 10 | 3 | 2 | 1 | | |
| 7 | CVS | 9 | 1 | 1 | | | |
| 8 | TOP | 9 | 8 | 4 | 2 | 2 | |
| 9 | TOP | 11 | 6 | 6 | | | |
| 10 | CVS | 11 | 1 | 1 | | | |
| 11 | TOP | 12 | 3 | 3 | | | |
| 12 | CVS | 10 | 2 | | 2 | | |
| 13 | TOP | 7 | 5 | 5 | | | |
| 14 | CVS | 10 | 6 | 3 | 1 | | 2 |
| 15 | TOP | 8 | 2 NI[6] | | | | |
| 16 | TOP | 11 | 7 | 7 | | | |
| 17 | TOP | 10 | 1 | 1 | | | |
| 18 | TOP | 11 | 4 Mat/NI | | | | |
| 19 | TOP | 9 | 11 | 6 | 2 | 1 | 2 |
| 20 | TOP | 9 | 4 | 3 | | | 1 |
| 22 | TOP | 7 | 4 | | 4 | | |

1 **maternal**: only maternal peaks present.

2 **fetal**: exclusively fetal PCR peaks.

3 **Mat/Fet**: both maternal and fetal STR peaks present;

4 **failed**: no PCR products visible due to either DNA extraction or PCR failure, or errors in manipulation of the cell clump into the microcentrifuge tube.

5 **Mat/NI**: (maternal/non-informative); TCC STR allele sizes identical to maternal, but no fetal reference tissue available

6 **NI**: (non-informative); TCC STR allele sizes identical to maternal

**Table 3.12 Molecular analysis of cell clumps isolated from transcervical cell samples**

| Patient | Procedure post-TCC sampling | Gestational Age | Fetal sex | Polymorphic STR marker, D21S11 | Dual colour XY-FISH | | |
|---|---|---|---|---|---|---|---|
| | | | | Clumps from lavage | Clumps from lavage | Single fetal cells in lavage * | Single fetal cells in aspirate * |
| 23 | TOP | 9 weeks. | male | n.d | **1 fetal** | **4.7%** | 0 |
| 24 | TOP | 9 weeks. | male | 2 maternal | 2 maternal, 2 failed | **7.1%** | **5.2%** |
| 25 | TOP | 9 weeks. | female | 1 maternal | n.d | n.d | n.d |

TOP: termination of pregnancy

"fetal": all nuclei in tested clump with fetal (male) XY-FISH result

"maternal": only maternal nuclei (two X signals) present.

"failed": failure of either DNA extraction, PCR or manipulation of the cell clump into the microcentrifuge tube/onto the slide.

"n.d": not done

* The proportion of single fetal (male) cells in the whole lavage or aspirate samples after removal of clumps.

**Table 3.13-Molecular analyses of cell clumps from cervical mucus and intrauterine lavage collected from the same patients**

| Patient | Procedure post TCC sampling | Gestational age | Fetal sex | Duplex PCR for D21S11 and Amelogenin | | Dual colour XY-FISH | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Aspirate clumps | Lavage clumps | Aspirate clumps | Lavage clumps | Single fetal cells in aspirate [a] | Single fetal cells in lavage [a] |
| 26 | TOP | 8 weeks. | male | - | - | 2/2 failed | **2/2 fetal** [a] | 0 | n.d. |
| 27[b] | TOP | 7 weeks. | male | **1/1 fetal** | **1/1 fetal** | **7/7 fetal** | **3/6 fetal, 1/6 feto-maternal,** 2 maternal | **6.7%** | **2.7%** |
| 28 | TOP | 10 weeks. | male | - | - | 1/1 maternal | n.d. | 0 | 0 |
| 29 | TOP | 8 weeks. | male | - | - | 4/7 maternal, 3/7 failed | **1/4 fetal,** 1/4 maternal, 2/4 failed | 0 | **2%** |

TOP: termination of pregnancy, "fetal": all nuclei with fetal (male) XY-FISH result, "maternal": only maternal nuclei (two X signals) present.

[a] The proportion of single fetal (male) cells in the whole lavage or aspirate samples after removal of clumps.

n.d not done

**Table 3.14-Summary of patients and cell clumps isolated from mucus aspiration or intrauterine lavage**

| Group | Number of TCC samples tested | Total number of cell clumps isolated | TCC samples containing fetal cell clumps | Total number of fetal cell clumps |
|---|---|---|---|---|
| Mucus aspiration | 22 | 90 | 7 (31.8%) | 13[a] |
| Intrauterine lavage | 3 | 8 | 1 (33%) | 1[a] |
| Aspirate and lavage | 4 | 31 | 3 (75%) | 15[b] |
| All groups | 29 | 129 | 11 (38%) | 29 |

[a] fetal origin proven by sex-independent PCR marker

[b] fetal origin proven by duplex PCR (correct diagnosis of male fetus and sex-independent PCR, case 27) or FISH for chromosomes X and Y (correct diagnoses of male fetuses, cases 26, 27 and 29).

In Group D, dual XY FISH performed on TCC samples, showed up to 7.1% single fetal male cells could be detected in samples retrieved from pregnant women with male conceptuses, even after suspected fetal clumps had been removed. By micromanipulation clumps consisting exclusively of fetal cells, as assessed by PCR or FISH analysis, were collected in 11 out of 29 patients investigated. In one case (patient 27, Table 3.13) using FISH, 7 out of 7 isolated fragments from the aspirate, and 3 out of 6 from the lavage, were found to contain only cells with two X and one Y chromosome. Furthermore, the PCR test for STRs and Y sequences confirmed the fetal origin in two further cell clumps isolated from both aspirate and lavage.

## 3.11. QF-PCR Assays refined for use on small numbers of cells

As shown by Section 3.8, aneuploidy detection using PCR of STR markers could be reliably achieved from extracted DNA samples. The successful detection of triploidy (Section 3.4.2.) also suggested that if single cells or tissue clumps of purely fetal origin could be consistently isolated the same assay could be applied to isolated cell clumps. In addition to the detection of aneuploidy, many existing PCR diagnoses could be adapted for use in a multiplex quantitative fluorescent (QF) PCR reaction. Further diagnostic assays were refined for use on cell numbers similar to those constituting the fetal cells clumps that could be isolated with micromanipulation (10-50). Each assay was first assessed using routinely extracted DNA then applied to minute amounts of DNA (200pg) and cell clumps from known sources.

### 3.11.1. Amelogenin

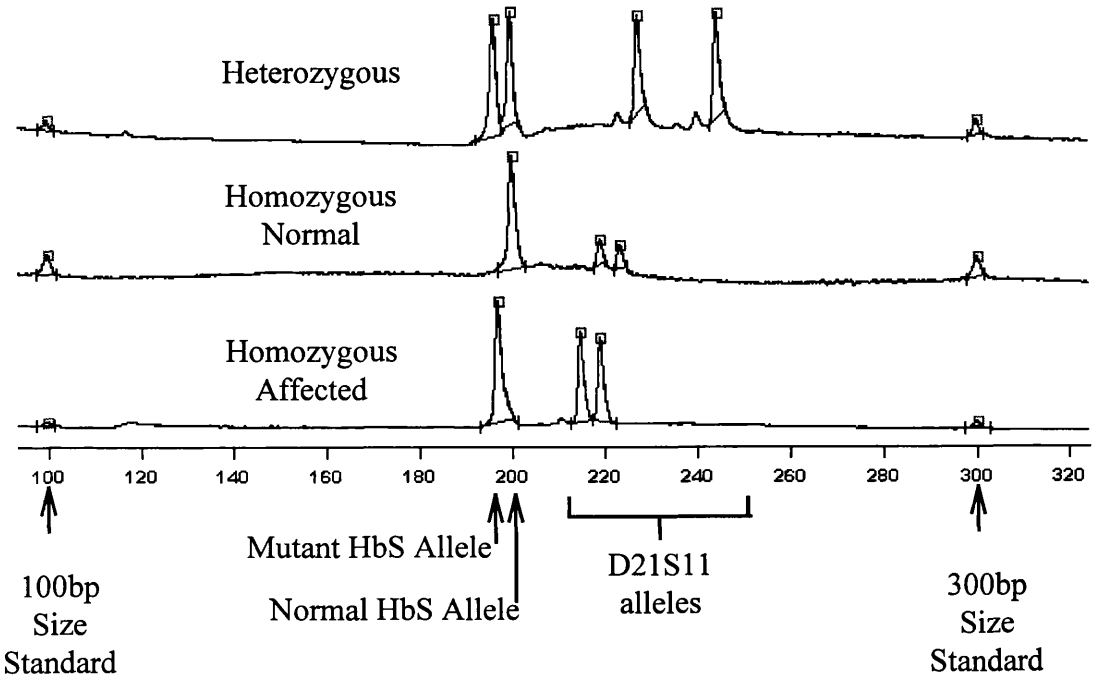
This reaction was found to be consistently reliable when used on DNA (Fig 3.14) and small groups of 10-50 cells. The primers could also be combined in multiplex QF-PCR reactions (see Section 3.13.5.3.).

### 3.11.2. Sickle cell anaemia (HbS)

The QF-PCR assay to detect sickle cell anaemia was first appraised in a blind study using DNA extracted from 30 peripheral blood or chorionic villus tissue samples previously tested for the presence of the HbS mutation using conventional ARMS PCR (provided by Dr. Mary Petrou, UCL Perinatal centre). These 30 samples were analysed

by multiplex QF-PCR with the common forward primer, both reverse ARMS primers and the chromosome 21 specific STR D21S11. Analysis of the results showed that all 30 samples (14 normal samples, 8 heterozygote samples and 8 homozygously affected) had been diagnosed correctly (Fig 3.17). All samples were found to be heterozygous for the STR marker, generating two PCR products with a ratio close to 1:1. Small aliquots of DNA (200pg), and groups of about 10-50 cells were also successfully tested with the same method. The three primers used in this assay were found to be compatible with the amelogenin and all STR primer sets enabling multiplex QF-PCR reactions to be performed.

**Figure 3.17 Fluorescent ARMS PCR for the analysis of HbS**

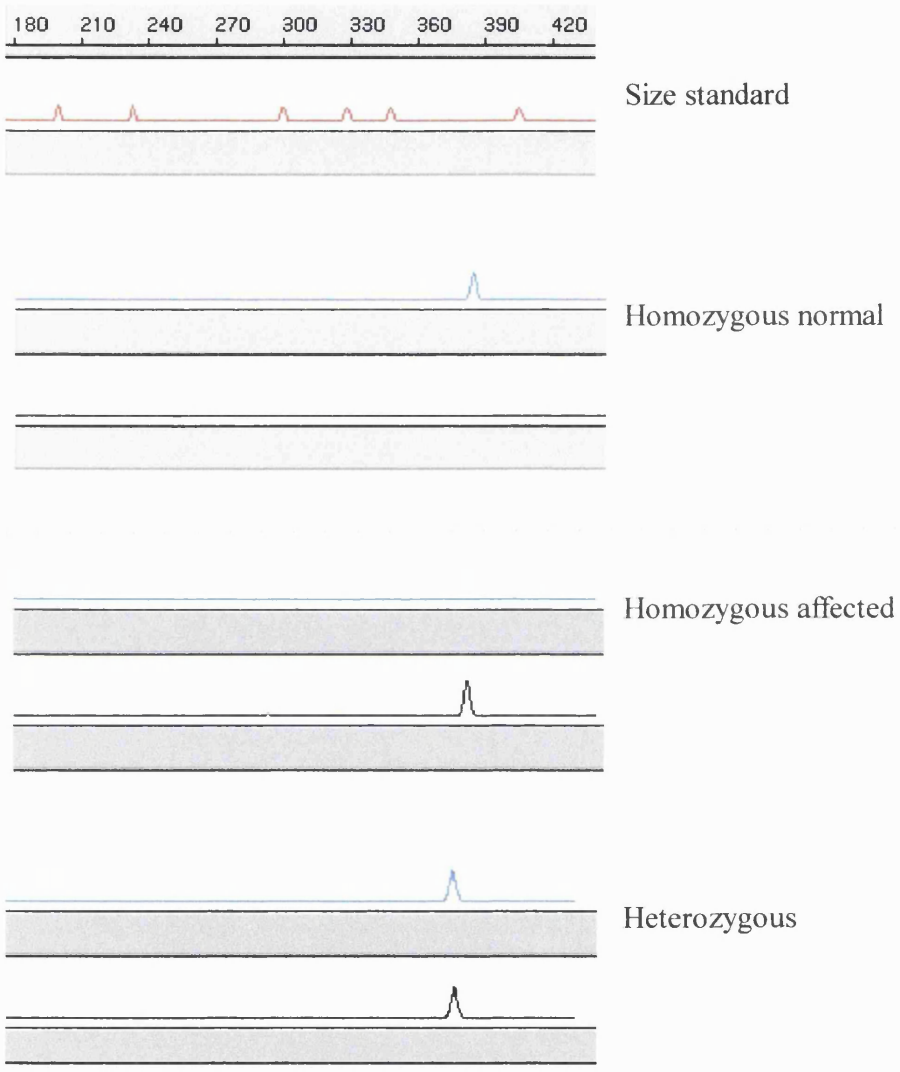



Detection of the single base change leading to sickle cell anaemia in a blind study using a duplex reaction combining analysis of the STR D21S11.

### 3.11.3. Beta Thalassaemia

In the initial blind study, all 34 DNAs tested for the thalassaemia mutation IVS1-110 were correctly identified. As anticipated, homozygous normal DNA generated one QF-PCR product corresponding to the normal DNA sequence. DNA from heterozygous carriers generated two QF-PCR products indicating the presence of both the normal and mutant sequences. The assay performed on homozygous affected DNA resulted in one QF-PCR product corresponding to the mutant DNA sequence (Fig 3.18). These results were replicated when clumps of 10-50 cells were exposed to the same assay with an increased cycle number. In multiplex QF-PCR assays, only primers for the STR D21S1414 were found to be compatible with the high annealing temperature necessary for the specificity of this ARMS reaction, and only when 'Stepdown' PCR was applied (Hecker and Roux, 1996). The majority of these PCR tests were performed in collaboration with Vincenzo Cirigliano.

**Figure 3.18** Detection of the IVS1-110 single base pair mutation causing beta thalassaemia




Three primers are included in a multiplex reaction. The forward primer specific to the normal base at its 3 prime terminus is labelled with blue fluorescence (FAM), and the forward primer specific to the mutant base is labelled with HEX (indicated by black). A blue peak therefore indicates the presence of the normal allele, and the black peak the presence of the mutant allele.

### 3.11.4. Cystic Fibrosis

Although the delta F 508 deletion leading to cystic fibrosis is only 3bp, this size difference in PCR products generated from flanking primers could easily be resolved using QF-PCR. An initial blind study on DNA extracted from 30 whole blood samples and clumps of 10-50 fibroblast cells showed the test to be accurate and reliable with correct diagnoses in all cases (Fig 3.19).

## 3.12 GROUP F

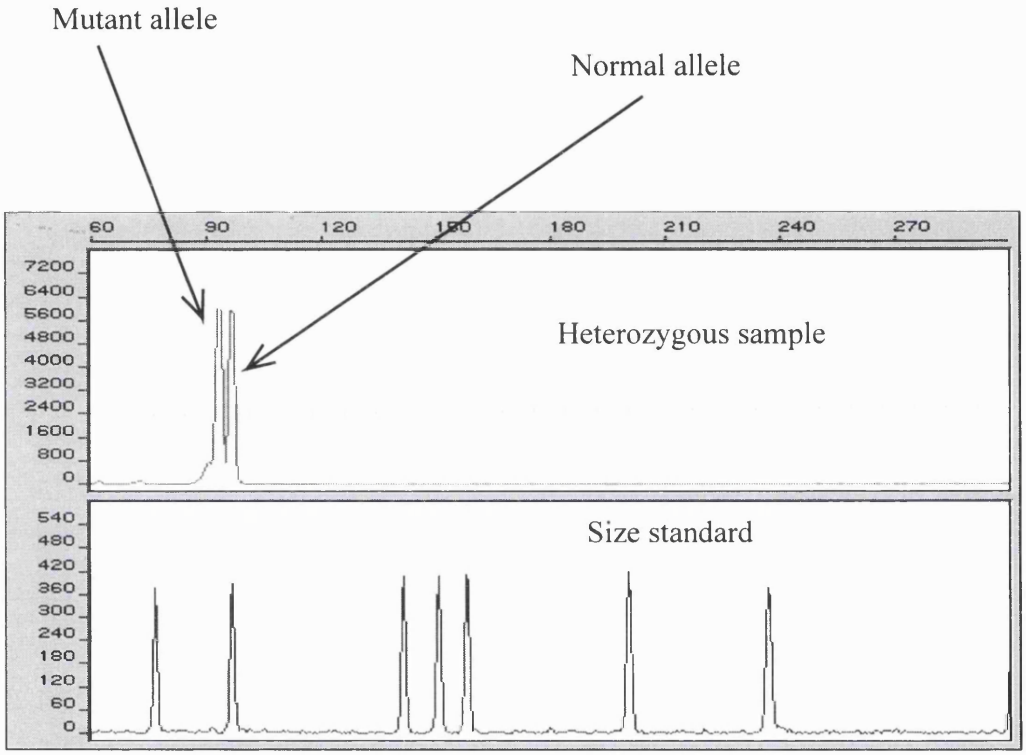
The aforementioned assays, refined for use on clumps of cells, were then applied to isolated trophoblastic elements from TCC samples collected from women carrying specific inherited genetic defects; cystic fibrosis, sickle cell anaemia, or beta thalassaemia. The assessment of purely trophoblast material, without the presence contaminating maternal cells, would allow the prenatal diagnosis of these fetal disorders.

### 3.12.1 Assessment of fetal CF status from TCC clumps

TCC aspiration samples were collected from 5 pregnant women at risk of delivering a fetus affected by cystic fibrosis, prior to CVS sampling. All these women were carriers of the delta-F deletion, as were their partners. Clumps of cells isolated from the TCC samples were stored until the results of standard CF diagnosis from the CVS sample was known. In four cases, the fetus was found to be a carrier of the deletion so, like the mother, was heterozygous for the ΔF-508 deletion. These cases were not tested further. In one case, the fetus was found to be homozygous affected. The TCC sample collected from this case was examined and cell clumps with the possible morphology of trophoblasts were collected using micromanipulation. Clumps of maternal squamous cells were also collected. In all, 26 suspected fetal clumps were isolated. A duplex QF-PCR reaction containing primers spanning the delta-F 508 deletion, and the STR D21S11 was performed on DNA extracted from these isolated cell clumps together with known fetal cells isolated from the remains of the cultured CVS sample, DNA prepared from peripheral maternal blood and positive and negative controls. The STR allele sizes in all tested clumps were the same as the maternal, with

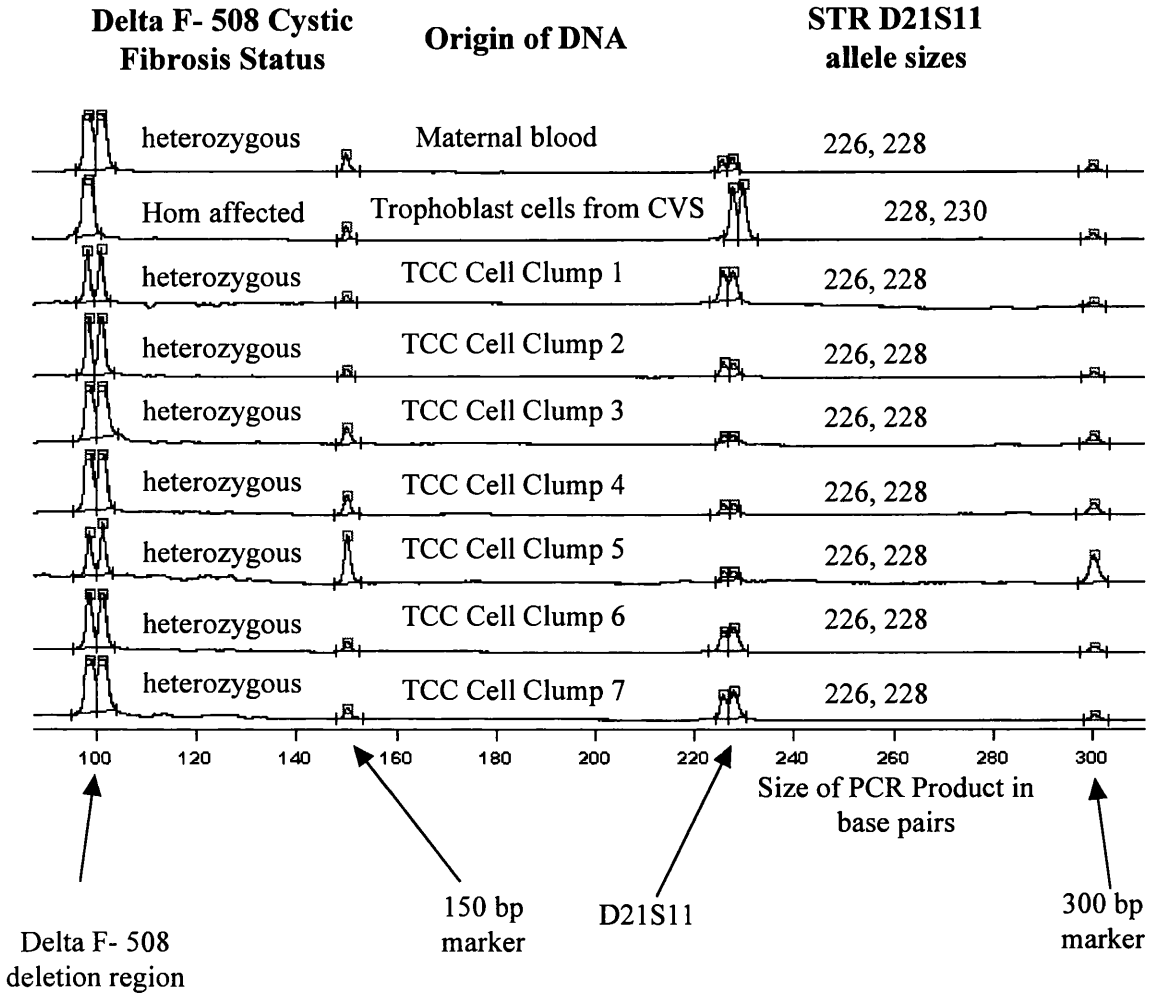
no evidence of the paternally inherited fetal allele (as determined by analysis of the placental DNA). The CF status in all clumps was heterozygous carrier- the same as that of the mother. All the clumps that had been isolated in this case were thus found to be of maternal origin (Fig 3.20).

**Figure 3.19** **Detection of the ΔF- 508 three base pair deletion causing cystic fibrosis**




Amplification with primers flanking the site of the 3pb deletion ΔF-508 generates PCR products of 100bp for the normal allele, and 97bp for the deleted form. The heterozygous DNA shown therefore produces two peaks of fluorescence indicating the presence of both normal and deleted alleles.

## Figure 3.20




Assessment of the origin of clumps of cells isolated from a TCC sample collected from a women carrying a fetus at risk, due to both parents being carriers of the delta F-508 3bp deletion, of cystic fibrosis. All isolated cell clumps had the morphology of trophoblast. From testing a clump of CVS cells the fetus was found to be homozygous (hom) affected with regards to the delta F-508 locus, and to have inherited a paternal STR allele of 230bp. In all 26 cell clumps were tested, 7 of which are shown. The heterozygous delta F-508 status, together with the absence of the paternal STR allele, shows that in all cases the cell clumps were maternal in origin.

### 3.12.2 Diagnosis of β-thalassaemia from isolated fetal trophoblasts

One of the couples in Group F of the study were at risk of having a child with beta thalassaemia. This was due to the mother carrying a 4bp deletion, and the father a 619bp deletion in two different regions of the haemoglobin gene. Seven clumps of cells with the morphology of trophoblast were isolated from the TCC aspiration sample collected from the woman at 12 weeks of gestation. Duplex PCR was performed as described (Section 3.8.1.) without prior knowledge of the status of the fetus. All seven clumps were shown to contain cells with the four base pair deletion present in the maternal genome (Fig 3.21). Three of the seven cell clumps showed only normal DNA when tested for the paternal deletion, while four were shown to contain both normal and deleted forms of DNA (Fig 3.22). This demonstrated that the three cell clumps without the paternal deletion were exclusively of maternal origin while the remaining four clumps contained fetal cells. These results suggested that the fetus was compound heterozygous, carrying both maternal and paternal deletions, and would thus be affected with beta- thalassaemia. This diagnosis was confirmed by comparison with the CVS result. Although the diagnosis was correct, the possibility that the fetal cell clumps were contaminated with maternal cells could not be excluded.

**Figure 3.21** **Assessment of the maternal 4 base pair deletion in isolated cell clumps**

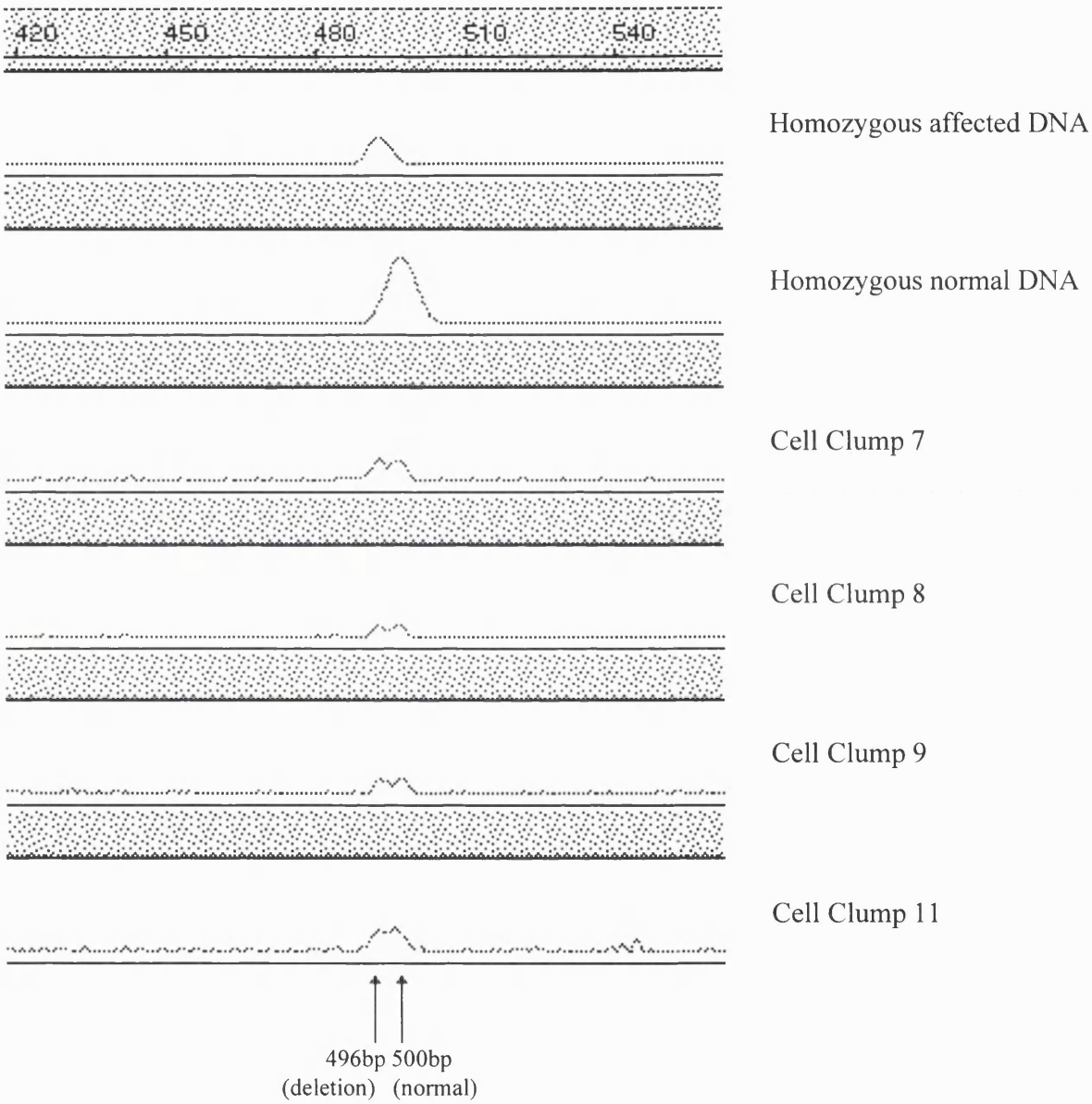
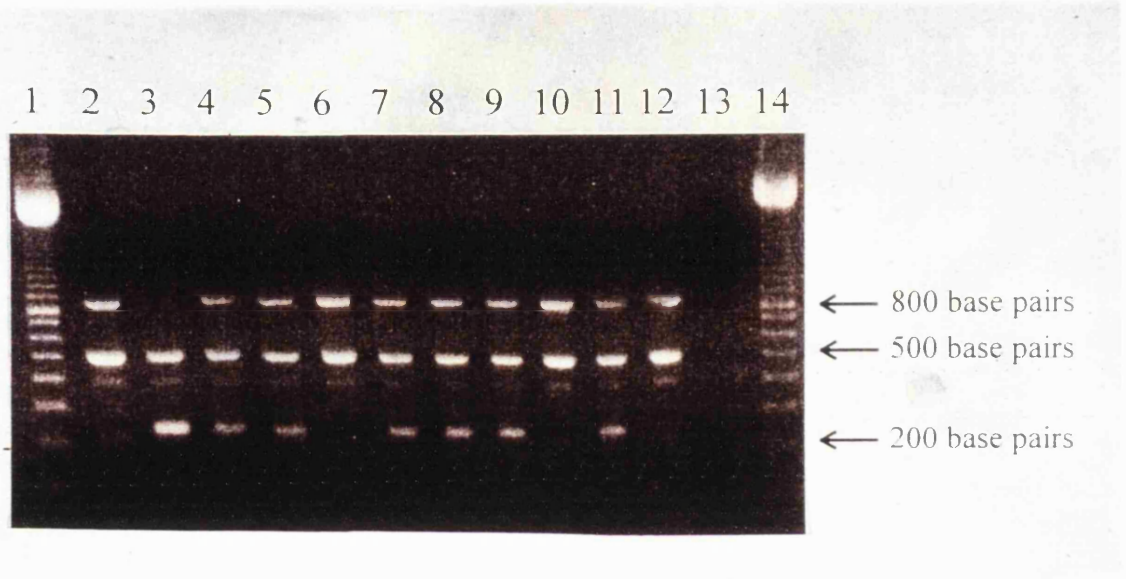


**Figure 3.22** **Assessment of the paternal Hb deletion in isolated cell clumps**




The ~500bp band seen in all lanes represents the products generated from the fluorescently labelled primers flanking the maternal 4pb deletion site. These products were resolved using QF-PCR (see Figure 3.21).

Lanes 1 and 14 contain 100Kb ladder; lane 2 homozygous normal DNA; lane 3 homozygous affected DNA; lane 4 heterozygous DNA; lane 5 placental DNA; lanes 6 to 12 contain the 7 tested cell clumps; lane 13 the negative control. The clumps in lanes 7, 8, 9 and 11 are here shown to contain the paternal Hb deletion (see text).

As shown by Table 3.14, the incidence of maternal DNA contamination in fetal TCC clumps isolated with micromanipulation is relatively commonplace. Maternal cells were obviously in vast abundance, and despite washing isolated cell clumps they were often still included in a micromanipulated aliquot. It may also be that decaying maternal cells become lysed, and their DNA released into the surrounding solution. By physically disagregating a TCC cell clump and using each of the washed constituent cells individually this problem may be avoided. The same PCR assays under investigation were thus attempted on single cells to assess their reliability.

## 3.13. Single cell QF-PCR assays

### 3.13.1. Sickle cell anaemia

The QF-PCR assay for the detection of HbS was then used on 190 single cells isolated from buccal washes and CVS cell cultures (trophoblasts) (Table 2.2). Buccal cells from normal (50), heterozygous carrier (50) and homozygous affected (50) individuals were tested, together with normal (20) and heterozygous (20) cells isolated from trophoblast cultures. One hundred and sixty one single cells were successfully amplified by QF-PCR and no significant differences in the success of the PCR results were noticed between the two cell types (buccal cells 123/150 [82%] and trophoblastic cells 34/40 [85%]).

One peak of fluorescent activity, corresponding to the wild type HbS allele, was observed in all 59 successfully amplified homozygous normal single cells (Fig 3.23).

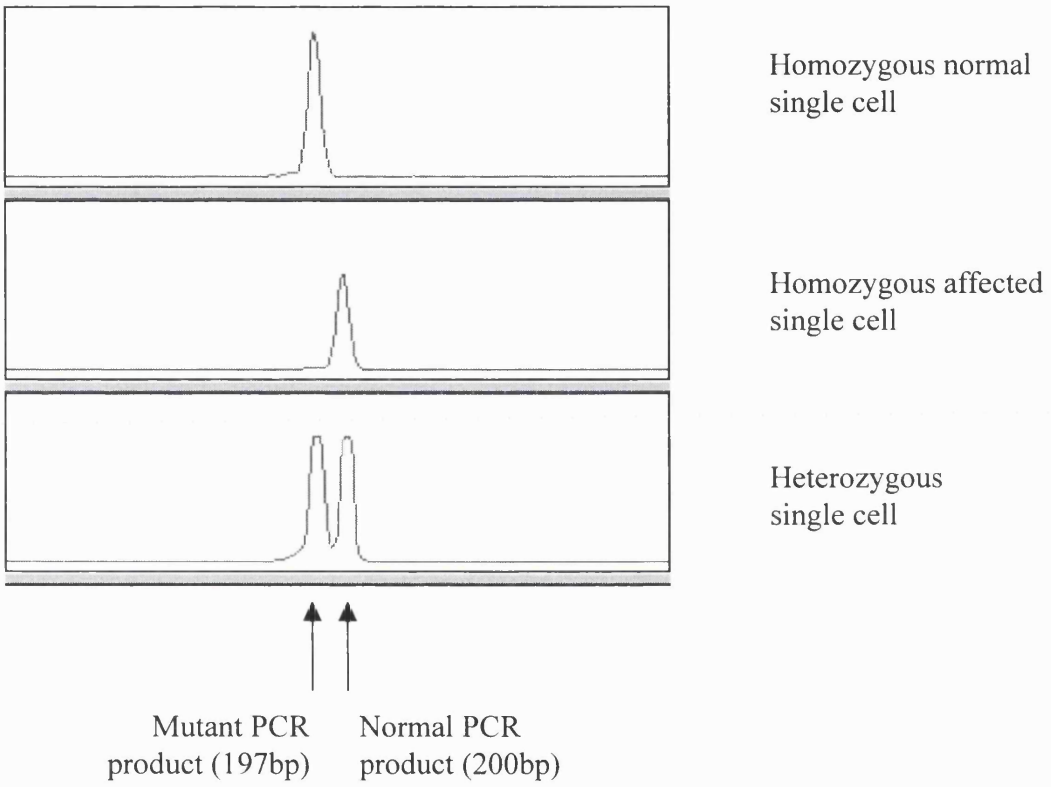
All of the 60 heterozygous single cells that were amplified showed two PCR products, indicating the presence of both normal and mutant DNA templates in each cell. No ADO was observed. However, preferential amplification was seen to occur, since the ratio of mutant to normal PCR product amounts ranged from 1:0.8 to 1:3.6 with a mean of 1:1.8 (Fig 3.24).

Of the 38 successfully amplified homozygous affected single cells, 35 displayed only one peak corresponding to the mutant strand of DNA (92%). The remaining 3 cells (8%) had a small amount of 'normal' PCR product together with a large 'mutant' peak. This suggests that the normal reverse primer anneals non-specifically to the mutant template sequence, but with low efficiency. This only

became apparent when a large amount of mutant PCR product was observed. The ratio of mutant to normal product in these three cases was 1:0.12, 1:0.10 and 1:0.08.

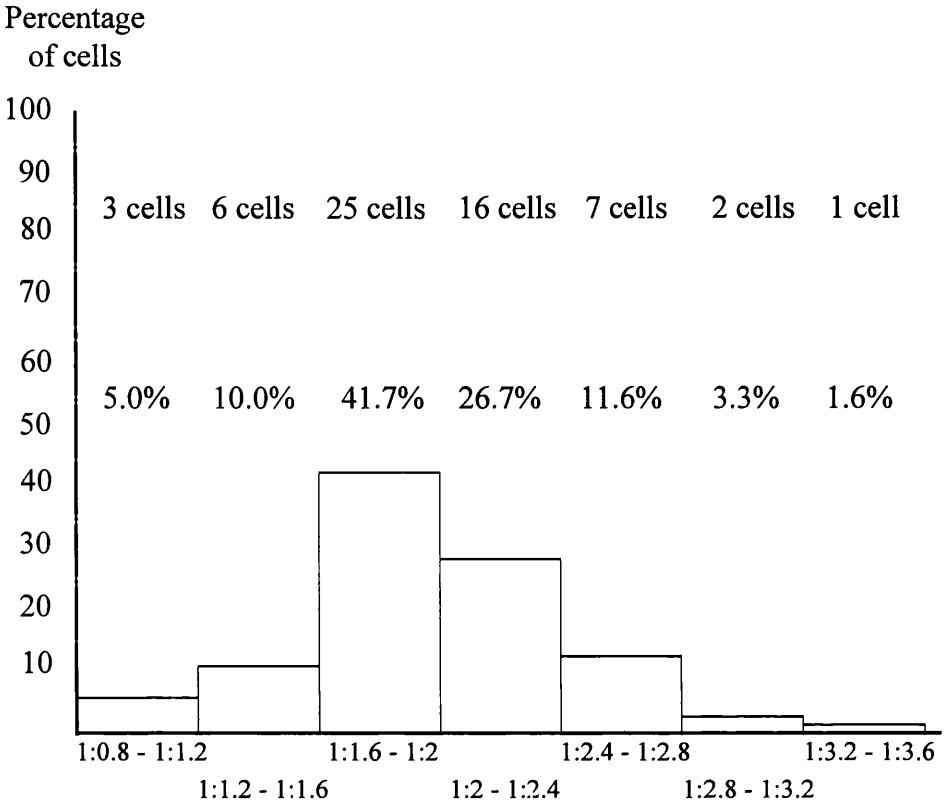
Despite the extra normal peak in a small minority of homozygous affected cells, and the variation in peak ratios in heterozygous cells, these two cell types are clearly distinguishable. The most extreme case of preferential amplification in heterozygote cells in favour of the mutant allele, resulted in a mutant to normal ratio of 1:0.8. In homozygous affected cells, the most extreme example of normal allele production resulted in a mutant to normal ratio of ratio of 1:0.12, a 6.7 fold difference. The PCR products from the two cells types are starkly different, and as a result the diagnosis of all 161 single cells was correct. The HbS primers were found to be compatible with all STR and amelogenin markers in multiplex assays (Fig. 3.25).

**Figure 3.23** **Detection of the HbS status from single cells**




Single buccal cells were isolated from homozygous normal, homozygous affected and heterozygous carrier individuals. These single cells were exposed to the QF-PCR ARMS assay to detect the single base substitution leading to sickle cell anaemia. As can be seen, normal cells produced one peak of fluorescence corresponding to the normal amplicon of 200bp. Cells from an affected individual produced only the smaller amplicon of 197bp, whilst heterozygous cells, as expected, generated both sized PCR products.

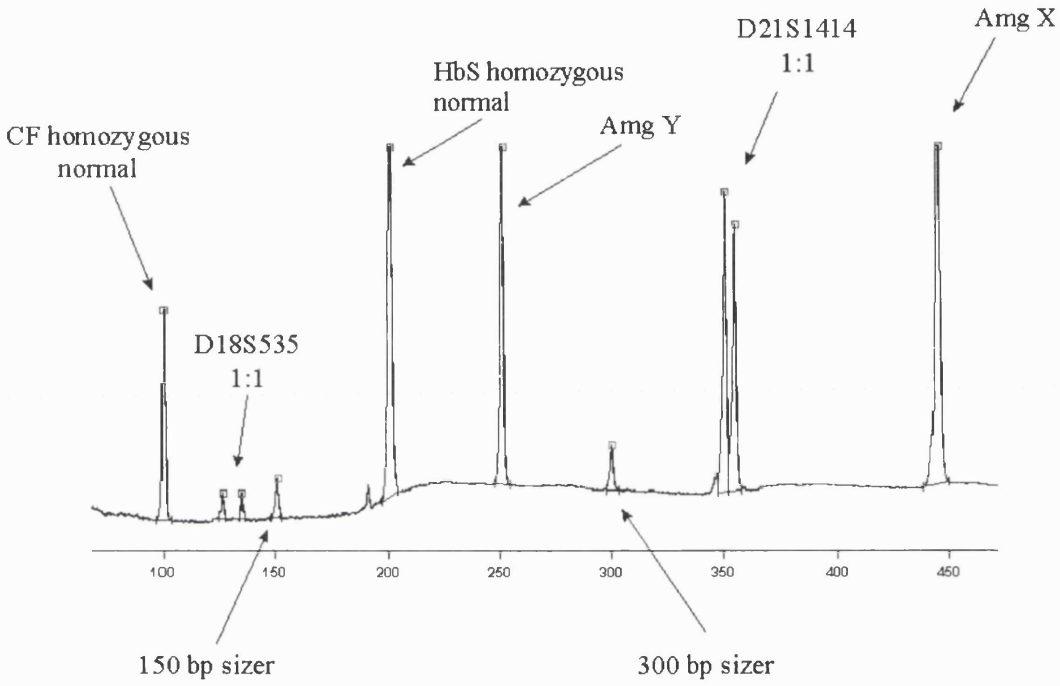
**Figure 3.24 Ratio of mutant to normal peak areas in single heterozygous sickle cell anaemia cells.**




Ratio of mutant to normal HbS allele as determined by division of the fluorescent peak areas

Heterozygous single cells were grouped according to the ratio of the areas of the two peaks generated, as shown on the X axis. The expected ratio should be 1:1, with any deviation from this figure due to preferential amplification.

**Figure 3.25** **Multiplex quantitative fluorescent PCR performed on a single cell.**

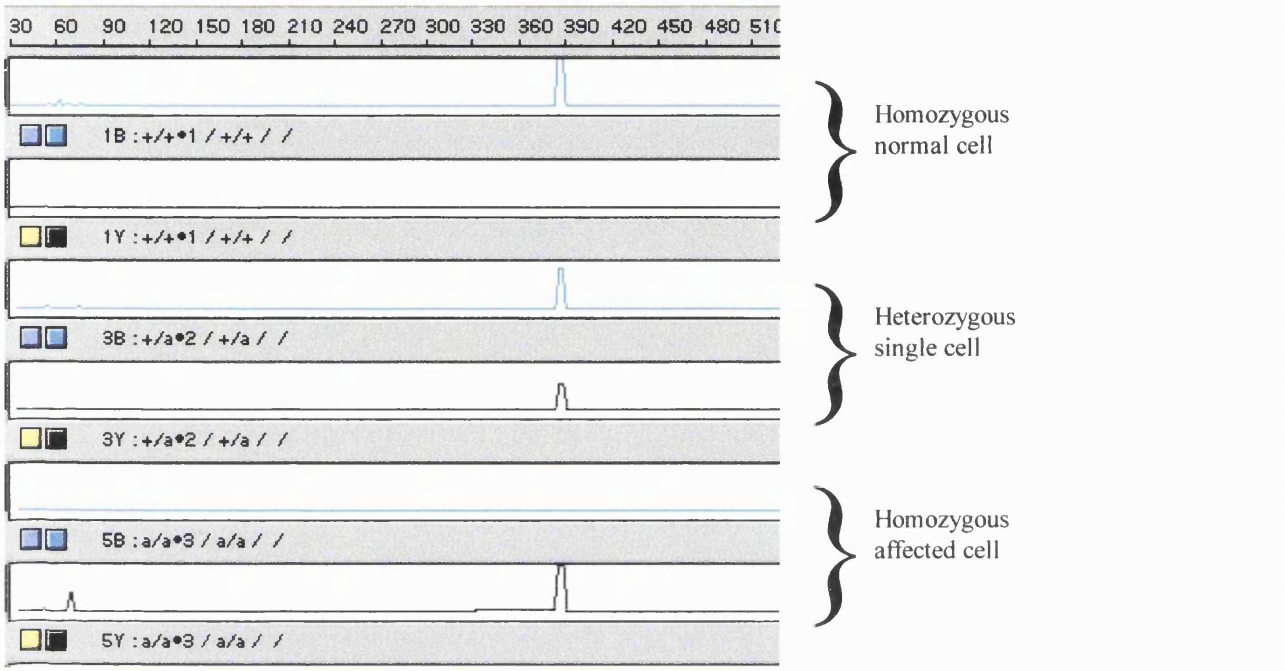



A single buccal cell was isolated from a normal individual and exposed to multiplex QF-PCR containing five primer sets. The results of the PCR show the cell to be male (with both X and Y products for the amelogenin), homozygous normal for sickle cell anaemia (with just the normal product at 200bp), homozygous normal for the ΔF-508 cystic fibrosis deletion (with just the normal 100bp product) and heterozygous for both the STR markers employed (D18S535 on chromosome 18 and D21S1414 on chromosome 21). The 'sizers' at 150bp and 300bp are included to enable accurate sizing of the PCR products.

### 3.13.2. Beta thalassaemia

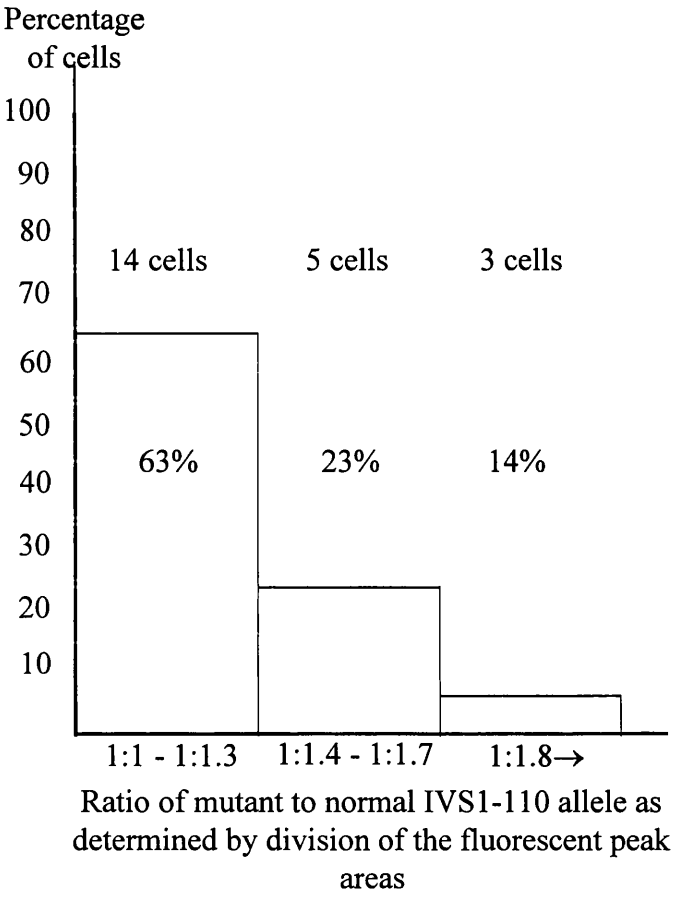
Of the 74 isolated single buccal cells tested, 57 produced PCR products (77%) (Table 2.2). All successfully amplified homozygous normal cells (19) generated a single peak of fluorescence corresponding to the normal allele. All homozygous affected cells (16) produced a single peak corresponding to the mutant DNA strand. When heterozygote cells (22) were tested, all produced two peaks of fluorescent activity indicating the presence of both normal and mutant DNA sequences in the starting template (Fig 3.26). ADO was not seen to occur; however, the ratio of the peak areas varied from the expected 1:1, with the QF-PCR product corresponding to the mutant DNA strand almost always more abundant. The ratio of mutant to normal ranged from 1:1.2 to 2.7:1 with a mean of 1.4:1 (Fig 3.27).

**Figure 3.26**




QF-PCR for the ARMS analysis of the IVS-110 single base substitution leading to beta thalassaemia performed on three single cells. The blue peak indicates the presence of a normal allele and the black peak the presence of an affected one. As can be seen, one single cell was homozygous normal, one homozygous affected and the last heterozygotic.

**Figure 3.27** **Ratios of peak areas in heterozygous IVS1-110 single cells.**

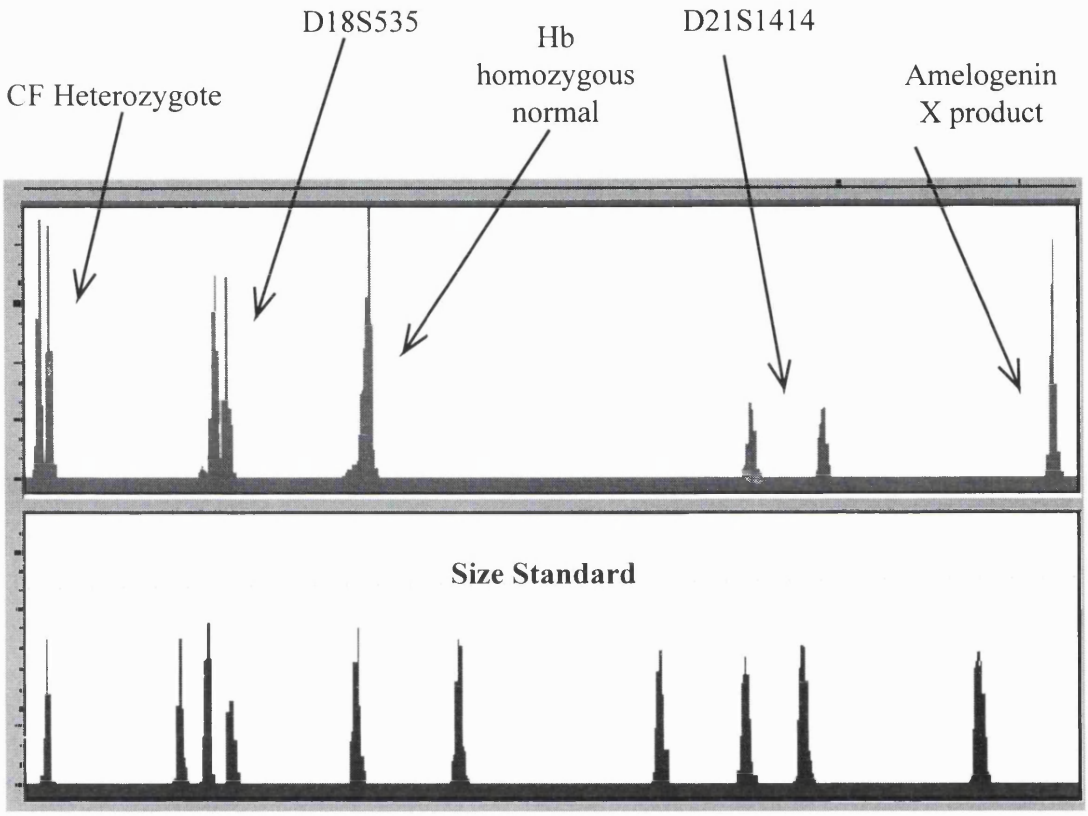



Heterozygous single cells were grouped according to the ratio of the areas of the two peaks generated, as shown on the X axis. The starting template ratio is 1:1 and so any deviation from this by the end of the reaction is due to preferential amplification.

### 3.13.3. Cystic Fibrosis

CF single cells from three sources were then isolated and examined with the same assay. These consisted of 16 buccal and 14 lymphocyte cells from 2 normal individuals; 17 transcervical squamous cells from a heterozygous carrier and 12 fibroblasts from a heterozygous cell line culture; and 18 fibroblasts from a homozygous affected cell line culture (Table 2.2). Of the 77 tested cells, 57 (74%) amplified successfully. The PCR products from the homozygous normal amplified cells (24) all contained one sized DNA fragment corresponding to the normal allele. The amplified homozygous affected cells (12) all resulted in one PCR product corresponding to the mutant DNA sequence comprising the deletion. The amplified heterozygous cells (21) all resulted in two PCR products representing both normal and mutant sequences. Occasionally the ratio of these two PCR products again varied from the expected 1:1 due to preferential amplification. Neither mutant or normal sequence was consistently favoured in the reaction, with the ratio of mutant to normal varying from 3.1:1 to 1:4.1 with a mean of 1:1.22. The primers for this reaction were shown to be compatible in multiplex reactions with the amelogenin and all STR markers (Fig 3.28).

**Figure 3.28** **Multiplex quantitative fluorescent PCR performed on a single cell.**




This multiplex QF-PCR was performed on a single squamous cell isolated from the TCC sample of a ΔF-508 deletion carrier. The presence of both normal (100bp) and deleted (97bp) amplicons demonstrates the presence of both alleles. The presence of the X with the absence of the Y amelogenin product shows the cells to come from a female. Only one peak is present as a result of the HbS primer set amplification corresponding to the normal sized allele. The individual was also heterozygous for both STR markers tested.

### 3.13.4. Amelogenin

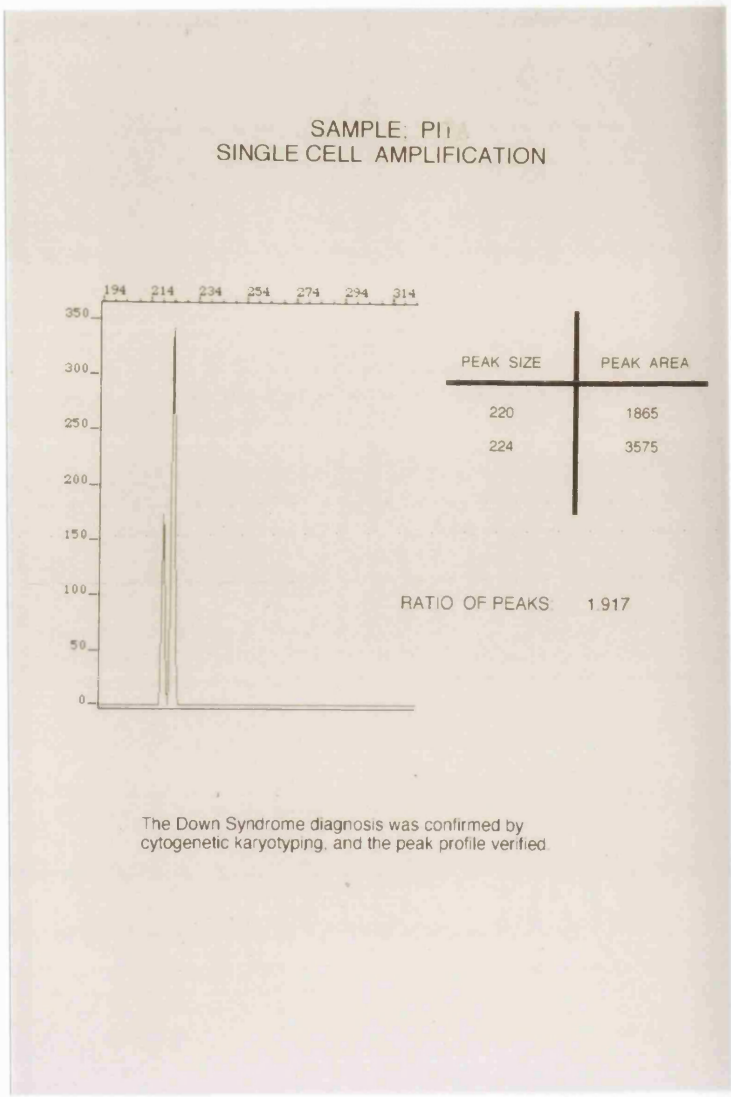
The amelogenin primers were used on 40 single buccal cells from male (20) and female (20) donors. Products of PCR amplification were seen in 35 of these cells (87.5%), all correctly indicating the presence/absence of the Y chromosome, together with the X chromosome specific sequence (Table 2.2). There was no Y-specific PCR product in any of the amplified 46XX cells (17). All PCR amplification products from the 46XY cells (18) contained both the X and Y specific DNA, but at varying amounts. Thus no ADO was observed, only preferential amplification. In subsequent experiments the amelogenin primers were shown to be unaffected by any other primer set when used in multiplex reactions.

### 3.13.5. Aneuploidy detection using STR markers from single cells

#### 3.13.5.1. Preliminary Assessment

Single lymphocytes were isolated by serial dilution from the blood of a Down syndrome patient. These single cells then had their DNA prepared (Section 2.2.2.1) and were exposed to 35 cycles of PCR, along with DNA extracted from whole blood and negative controls. The DNA profile from whole blood was found to be two alleles with a ratio of 2:1 (trisomic di-allelic). In 60% of cases, single cells amplified to give the same profile, with a consistent 2:1 ratio (Fig 3.29). Twenty five percent of single cells tested failed to produce any amplification products. The remaining 15% of PCR amplified single cells produced the same two alleles as expected but at ratios different from 2:1, ranging from 1:1.2 to 1:3.1. This is thought to be due to preferential amplification of one of the two alleles during the early stages of PCR.

**Figure 3.29** **QF-PCR for D21S11 performed on a single trisomy 21 lymphocyte**




**The ratio of almost exactly 2:1 demonstrates the presence of 3 copies of the STR sequence and therefore 3 copies of chromosome 21.**

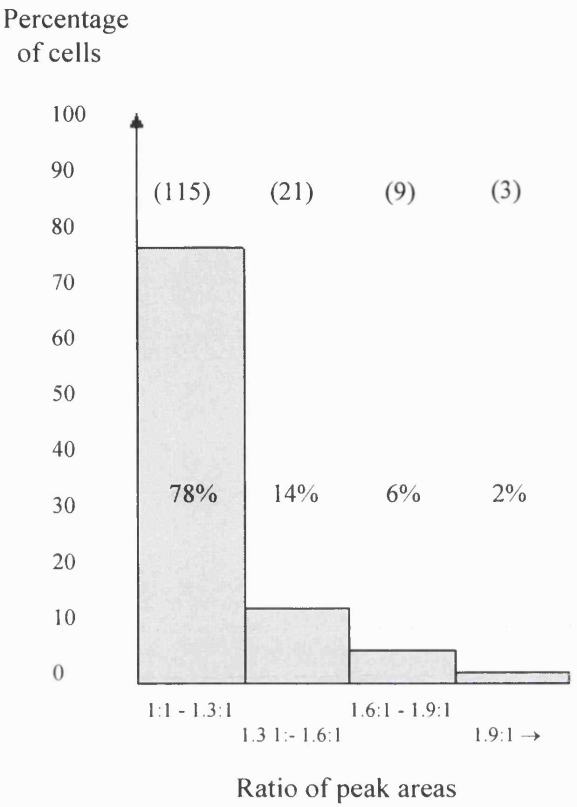
### 3.13.5.2. Single cell QF-PCR assessment with one STR marker

Single STR markers were first tested individually by QF-PCR on normal and trisomic cells (Table 2.2). Successful amplification was achieved in about three quarters of the tests, with no significant differences between the cell types ranging from 35/50 [70%] in normal lymphocytes, to 52/70 [74%] in trisomic lymphocytes, 113/150 [75%] in buccal cells and 65/80 [81%] in trisomic trophoblasts.

All normal cells employed for this work were heterozygous (di-allelic) for the particular STR marker tested, so that a PCR product ratio of 1:1 was expected in each case of single cell QF-PCR. To obtain a quantitative ratio, for each QF-PCR reaction, the major peak (the PCR product of highest fluorescent activity) was compared to the minor peak. One hundred and forty eight normal cells produced PCR products, all with two DNA peaks corresponding to the two expected STR alleles. However, only 115 (78%) had peak areas close to the anticipated 1:1 ratio (1:1 to 1.3:1) (Fig 3.30). The remaining 33 cells (22%) had all undergone preferential amplification producing skewed peak area ratios. In some cases, this resulted in the STR ratio becoming 2:1, incorrectly suggesting the amplification of a trisomic cell. In these cases of preferential amplification neither allele was consistently larger or smaller and the most extreme example resulted in a peak ratio of 6:1.

The 117 successfully amplified trisomic cells were trisomic for chromosome 21, 18 or 13. For each cell line an STR marker specific for the trisomic chromosome was employed. In every case, from the control DNA samples amplified previously, it was known that these cells were trisomic di-allelic (peak area ratio 2:1). To assess the maintenance of these ratios, the allele expected to be twice as abundant was compared to the allele which should have been present at half the amount. All cells displayed both expected allele sizes, showing that ADO had not occurred (Fig 3.31). The ratio of these alleles was as expected within acceptable bounds (1.7:1 to 2.3:1) in 89 cells (76%). Preferential amplification had thus occurred in 28 tests (24%), producing ratios as extreme as 10:1 in one case, and in three cases creating a peak area ratio close to 1:1, erroneously indicating a normal disomic cell.

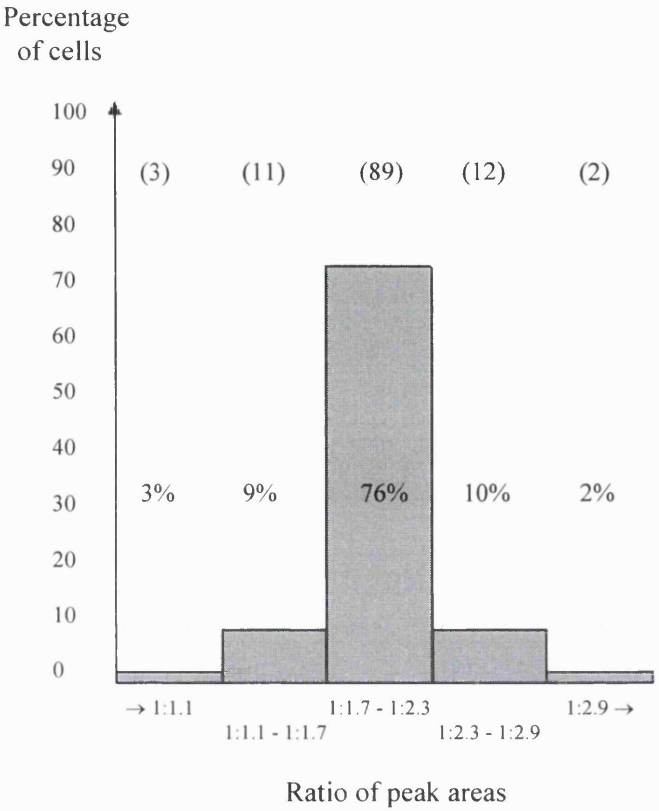
**Figure 3.30** Ratio of STR peak areas in di-allelic (heterozygote) normal cells.




Numbers in brackets indicate actual cell number.

Heterozygous single cells were grouped according to the ratio of the areas of the two peaks generated, as shown on the X axis. The starting template ratio is 1:1 and so any deviation from this by the end of the reaction is due to preferential amplification.

**Figure 3.31** Ratio of STR peak areas in di-allelic trisomic cells.




Numbers in brackets are actual cell numbers.

Di-allelic single cells were grouped according to the ratio of the areas of the two peaks generated, as shown on the X axis. The starting template ratio is 2:1 and so any deviation from this by the end of the reaction is due to preferential amplification.

### 3.13.5.3. Multiplex single cell QF-PCR

Trisomy detection in a single cell could only be determined with confidence when a tri-allelic profile was generated since QF-PCR using a single STR marker could only be relied upon to indicate chromosome aneuploidy accurately in around two thirds of di-allelic cells. To see if these results for a single STR would be affected by the inclusion of further primer sets, multiple four STR markers were next amplified in the same reaction (D21S11, D21S411, D21S1412, and D18S535). This multiplex QF-PCR assay was tested on over 41 individual cells obtained from each of two trisomy 21 trophoblast cell cultures (Table 3.15). Both cell lines were heterozygous for the chromosome 18 marker D18S535, producing a normal di-allelic peak ratio of 1:1. One cell line was trisomic di-allelic (peak ratio 2:1) for all three chromosome 21 markers, the other was trisomic di-allelic for one STR, and trisomic tri-allelic (peak ratio 1:1:1) for the other two. Of the 82 cells tested, 6 did not produce any PCR products at all (7%). PCR products could be observed from the other 76 cells (93%), but only in 56 of these were all four STR markers represented (74%), with some makers more consistently amplified than others (Table 3.16).

| | | STR marker | | | |
|---|---|---|---|---|---|
| | | **D21S11** | **D21S1411** | **D21S1412** | **D18S535** |
| Cell line A n=40 | Number of cells and (%) with PCR products | 21 (52.5%) (tri-allelic) | 28 (70%) (di-allelic) | 35 (87.5%) (tri-allelic) | 40 (100%) (di-allelic) |
| | Percentage with correct ratio | 76.2 | 72.2 | 51.4 | 60 |
| Cell line W n=36 | Number of cells and (%) with PCR products | 23 (63.9%) (di-allelic) | 21 (58.3%) (di-allelic) | 31 (86.1%) (di-allelic) | 34 (94.4%) (di-allelic) |
| | Percentage with correct ratio | 82.6 | 42.9 | 32.3 | 73.5 |
| Both cell lines n=76 | Number of cells and (%) with PCR products | 44 (58%) | 49 (65%) | 66 (87%) | 74 (97%) |
| | Percentage with correct ratio | 79.5 | 42.4 | 66.2 | 56.4 |

**Table 3.15** **Results of multiplex QF-PCR assays with four STR markers performed on single trisomic cells. Cells with no PCR products at all (8%) were excluded from this table.**

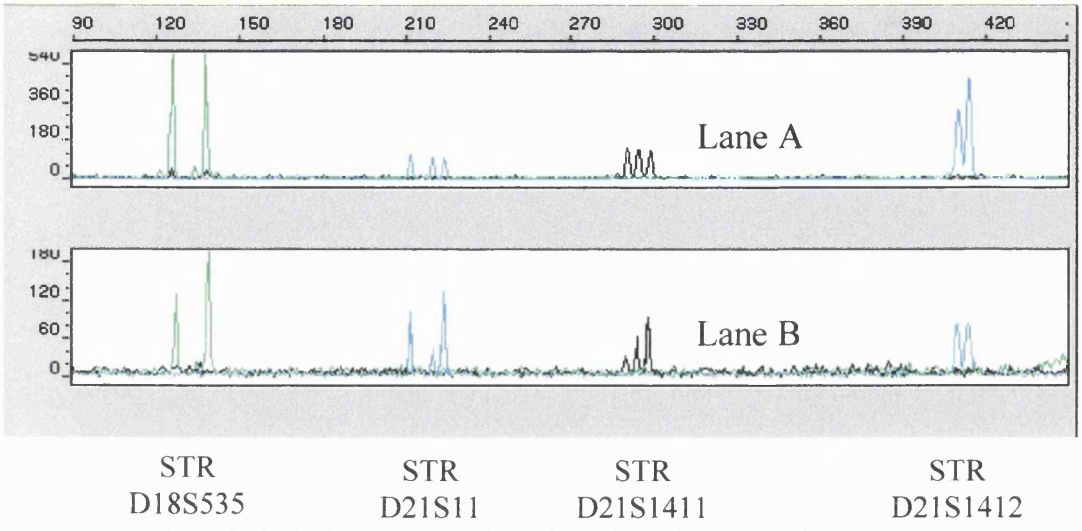
**Table 3.16 Trisomy 21 single cells tested by QF-PCR with four STR markers in a multiplex reaction.**

| | Concordance of STR marker ratios in each multiplex QF-PCR assay. | | | | |
|---|---|---|---|---|---|
| | 4/4 | 3/4 | 2/4 | 1/4 | 0/4 |
| Cell line A* | 2 | 6 | 17 | 13 | 2 |
| Cell line W* | 3 | 9 | 12 | 10 | 2 |
| Total* | 5 | 15 | 29 | 23 | 4 |

*Number of cells displaying correct STR PCR product ratios.

Even when products were generated from STR markers, these were not always at the expected ratio. Of the 76 cells that did amplify, all four STR markers maintained the correct ratios within acceptable bounds in only 5 cells (Table 3.16). In four cells, despite fluorescent PCR products representing all four STR markers being visible, none were at the expected peak ratio (Fig. 3.32). The D18S535 alleles, expected in both cell lines to produce a ratio of 1:1, were sometimes at 2:1, erroneously indicating trisomy of chromosome 18. Trisomic di-allelic STRs on chromosome 21, expected to have a ratio of 2:1, were sometimes observed at 1:1 suggesting a normal diploid cell. Tri-allelic STRs were at the anticipated 1:1:1 in around 65% of cases. In the remaining 35% of cells, ratios varied greatly (Fig 3.32).

**Figure 3.32**




**Lanes A and B** show the PCR products generated from the multiplex QF-PCR assay performed on two single cells, from the same trisomy 21 cell culture. The products in **Lane A** are all at the correct ratios (D18S535 di-allelic at 1:1; D21S11 tri-allelic at 1:1:1; D21S1411 tri-allelic at 1:1:1; and D21S1412 di-allelic at 2:1). **Lane B** shows the PCR products of all 4 loci to have undergone preferential amplification (D18S535 di-allelic at 1.8:1, erroneously indicating trisomy 18; D21S11 tri-allelic at 2.9:1:4.1; D21S1411 tri-allelic at 1:2.1:3; and D21S1412 di-allelic at 1:1.2 erroneously indicating disomy 21).

## 3.14. Magnetic Activated Cell Sorting

When the positively sorted fraction of cells was examined on a microscope slide, stark differences were observed according to which magnetic beads were used. When Minimac beads were employed, the sorted fraction after MACS contained no cells. This was consistent irrespective of what cell type or mixture was used, and what antibody was added. Increasing the antibody concentrations, the cell numbers and the incubation and separation times had no effect.

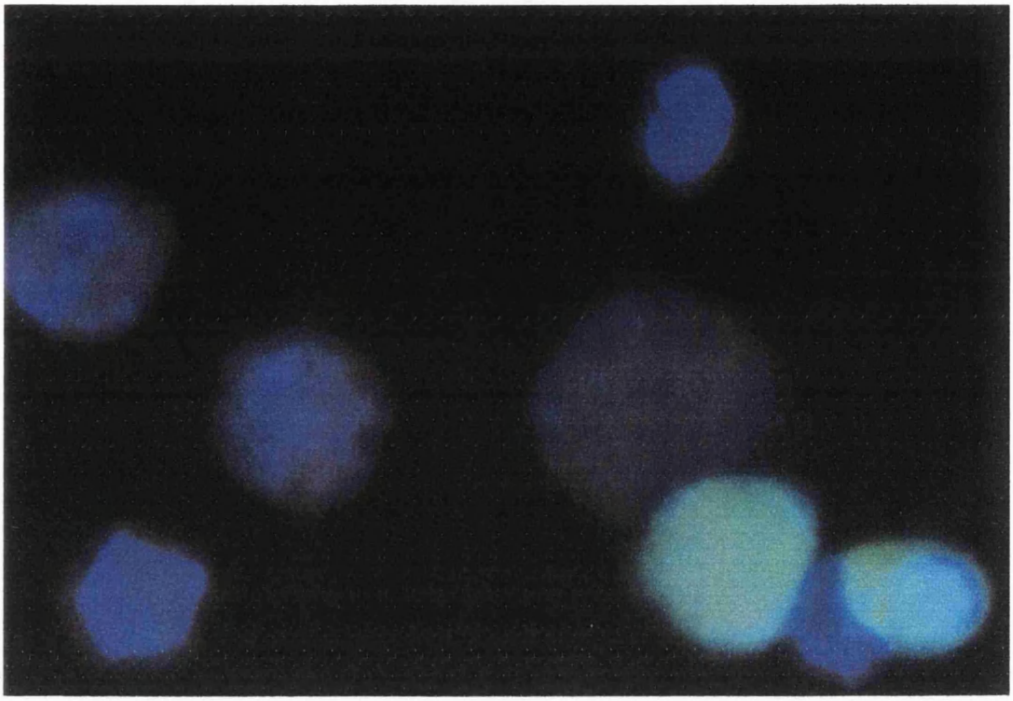
When Serotec beads were employed however, the sorted fraction contained numerous cells. This was seen to be the case when any antibody was used on any cell type or mix, and to occur even when no primary or secondary antibody was added. Decreasing the antibody amount, the cell numbers or the time of the exposure to the magnetic field had no effect; large numbers of cells, particularly cell clumps, were seen to always be in the positive fraction. Increasing the concentration of blocking agents, or the length of wash steps again had no effect. No way could be found to prevent cells from being collected with the sorted fraction from negative controls.

## 3.15. *In situ* RT-mRNA PCR

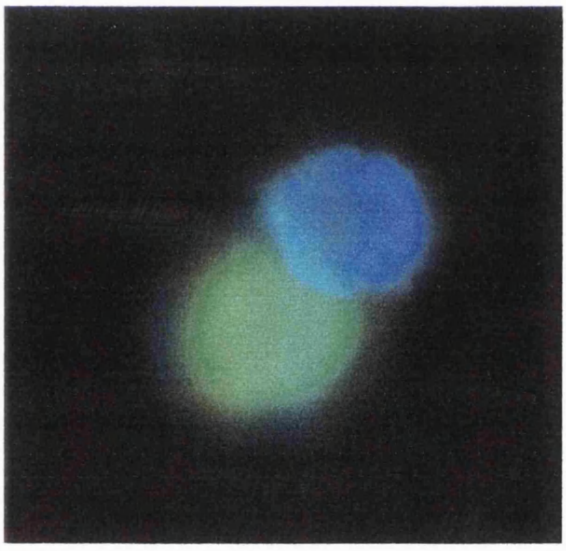
Initial reactions were performed using nucleotides in the reaction labelled directly with fluoroscein. This would enable visualisation of β-HCG mRNA positive cells without further signal development. All slides however, including the negative control, which had no EZ rTth enzyme added, showed some cells with cytoplasmic fluorescent signals (Fig 3.33), independent of cell type. It was thought that the fluorescently conjugated nucleotides may adhere to the membrane of some intact cells. To avoid this possibility nucleotides labelled with biotin were employed. After the RT-PCR reaction, the biotinylated nucleotides were visualised by the addition of fluorescein conjugated avidin. This resulted in the same labelling of many cells, even without the addition of enzyme. Buccal cells, lymphocytes and trophoblasts were all equally labelled. Increasing the annealing temperature of the PCR reaction to increase primer annealing specificity had no effect. Two bright fluorescent spots were observed in the nucleus of many cells indicating the annealing of the primers to the complementary sequence in the genome, and the production of labelled amplicons by primed in situ PCR (PRINS). No way could be found to prevent aspecific labelling of some cells on the slide.

**Figure 3.33 In situ RT-PCR**

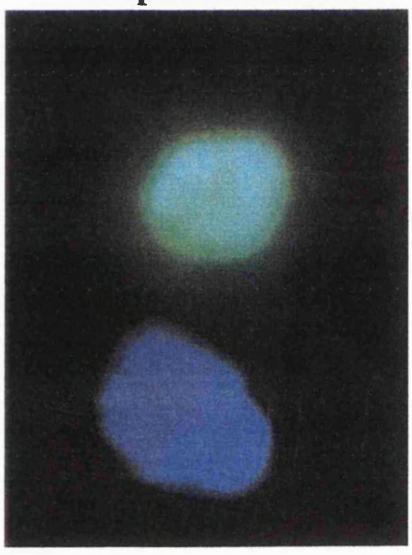
**Lymphocyte cells**



**Buccal cells**



**Trophoblast cells**



Cells exposed to in situ reverse transcription PCR for detection of the mRNA coding for the beta subunit of human chorionic gonadotrophin. The amplified products, detected with the use of fluorescently conjugated nucleotides, appear to be specific to certain cells, but not to cell types.

## 3.16. SUMMARY AND SAFETY

During the course of this thesis almost all TCC samples have been examined microscopically soon after their retrieval. The total volume in conjunction with the cellularity and the pattern of cell types present in the samples were stratified semi-quantitatively on an arbitrary scale "poor-average-good". The sample qualities grouped into these three categories were compared with patient variables (Table 3.17). A 'good' sample consisted of higher absolute and relative numbers of non-squamous-epithelial cells, good amount of mucus in aspirates and high volume of returned fluid in lavage cases, a high nucleated cells:erythrocytes ratio and the presence of multinucleated cells and non-squamous tissue clumps.

Aspirate samples taken before TOP were slightly more often classified as "average" or "good" than the ones taken before CVS. Mucus samples from multiparous patients and from patients with low placentae were more often classified as poor; for the lavage samples, although the numbers were small, these differences were not noted. The quality of the mucus samples improved with gestation from 6 to 13 weeks; this tendency was not as clear in the lavage samples. The yield from the mucus aspirations generally improved with individual operator training and experience in the sampling technique; it showed a correlation with different operators (operators A and B, Table 3.17). There was a tendency for better samples to be obtained by lavage and when cervical priming with prostaglandins (in the TOP cases) was used.

It should be noted however that the assessment of sample quality was subjective, and the differences noted were therefore not analysed for statistical significance.

**Table 3.17 Sample quality and cell yield by patient group, gestational age, placental site, past obstetric history, operator and cervical priming with prostaglandin**

| | Mucus aspiration | | | | Intrauterine lavage | | | |
|---|---|---|---|---|---|---|---|---|
| | cases | poor | average | good | cases | poor | average | good |
| total | 153 | 66 (43%) | 45 (29%) | 42 (27%) | 42 | 13 (31%) | 16 (38%) | 13 (31%) |
| pre-TOP | 53 | 19 (36%) | 19 (36%) | 15 (28%) | 42 | 13 (31%) | 16 (38%) | 13 (31%) |
| pre-CVS | 100 | 47 (47%) | 26 (26%) | 27 (27%) | n.d. | n.d. | n.d. | n.d. |
| 6-8 weeks | 28 | 14 (50%) | 10 (36%) | 4 (14%) | 22 | 7 (32%) | 10 (45%) | 5 (23%) |
| 9-10 weeks | 39 | 17 (44%) | 12 (31%) | 10 (26%) | 10 | 4 (40%) | 2 (20%) | 4 (40%) |
| 11+ weeks | 80 | 32 (40%) | 23 (29%) | 25 (31%) | 10 | 2 (20%) | 4 (40%) | 4 (40%) |
| high placenta | 84 | 35 (42%) | 25 (30%) | 24 (29%) | 7 | 2 (29%) | 2 (29%) | 3 (43%) |
| low placenta | 23 | 13 (57%) | 5 (22%) | 5 (22%) | 2 | 1 (29%) | 0 | 1 (29%) |
| primigravida | 40 | 12 (30%) | 16 (40%) | 12 (30%) | 15 | 5 (33%) | 6 (40%) | 4 (27%) |
| multigravida nullipara | 20 | 4 (20%) | 6 (30%) | 10 (50%) | 17 | 5 (29%) | 7 (41%) | 5 (29%) |
| multigravida multipara | 63 | 31 (49%) | 17 (27%) | 15 (24%) | 8 | 2 (25%) | 2 (25%) | 4 (50%) |
| operator A[a] | 33 | 16 (48%) | 8 (24%) | 9 (27%) | n.d. | n.d. | n.d. | n.d. |
| operator B[a] | 10 | 8 (80%) | 2 (20%) | 0 | n.d. | n.d. | n.d. | n.d. |
| operator C[b] | 22 | 5 (23%) | 13 (59%) | 4 (18%) | 23 | 6 (26%) | 9 (39%) | 8 (35%) |
| prostaglandin | 14 | 1 (7%) | 7 (50%) | 6 (43%) | 12 | 2 (17%) | 5 (42%) | 5 (42%) |
| no prostaglandin | 124 | 58 (47%) | 33 (27%) | 33 (27%) | 13 | 5 (38%) | 4 (31%) | 4 (31%) |

The first column lists selected patient parameters (gestational age, placental site, past obstetric history), operator and treatment (prostaglandin: cervical priming before termination of pregnancy). Not all data items were available from all patients. *Mucus*: poor=minimal cell number mostly squamous epithelial cells or macroscopically absent mucus or saline only; average = small to average amount of mucus and cellularity; good=copious amount of mucus with multiple tissue fragments by low power microscopy. *Lavage*: poor=very low volume (<0.2ml), low cell number, or mostly erythrocytes; average= little to average volume (0.2-1ml) and cellularity; good=high volume (>1ml) or very good cellularity. [a] Samples taken from ongoing pregnancies; [b] samples taken from termination cases. All lavage samples were obtained prior to elective TOP.

In Group B 130 women were sampled before transcervical CVS. Mucus from the external cervical canal was obtained by aspiration. In order to assess the risk attributable to the TCC sampling, a follow-up was conducted. 145 control cases were selected from a comparable group of pregnant women receiving transcervical CVS under the same circumstances, but with no prior TCC sampling. The diagnostic indications, including the maternal age, were similar in the two groups. In Table 3.18 the follow-up data is shown with comparable outcomes in both groups.

**Table 3.18 Follow-up on 130 study cases (cervical mucus aspiration immediately before transcervical chorionic villus sampling) and 145 control cases.**

| | **Study group (n=130)** | **Control group (n=145)** |
|---|---|---|
| **Follow-up complete** | 70 | 79 |
| **Normal pregnancies** | 56 | 62 |
| **Miscarriages** | **none** | **4** (at 16, 16 and 15 weeks, one unknown) |
| **Stillbirths** | **2** (at 40 and 27 weeks) | **none** |
| **Terminations of affected pregnancies** | 12 (3 affected karyotypes, 2 blood disorders, 3 metab. disorders, 4 others) | 13 (3 affected karyotypes, 5 blood disorders, 4 metabolic disorders, 1 other) |

As can be seen, there was no difference in both groups with respect to miscarriages after the procedures or other adverse outcome.

However, due to logistic difficulties almost 50% of the study and control groups were not followed up.

# Chapter 4

# Discussion

***Repeated observations of the cellular composition of human endocervical mucus from the earliest weeks of pregnancy, using various staining techniques, have revealed not only cells of maternal origin, but also chorionic cells which have been shed into the fairly static cervical mucous plug.***

***I have used the fluorescein dye test on smears taken with a cotton swab from the mid-cervical mucus of thirty patients in the first, second and third trimesters of pregnancy. In eighteen cases quinacrine hydrochloride revealed in a proportion of the interphase chorionic nuclei a single fluorescent spot, indicative of Y chromosome material, and thus of a male conceptus. In the other twelve patients smears developed no fluorescent spot, indicative of a female conceptus. Six of the eighteen have now been delivered of boys and four of the twelve have produced girls. These observations are being extended.***

Landrum B. Shettles (1970) *Nature* Volume **230**; page 52

# Discussion

Since Shettles' initial report in 1970, research into the retrieval of trophoblast cells from endocervical canal have produced conflicting results. It is known that during placental development, as the primary chorionic plate fuses with the trophoblast shell, the villi over the abembryonic pole degenerate to form the chorion laeve. The rapid reduction in overall trophoblast volume in this region does suggest that the cells may migrate as well as degenerate, and with the advent of recent molecular methods such as PCR and FISH it has become possible to trace the passage of these cells into the endocervical canal.

If trophoblast cells were to be ubiquitously shed into the uterine cavity during pregnancy, apart from providing a valuable source of fetal material for potential non-invasive prenatal diagnosis, this would raise interesting physiological questions: The manner of cell passage through the decidua capsularis, the stage(s) during pregnancy which facilitate this migration and the viability and exact nature of the extruded placental cells may all have important implications.

I will first discuss the results of this study with a view to the clarification of the presence of trophoblast cells in TCC samples and will then propose mechanisms in which their passage may be achieved.

## 4.1. TCC Sample cell heterogeneity; Microscopic observation.

All TCC samples collected in this study were seen to contain heterogeneous cell populations. Squamous cells were ubiquitously present due largely to the passage of the catheter dislodging maternal epithelial cells lining the vagina and cervix in addition to the collection of naturally shed cells that accumulate in and around the viscous mucous plug. Leukocytes and erythrocytes were seen with extreme rarity in aspiration samples, however their presence was common in cytobrush and lavage samples. The presence of blood cells in TCC samples may indicate the damage of maternal tissue during the collection procedure, particularly with the cytobrush method. However, a dense capillary network permeates throughout the maternal decidua lining the uterine cavity and extends to the extremities of this tissue. Blood cells may pass into any solution washing the decidua, as in the case of intrauterine lavage sampling, without any actual tissue damage.

Early on in this study it was observed that retrieval of TCC samples by cotton swab is an unsuitable method because cells remain trapped on the swab, and only a low percentage of fetal cells can be recovered from most specimens. This suggests that some of the negative evaluations of the TCC procedure previously published in the literature may be due to the different methods applied to retrieve the cell samples.

## 4.2. Groups A and B; Comparison of Aspiration versus Lavage

As a method for identifying fetal DNA in TCC samples, the detection of Y specific DNA has the limitation of being useful only for pregnancies with male fetuses. The absence of Y-specific PCR products in a TCC sample, collected from a woman with a female fetus, does not indicate the presence of trophoblast cells. Fetal cells may or may not be present in the sample but would not be detected with this method. The XY PCR assessment of TCC samples collected from 'female' pregnancies is useful merely as an indicator of the accuracy of the method regarding the incidence of false positive results.

Using PCR to amplify the amelogenin region of the sex chromosomes with ethidium bromide PCR product detection, Y-sequences were identified in 56.3% of the aspirate samples collected from male pregnancies. All lavage samples collected from women with a male fetus were shown to contain Y-specific DNA. There were 7 TCC samples in which no Y-specific DNA sequence could be identified despite the fact that the fetus was male (false negative results). The inability to detect male DNA in these TCC samples could be due to a number of reasons. There may have been no fetal (trophoblast) cells in the samples due to the absence of cells shed into the cervix from the placenta. Alternatively there may have been no fetal cells in the samples due to collection method inconsistencies. Finally, fetal cells may have been present in the collected samples, but were below the threshold of detection sensitivity in the tested aliquots.

No false positive results were seen in any of the 32 samples collected from women with female fetuses showing the PCR assay utilised to be a reliable one. In addition, this indicates that spermatozoa contamination does not appear to be a problem when using XY PCR, even when extracting the DNA from whole TCC samples. This may be due to the absence of spermatozoa in all tested TCCs or the sensitivity of the XY PCR assay being low enough to avoid detecting the minute amount of DNA present in any possible spermatozoa present. It is also likely that the employed DNA extraction methods would not be sufficient to expose the highly condensed sperm DNA

to PCR primers, as the nucleus in sperm is highly condensed and not accessible to PCR reactions using standard extraction protocols (Rank et al., 1991).

In later studies, fluorescent PCR for the amelogenin region was employed using primers flaking a region within the originally amplified site, producing smaller products of amplification. This facilitated the inclusion of other PCR primer sets in multiplex fluorescent PCR assays and provided increased sensitivity of Y chromosome detection.

FISH can also be employed for the detection of the Y chromosome within nuclei. Using this approach, male cells found in TCC samples collected from women with male fetuses indicates the presence of fetal cells. This procedure is again only informative for approximately 50% of TCC samples; those from male gestations.

When using a single probe for FISH, it is expected that, in some cases, false positive and false negative results will be obtained. False negative results are due to overlapping signals or inefficient probe hybridisation to the target DNA of a nucleus. False positive results arise when background fluorescence, such as from fluorescing debris, causes an apparent fluorescent signal to be recorded. In a large study in 1986, Thomsen and Niebuhr showed that their results with a single Y probe had a 0.65% false-positive rate and an incidence of 11% false-negatives. In a diagnostic test, or a protocol to detect male cells in very low concentration, this would be unacceptable. Thus for the purposes of this study, when sexing cell nuclei, dual coloured FISH with two probes was performed, one probe specific to the X chromosome and one to the Y chromosome, each labelled with a different fluorescent marker for subsequent detection. For a cell to be defined as 'male', it would have to have one of each signal. Thus for a false positive result to occur (an apparent XY cell in an exclusively XX cell population) the X probe would have to hybridise erroneously to only one X chromosome, in addition to an apparent Y fluorescent signal occurring within the same cell nuclei. The chances of this occurring are less than 0.07% according to the same figures. During the course of the present study, no false positive results have been recorded in control samples (with over 10000 nuclei scored). Therefore a very low incidence of XY nuclei, even a single cell detected in a TCC sample with a background of over 300 female cells, is sufficient evidence of the presence of a fetal cell, and not the result of an artefact.

The FISH technique for the sex chromosomes has the advantage over PCR that it enables the detection of chromosome numerical abnormalities such as triploidy or Klinefelters Syndromes. The percentage of XY (fetal) cells in a positive sample can be calculated giving an indication of the number of fetal cells present. Also, any spermatozoa contamination will be apparent, due to these nuclei displaying only one Y or X fluorescent signal. In the course of this study, sperm contamination was observed in 5 TCC samples examined by FISH although no spermatozoa were seen in freshly collected TCC samples analysed by light microscopy. Possible sperm contamination could be avoided by asking pregnant women to refrain from intercourse before TCC samples are retrieved for prenatal diagnosis.

Using dual FISH for the X and Y chromosomes, male cells were detected in 58.3% of TCC aspiration samples collected from women with male fetuses in Groups A and B, ranging from 0.2-2%. The 5 false negative results may indicate the total absence of fetal (trophoblast) cells in the TCC samples. The false negatives may however be due to TCC collection method inadequacies. It is also possible that trophoblast cells were present in the collected samples, but at a concentration too low to be detected by FISH. In most cases, approximately 300 cells were scored per TCC sample examined, so if cells from a male fetus were present at a concentration less than 0.3% they would not have been seen.

There were no false positives in any of the 27 examined samples which were collected by aspiration or lavage from pregnant women with female fetuses. These 'successfully' sexed female pregnancies by FISH were merely a result of the absence of nuclei containing X and Y chromosomes, which may be due to the absence of fetal cells in the sample. These results do however highlight the reliability of the FISH assay.

Overall there was a good correlation between the results of the FISH and PCR tests for the presence of the Y chromosome, indicating that fetal cells are indeed present in the endocervical canal of at least 50% of women with a male fetus.

The presence of fetal cells in TCC samples can also be determined by detecting a paternally inherited fetal STR allele which is absent in maternal DNA. This method of fetal DNA detection is useful as it can highlight the presence of trophoblasts in TCC samples collected from women with female fetuses.

In cases that tested positive with this method, one STR marker in the fetal sample differed from those present in the mother and, consequently, must have been inherited from the father; the other allele was the same size as one of the maternal markers. The fetal STR marker was also detected in the corresponding TCC samples, together with the two STR sequences of maternal origin (Figs 3.6, 3.12). This was clear evidence that the cells collected from the endocervical canal contained two populations of cellular elements, one derived from the mother and the other from the fetus. The paternal STR was seen in 13.8% and 25% of examined aspiration and lavage samples respectively. The frequency of TCC samples with detectable fetal cells using this STR allele detection method was considerably lower than that using PCR for Y specific sequences. This is due partly to the technique of STR identification being a relatively insensitive one: In control experiments, using artificial mixes, the presence of DNA at a concentration below 2.5% compared to the majority DNA was not detectable. Amplification of the abundant DNA was favoured in the reaction to such an extent that the amount of generated PCR product specific to the minority DNA did not exceed the levels of background fluorescence, aspecific fluorescent signals and stutter bands (Section 3.1.3.1.). In addition to this relative insensitivity, 20% of cases were non-informative, since the same STR markers were detected in both maternal and fetal samples, so even if fetal DNA were present it could not be distinguished (although this figure should be compared to the 50% of pregnancies with female fetuses and are thus non-informative using Y-chromosome detection). Removing non informative and 'possible' cases from the data, 22% of aspiration and 33% of lavage samples were shown to have the fetal STR allele. In later stages of this study two STR markers were utilised to overcome the problem of non-informative cases (Section 3.5.).

## 4.3. Preliminary Assessment

From preliminary studies on samples in Group A and B, it was concluded that TCC samples do in many cases contain fetal cells. Lavage appears to be a more effective technique for the retrieval of fetal cells, but has the disadvantage of being a considerably more 'invasive' procedure. Aspirated cervical mucus does yield fetal cells in many cases. However, these cells in the mucus are relatively inaccessible to DNA extraction, are not conducive for the preparation of slides for FISH and are more difficult to stain and examine.

During the early stages of this research the mucus was actively removed from TCC samples prior to analysis. From later studies, with the aid of acetyl cystine (which destroys the sulphur bonds in mucus causing it to liquefy) it seems that many cells actually reside within the mucus plug. The removal of the plug therefore resulted in the loss of fetal cells from the TCC samples and may account for some of the early false negative results, particularly with the aspiration technique.

The contrast in the percentage of TCC samples shown to contain fetal cells using XY and STR-PCR methods shows that the majority of TCC samples which do contain fetal cells, do so at a concentration of less than 2.5%. This low fetal cell level renders them beyond the threshold of detection using STR analysis but detectable when using XY PCR.

## 4.4. Cytobrush versus Lavage

Using conventional microscopy and staining trophoblast specific monoclonal antibodies, syncytiotrophoblastic cellular elements could be seen in four of eleven TCC samples collected by lavage and in one sample collected by cytobrush (Group C). The number of syncytiotrophoblastic cells may be higher than that reported, as only a small aliquot of each transcervical cell sample was analysed by phase-contrast microscopy or after conventional staining. Cytotrophoblast cells were also detected in several TCC samples however, immunoperoxidase or immunofluorescent staining of these cells, using antibodies raised against trophoblast antigens, is still hampered by technical difficulties. In controls, only a small proportion of cytotrophoblast cells demonstrated specific staining. No McAb has yet been consistently shown to be completely specific for trophoblast cells so these data are only suggestive evidence that trophoblasts were present in the samples. Although a good indication, identification of fetal cells using monoclonal antibodies is unreliable without supporting molecular evidence.

A good correlation was observed between the results of the PCR amplification of X- and Y-specific sequences in TCC samples obtained by both techniques, and the corresponding villous placenta cells collected after termination of pregnancy. Y-specific DNA was detected in all cases where the fetus was male.

Fluorescent in situ hybridisation, using Y and X probes on transcervical cells in interphase provided clear evidence for the presence of fetal cells derived from male

fetuses; however, in three cases (2 cytobrush and 1 lavage), 46, XY cells could not be detected in the TCC samples. This is most likely due to the low concentration of fetal cells and the limited number of nuclei analysed. The FISH method is limited by the number of cells present under a coverslip (200-300) and so small concentrations of fetal cells (>0.3%) may not be observed. The TCC samples collected in each case were also divided up for each of the assays applied. Therefore only a small aliquot was available for assessment with each technique.

The high percentage of fetal cells in some samples is due to the presence of clumps of male trophoblast cells on the slide, so causing an artificially large percentage of fetal cells present in the sample to be calculated. These were seen as large numbers of adjacent nuclei, all with both the X and Y chromosome. The potential problem of contaminating spermatozoa in the TCC samples was again not evident.

The results of testing transcervical cells for the presence of paternally derived (and thus fetal) short tandem repeat sequences must be assessed in view of the low sensitivity of the detection method. Chromosome 21-derived short tandem repeat sequences of fetal origin were observed in about 50% of the lavage samples but in only one of the samples collected by cytobrush; however three samples retrieved by this technique provided non-informative results because the same patterns of peaks were seen in maternal and fetal specimens. This is in concordance with the low percentage of male cells recorded with FISH experiments (less than 2% in all but one cytobrush sample).

The findings of this comparative study show that cells of fetal origin are present in transcervical cell samples, and in a sufficient number to warrant isolation. It can be seen that the lavage method of TCC retrieval provides more fetal cells suitable for analysis than does the cytobrush method. The collection of TCC samples by cytobrush was found to increase the number of maternal cells and consequently reduced the percentages of trophoblastic cellular elements. Cytobrush samples also contained more debris and endocervical cells than samples collected by lavage, a factor favouring the latter technique. This is not surprising given the abrasive contact with maternal epithelium using the cytobrush procedure. For these reasons the TCC cytobrush sampling method was abandoned for future research. However, this comparison is based on the analysis of small numbers of samples (Table 3.4) which may not be statistically significant.

should be able to be repeated without any increased risk to mother and fetus.

## 4.5. Aspiration followed by Lavage

Contrary to invasive prenatal sampling techniques, where repeat passage of the needle results in significantly increased risks to the fetus, non-invasive TCC sampling . Sequential sampling was thus undertaken assessing the increased likelihood of obtaining fetal cells, and comparing two collection techniques performed on the same patient (Group D). This comparative study between TCC samples collected by aspiration and subsequent lavage showed that trophoblastic cells could be seen in both types of samples with a similar frequency (eight out of thirteen; 61.5%). Based on the cumulative analysis of both samples, the correct diagnosis of the sex of the fetuses was achieved by FISH in 12 out of 13 pregnancies with male fetuses and no false positive results were recorded in the 9 pregnancies with a female fetus.

These findings appear to be contrary the far greater success of the lavage technique compared to aspiration in the retrieval of fetal material as seen in the earlier part of this study. However only the FISH technique was employed to identify fetal cells, a method which can only identify the presence of cells at concentrations higher than ~0.3%. If fetal cells were present, but in lower concentrations, they would not be detected. In addition, the aspiration procedure, performed prior to lavage sampling, may have removed fetal cells which would otherwise have been collected by lavage had it been the first or only method undertaken. Fetal cells were shown to be present in over 60% of aspiration cases- an increase from the previous work. At this stage of the research acetyl cystine had been introduced as a mucus lysis agent, releasing cells bound in the mucus plug and rendering them accessible to molecular assessment. This may explain the increased number of aspiration samples found to contain fetal cells.

## 4.6. Pipelle Aspiration

The alternative method of aspiration employing a catheter containing an internal piston (Pipelle), proved to be equally successful at retrieving fetal cells as shown by the detection of fetal cells in 68% of TCC samples from male gestations (Group E). The low frequency of fetal STR markers in TCC samples (29.6%) reflects the reduced sensitivity of this assay as compared to the PCR detection of Y-derived sequences, and the large number of non-informative fetuses (12) which had inherited paternal alleles of the same repeat number to those present in the mother. The simplicity of the sampling procedure using this device, where no syringe need be

attached and the disposable device is totally self-contained, suggests that it would be the operator method of choice for aspiration collection.

## 4.7. Comparison of TCC sampling techniques; Conclusions

Fetal cells have been identified in TCC samples retrieved by all three of the techniques mentioned. Cell yield and sample quality were assessed subjectively and using monoclonal trophoblast-specific antibodies, and were correlated with patient and clinical parameters. Arbitrary sample quality assessment is not truly reflective of fetal cellular content. There was a tendency for better samples to be obtained by lavage, although mucus aspiration samples showed improvement with gestational age.

Different operators and cervical priming with prostaglandin before termination of pregnancy also seemed to influence the quality of the samples.

Aspiration is considered the least invasive method of TCC retrieval and is the only procedure which has been performed on women intending to continue with their pregnancy. When trophoblastic cells have been located in aspiration samples, they have been present at low concentrations (0.2-7.1%). Aspiration samples are also subject to the greatest variation in quality from one operator to another.

The cytobrush technique, although providing samples which produced a good concordance with fetal sex, resulted in a marked increase in the number of maternal epithelial and blood cells present in the TCC sample. This resulted in a relative decrease in the concentration of fetal cellular elements compared to the majority of maternal cells. As a consequence XY cells could not be easily located using FISH and the paternally inherited STR allele could not be identified using STR.

Lavage using between 5-10ml of saline, provided TCC samples from male pregnancies that always contained fetal cells, as judged by PCR amplification of Y-derived DNA sequences. Analysis by dual-colour FISH had the advantage of providing information of the incidence of 46,XY cells in each sample since cells with Y fluorescent signals can be clearly identified and counted. It has been shown that when 'male' cells were detected in samples obtained from pregnant women with male fetuses, the percentages of these cells varied from 0.2-50%. This variability probably reflects the casual inclusion of clumps containing a large number of 46,XY cells in the small samples

used for the FISH test. The lavage technique causes the washing of the decidua capsularis and irrigation of the entire uterine cavity, as shown by ultrasound examination (Dr. P. Soothill and Dr. J. Kingdom, personal communication). This method may collect more fetal cells because, as trophoblasts enter the uterine cavity, they may not fall into the mucus plug at the site of the internal os, which is the only region sampled with aspiration. Cells may remain attached to the decidua capsularis, or become adhered to the decidua parietalis. These trophoblast cells will only be collected with an intrauterine lavage technique.

### 4.7.1. Safety

An important question that must be answered is whether the collection of TCC samples is associated with maternal or fetal risks. Although the most successful approach to retrieving fetal cells with TCC sampling is with lavage, the semi-invasive nature of this technique suggests that it may have a higher risk of procedure related pregnancy complications associated with it, particularly infection. Although compared to a CVS this 'invasiveness' is minimal, the possibility of introducing infection due to the passage of liquid into the uterine cavity, or causing pressure on the developing fetal sac must be considered. For these reasons lavage samples have not been retrieved from pregnant women intending to continue with gestation. The safety of the aspiration sampling procedure was assessed by following patients who had received pre-CVS TCC sampling together with a second comparable control group of patients who had no TCC samples taken but had undergone CVS. There was no difference in both groups with respect to miscarriages after the procedures or other adverse outcome. However, the fact that almost 50% of the subjects in this study were not followed up does not allow firm conclusions to be drawn about the risks of this procedure, with further trials necessary for a more definitive answer.

## 4.8. Detection of Fetal aneuploidies using FISH

Any de novo chromosome aneuploidies affecting a fetus are obviously absent in the mother. The FISH technique provides a unique opportunity to diagnose fetal abnormalities from a TCC sample, even if a small number of trophoblasts are present in an overwhelming population of maternal cells. A fetus with Down Syndrome was identified using FISH with two probes, each specific to chromosome 21 but labelled

with a different fluorochrome, performed on the TCC sample. This diagnosis was based on the finding that 0.8% of cells in the sample had three doublet FISH signals. This proportion of trisomy cells is sufficient for diagnosis due to the low occurrence of false positive results using the dual FISH technique (Davies et al., 1995), as negligible numbers of apparent trisomy 21 nuclei are observed in normal samples. This was verified by the absence of any false positive results in over 4000 nuclei scored from 13 samples collected from women with normal fetuses, as confirmed by the presence of normal karyotypes in cultured chorionic villous cells. All these tests were performed without prior knowledge of the status of the fetus.

Fetal triploidy was also diagnosed using FISH on the whole TCC samples, subsequently confirmed by tests on placental tissue. This diagnosis was further substantiated by FISH and QF-PCR tests performed on clumps of fetal trophoblast cells isolated by micromanipulation.

A fetus with a karyotype of 47,XYY was also identified using FISH on an isolated clump of trophoblast cells. This confirmed the diagnosis reached by standard CVS culture and karyotyping. As this patient had undergone a CVS procedure prior to TCC sampling, it is however conceivable that the fetal cells detected had been released due to the passage of the CVS catheter, particularly given the late age of gestation at the time of TCC sampling.

In the TCC sample collected from a woman known to be carrying a fetus with the karyotype 48XYY+13, no abnormal cells were found in over 900 examined. If fetal cells were present in this sample, then they were at a concentration less than 1:1000. As this experiment was performed at the time when cervical mucus was being discarded, it may be that fetal cells within the mucus plug were lost. It should be noted that this sample was collected at 15 weeks of gestation, a time when the decidua capsularis has fused with the decidua parietalis eradicating the uterine cavity. Trophoblastic cells cannot therefore be shed into the uterine cavity at this stage of gestation. It is also interesting that the patient had undergone a CVS procedure 2 weeks previously; a procedure thought likely to cause the artifactual release of trophoblasts into the cervical canal. Other groups have also detected chromosome aneuploidies using FISH on TCC samples (See later text; Table 4.2).

## 4.9. Fetal Rh(D) determination

In the pilot study to assess the potential for fetal Rh(D) type determination from TCC samples, in the majority of the cases the PCR results on TCCs were in agreement with those obtained by testing fetal tissues. However, in two cases, the TCC samples retrieved by aspiration or lavage appeared to be Rh(D) negative while the fetuses were positive. These erroneous results may be due to the lack of fetal cells in the TCC samples, or the inability of the technique to identify trace amounts of Rh(D) positive DNA in a majority Rh(D) negative population. The clinical implications of a false negative diagnosis are such that this technique is not yet a viable option for the prenatal diagnosis of Rh incompatibility (Bennett et al., 1995).

## 4.10. The isolation of pure fetal cells

The detection of fetal cells in TCC samples can be facilitated by the identification of a paternally inherited DNA sequence, absent in the mother, even in the presence of an overwhelming majority of maternal cells. Without enrichment or isolation of fetal cells prenatal diagnoses of selected disorders can also be performed from whole TCC samples. This has been demonstrated by the identification of fetal chromosome aneuploidies, and Rh(D) type. However, the prenatal diagnosis of single-gene disorders (eg autosomal recessive and X-linked conditions) requires the use of fetal cells free of maternal contaminants.

Attempts at isolating purely fetal cells using MACS failed due to technical difficulties. When using Minimac beads, the positively sorted fraction did not contain any cells, irrespective of cell type or antibody applied. Due to their small size, Minimac beads require a metallic mesh around them to generate sufficient magnetic attraction to enforce movement of bead, and any cells adhered to it, to the magnetic source. Without this mesh, it appears that they cannot be used. The mesh is incompatible with cell clump migration, due to the small diameter of the mesh pores so, without some form of cell disagregation, this technique cannot be used for TCC sample trophoblast selection.

The Serotec beads, although strongly attracted by the magnetic field, caused cells in solution to stick and move with them, even without the presence of any primary or secondary antibodies. This may be due to some form of non antibody dependant adhesion between the beads and cell surfaces. This would have to be non cell type specific as

buccal, lymphocyte and trophoblasts were all affected equally. Another explanation is the physical collision of the beads, causing the movement of the cells, as they were pulled toward the magnet. No method of bead preparation or antibody blocking was found to affect the movement of unlabelled cells into the expected positive MACS fraction.

There has been only limited success in raising an antibody reactive with all trophoblast populations but unreactive with other cell types (Anderson et al., 1987; Hsi and Johnson, 1992). Several antibodies react mainly with syncytiotrophoblast in uterophlacental tissues. In contrast other antibodies which are reactive with all villous and extravillous trophoblast proliferations detect antigens also expressed by various epithelial cells, including endometrial epithelium and/or ecto-cervical and endocervical epithelial cells. To date, only one antibody has been described which demonstrates reactivity with all trophoblast populations but not with most other epithelial cell types. This McAb, FD0161G has been claimed to identify trophoblast cells in maternal peripheral blood (Mueller et al., 1990). It was not however made available to other researchers in the field, so this research remains unsubstantiated. It may well be that even if the MACS technique had been successful, a battery of McAbs would have to have been applied for the isolation of trophoblast cells from TCC samples.

Trophoblast populations in the first-trimester human placenta do not express classical class I or class II MHC antigens. Extravillous trophoblast within the cytotrophoblast columns, shell, and in uterine decidua and arteries expresses the non-classical molecule HLA-G, although villous syncytiotrophoblast and cytotrophoblast are HLA-G-negative. HLA-G-reactive McAbs may be a useful addition for the identification of extravillous trophoblast in TCC samples but would fail to detect cytotrophoblast originating from the chorionic villi.

Attempts to identify trophoblast specific β-HCG (the beta sub-unit of human chorionic gonadotrophin) mRNA were also thwarted by technical problems. Some cells were clearly labelled irrespective of their type, or the presence/absence of the RT- PCR enzyme (Fig. 3.33). The technique of *in situ* RT- PCR is a new one, and to date has only been performed on tissue sections. Attempts to apply it to fixed cell solutions were not successful. The presence of two bright nuclear signals in many nuclei on slides exposed to the enzyme, indicated the successful annealing and PRINS extension of the β-HCG primers. Aspecific primer binding, or expression of β-HCG in all cell types cannot be an explanation for the labelling of all cells, as this occurred even in the absence of enzyme.

In addition not all cells were labelled. Increasing the annealing temperature also had no effect. As the mRNA which was to be detected is cytoplasmic, cells had to remain intact and could not be treated with hypotonic solution as is the normal preparation for FISH/PRINS slides. It may be that the intact cells' membranes or cytoplasm had an affinity with free nucleotides, so became labelled. Endogenous nucleases still present and functional within the cells may have also incorporated labelled nucleotides during DNA repair or mRNA synthesis. This would have resulted in the labelling of some cells.

There may also have been some common property of all cells which were fluorescently labelled. They may have been cells undergoing necrosis or some other biological process which may affect the membrane and its affinity with, or permeability to, the fluorescent molecules or nucleotides. For whatever reason, the inability to specifically label cells according to type meant this method could not be used in attempts to identify trophoblasts.

Using micromanipulation, clumps consisting exclusively of fetal cells, as assessed by PCR or FISH analysis, were successfully isolated from TCC samples collected by lavage and aspiration. Although a laborious process, the micromanipulation technique is a simple one that can be performed without special equipment. The origin of an isolated cell clump cannot however be determined with certainty without molecular assessment, and frequently a clump of cells with the morphology of trophoblast was found to be of maternal origin. Utilising XY FISH and PCR assays performed on TCC samples collected from male gestations, and polymorphic STR markers (in informative cases), the origin of an isolated cell clump could be resolved.

Having shown that it was possible in some cases to isolate clumps of cells exclusively of fetal origin, potential diagnostic application were assessed.

## 4.11. Chromosome aneuploidy detection using STR quantification

The detection of chromosome aneuploidy using STR QF-PCR amplification is based on the incorporation of fluorochromes into the products of PCR amplification *via* oligonucleotide primers specific for each STR and on the assumption that, within the early exponential phase of PCR amplification, the amount of specific STR produced is proportional to the quantity of the initial target sequence (Ferre, 1992). The optimal

number of PCR cycles was evaluated in order to obtain, using DNA from heterozygous subjects, two peaks of equal fluorescent activity corresponding to the presence of two different alleles at one locus. When the fluorescent PCR products were examined by an automated DNA sequencer, in normal heterozygotes the ratio of the fluorescent activity of the two peaks should be close to 1:1. If the STR marker is highly polymorphic, few normal subjects should be homozygotes and show one peak of activity.

In a trisomic patient the three doses of an STR marker can be detected either as a three peaks of fluorescent activities with a ratio 1:1:1 (trisomic triallelic) or as a pattern of two peaks with a ratio 2:1 (trisomic diallelic). Due to the high polymorphism of the STR markers very few trisomic patients should show a single peak of fluorescent activity and, in the course of this investigation, this was never seen to occur.

With the DS21S11 marker alone, normal amniotic and peripheral blood samples produced two fluorescent peaks (normal diallelic) with ratios very close to 1:1 and a small standard deviation. All chromosome 21 trisomic samples showed either three peaks (trisomic tri-allelic) with ratios 1:1:1 or two peaks (trisomic di-allelic) with a ratio 2:1. Out of 134 samples investigated, only two produced uninformative patterns with more peaks than expected or unusual ratios (e.g. a ratio 4:1 for a DS sample). This showed that QF-PCR could be used with confidence on genomic DNA to detect chromosome aneuploidy.

QF-PCR was then improved to include several STR markers on the same and other chromosomes. It was shown that each STR used in a multiplex reaction amplifies the specific region of DNA independently of any other primer sets in the same assay. The two studies assessing the reliability of multiplex tests revealed a good concordance when comparing QF-PCR results with those obtained from full karyotypes. Of the 161 DNAs tested, only 4 produced spurious results: one trisomy 21 sample produced a ratio of 1:1.6 at Locus A with the MBP STR primer set. This was in contrast to the expected 1:1 ratio observed at Locus B, and to the 1:1 ratio seen with D18S535. Little is known about the STRs that map within the MB P locus and it is therefore difficult to speculate on the cause of this unexpected finding. Three normal samples also produced an apparently false-positive result when tested with one of the markers specific for chromosome 13. The most likely explanation is that these findings were caused by contaminating maternal cells, or a duplication of the DNA sequence detected by this chromosome 13 primer set. In the vast majority of cases QF-PCR for the detection of

chromosome aneuploidy was shown to be reliable, particularly when including more than one primer set for each chromosome under investigation. A recent clinical trial has confirmed these findings (Verma et al., 1998). Two clinical applications of this technique have also been reported (Pertl et al., 1997) where ultrasound scan abnormalities were confirmed by the identification of a triploid fetus and one with trisomy 18. Full karyotype analysis of fetal material acquired after TOP confirmed these diagnoses.

A large number of human tetranucleotide repeats are now available and can be employed for QF-PCR tests. Tetranucleotide repeats have been shown to have better stability during PCR amplification than dinucleotide markers (Utah Marker Development Group, 1995). Some STR markers have been shown to perform better than others. The two STRs amplified by primers in the MBP region demonstrate a greater variation from the expected 1:1 ratio in heterozygotes, particularly at Locus A (Mansfield et al., 1993; Pertl et al., 1996), and are thus no longer used.

Protocols were then successfully refined to test DNA extracted from small numbers of cells (10-50) with STR quantitative assays for the presence of chromosome aneuploidies. No alterations of the anticipated ratios of the fluorescent PCR products were observed, even when the concentration of the DNA template was less than 100pM, with the assays seen to be consistent and reliable. In addition QF-PCR can be utilised to incorporate sets of primers for numerous loci in multiplex reactions. Such additional sets could include primers for the assessment of deletions, single base changes and sex chromosome detection. It was possible to combine several QF-PCR amplifications into a single multiplex reaction and assess, at the same time, the correct number of autosomes in a cell clump, the presence of chromosomes X and Y -derived sequences, and specific single gene disorders (Fig. 3.23). The single base change causing HbS can be assessed with confidence using an adapted ARMS protocol. A similar adaptation of the ARMS procedure was used to successfully diagnose the IVS1-110 single base substitution causing beta-thalassaemia. The 3bp deletion delta F-508 leading to cystic fibrosis could be readily detected from single cells using QF-PCR. The amelogenin region of the sex chromosomes could confidently be employed to assess the presence or absence of the X and Y chromosomes.

QF-PCR amplification of DNA prepared from a clump of trophoblast cells isolated from an aspiration sample was successfully employed to confirm a diagnosis of fetal triploidy from clumps of cells isolated from a transcervical sample collected from a pregnant woman at 10 weeks of gestation. This was achieved using both FISH and PCR.

Clumps of isolated fetal cells were also successfully employed to detect a paternally inherited haemoglobin gene deletion. This was achieved together with the identification of a 4bp maternal deletion in the same cell clumps and lead to the diagnosis of a compound heterozygote fetus. This conclusion was confirmed by routine prenatal diagnosis. This result, together with other successful beta-thalassaemia diagnosis from fetal TCC cell clumps isolated by micromanipulation (Adinolfi et al., 1997) confirms that prenatal diagnosis with isolated cell clumps is possible. The presence of maternal cell/DNA contamination in a large number of samples is however a problem as it may result in misdiagnosis. The inclusion of STR markers in a duplex reaction can determine the origin of a cell clump, as demonstrated by the attempted diagnosis of cystic fibrosis and subsequent determination of the maternal origin of all isolated samples. They will also identify contaminating maternal DNA if present. In such a situation (fetal cells isolated together with maternal cell contamination) a misdiagnosis can be avoided, but a definitive diagnosis cannot be made unless fetal cells are obtained absolutely free of maternal contaminants.

There are various explanations for the presence of maternal cells isolated together with trophoblastic cellular elements from TCC samples. The sample itself is collected from a region dense with maternal cell types. These will obviously be collected in the TCC sample and may become attached to the isolated cell clump.

Also, if trophoblastic villi are breaking through the decidua capsularis these may have maternal cells attached to them which are also collected when the cell clump is isolated by micromanipulation. It has been observed that during implantation syncytiotrophoblast envelope maternal epithelial cells and that many of these cells are apparently destroyed through phagocytoses. If the same invasive procedure allows trophoblast cells to pass through the epithelial layer of the decidua capsularis, then maternal cells (or DNA) may well exist within the bounds of an apparently intact syncytial bud. This may explain the presence of high proportions of maternal contamination despite the careful washing of isolated cell clumps (Table 3.14). In addition ageing maternal cells in TCC samples may become ruptured or lysed,

releasing their DNA into the solution of the TCC sample. This DNA would not be visible and could easily be removed with a desired cell clump.

For the certain knowledge that no maternal cell will be included in a PCR, a clump of cells will have to be broken up and assessed individually. This may be achieved by testing single cells isolated after digestion of cell clumps with enzymes such as collagenase or trypsin (Section 2.2.7.2.b.).

## 4.12. Assessment of QF-PCR assays on single cells

The phenomenon of ADO from single cell PCR, where one allele present in a cell is not represented by the products of PCR, has been previously documented (Finlay et al.,1995a; b; Ray et al., 1996). However, probably as a result of the DNA extraction procedure (El-Hashemite and Delhanty, 1997) and the sensitivity of QF-PCR product detection, in the course of this research ADO was not observed. Due to the small number of informative cells used in this study (heterozygotic cells), it cannot be claimed that ADO does not occur, but from these results it can be seen that ADO has been reduced to an incidence of less than once in 250 reactions.

Preferential amplification was seen to be a frequently occurring phenomenon, affecting around a quarter of single cells (see later text and Figure 3.32). No specific allele was consistently larger, suggesting that this was a random process. Preferential amplification was not observed when 10 or more cells were amplified. Presumably, this was because any single cell within the clump undergoing preferential amplification was counterbalanced by the normal amplification of the majority of remaining cells or the preferential amplification of another cell in favour of the other allele. Each primer set was initially assessed individually in a QF-PCR assay before combining primer sets into multiplex reactions.

The single base change causing HbS could be assessed from a single cell with confidence using the adapted ARMS protocol. A small level of aspecific annealing between the normal primer and the mutant sequence was observed in some cases, particularly when the mutant sequence was over amplified. However this did not compromise diagnoses as there was no overlap in the resulting ratios. The most extreme case of PA in a heterozygote cell, favouring the mutant allele, led to a mutant to normal ratio of 1:0.8. The largest incidence of normal PCR product generation from a homozygous affected cell results in a ratio of 1:0.12; a 6.7 fold difference.

A similar adaptation of the ARMS procedure could be used to successfully diagnose the IVS1-110 single base substitution causing beta-thalassaemia. No such problems with aspecific product generation were observed with this assay.

Preliminary studies suggest that the QF-PCR ARMS test on single cells can be applied to other single base substitutions causing beta thalassaemia such as IVS1-5 (Sherlock et al., 1997a).

The 3bp deletion ΔF-508 leading to cystic fibrosis could also be readily detected from single cells using QF-PCR. The 3bp difference in the products of normal and affected DNA could readily be resolved.

The amelogenin region of the sex chromosomes could confidently be employed to assess the presence or absence of the X and Y chromosomes, in conjunction with all the aforementioned primer sets, with the exception of the β-thalassaemia IVS-110 mutation.

### 4.12.1. Aneuploidy detection.

It was observed that when a single STR marker was employed in QF-PCR assays on single cells, trisomy could be readily detected if the marker produced a trisomic tri-allelic pattern. However, in cases of di-allelic trisomic samples the expected ratio of 2:1 was seen in only 76% of cases. Due to preferential amplification, in the other cases the ratios became skewed and the resulting patterns were not concordant with cytogenetic diagnosis (Figs 3.30, 3.31, 3.32).

Increasing the number of STR markers used in a single cell QF-PCR multiplex increases the chances of a trisomic STR marker being tri-allelic, so reduces the chances of misdiagnosis. However it may well be the case, as in this study, that a trisomic cell line has only di-allelic STR markers on the trisomic chromosome. In two amplified cells from this cell line, all three STR markers were di-allelic with incorrect ratios (Tables 3.15, 3.16). The use of QF-PCR with the STR markers on single cells with a view to aneuploidy detection may not be reliable. The polymorphic nature of the STR repeat markers however, mean that they can be employed to determine the origin and purity of amplified DNA. Individual cells can be identified by comparison of STR alleles sizes. With prior knowledge of maternal and paternal DNA a cell isolated from

a TCC sample can be seen to be fetal. In addition contaminating DNA can be readily identified (Findlay et al., 1995a; 1995b).

When single cells were examined, the success rate of PCR amplification was near 75%, with no significant difference between the type of cell tested. The absence of QF-PCR products could be due to either the lack of single cells in the starting mixture, or to the failure of amplification. The first hypothesis was supported by the total absence of any QF-PCR products in about 8% of cells tested in the STR multiplex QF-PCR reactions. It is of interest that in those reactions which did yield PCR products, many did not have all the STR markers amplified (Table 3.15). The causes of apparent failure of one primer set to generate products in a multiplex PCR reaction are not clear. The DNA chain in this region may be damaged or broken, there may be some structural phenomena preventing access of the primers to the specific site in question, or, by random chance, amplification at this locus may be inefficient during the early cycles of PCR resulting in insufficient product generation to enable detection.

Multiplex fluorescent PCR has been shown to be possible on single cells. In the right conditions primers operate independently of each other. Analysis of sex chromosome content, amplifying the amelogenin region of the sex chromosomes, is reliable and compatible with STR amplification, CF delta F-508, and HbS assessment.

### 4.12.2. Whole genome amplification

An alternative approach for multiple loci assessment from a single cell, would be to employ some form of whole genome amplification (WGA) (Zhang et al., 1992). Such a technique would aim to pre-amplify the entire genome of the cell producing numerous copies of every DNA sequence. Various repeatable PCR assays could then be undertaken rather than attempting all simultaneously in a single multiplex. Work is currently underway to investigate the possibilities of using various WGA methods on single cells, and their effects on sequence fidelity (Wells et al., 1996).

These QF-PCR assays refined for use on single cells have potential applications in other fields. Non-invasive prenatal diagnosis using fetal cells isolated from maternal blood, due to the minute proportion of fetal material, would have to be performed upon a single or very few cells. Polymorphic STR markers have been used to investigate whether isolated nucleated red blood cells are indeed

fetal or maternal in origin (Eggeling et al., 1997). With the known presence of maternal nRBCs in any 'fetal' enriched sample of maternal blood, it is likely that with the exception of chromosome aneuploidy detection using FISH, any prenatal diagnosis will have to be performed on a single cell.

Preimplantation diagnosis (PGD) is technique enabling the diagnosis of affected embryos before pregnancy ensues. Embryos are generated by superovulation and *in vitro* fertilisation (IVF) procedures and remain in culture until day three post-fertilisation , by which time they typically consist of 6-10 cells (blastomeres). For PGD one or two blastomeres are removed and subjected to genetic analysis (Handyside et al., 1990; Handyside and Delhanty, 1997). At the cleavage stage all cells are totipotent and consequently the development of the embryo should not theoretically be impaired by this loss of material. Fluorescent in situ hybridisation (FISH) and PCR can be used to test these single cells prior to selective transfer to the uterus (Delhanty, 1994; Munne and Weier 1996). If the blastomere(s) biopsied are shown to be unaffected by a genetic disorder then it can be inferred that the rest of the embryo is also free of the disease. Only unaffected embryos are transferred to the mother's uterus, and consequently any pregnancy resulting from the procedure must be unaffected. Tests on single cells by standard PCR are restricted by the amount of DNA available and limited to a single reaction. They must therefore be targeted to a single specific inherited disorder (Liu et al., 1992). The reliability of the FISH technique is limited by the number of specific probes that can be successfully used on a single cell and to the detection of only major chromosome disorders.

Using the QF-PCR multiplex assays described in this thesis, with prior knowledge of maternal and paternal DNA, cell origin determination could be combined with specific gene defect analysis, contamination detection and sexing. STR QF-PCR from a single blastomere will accurately indicate a haploid genome, and uniparental disomy.

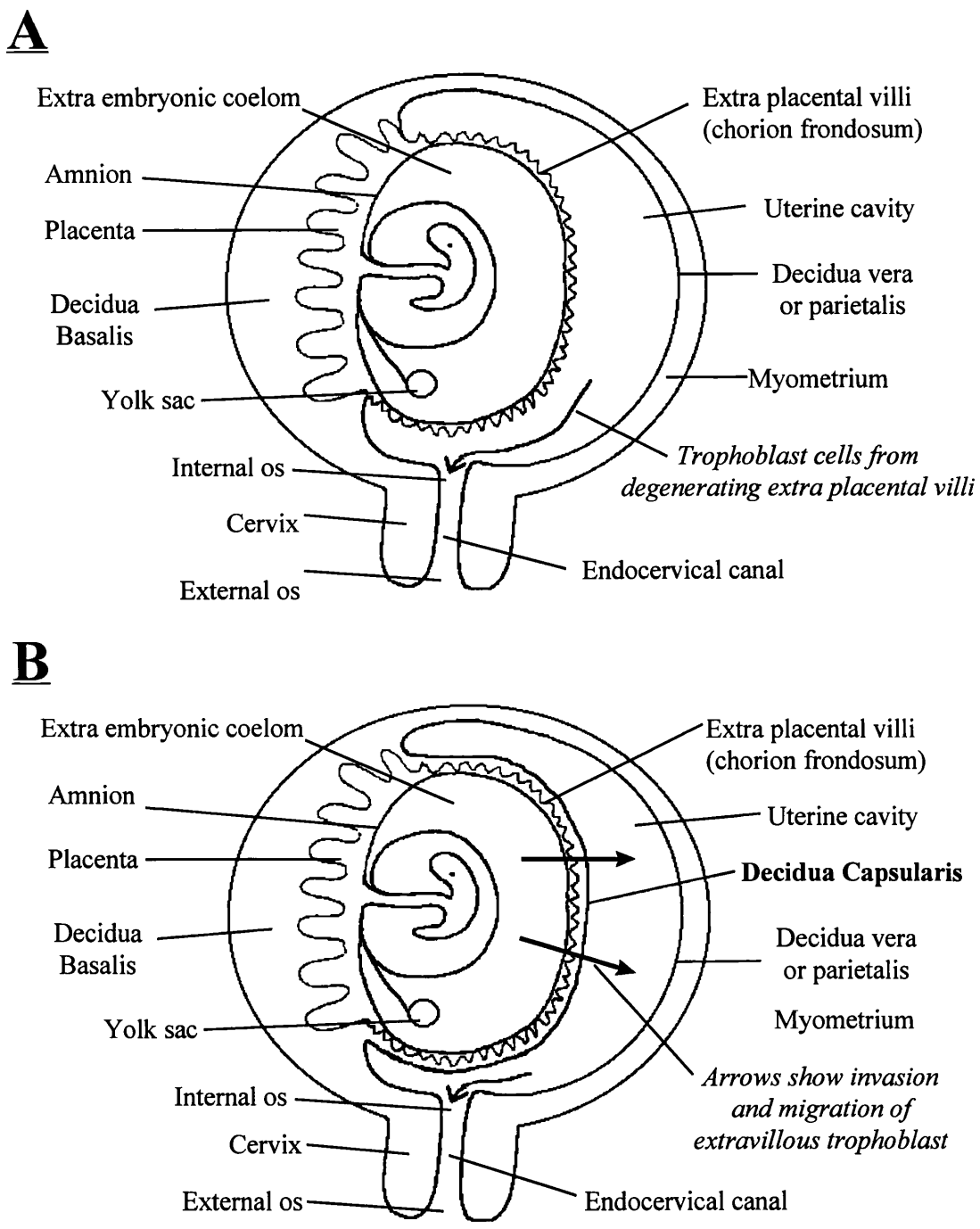
The polymorphic nature of the STR markers, combined with the apparent elimination of allele drop-out lends these QF-PCR assays to forensic applications where knowledge of the genetic origin of a minute sample of cells is required.

## 4.13. The passage of trophoblast cells into the uterine cavity

It has been shown that trophoblastic cells can, at least in some cases, be retrieved from the uterine cavity and endocervical canal of pregnant women. The mechanism whereby fetal cells appear in the uterine cavity and endocervical canal is of clinical and biological interest. Some explanation must be offered as to how these cells can apparently migrate through the maternal decidua capsularis into the uterine lumen.

The view that appears to have been held by Rhine and other early workers in this field was that the gestation sac in utero was covered by fronds of villi. As this part of the chorion frondosum degenerates to become the chorion laeve, trophoblast cells drop off into the uterine cavity (Fig 4.1). However, this hypothesis does not take into account the fact that anatomically the villi in the wall of the gestation sac are covered by the decidua capsularis. The process of formation of the chorion laeve is one of avascular necrosis and degeneration, rather than exfoliation, since the villi are not directly exposed to the uterine cavity.

## Figure 4.1 Migration of trophoblast into the uterine cavity




Migration of trophoblast through decidua capsularis into the uterine cavity: A) Incorrect display of uncovered chorion facing the uterine cavity- the situation assumed by early workers in the field. B) Correct anatomical situation with decidua capsularis covering the chorion toward the uterine cavity.

### 4.13.1. Invasion of trophoblast through the decidual capsularis?

At implantation the conceptus leaves the uterine lumen and becomes embedded in the uterine mucosa. This process involves the dissolution of the zona pellucida, adhesion between the blastocyst and the endometrium, trophoblastic penetration, and migration of the blastocyst into the endometrium. Although even at this stage the blastocyst has a polarity or orientation, there is nothing to suggest that the polar trophoblastic cells are in any way different to the mural trophoblasts (Figs 1.2, 1.3). At the site of implantation proliferating endometrial epithelial cells encapsulate the blastocyst in the maternal mucosa. This region defines the decidua capsularis in later gestation. Up to the eighth week of development, cytotrophoblast and syncytiotrophoblast villi invasion result in the blastocyst resembling a ball of wool within the maternal decidua, with the villi tree network perforating all through the intervillous space surrounding the conceptus (Fig. 1.3). Although there may be slightly less proliferation from the abembryonic trophoblast, there is still no reason to assume that the trophoblastic villi all around the blastocyst are not identical. During this proliferative phase of the reproductive cycle, the uterine interstitial matrix is characterised by a dense network of cross-linked fibrillar collagens including types I, III, IV, & VI as well as fibronectin. Type IV collagen, which is particularly abundant and is typically responsible for cross connecting large fibrils of the major collagens, decreases dramatically with decidualisation and is ultimately only found in association with blood vessels. From animal studies it has been suggested that this removal of type IV collagen, by specific proteases released from differentiated decidual cells, could reduce cross linking between major interstitial collagen types without destroying them, and thus lead to loosening of the stroma without compromising its overall strength (McLaren and Nilsson 1971; Kisalus et al., 1987; Mulholland et al., 1992). This enables the vast network of invading trophoblastic villi to percolate throughout the intervillous space. Although up to the eighth week of gestation this villi tree network covers the whole embryo, almost all research from this stage of development onwards has centred on the trophoblast at the basal region, as this goes on to become the exclusive site of maternal-fetal exchange. The chorion frondosum at the decidua basalis is known to degenerate with increasing fetal development to become the chorion lavae; it is the fate of this degenerating trophoblast that is the concern of this thesis.

Prior to any villous degeneration, the trophoblast filled intervillous space is bordered internally by the chorionic plate enclosing the amniotic cavity membrane, and

externally by the epithelial layer of the decidua capsularis. It is only this layer of epithelium that lies between the trophoblast villi and the uterine cavity. Trophoblastic invasion has breached the epithelial layer before, at implantation, it may well be the case that this phenomenon is repeated. As with initial implantation (Section 1.3), the epithelial cell layer may be traversed without cellular destruction. Lindenberg and co-workers in 1986 cultured hatched blastocysts, obtained after IVF, on monolayers of human endometrial epithelium. Trophoblast cells produced long slender processes that penetrated the intercellular spaces between adjacent endometrial cells. There was no evidence of phagocytoses by the trophoblast, and the endometrial cells were displaced laterally where they formed a multilayer, three to four cells thick. In the ferret, the first penetration of epithelium is seen as the projection of a thin fold of syncytium between adjacent epithelial cells. Initially the process is ectoplasmic but as they enlarge and progress to the basal lamina, the cytoplasm is found to contain the usual array of organelles. The trophoblastic membrane is observed to share both apical junction complexes and punctate desmosomes with the lateral membrane of adjacent epithelial cells resulting in unharmed epithelial cells adjacent to the trophoblast (Enders and Schlafke, 1972). It may be the case that this occurs with human trophoblast, not only at implantation but at the decidual capsularis during proliferation. Both ICM and trophoectoderm cells express E-cadherin (ovumorulin)- one of a family of $Ca^{2+}$ dependant cell adhesion molecules- and as embryos begin to implant, E-cadherin has also been detected on uterine epithelial cells and at implantation sites between the mural trophoectoderm and uterine epithelium (Damjanov et al., 1986; Kadokaw et al., 1989). This would suggest that interactions between the villi and decidual capsularis epithelium could be facilitated and the passage of trophoblast cells into the uterine cavity may occur without visible physical rupture of the decidua capsularis epithelial layer.

During early embryonic development, interactions between the blastocyst and the decidualising mucosa are vital. Where decidual response is inadequate (i.e. in an ectopic pregnancy) invasion is much more aggressive and penetrating. It has been suggested that the decidua has a restraining influence on trophoblast tissue (Burrows et al., 1994; 1995). As the decidua capsularis becomes further compressed and diminished with continued embryonic expansion, it may be that this restraining effect is lessened allowing penetration of the trophoblastic villi through the epidermal layer in an invasive manner into the uterine cavity. This is in concordance with the observation that first trimester human trophoblast cells have been shown to share *in vitro* invasive properties with malignant cells (Graham & Lala 1991).

### 4.13.2. Mechanical Rupture of the Decidua Capsularis?

A second explanation is that the stretching of the decidua capsularis caused by the embryonic expansion (increase in size of the uterine cavity, increase in size of the embryo proper, and increased proliferation of the trophoblastic shell) may cause ruptures in the continuity of the decidua capsularis. It is not uncommon to see lesions in the decidua capsularis in first trimester placentas (personal communication Dr. Kaufman). Such compromises in capsularis integrity would allow the passage of trophoblast cells into the uterine cavity. The decidua capsularis epithelium is an actively dividing tissue. Should such ruptures occur they would soon be re-enclosed by new cells. The short lived disruptions in the epithelial layer may however be present for a sufficient time to allow trophoblast cells to enter the uterine lumen.

### 4.13.3. Oxygen deprivation

The chorion frondosum degeneration under the decidua capsularis may well be accompanied by increased villous sprouting and budding enabling the release of more free villous trophoblast. This may be the case, as a result of insufficient oxygen supplies to the villi underlying the decidua capsularis with increasing physical restriction. Histological studies unanimously report that the amount of villous cytotrophoblast is increased in all those pathologic conditions that are thought to be related to intrauterine hypoxia (Fox, 1970; Piotrowicz et al., 1969). Pathohistologic (Alvarez et al., 1970) and experimental (Tominaga and Page, 1966; Ong and Burton, 1991) evidence has also shown that the villous syncytiotrophoblast is affected by hypoxia; it is reduced in thickness and produces increased numbers of syncytial knots. These changes under conditions of low oxygen tension are thought to be due to degenerative processes (Ong and Burton, 1991) and this placental adaptation is thought to be aimed at improving oxygen diffusion exchange. It has also been shown that chorionic hypoxia results in poor fibrosis of villi (Benirschke K, and Kaufmann P, 1990). The apparent degeneration of syncytiotrophoblast cells under the decidua capsularis is thought to be due to decreased oxygen supply as the structure of villous trees constituting the chorion frondosum around the abembryonic pole is compressed against the chorionic plate on one side and the decidua capsularis on the other. This may lead to the shearing of entire, profusely budding, villous trees, weakened by poor fibrosis and thinning, under the increasing compression pressure. These free buds and trees of trophoblast, unattached at the

chorionic plate, will readily pass through any rupture in the decidua capsularis or, should their invasive characteristics prevail, actively traverse the epithelial membrane.

Alvarez et al., (1970) studied the number of syncytial sprouts in different regions of the placenta. They found the least number at the basal plate, more in the intermediate zone, and the greatest number at the chorionic plate. A relationship was described between the number of sprouts and the oxygen tension, which was higher at the basal plate than at the chorionic plate, confirming that syncytial 'sprouting' is a response to a low oxygen supply. In many electron microscopy studies of the human placenta, large (2-15μm) mushroom like membrane-lined protrusions of the apical syncytioplasm, largely or completely devoid of organelles, have been described. Other studies have shown that these protrusions are not a specific feature of syncytiotrophoblast, but are common features of most epithelia and sometimes other cells (Kaufmann, 1975). In vitro studies have shown that the protrusions are produced under degenerative conditions. In particular, disturbances of glycolysis seem to be important, whereas hypoxia or experimental blockage of oxidative phosphorylation does not induce their production (Kaufmann 1975). These phenomena have also been described to be a characteristic feature in areas of focal syncytial necrosis in the pre-eclamptic placenta (Jones and Fox, 1981).

### 4.13.4. Residual cells?

There is also the possibility that trophoblast cells recovered from TCC samples have occupied the uterine cavity for a long period of time, particularly within the mucus plug. As implantation progresses, the epithelium has to regenerate to enclose the blastocyst. At this time (between days 4 and 12 post conception) trophoblasts are proliferating and budding. Before total encapsulation it may be that some villi are shed from the growing trophoectoderm into the uterine cavity and remain in the developing mucus plug. Even as the endometrial epithelium is growing over the site of implantation, proliferating villi may exceed the periphery of the maternal regenerating epithelium so protrude into the uterine lumen. Should this be the case these villi may remain spanning the decidua capsularis for a time before being shorn off and released.

It is unlikely that this hypothesis is the only explanation for the presence of trophoblast cells in TCC samples as it has been shown that at least some cells are viable and can be cultured (Rhine et al., 1977; 1979; Ishai et al., 1995; Massari et al., 1997) and would thus have to be cytotrophoblasts rather than syncytiotrophoblasts since mitosis has

never been observed within syncytial nuclei and even transcription seems to be reduced (Kim and Benirschke 1971).

### 4.13.5. Passage of trophoblasts at the edge of the forming decidua capsularis?

The process of chorion frondosum degeneration as it becomes chorion lavae is a progressive one, beginning at the abembryonic pole and finally including 70% of the chorion. During this occurrence there comes a point were there is a meeting of chorion lavae and persisting chorion frondosum; at the circular region where decidua basalis, parietals and capsularis all meet (Fig 4.1). It may be that trophoblast continues to invade the uterine cavity from this region, where trophoblasts are last to degenerate and are still highly proliferative.

The passage of trophoblast cells into the uterine cavity may not be a ubiquitous occurrence. The inability to retrieve fetal cells from some TCC samples may be due to failure in collection technique, cell detection techniques, or just their being present in too small a concentration. It may however indicate that fetal trophoblast cells are shed only in some cases, maybe due to sporadic lesions in the decidua capsularis not occurring in all pregnancies.

It is of interest to note that during this study cytotrophoblast cells as well as syncytiotrophoblast buds were observed in the TCC samples. This finding has also been confirmed by other studies (Bulmer et al., 1995; Miller & Brigs, 1997). This observation is unexpected as syncytiotrophoblast form the outer extremities of developing trophoblast and cytotrophoblasts lie beneath. Although migrating trophoblast buds will only consist of syncytiotrophoblasts since trophoblast cells, retrieved from the lower uterine segment, derive from regressing chorionic villi, it may be expected that all trophoblast subpopulations would be represented. Cytotrophoblasts may find their way into the uterine cavity from villous islands and columns (Section 1.5.2.) which are in direct contact with decidua capsularis (Fig. 1.4).

There is also the possibility that cells identified as cytotrophoblast in this study may have actually been maternal cells. Single cytotrophoblasts are sometimes difficult to distinguish from macrophages and groups of cytotrophoblast from inflamed endocervical epithelial cells or metaplastic squamous cells (Bulmer et al., 1995). Definitive identification of a single cell, or cell clump, as fetal must be achieved by

molecular methods; visual and even monoclonal antibody staining with presently available 'trophoblast specific' antibodies is insufficient. However, in GROUP D, dual XY FISH on the remainder of TCC samples after exhaustive 'fetal' clump removal showed up to 7.1% of single isolated nuclei were fetal. These were almost certainly cytotrophoblasts given their large, irregular, pleomorphic hyperchromatic nuclei. The ability of other research groups to culture trophoblastic elements from TCC samples (See later text; Table 4.3) is further confirmation of the presence of this cell type.

## 4.14. Subsequent reports of TCC sampling

Since the initiation of this study there have been a number of publications on this topic. In general these reports have confirmed the findings of this thesis.

In 1995 the culture and karyotype of trophoblast cells in TCC samples was attempted together with dual XY FISH (Ishai et al., 1995; See Tables 4.1, 4.3). TCC samples were collected by intrauterine lavage with 10ml of saline from 54 women in the first trimester of pregnancy (between 6-12 weeks gestation) prior to elective TOP. Of these 54 samples, 4 were discarded due to the inability to retrieve placental reference material. Dual XY FISH was performed on all samples and the sex of the fetus was correctly predicted in all cases; 29 males and 21 females (as determined by the same procedure performed on placental tissue) (Table 4.1). Both short term (direct preparations) and long term (standard CVS protocol) cell culture were attempted on 34 samples. Although growing cells were seen in 28 long term cultures, full karyotypes were only possible in 19 long term cultures and 6 short term cultures. The karyotype in all cases was in concordance with that obtained from placental samples. The viable nature of the collected trophoblast cells in this study shows them to be cytotrophoblasts.

In 1995 Bulmer et al., examined in detail the histology of 11 aspiration and 13 endocervical lavage samples using phase contrast microscopy, Giemsa DNA staining and immunohistochemistry with a panel of monoclonal antibodies. One aspiration and one lavage sample were excluded due to unsatisfactory cellular content. All the samples collected by aspiration were seen to contain the same variety of maternal cell types: ectocervical squamal cells, both singly and as sheets of cells; endocervical columnar epithelial cells observed singly and in 'gland-like' structures; macrophages, notably

histocytes; lymphocytes and polymorphs. Three of the ten samples were seen to contain both syncytial fragments and single and small groups of cytotrophoblasts. A further sample, although apparently devoid of syncytia did clearly contain cytotrophoblast cells. These findings were confirmed by cell type characterisation using monoclonal antibodies and avidin-biotin-peroxidase staining. The monoclonal antibodies used were directed against extravillous trophoblast with no reactivity to cytotrophoblast (BC1, NDOG5), against villous syncytiotrophoblast with no cross reactivity (NDOG1), against macrophages (KP1), against leukocytes (LCA), against epithelial cells (EMA), and against epithelial cells with reactivity to villous and extravillous trophoblast (5D3). These assays were used for both positive identification and negative exclusion of the presence of trophoblast cells.

The 12 lavage samples were examined in the same manner and were all seen to contain squamal and columnar epithelial cells. Macrophages, lymphocytes and polymorphs were also ubiquitous. Five of the samples were seen to contain multinucleated syncytial fragments these 5 with an additional 4 (9/12) were seen to include cytotrophoblast cellular elements.

Kawamura et al., (1995) retrieved 24 intrauterine lavage samples using 10ml of saline from 24 women prior to elective TOP; 9 due to a 'missed abortion', and 15 for social reasons. FISH for the Y chromosome was performed and Y bearing nuclei were observed in all 13 male pregnancies, as confirmed by reference to placental material, with no false positive results. Four of these samples contained a very high proportion of male cells and visible villi so were considered to have been 'mini-CVS'. The remainder of positive samples contained between 5.8-23.5% fetal cells.

Bahado-Singh et al. (1995) performed endocervical lavage with 3ml of saline on 20 pregnant women with fetuses at 7-10.5 weeks gestational age, prior to elective TOP. Cells of trophoblastic origin were identified morphologically using light microscopy in 50% of cases. Cell cultures were established from five of these samples and trophoblast cells visualised by staining with the placental specific antigen alpha human chorionic gonadotrophin (Table 4.3). Trophoblast cells were confirmed to be present in one sample, two were ambiguous and three negative. Without the application of more sensitive methods of fetal cell detection such as FISH or PCR for the detection of Y-specific DNA sequences it may well be that fetal cells although present, were not detected in these samples. The authors stated that the possibility of intrauterine lavage

effectively representing a 'mini-CVS' could not be unequivocally ruled out. However, this is extremely unlikely as the trophoblast villi are enclosed by the decidua capsularis.

Briggs et al. (1995) studied TCC samples obtained by aspiration from beyond the internal os and inter-uterine lavage, from 150 women at 7-17 weeks of gestational age, prior to elective TOP; 117 by aspiration of cervical mucus and 33 by endocervical lavage. Syncytial vesicles were observed in 39% of lavage and 26% of aspiration samples, with most syncytia being apparent in samples taken from women at 9-10 weeks gestation. No syncytia were observed in samples taken from pregnancies with a gestational age greater than 14 weeks. Twenty aspiration samples were selected and assayed blind by FISH and PCR for Y-specific DNA sequences. Only six of these 20 had been seen to contain syncytia. The results were compared to similar assays performed on placental material obtained after TOP (Table 4.1). Male fetuses were correctly predicted in 12 of the 13 samples collected from male gestations, with one false negative. Female fetuses were correctly predicted in 6 of the 7 samples collected from female gestations, with one false positive. There was total concordance between FISH and PCR results. The authors confirmed that the existence of male cells in TCC samples collected from women with male gestations despite the apparent absence of syncytia in these samples showed there to be a source of fetal material other than syncytiotrophoblasts.

These results were corroborated in a further publication (Miller and Briggs, 1996) where further TCC samples were collected by aspiration and lavage from pregnant women between 7 and 17 weeks of fetal gestation and examined microscopically for evidence of syncytiotrophoblast structures. Syncytiotrophoblasts were observed in 39/137 (38%) of aspirates and 17/34 (84%) lavage samples. Despite the apparent absence of syncytiotrophoblast structures the sex of the fetus was correctly predicted in 84% of tested aspirated samples (72/86) by PCR and FISH for Y specific sequences, confirming once more that cytotrophoblast cells must be an addition source of fetal material in TCC samples.

Maggi et al., in 1996 used 'vigorous' intrauterine lavage with 5ml of saline to collect 86 samples from pregnant women prior to termination of pregnancy in the first trimester in two phases of study. Samples were examined microscopically and then cultured in an attempt to karyotype trophoblast tissue. In Phase 1, 15 samples were

collected and 12 were seen to contain villi. From these 12, long term cultures were set up. Pure male and female karyotypes were found in one and seven cases respectively, while a mixture of XX and XY metaphases was observed in the remaining four, indicating there to be fetal metaphases in the presence of contaminating maternal cells (Table 4.3). To avoid this maternal cell contamination Phase 2 of the study, consisting of 71 samples, used a semi-direct culture protocol. Villi were observed in 60 of these samples so cultures were established from these. Chorionic villus karyotypes were determined in 40 cases (66%); 24 female and 16 male (Table 4.3). Included in these 40 successfully karyotyped lavage samples were four aneuploid trophoblast cell lines; Trisomy 13, Trisomy 15, Trisomy 16 and mosaic Trisomy 12 (Table 4.2). Only the results from 27 of the 40 samples could be confirmed against placental tissue retrieved after TOP but these were all correct and included the 4 chromosome aneuploidy cases. In all, 83.7% of the examined samples contained villi, with the amount varying from 1-32mg, with an mean of 20mg. Although, without confirmation of fetal sex with reference to analysed placental tissue in all cases, no accurate assessments of fetal sex prediction accuracy can be drawn, it is clear that in this study fetal trophoblast material was retrieved and successfully cultured from TCC samples. Indeed the skewed ratio of female to male TCC cultures (24:16) suggests that some male pregnancies may have been missed and maternal cells karyotyped. However, the absence of XX/XY mixed cultures in Phase 2 of the study is evidence contrary to this assumption.

Massari et al., (1996) collected endocervical/lower uterine lavage samples from 39 pregnant women prior to 1st trimester CVS sampling. Prenatal diagnosis was being provided as each woman was at high risk of an affected pregnancy; 37 of chromosome aneuploidies due to maternal age; one of SMA1 due to an affected father; and one of myotonic dystrophy due to an affected father. Lavage was performed using 2-3ml of saline, and other than those with affected fetuses, all pregnancies continued to term. Using standard phase contrast microscopy placental villi were observed in 17 of the 39 samples, with single syncytiotrophoblasts seen to be present in 21. PCR was performed on DNA extracted from these samples for Y chromosome specific DNA and for 3 polymorphic STR markers (D21S11, D5S127 and D5S663). Fetal DNA was identified in 17 of the 39 samples using this method; 7 samples showing the presence of male DNA (all confirmed to be TCC samples from male gestations with no false positives); and 10 further pregnancies displaying evidence of 1 or more paternal STR alleles (Table 4.1).

The 17 fetal-positive samples were tested by FISH for the presence of chromosome 21. One sample contained cells with three FISH signals indicating the fetus to have Down Syndrome (Table 4.2). DNA from this sample was further tested using a semi-quantitative PCR method comparing relative amounts of SOD1 and control sequence generated by simultaneous duplex PCR. The 1.5X overproduction of SOD1 further indicated the presence of trisomic fetal DNA. This diagnosis of Down Syndrome was confirmed by a standard karyotype performed on the CVS tissue. All other samples were found to only contain cells with two FISH signals and thus two copies of chromosome 21 suggesting a normal fetus. This was confirmed by corresponding karyotype results from cultured CVS material.

The STR marker D5S127 is closely linked to the SMA1 triplet repeat expansion and was informative for linkage in the SMA1 risk pregnancy in the study. The presence of the STR allele linked to the normal chromosome 5 from the affected father in the lavage sample showed, by exclusion, that the fetus was unaffected. This was confirmed with results from standard prenatal analysis.

DNA from the lavage sample collected from the pregnancy at risk of myotonic dystrophy was digested with EcoR1, electrophoretically separated, and probed with MDY-1 which contains the CTG repeat (Novelli et al., 1993). Only normal sized repeat numbers were observed so the fetus was predicted to be normal. This was confirmed by routine CVS diagnosis.

Six of the pregnant women in this study also had samples collected by vaginal and cervical swabs subsequent to endocervical lavage. All the lavage samples from these cases were shown to contain fetal DNA. Analysis of the swab material using the same PCR techniques demonstrated fetal DNA to be detectable in only 2 of the 6. The authors concluded that compared to swabs, lavage sampling was a more effective method of fetal trophoblast retrieval.

A the time of publication it was thought that Massari et al., (1996) had been the first group to carry out lavage on ongoing pregnancies. However, since this report it has come to light that from the second half of 1992 uterine lavage with 20ml of saline has been performed in Taiwan (Chou et al., 1997) employing Y chromosome specific PCR for fetal sexing. Traditional Asian culture believes the 'blood line' of a family can only pass through male offspring so the desire for at least one boy in a family is high. This need, coupled with economic factors limiting progeny number, sadly results in the desire to abort female fetuses. Although no detailed data has been published, it is believed that

more than 20000 procedures have been completed (Hsi and Adinolfi, 1997). No information regarding exact sample number, the safety of the procedure or success rates of sexing are available, however one case of fetal limb deformities has been reported (Chou et al., 1997).

Unsuccessful results were reported by Overton et al., in 1996 who collected 204 TCC samples from pregnant women in the first trimester prior to elective termination of pregnancy; 87 by endocervical lavage, 66 by endocervical swab, and 51 by swab from the lower uterine pole (Table 4.1). Samples were examined by either dual XY FISH or Y-PCR employing 3 techniques; amplification of the amelogenin region of the sex chromosomes, one step PCR amplification of Y-specific DNA and nested PCR for the same Y-specific region.

From XY FISH performed on the lavage samples obtained from male pregnancies, correct prediction of fetal sex was achieved in 40% (6/15) with 9 false negatives. With the swab samples the success rate was 27% (4/15). When present, the percentage of male cells in lavage samples ranged from 4.4-24.8%, and in swabs from 0.7-3.4%. The correct prediction of a female fetus was achieved in 95% of lavage (20/21) and 90% of swab (19/21) samples, with false positive results in 1 and 2 cases respectively. It should be noted that when testing TCC samples for evidence of the Y chromosome, the correct prediction of a female fetus does not necessarily indicate the presence of fetal cells as the same conclusion would be drawn in the absence of fetal material. The false positive results obtained with XY FISH (a female fetus predicted as a male due to the presence of XY nuclei in the TCC sample) could not be due to the presence of contaminating spermatozoa in the TCC samples, as a haploid genome will only produce a single FISH signal. Rather these erroneous results are due to the inadequate FISH protocol employed, or inexperience of the operator as indicated by the presence of 0.5% XY cells in control female lymphocyte preparations. This false positive rate is unacceptable for the identification of minority XY cell populations and is more than 100 fold greater than that experienced in our laboratory (C. Conn, A. Halder, D. Wells data not shown).

With PCR, Y specific DNA was detected in 28-79% of samples from male pregnancies depending on the collection and PCR technique applied. However, false positive results were observed in 14-45% of female pregnancies. This may be due to the presence of contaminating spermatozoa in these samples; however due to the standard DNA extraction procedures applied and the hypercondensed inaccessible nature of sperm

nuclei this in unlikely. The PCR assays used had been refined for the detection of 2pM of male DNA in 5ul solution, which constitutes approximately 1/4 of a diploid genome (Wachtel and Tiersch, 1993). It may well be that the false positive results were artefacts of the ultra-sensitive PCR methods applied. The authors noted that under ultrasound intrauterine lavage had been seen to cause 'tenting' of the fetal membranes due to the vacuum created. They postulated that this may result in a small placental biopsy, but this could only be the case if the decidua basalis becomes ruptured.

In 1997 Daryani et al., took intrauterine lavage samples using 5-8ml of saline from 21 pregnant women between 9 and 14 weeks of fetal gestation prior to elective termination of pregnancy. Dual FISH for the X and Y chromosomes was performed in a blind study in an attempt to predict the sex of the fetus (Table 4.1). Four samples did not contain enough material to analyse so were discarded. Of the remainder, correct sex prediction was achieved in 81.3% with 2 false negatives (prediction of a female fetus from a male pregnancy) and one false positive (prediction of a male fetus from a female pregnancy). In those lavage samples correctly diagnosed as male, the percentage of nuclei with both X and Y signals ranged from 3.6-7.8%. It was noted in this study that syncitial clumps did not hybridise well to FISH oligonucleotide probes possibly resulting in the false negatives, and that the mucus present in most samples inhibited efficient FISH probe hybridisation.

## 4.15. Conclusion

The results of this thesis clearly demonstrated that fetal cells are present in TCC samples collected from the majority of pregnant women between 6-13 weeks gestation. It is still uncertain as to whether the shedding of trophoblast cells into the endocervical canal is a ubiquitous physiological phenomenon which takes place in all normal human pregnancies or whether they can be found only in certain, albeit the majority, of cases. Before any clinical application of this methodology, it remains to be seen if the current methods employed for TCC collection and fetal cell detection allow them to be used for prenatal diagnostic tests. As most research has been carried out upon samples collected prior to elective TOP it must also be established that fetal cells can be retrieved from ongoing pregnancies with a similar frequency. The detection of clumps of syncytio- and cytotrophoblastic cells in samples retrieved by aspiration of endocervical mucus cannot be due to a mini-CVS or placental biopsy, as suggested (Fisk 1995; Overton et al., 1996), since villi are covered by the decidua capsularis. With this method of collection, the point of the catheter does not reach the placental tissues (Rodeck et al., 1995).

The presence of fetal cells in the uterine cavity and endocervical canal of pregnant women has been confirmed by reports from other authors (Table 4.1). The majority of studies have relied on the detection of Y-chromosome specific DNA in TCC samples collected from women with male fetuses using either FISH or PCR (Table 4.1). In addition to this technique of identifying fetal material, fetal aneuploidies have also been detected (Table 4.2).

**Table 4.1 Incidence of Trophoblastic cells in TCC samples retrieved from mothers with male fetuses.**

| Methods for collection | | Test by | | Authors |
|---|---|---|---|---|
| | | FISH | PCR | |
| 1 | Transcervical swabs | ... | 9/9 | Griffith-Jones et al., 1992 |
| 2 | Endocervical lavage (10ml) | 7/7 | 6/6 | Adinolfi et al., 1993 |
| 3 | Endocervical lavage (10ml) | 5/6 | 6/6 | Kingdom et al.,1995 |
| 4 | Cytobrush | 4/6 | 6/6 | |
| 5 | Endocervical lavage (10ml) | 6/6 | ... | Adinolfi et al., 1995b |
| 6 | Aspiration | 1/1 | ... | |
| 7 | Aspiration [a] | 2/5 | 7/14 | Adinolfi et al., 1995c |
| 8 | Endocervical lavage (5ml) | 1/1 | 2/2 | |
| 9 | Aspiration | 8/13 | ... | |
| | followed by lavage (5ml)[b] | 8/13 | ... | |
| 10 | Lavage (10ml) | 8/8[c] | ... | Ishai et al., 1995 |
| | | 21/21[d] | ... | |
| 11 | Aspiration | 12/13 | 12/13[e] | Briggs et al., 1995 |
| 12 | Lavage (3ml)[f] | ... | 7/7 | Massari et al., 1996 |
| 13 | Uterine lavage (20ml)[a] | 5/5 | ... | Chang et al., 1996 |
| 14 | Swab and lavage | f | f | Overton et al,. 1996 |
| 15 | Lavage (5-8ml) | 10/12 | ... | Daryani et al., 1997 |

a) All samples were collected before TOP with the exceptions of samples in 7 and 13; b) In total, 12 out of 13 samples were Y positive; c) Double colour FISH; d) Single colour FISH; e) One false positive; f) Out of 39 samples tested 17 contained fetal DNA; f) See text.

**Table 4.2 Fetal chromosome aneuploidies detected in TCC samples**

| Collection method | Detection Method | Aneuploidy | Authors |
|---|---|---|---|
| Intrauterine Lavage | FISH | Trisomy 18 | Adinolfi et at., 1993 |
| Aspiration followed by endocervical Lavage | FISH and QF-PCR | Triploidy | Tutschek et al., 1995 |
| Intrauterine Lavage | Cultured Karyotype | Trisomy 13<br>Trisomy 15<br>Trisomy 16<br>Trisomy 12 (mosaic) | Maggi et al., 1996 |
| Endocervical Lavage | FISH | Trisomy 21 | Massari et al., 1996 |
| Aspiration<br>Aspiration followed by endocervical Lavage | FISH<br>FISH<br>FISH and QF-PCR | Trisomy 21<br>47XXY<br>Twin chimerism | Sherlock et al., 1997 |

The ability to culture *in vitro* trophoblasts present in TCC samples shows that viable cytotrophoblast cells are shed into the endocervical canal and suggests that future research, with appropriate selection of culture media, may enable a full fetal karyotype to be obtained from TCC samples (Table 4.3).

**Table 4.3. Summary of studies attempting to culture Transcervical Cell Samples.**

| Methods for collection | | Results | Authors |
|---|---|---|---|
| 1 | Endocervical lavage | 36 cultures /66 attempted; 16 with polymorphic differences/17 scored | Rhine et al., 1977 |
| 2 | Endocervical lavage | 37 cultures /53 attempted: 26/37 contained fetal chromosomes | Rhine and Milunsky 1979 |
| 3 | Endocervical lavage | 0 male metaphases out of 9 successful TCC cultures from male fetuses | Goldberg et al., 1980 |
| 4 | Intrauterine lavage | 5 cultures; 1 fetal, 2 maternal, 2 equivocal | Bahado-Singh et al., 1995 |
| 5 | Intrauterine lavage | 19 metaphase cultures / 38 attempted; 6/6 direct preparations | Ishai et al., 1995 |
| 6 | Intrauterine lavage | 52 metaphase cultures / 86 attempted; 47/52 purely fetal 5/52 fetal & maternal | Maggi et al., 1996 |

TCC sampling is relatively easy, quick (<3min), cheap and painless. Due to the non/semi-invasive nature of the sampling procedure, there need be no limit to the number of TCC samples retrieved; Endocervical samples can be collected at early stages of gestation, and a second sample can be collected after an interval of only one week, if the first specimen does not contain fetal cells (Ishai et al., 1995). The procedure is still limited by the small proportion of fetal cells retrieved, and the current inability to isolate pure fetal samples with confidence. Although shown to be successful, the micromanipulation of cell clumps with apparent trophoblast morphology is a laborious task and impractical for implementation on a large clinical scale. The introduction of automatic scanning methods for the detection of FISH signals should greatly increase the number of cells that can be analysed in each sample. This may enable fetal

aneuploidy screening or the identification of fetal cells from male gestations for subsequent analysis. Equally advantageous should be the use of immunohistochemical staining with McAbs raised against placental antigens which prove to be more specific than those currently available, followed by FISH tests. This approach or the detection through reverse transcriptase PCR (RT-PCR) of placental specific mRNA should allow the identification of fetal cells derived from either male or female fetuses. Identified fetal cells could then be exposed to FISH assays for chromosome aneuploidy assessment or removed from the slide using micromanipulation for subsequent PCR analysis. Until such isolation techniques are established, the use of fetal cells in the endocervical canal for prenatal diagnostic tests should remain only as a research tool.

# Chapter 5

# Bibliography

# Bibliography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