An Investigation of Conceptuses with Placental Chromosomal Mosaicism for the Presence of Germline Mosaicism

by

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Abstract

Confined placental mosaicism (CPM) is detected in approximately 1-2% of viable pregnancies and has been defined as a dichotomy between the chromosomal constitution of the placental and embryonic/fetal tissues. An investigation for the presence of trisomy in the germ cells was initiated in cases diagnosed with CPM since we suspect that the stroma of the placenta and the primordial germ cells (PGCs) have a common developmental origin. Placental and fetal tissues from one conceptus diagnosed with CPM for trisomy 7 and two conceptuses with CPM for trisomy 16, were analyzed using fluorescence *in situ* hybridization (FISH).

Post-termination conventional cytogenetic analysis of amnion and chorionic stroma from case 1 showed only diploid cells in the amnion and mosaicism for trisomy 7 in the stroma. FISH analysis of formalin fixed - paraffin embedded tissues showed significant levels of trisomy in the placenta while all fetal tissues analyzed including testis were diploid. For Case 2, high levels of trisomy 16 were detected using FISH in both chorionic stroma and cytotrophoblast of the placenta while fetal somatic tissues where diploid. FISH analysis of meiotic oocytes showed significant levels of trisomy 16. In case 3, prenatal metaphase analysis of cytotrophoblast and stroma from chorionic villus sampling showed only 47,XX,+16. Culture of placental villi after termination showed only trisomy 16 while cultures of multiple fetal tissues showed no evidence

of trisomy. FISH analysis of formalin fixed - paraffin embedded lung and meiotic oocytes also failed to show evidence of trisomy 16. The results from Case 2 suggest that conceptuses diagnosed with mosaicism in the chorionic stroma are at risk of exhibiting chromosomal mosaicism in their germ cells. Future studies in similar cases are needed to understand the effects this may have on human fertility in the reproductive period.

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1 Introduction

1.1 Chromosomal Mosaicism

Constitutional chromosomal mosaicism is defined as the presence of two or more cell lines with different chromosomal complements in an individual. In most instances the mosaic individual presents with two cell lines - a normal diploid cell line and an aneuploid cell line. This usually arises from a post-zygotic mutational event in early embryonic development. Mosaicism can occur in both chromosomally normal and abnormal conceptions as a result of a mitotic error such as nondisjunction, anaphase lag or a structural rearrangement. Different types of constitutional mosaicism can arise depending on the exact timing of the mutational event, cell lineage involved and viability of the mutant cell line. The mosaicism can be generalized affecting both the fetus and the placenta, or confined to the placenta or the embryo/fetus proper (Kalousek and Dill, 1983).

1.2 Generalized Mosaicism

In cases of generalized mosaicism, multiple tissues of both embryonic and extraembryonic origin are affected. This occurs when the viable mutant cell line arises at the first postzygotic division or shortly thereafter (Kalousek and Vekemens, 1996). An early mutational event results in a

greater proportion of mutant cells in the developing morula, thus increasing the possibility that tissues of both embryonic and extraembryonic origin are affected (Kalousek, 1993).

Data from amniocentesis performed in the second trimester of pregnancy in women 35 years of age have been used to estimate the prevalence of generalized mosaicism. This procedure makes available cells derived from the amniotic membrane, skin and epithelial cells of the gastrointestinal, urinary and respiratory tracts of the fetus for cytogenetic analysis. Several studies have shown that approximately 0.1 to 0.3 per cent of viable pregnancies are generalized mosaics (Hsu et al., 1984; Worton and Stern, 1984; Bui et al., 1984). Approximately 70% of these positive findings have been confirmed in fetal tissues (Hsu et al., 1984). Failure to confirm prenatally diagnosed mosaicism in the fetus following pregnancy termination has been explained by contamination of amniotic fluid by chorionic cells during the amniocentesis in gestations which exhibit chromosomal mosaicism confined to the placenta (Kalousek et al., 1987).

1.3 Confined Mosaicism

Before the studies of Kalousek and Dill (1983), mosaicism observed in placental tissue was interpreted to represent the entire conceptus. However, since the timing

of the mutational event and the cell lineage involved determines the distribution of mutant cells in the conceptus, Kalousek and Dill postulated that a postzygotic nondisjunctional event can produce mosaicism confined to either the placenta or the fetus but not necessarily both. They showed that some infants diagnosed with unexplained intrauterine growth restriction (IUGR) exhibited aneuploidy in tissues of extraembryonic origin while fetal tissues were diploid. For example, analysis of the term placenta from a gestation with unexplained IUGR revealed trisomy 22 in cultured chorion with no evidence of aneuploidy in amnion, cord blood or skin.

Confined placental mosaicism (CPM) has been defined as a dichotomy between the chromosomal constitution of the placental tissues and embryonic/fetal tissues. CPM is most often prenatally diagnosed in chorionic villus sampling (CVS) at 9 to 12 weeks of gestation. CVS involves obtaining a small piece of chorionic villus for cytogenetic analysis which could either be processed by short term incubation (direct preparation) or the culture method. The direct preparation involves cytogenetic analysis of rapidly dividing cells derivet from the trophoblast lineage; the long term culture allows the analysis of villus stroma (Sadler, 1995). CPM has been reported in approximately 1 to 2% of viable pregnancies analyzed by CVS followed by a failure to confirm mosaicism in fetal tissues (Ledbetter et al., 1991; Wang et al., 1993). Also, several studies have

shown that mosaicism in the placenta persists to term in the majority of cases analyzed (Schwinger et al. 1989; Kalousek et al. 1991; Henderson et al., 1996).

A number of hypotheses have been proposed to explain the more frequent occurrence of chromosomal mosaicism in the placenta when compared to the fetus. Kalousek and Dill (1983) proposed that the incidence of mosaic placentas should be higher than the incidence of mosaicism in fetuses because the embryo is derived from three or four cells of the mammalian blastocyst while the remaining cells give rise to the extra-embryonic membranes (Markert and Petters, 1978). A second hypothesis is that trisomic cells have a growth disadvantage in fetal cell lineages and selection of normal cells during embryologic development would ensue, while the abnormal cells are tolerated in the placenta (Gosden, 1993).

CPM has been described to exist in three different forms depending on the placental tissues involved (Kalousek 1990). Mosaicism confined to the trophoblast cell lineage of the placenta is designated as Type I CPM. Certain autosomal trisomies such as those for chromosomes 3, 7, 11, 13, 16 and 18 are most common in Type I CPM. Rare trisomies include 5, 10, 14, 17 and 19, while no cases of chromosomes 1, 4 and 6 have been described. For sex chromosome aneuploidies, monosomy for chromosome X in a complete or mosaic form in the placenta is most common. (Godsen et al, 1995).

Aneuploid fetuses may also exhibit type I CPM. Kalousek et al. (1989) have reported non-mosaic trisomy 13 or 18 in fetal tissues, amnion and placental stroma while a diploid cell line was present in the cytotrophoblast.

Type II CPM represents chromosomal mosaicism confined to the chorionic stroma. The frequency of Type II CPM is similar to that reported for Type I, although the frequency of specific chromosomal trisomies involved differ (Godsen et al. 1995). The most commonly observed trisomies include chromosomes 2, 7, 8, 9, 12, 16, 18 and 21. Trisomies 3, 5, 10, 11, 13, 14, 15, 17, 20 and 22 are rare while 1, 4, 6 and 19 have not been reported. Sex chromosome aneuploidy mainly involves monosomy of the X chromosome (ibid.).

Mosaicism involving both the trophoblast and stroma of the placenta has been designated as Type III CPM. This is the least common form of CPM and usually involves trisomies for chromosomes 7, 15,16 and 22. Aneuploidy for chromosomes 2, 9, 12, 18, 20 and 21 are occasionally observed and trisomies for the remaining chromosomes have not been reported (Godsen et al., 1995).

1.4 Clinical Consequences of CPM

The possibility of poor pregnancy outcome as a result of chromosomal mosaicism confined to the placenta was first suggested by Kalousek and Dill (1983) when they found CPM in two out of nine pregnancies with unexplained IUGR. These

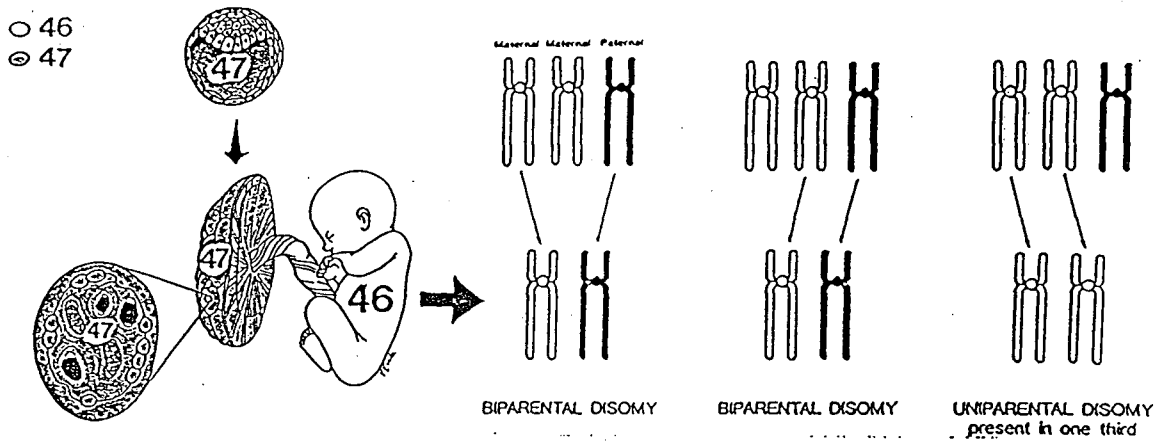
findings have been further supported by Kalousek et al. (1991) when they reported 6 of 34 term pregnancies diagnosed with CPM at 9 to 12 weeks gestation, with fetuses small for gestational age. A number of reports have followed in which a positive correlation of CPM and fetal manifestations including intrauterine death of chromosomally normal fetuses or IUGR have been found (Wolstenholme et al., 1994). Johnson et al. (1990) evaluated 4319 pregnancies by both direct and long-term culture CVS techniques and found that 16.7% of pregnancies diagnosed with CPM ended in fetal loss versus 2.7% of the non-CPM group. These findings have been further supported by Breed et al. (1991) who found a similar rate of 16.6% of pregnancy loss in the CPM group. Additional evidence suggesting that CPM may affect perinatal outcome was reported by Kalousek et al. (1989). They found intrauterine survival of chromosomally abnormal fetuses with trisomy 18 or 13 may be enhanced by the presence of a normal cell line confined to the trophoblast of the placenta. However, other studies suggest that the effect of CPM is minimal or non-existent (Schwinger et al., 1989). Many of these studies attempt to correlate the pregnancy outcome with the prenatal CVS result. However, since the anueploid cell line in the placenta does not persist to term in all pregnancies diagnosed with CPM prenatally, the outcome of the pregnancy should be compared to the extent of chromosomal mosaicism in the term placenta (Henderson et al., 1996).

The effect of CPM may depend on the type of chromosomal abnormality, the placental tissues affected and the percentage of aneuploid cells in the placenta. For example, CPM for trisomy 16 is most often found as type III and is more frequently associated with pregnancy complications than trisomies involving other chromosomes (Kalousek and Barrett, 1994). CPM type I seems to be associated with IUGR and spontaneous abortion (Johnson et al., 1991) while the effect of CPM II has not been clearly defined (Kalousek et al., 1987; Robinson et al., 1997). Further, Robinson et al. (1997) have found that the level of trisomy in the term trophoblast was higher when the origin of the extra chromosome was meiotic as compared to cases in which the trisomy was a result of a mitotic error. Also, a meiotic origin of trisomy was correlated with a high frequency of IUGR.

1.5 Confined Placental Mosaicism and Uniparental Disomy

The occurrence of a diploid fetus/newborn with a high level or non-mosaic complete trisomy in both placental lineages most likely results from a trisomic zygote rescue (Kalousek and Vekemans, 1996). In such cases it is thought that a meiotic non-disjunction event gives rise to a trisomic zygote. Loss of one chromosome from a postzygotic mitotic error results in the formation of a diploid cell line (Figure 1.1).

Figure 1.1: Illustration of a trisomic zygote rescue. CPM type III usually results from meiotic nondisjunction followed by a post-zygotic loss the extra chromosome in an embryonic progenitor cell. Depending on which chromosome is lost, the diploid fetus may be affected with uniparental disomy. (From Kalousek, 1994)



When the trisomic chromosome is lost in the trophoblast, Type I CPM with a non-mosaic aneuploid fetus results (Kalousek et al., 1989). When the trisomic chromosome is lost in the embryonic progenitor cell, a diploid fetus with Type III CPM develops. Theoretically, in one third of these cases, the consequence of the mitotic error will be uniparental disomy (UPD) in which both chromosomes of a pair originate from the same parent (Hall, 1990).

1.6 Clinical implications of UPD

When considering the phenotypic consequences of UPD in the fetus and the placenta several different factors must be taken into account: (1) the effect due to imprinted gene(s); (2) metabolic effects due to a mutated allele being present in homozygous form; and in the case of fetal UPD (3) the effect of placental trisomy on the placental function during intrauterine development.

Genomic imprinting refers to the differential genetic marking of genes which is dependent on parental origin. A number of loci in the mammalian genome are only expressed from one chromosome and are thus said to maintain a "parental imprint". The role of imprinted genes in development was demonstrated by Surani et al. (1984) and Barton et al. (1984) by the production of androgenic and gynogenic mice. Androgenic mice were constructed by removing the female pronucleus from a zygote and replacing

it with a male pronucleus. The embryo contained a diploid set of paternally derived chromosomes and gave rise to predominantly extra-embryonic placental tissues with severely stunted embryos. For gynogenic gestations which only contained maternally derived chromosomes, development of the embryo proper was normal to early somite stages and died *in utero* with unusually small extraembryonic placental tissue. These studies suggest that certain genes required for placental development are expressed from the paternally derived chromosomes while those on the maternal homologues are inactive. Conversely, early development of the embryo proper is dependent on genes preferentially expressed from the maternal homologues.

Two extensively studied examples of human disorders caused by uniparental disomy of imprinted genes are Prader-Willi syndrome (PWS) and Angelman syndrome (AS). Individuals affected with PWS have mental retardation, hypophagia, obesity and are hypoactive while AS is characterized by mental retardation, hyperactivity, seizures and a happy puppet-like appearance with bouts of inappropriate laughter (Nicholls, 1993). The role of genomic imprinting in the pathogenesis of these syndromes was suggested when a deletion in the 15q11-13 region of the paternal allele was described in patients with PWS while a deletion on the maternal chromosome in the same region was observed in patients affected with AS (Knoll et al., 1989; Williams et al., 1990). Further evidence for a role of

genomic imprinting comes from the observation that UPD for chromosome 15, in which both homologues are of maternal origin causes PWS while paternal UPD for chromosome 15 leads to AS (Nicholls et al., 1989; Malcolm et al., 1991). Also, PWS has been reported in cases of CPM 15 with maternal UPD for this chromosome pair, suggesting that this arose due to a trisomic zygote rescue (Purvis-Smith et al., 1992; Morichon-Delvallez et al., 1993).

Depending on the mutational event involved in the formation of UPD, an individual may have heterodisomy in which the uniparental pair is made up of two homologous chromosomes, or isodisomy in which the pair contains duplicates of the same chromosome. The latter case provides a genetic mechanism in which an autosomal recessive disease cqn be inherited from a single carrier parent. For example, UPD 7 has been associated with cystic fibrosis (Voss et al., 1989) and Bloom syndrome has been associated with UPD 15 (Woodage et al., 1994). Presumably, the affected child has acquired two copies of a single chromosome from one parent who is a carrier of the mutant gene.

UPD of chromosomes other than 15 has been described in which trisomy mosaicism was present in the placenta(Langlois et al., 1995; Kalousek et al., 1993). In such cases, the phenotypic effects of UPD may be due to imprinting effects or the functional insufficiency of the trisomic placenta or both. The best studied example of this is CPM 16 which most often arises by a nondisjunction event in meiosis followed

by trisomic zygote rescue. Kalousek et al. (1993) and Bennett et al (1995) have reported a high incidence of IUGR and intra-uterine death in CPM 16. It has been found that a high level of trisomy 16 in the placenta rather than UPD 16 is associated with IUGR (Kalousek and Vekemans, 1996). IUGR is found in cases with biparental inheritance and high levels of trisomy 16 in the placenta, while normal birth weight has been recorded in a patient with UPD 16 and low levels of trisomy 16 in the placenta (Kalousek and Barrett, 1994).

1.7 Human Embryology

1.7.a. Cleavage and Blastocyst Formation

To understand the concept of confined mosaicism, the differentiation of embryonic and extraembryonic tissues in early human embryology must be considered. Human development begins with the union of the spermatozoon and the oocyte to give rise to the zygote. The act of fertilization releases the ovum from a depressed metabolic state and the zygote begins a series of mitotic divisions. The resulting cells are known as blastomeres and become smaller with each cell division. At the eight cell stage, the loosely arranged clump of cells undergoes a process of compaction in which they maximize their contact with each other and form tight junctions. At approximately three days

after fertilization the embryo undergoes the fourth cleavage to form the 16 cell morula. The blastomeres at this stage acquire their identity as a result of their position in the morula. The outer surrounding cells will develop into the outer cell mass while the inner cells of the morula will constitute the inner cell mass. A stage of cavitation follows in which the blastocyst forms a fluid filled cavity referred to as the blastocele. The inner cell mass is located at one pole and will eventually give rise to the embryo/fetus proper as well as the extraembryonic structures including the amnion, yolk sac and stroma of the chorion. The outer cell mass flattens to form the epithelial wall of the blastocyst known as the trophoblast, which will eventually form the cytotrophoblast and syncytiotrophoblast of the placenta (Sadler, 1995; Carlson, 1994).

1.7.b. Formation of the Embryonic Cell Lineages

At eight days development the cells of the inner cell mass differentiate into two layers: 1) the epiblast, a layer of high columnar cells; and 2) the hypoblast, a layer of small cuboidal cells adjacent to the blastocele. Together these layers form the bilaminar disk (Carlson, 1994).

The epiblast contributes cells to the formation of embryonic as well as extraembryonic tissues. The first tissue observed to derive from the epiblast at eight days is the amnion, which ultimately encloses the embryo proper in a

fluid filled sac called the amniotic cavity. The extraembryonic mesoderm has been described to arise from the caudal region of the epiblast (Luckett, 1978). However the origin of the extraembryonic mesoderm in the human is controversial since Enders and King (1987) described development of the extraembryonic mesoderm from the hypblast. It now seems that both the epiblast and hypoblast contribute to the formation of the extraembryonic mesoderm (Carlson, 1994).

The embryonic ectoderm is derived directly from the epiblast cells while the embryonic mesoderm and endoderm are formed through a process of cell migration called gastrulation. Gastrulation begins in the third week of development when the primitive streak appears as a short linear depression on the dorsal surface of the epiblast. Cells of the epiblast migrate in the direction of the primitive streak and slip beneath it through a process of invagination to form the embryonic mesoderm and endoderm (ibid.).

1.7.c. Formation of the Yolk Sac

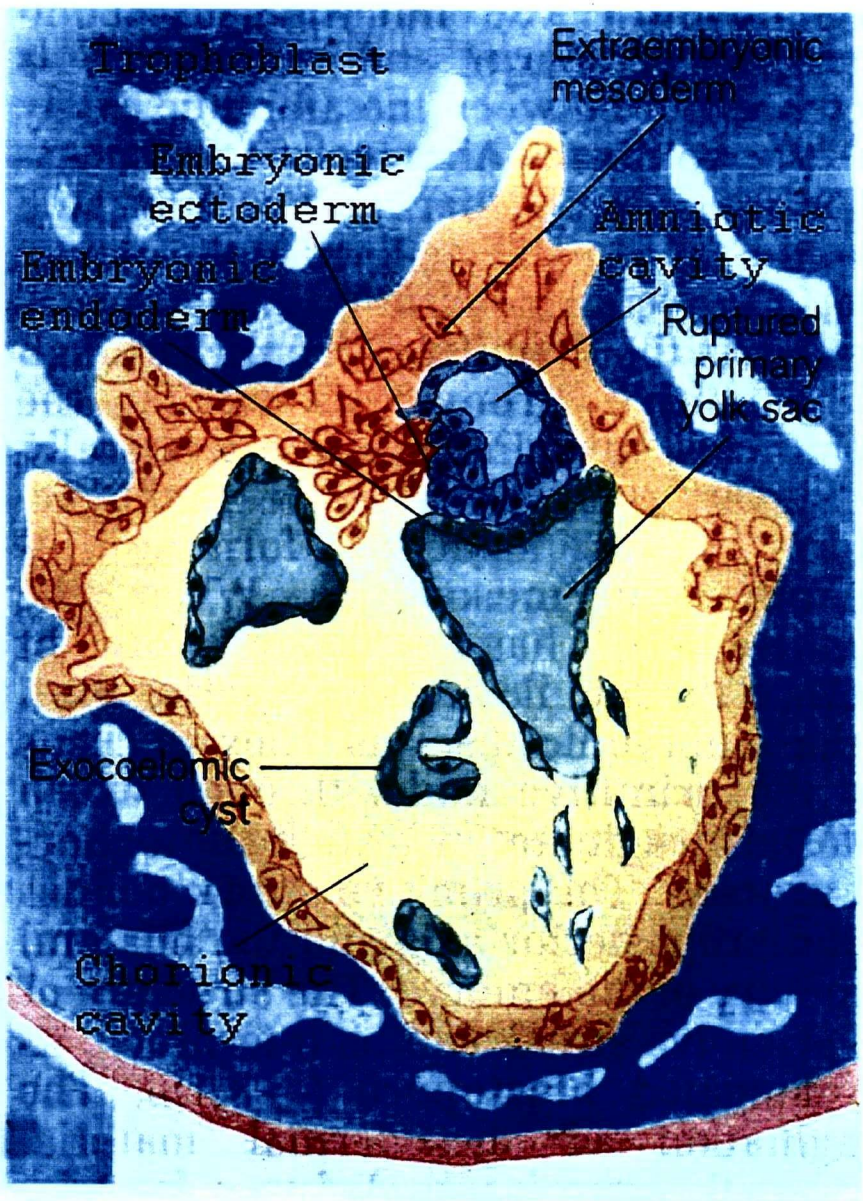
It is generally accepted that flattened cells originating from the hypoblast and lining the inner surface of the cytotrophoblast form the primary yolk sac during the ninth day of development (Luckett, 1978). In the thirteen day old embryo, the multipotent extraembryonic mesoderm

surrounds the primary yolk sac and amnion. This is followed by the rupture of the primary yolk sac at 14 days; repair then occurs to form a smaller secondary yolk sac (Luckett, 1978) (Figure 1.2).

1.7.d. Placental Development and Hematopoiesis

The placenta consists of chorion, amnion and the umbilical cord. The embryologic origin of the chorion can be traced to two cell lineages: 1) the trophoblast and 2) the extraembryonic mesoderm. The cytotrophoblast and syncytiotrophoblast which begin to invade the uterine wall at eight days postfertilization are derived from the trophoblast cell lineage (Carlson, 1994). The extraembryonic mesoderm contributes to the formation of the villus stroma, chorionic plate, the mesoderm of the umbilical cord, secondary yolk sac and amnion. During the umbilical cord, the amnion becomes constricted around the body stalk to form a cord with two veins and one artery, which connect the embryonic circulation with the placenta (Drews, 1995).

Figure 1.2: Rupture of the primary yolk sac to form the secondary yolk sac at 14 days of development. Notice the multipotential extraembryonic mesoderm surrounding the ruptured yolk sac. Note: embryonic ectoderm = epiblast; embryonic endoderm = hypoblast; trophoblast is blue surrounding the extraembryonic mesoderm (Modified from Drews, 1995)



At the third week of development the mesoderm of the villi and the secondary yolk sac begin to form blood vessels which will become continuous with the intraembryonic circulatory system. At the same stage of development blood islands begin to form in the mesoderm of the yolk sac which is the sight of embryonic hematopoiesis up to the sixth week of development (Drews, 1995). Hematopoiesis is then taken over by the liver followed by the spleen and bone marrow. It has been generally accepted that the primitive blood cells from the secondary yolk sac serve as progenitors for all hematopoiesis in later life (Schwartz et al., 1991).

However, recent evidence from studies in the mouse suggest that hematopoietic contribution from the yolk sac progenitor cells is transient and definitive hematopoiesis is autonomously initiated in the para-aortic splanchnopleura, also known as the aorta-gonad-mesonephrous (AGM) region. Dickson et al. (1995) reported that TGFb1 mutant mouse embryos, which die as a result a developmental deficiency of endothelial differentiation and hemopoiesis in the yolk sac, appeared to have erythrocytes in the para-aortic regions within the embryos. Medvinsky and Dzierzak (1996) demonstrated that the AGM region exclusively initiates long-term repopulating hematopoietic stem cells (LTR-HSC) independently from the yolk sac. Cultured0tissues from the yolk sac, liver and AGM region at 10 days gestation were transplanted into lethally irradiated mouse recipients, and only the AGM was able to contribute to the recipient

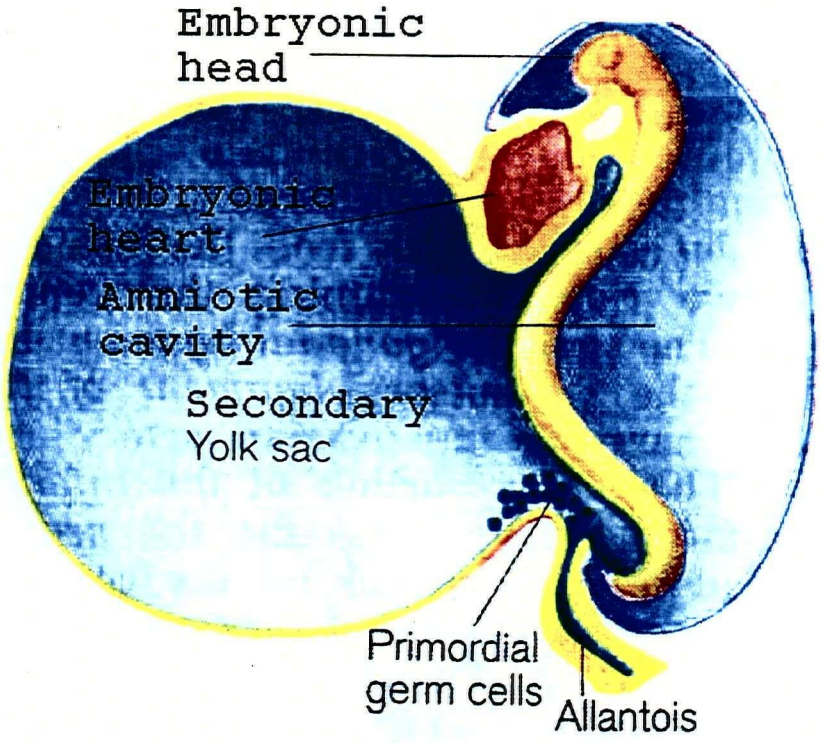
definitive circulation. Also, Muller et al. (1994) have identified hematopoietic stem cells in the AGM before they appear in the fetal liver, suggesting that the AGM is the major contributor of progenitor cells to the liver for fetal hematopoiesys in the mouse.

It is important to realize that although the mouse has been used extensively as a model for early human hematopoiesis and other aspects of embryological development, these results must be taken with caution. The embryological events leading to the establishment of the definitive hematopoietic system in the human are not well understood. However, Huyhn et al. (1995) have shown that progenitor blood cells are present in extrahepatic embryonic tissue at 30 to 35 days of development, before hematopoiesis predominates in the human fetal liver. These observations are in agreement with those from the mouse which suggest that progenitor blood cells found in the liver originate from extrahepatic embryonic tissues such as the AGM region.

1.7.e. Primordial Germ Cells

The first identifiable primordial germ cells (PGCs) are observed in the secondary yolk sac endoderm at four weeks of development in the human (Figure 1.3) (Fujimoto et al., 1977).

Figure 1.3: Illustration of the human embryo at four weeks development. The primordial germ cells are first identified in the endoderm of the secondary yolk sac. (Modified from Drews, 1995)



The PGCs then separate from the endoderm and migrate by amoeboid movements into the developing embryo. They arrive at the primitive gonads at the fifth week and begin to invade the genital ridges at the sixth week of development. The genital ridges consist of a population of cells derived from the coelomic epithelium and cells from the mesonephric ridge (Carlson, 1994). Early in the sixth week the primitive sex cords grow into the genital ridge from the mesonephrous, and approximately 1000 to 2000 PGCs migrate into sex cords. There are no distinguishable male or female characteristics of the gonad at this stage, hence it is referred to as the indifferent gonad. In the absence of testis differentiation signals, the gonad undergoes ovarian development. In the ovary PGCs are known as oogonia. They undergo a series of mitotic divisions while the surface epithelium of the ovary gives rise to another population of cells called follicular cells. The number of germ cells in each ovary reaches approximately 7 000 000 at eighteen weeks of development. Most of these cells die by apoptosis, and by the seventh month only 400 000 viable oocytes remain (Sadler, 1995).

The first meiotic figures in the female gonad have been reported as early as eight weeks of development (Baker, 1963). Once meiosis is initiated the oocytes enter into prophase I, which is characterized by five stages. The first stage is known as leptotene, in which the chromatin begins to condense and chromosomes begin to take form. This is

followed by the initiation of pairing between homologous chromosomes in the zygotene stage and the completion of pairing and formation of synaptonemal complexes (SC) during pachytene. In diplotene, the lateral elements of the SC's begin to separate and the oocytes then arrest in dictyotene when the lateral elements disperse forming a fine background of chromatin threads (Speed, 1985). The oocytes remain in distyotene until ovulation, when meiosis I completed and meiosis II occurs, resulting in the formation of haploid gametes.

In the male embryo, the germ cells are surrounded by the developing sex chords which eventually give rise to the seminiferous tubules of the testis. Within the tubules the PGCs proliferate to form the spermatogonia which are accompanied by Sertoli cells derived from the surface epithelium of the gonad. The Leydig cells derived from the original mesenchyme of the gonadal ridge are first recognizable in the interstitium surrounding the tubules at 6 weeks development (Trainer, 1987). Unlike oogonia, the spermatogonia due not enter into meiosis until puberty.

1.8 Gonadal Mosaicism

1.8.a. Genetic Consequences of Gonadal Mosaicism

Germline mosaicism has been suggested in families where phenotypically normal parents have more than one child affected with a dominantly inherited disorder (Hall, 1988). These disorders include osteogenesis imperfecta (Byers et al., 1988), Duchenne muscular dystrophy (Edwards, 1986), neurofibromatosis type I (Ricardi and Lewis, 1988)and incontinetia pigmenti (Kirchman et al., 1995). Molecular analysis for the presence of mutations in the affected children and phenotypically normal parents suggests that the mutation may be confined to the germline or it may be found in somatic as well as germ cells. Raghunath et al. (1995) described a family in which two children severely affected with osteogenesis imperfecta carried the mutation for which the mildly affected father was mosaic in blood, saliva, hair root bulb cells and fibroblasts. Conversely, Prior et al. (1992) detected a deletion in the DMD gene in two out of three siblings but not in the leukocytesof the mother who theoretically should be the carrier.

It has been suggested that the apparent confined germline mosaicism reported in several families is a result of an early mutation in the progenitor cells of the gametes

but not somatic cells (Hall, 1988). Another scenario is that the mutation occurs before the allocation of somatic and germ cells, accounting for cases in which the parent is mosaic in both cell lineages. Further, apparent confined germline mosaicism may be due to the inability of somatic cells to tolerate the mutant cells. Also, since most studies only examine blood and/or skin of mosaics, it is possible that the mutation is present in other somatic tissues which have not been sampled (ibid.).

1.8.b. Germline Chromosomal Mosaicism

The occurrence of individuals with germline chromosomal mosaicism has been scarcely reported in the literature. There have been reports of recurrence of trisomy 21 in families with phenotypically normal parents. Given that the expected frequency of trisomy 21 is 1 in 600 to 800 livebirths (McKusik, 1994) there has been speculation that families with multiple affected pregnancies either have genetic and/or environmental predisposing factors to nondisjunction or have germline chromosomal mosaicism (Pangalos et al., 1992). Cytogenetic analysis of skin fibroblasts and/or blood leukocytes has shown parental mosaicism in some cases (Harris et al., 1982; Uchida and Freeman (1985); Neilson et al., 1988; Pangalos et al., 1992). A comprehensive analysis of multiple somatic tissues

and gametes however is lacking in the literature due to the difficulty of obtaining multi-lineage tissue samples.

1.9 Aims of the Study

The purpose of this study is to explore the possibility that gestations diagnosed with type II and type III confined placental mosaicism will also exhibit gonadal mosaicism. The embryologic origin of the PGCs in the human is not fully understood. They are first observed in an extraembryonic location in the endoderm of the secondary yolk sac (Fujimoto et al., 1977); however it is not known whether they are direct descendants of the primary yolk sac endoderm (i.e. hypoblast) or another cell lineage. Clonal analysis of epiblast cells in the mouse have shown that the same clones which give rise to the primordial germ cells also contribute to the extraembryonic mesoderm (Lawson and Hage, 1994). This suggests that the extraembryonic mesoderm and the PGCs have a common embryologic origin in mammals.

In the developing human, the primary yolk sac ruptures at 14 days of development and at the same time is surrounded by multipotent extraembryonic mesoderm. We propose that the extraembryonic mesoderm is involved in the repair of the primary yolk sac to form the secondary yolk sac and in doing so, contributes to the formation of the PGCs. Thus we suggest that conceptuses with an aneuploid cell line in the

stroma of the placenta may also exhibit aneuploidy in the oogonia/spermatogonia.

A comprehensive analysis using fluorescence *in situ* hybridization was undertaken in spontaneously or therapeutically terminated pregnancies diagnosed with CPM, to test for chromosomal mosaicism in placental and fetal tissues, including the germ cells. When possible, the analysis was performed on the trophoblast and stromal lineages of the placenta separately, as well as the brain, lung, kidney, cord blood and germ cells. This study may lead to a greater understanding of the developmental aspects of CPM and the embryological origin of the PGCs. It may also provide insight into human fertility in the reproductive period in individuals whose placentas exhibited chromosomal mosaicism.

2. Materials and Methods

2.1 Source of Specimens

The three mosaic gestations included in this study were collected for analysis on the basis of diagnosed CPM types II or III after conventional cytogenetic analysis of placental and/or fetal tissues. The mosaic gestations for cases 1 and 3 were formalin fixed and paraffin embedded after pregnancy termination in 1993 and 1991 respectively. Tissue sections of cases 1 and 3, as well as all formalin fixed - paraffin embedded control samples were obtained from the B.C. Children's Surgical Pathology and Embryofetopathology Laboratories. Fresh tissues for Case 2 were obtained from Dr. David Bick at the Genetics and IVF Institute, Fairfax, VA. All fresh tissues used for control samples were obtained from the B.C. Children's Hospital Embryofetopathology Laboratory.

The gestational age of mosaic conceptuses and control samples is defined as the period of time elapsed since the date of the last menstrual period and is two weeks greater than the developmental age (i.e. if developmental age is 9 weeks than gestational age is 11 weeks). When possible, the gestational age was determined from information provided by the mother. If information of gestational age was not provided by the mother, it was calculated as two weeks greater than the developmental age. The developmental age

was established by measurements of fetal hand and foot lengths.

2.2 Sampled Tissues

2.2.a. Tissues Sampled for Cytogenetic Analysis

For formalin fixed - paraffin embedded specimens, tissue sections from the chorionic villi, kidney, lung and gonad were sampled for FISH analysis. The brain, kidney, lung, chorionic villi, cord blood and gonad were sampled for fresh tissues analyzed by FISH analysis. Disomic controls were used to establish the minimum proportion of nuclei with three signals required for a diagnosis of trisomy. Cut-off values were calculated separately from disomic controls for each formalin fixed - paraffin embedded and fresh tissue analyzed.

Conventional cytogenetic analysis of metaphases using trypsin G-banding was performed on the amnion and chorionic plate for case 1; amnion, chorionic plate and villus stroma for case 2; and the amniotic fluid, amnion, skin, blood, kidney, spleen, cytotrophoblast, and villus stroma for case 3.

2.2.b. Tissues Sampled for Microsatellite Analysis

Molecular analysis of amnion, villus stroma, cytotrophoblast, lung, adrenal gland and parental blood of case 2 was performed in the Medical Genetics Laboratory of Dr. Wendy Robinson in the B.C. Research Institute for Child and Family Health. Molecular analysis of fetal and parental blood was performed on case 3 and published by Kalousek et al. (1993).

2.3 FISH analysis of Fresh Tissues

2.3.a. Preparation of Tissues for FISH

i. Enzymatically Digested Placental Tissues

Fresh chorionic villi were separated into trophoblast and stromal tissues via enzymatic digestion. Samples of 30 to 50 mg of chorionic villi were washed in transport medium which consisted of minimum essential medium (Gibco BRL, Grand Island, NY) and 4% (v/v) of antibiotic-antimyocotic solution (Gibco BRL, Grand Island, NY) to remove contaminating blood. Maternal decidua was identified using a Nikon (Japan) dissecting microscope with 30X magnification and removed from the villi with forceps. The villi were then digested with 1 ml of collagenase type 1A (1mg/ml; Sigma Chemical Company, St. Louis, MO) for 10 to 20 min.

The solution was vigorously agitated and allowed to sit for 2 min, and the cell suspension of trophoblast was placed in a 15 ml polystyrene conical centrifuge tube (Falcon, Fair Lawn, NJ, USA). The cell suspension was centrifuged at 1000 rpm for 5 min, and the supernatant was removed. The pellet was resuspended in 1% (w/v) sodium citrate solution (Fisher Scientific, Fair Lawn, NJ) prewarmed to 37°C and allowed to sit for 20 min at room temperature. Then the cell suspension was centrifuged at 1000 rpm for 5 min, and the pellet was fixed in 3:1 (v/v) methanol (Baker, Phillipsburg, NJ)/glacial acetic acid (BDH, Toronto, ON). The fixed cells were washed in 3:1 methanol/ glacial acetic acid twice and a cell suspension was made for slide preparation. The trophoblast cell suspension was then dropped on twin frosted microscope slides (FGR Steinmetz Inc., Surrey, BC) cleaned with methanol. Slide preparations were air dried and then dehydrated in ethanol series (70%, 80%, 90% and 100% for two min each). Enzyme pretreatments were carried out with 0.000075% (w/v) trypsin (DIFCO BACTO, Detroit, MI) for 2 to 4 sec, washed in phosphate buffered saline solution (PBS) (GIBCO BRL, Grand Island, NY) twice for 5 min and treated with 10% formalin (Fisher Scientific, Fair Lawn, NJ) for 10 min. They were then washed twice in PBS for 5 min and placed in 50% formamide (Fisher Scientific, Fair Lawn, NJ) in 2X SSC for 15 min at room temperature. The 2X SSC consists of 0.15 M sodium chloride and 0.015 M sodium

citrate solution with a pH of 7 (Fisher Scientific, Fair Lawn, NJ).

The remaining villus stromal material from the collagenase digested villi was washed twice in PBS and placed in -70°C freezer for at least 2 h. Then it was minced with two scalpel blades, placed in a 15 ml centrifuge tube and washed with 0.9% sodium chloride (w/v), pH 1.5. The digestion with 0.125% (w/v) pepsin (Sigma Chemical Company, St. Louis, MO) in 0.9% sodium chloride solution, pH 1.5 at 37°C for 10 to 15 min followed. The solution was then agitated vigorously, allowed to stand for 2 min and the supernatant was placed in a new 15 ml centrifuge tube. This was then washed twice with PBS and the cell suspension was dropped on silanized precleaned slides (Fisherbrand Superfrost/Plus, Fisher Scientific, Fair Lawn, NJ) and allowed to air dry overnight. Microscope slides were then placed in 10% formalin for 2.5 h, washed in PBS twice for 5 min and dehydrated in ethanol series.

ii. Touch Preparations

Fetal lung and kidney were washed in transport medium and sliced in half. The freshly sliced tissue was touched onto precleaned silanized microscope slides and allowed to air dry. Blood dropped out of the umbilical cord onto the end of a precleaned silanized microscope slide was smeared across the slide with the end of a second slide and air

dried. Fetal brain tissue was smeared across precleaned silanized microscope slides and allowed to air dry. All touch preparations were then placed in 100% methanol for 5 min and allowed to air dry. They were then placed in 3:1 methanol/glacial acetic acid for 5 min, air dried and dehydrated in ethanol series. Slide preparations were treated with 0.00005% trypsin (w/v) for 2 to 4 sec, washed in PBS twice for 5 min and placed in 10% formalin for 10 min. This was followed by two PBS washes for 5 min and incubation in 50% formamide in 2X SSC for 15 min at room temperature prior to DNA denaturation for FISH.

iii. Squash Preparation of Oocytes

The fetal ovary was cut in 4 to 6 pieces and each piece was placed in a drop of PBS on precleaned silanized microscope slides. A second slide was placed on top of the tissue which was then squashed. The second slide was carefully removed and the oocytes were allowed to air dry on the bottom slide. The slide preparations were placed in 100% methanol for 5 min, air dried, followed by 3:1 methanol/glacial acetic acid for 5 min and air dried. The slides were then dehydrated in ethanol series. Enzyme pretreatments were carried out with 0.00075% trypsin (w/v) for 4 to 8 sec, followed by washes in PBS twice for 5 min and treatment with 10% formalin for 10 min. They were then washed twice in PBS for 5 min and placed in 50% formamide in

2X SSC for 15 min at room temperature prior to the DNA denaturation for FISH.

2.3.b. Single Colour FISH

One colour FISH was performed on all fresh tissues except oocytes. The slide preparations were denatured in 70% formamide in 2X SSC at 70°C for 5 min. The slides were then dehydrated in -20°C prechilled ethanol series and allowed to air dry. A hybridization mixture of 15 µl Hybrizol VI (Oncor, Gathersburg, MD) + 0.75 µl digoxigenin labeled centromeric DNA probe mixture (Oncor, Gaithersburg, MD) was denatured at 70°C for 5 min and then placed on ice. The hybridization mixture was applied to each slide, covered with a 22 X 22 glass coverslip, sealed with rubber cement and incubated overnight at 37°C. On the next day, the rubber cement was removed, and microscope slides were placed in 2X SSC until the coverslips could be easily removed. Post-hybridization washes consisted of 65% formamide in 2X SSC at 43°C for 15 min followed by two washes in 2X SSC at 37°C for 8 min each. The slides were then washed three times for 2 min in phosphate buffered detergent (PBD) which consisted of 0.1 M sodium phosphate dibasic anhydrous (Fisher Scientific, Fair Lawn, NJ), 0.06 M sodium phosphate monobasic (Fisher Scientific, Fair Lawn, NJ) and 0.1% Triton X-100 (v/v) (Sigma, St. Louis, MO).

Immunodetection of hybridized probe was carried out with a digoxigenin antibody system. All antibodies were dissolved in PMN solution which consists of PBD with 0.048% instant skim milk powder (w/v) (Nestle, Don Mills, ON) and 0.02% sodium azide (Sigma, St. Louis, MO). 60 µl of 0.4 µg/ml anti-digoxigenin (sheep) IgG in (Boeringer Mannheim, Laval, QE) was placed on the microscope slides, covered with plastic coverslips and incubated for 15 min at 37°C. The slides were then washed in PBD three times for 2 min and incubated with 60 µl of 2 µg/ml rabbit anti-sheep IgG (Pierce, Rockford, IL) for 15 min at 37°C. This was followed by three PBD washes and incubation with 60 µl of 20 µg/ml anti-rabbit IgG fluorescine-isothiocyanate (FITC). The slides were then washed three times in PBD, and the nuclei were counterstained with 2 µg/ml propidium iodide (Sigma Chemical Company, St. Louis, MO) in vectashield mounting medium (Vector Laboratories, Burlingame, CA). If needed, the fluorescent signals were amplified by repeating the last two incubations of the antibody treatments.

The nuclei were analyzed under a Nikon (Japan) fluorescence microscope at 600X magnification. For each sample approximately 500 nuclei were scored, and the proportion of nuclei displaying one, two, three and four or more signals were recorded. Interphase nuclei with disrupted or overlapping nuclear membranes were not scored.

2.3.c Two-Colour FISH

Two colour FISH was utilized in order to visualize two different chromosome specific centromeric DNA probes per nucleus. One probe was specific for the chromosome of interest, and the second probe was used as an internal control. In order to select oocytes with unpaired chromosomes, only oocytes with two hybridization signals for the internal control were scored for the test probe.

The slide preparations were denatured as described in section 2.3.b. The hybridization solution contained 1.5 µl of digoxigenin labeled centromeric DNA probe mixture + 1.5 µl of biotin labeled centromeric DNA probe mixture(Oncor, Gathersburg, MD) and 27 µl of Hybrizol VI. The hybridization solution was denatured at 70°C, applied to the slide preparation, covered with a 22 X 60 mm coverslip, sealed with rubber cement and incubated overnight at 37°C.

The post-hybridization wash was carried out as described in section 2.4.b., and slides were washed three times in PBD for 2 min. All antibodies applied to the slide preparation were dissolved in PMN. A mixture of 30 µl of 5 µg/ml FITC-avidin (Vector Laboratories, Burlingame, CA) and 30 µl of 1 µg/ml mouse anti-digoxigenin (Sigma Laboratory, St. Louis, MO) per slide was chilled on ice for 5 min. This was applied to each slide and incubated at 37°C for 15 min. The slides were washed three times in PBD and incubated with 60 µl of 2 µg/ml anti-mouse Ig-digoxigenin, $F(ab)_2$ fragment

(Boehringer Mannheim, Laval, QE), per slide for 15 min followed by 3 more PBD washes. The next antibody applied to each slide was 60 µl of anti-dig-rhodamine, Fab fragment (Boehringer Mannheim, Laval, QE) which was incubated for 15 min at 37°C followed by three PBD washes. Then 60 µl per slide of 5 µg/ml biotinylated anti-avidin D (Vector Laboratories, Berlingame, CA) was applied and incubated for 15 min. The slides were washed in PBD and incubated with 60 µl of 5 µg/ml FITC-avidin (Vector Laboratories, Berlingame, CA) for 15 min and washed with PBD as before. The nuclei where then counterstained with 0.04 µg/ml 4,6-diamino-2-phenylindole dihydrochloride hydrate (DAPI) (Sigma Chemical Companies, St. Louis, MO) in 2X SSC for 4 min. The slides were washed three times in PBD for 5 min, and 22 X 60 mm coverslips were mounted with vectashield mounting medium.

Approximately 100 to 500 oocytes were analyzed per sample with a Zeiss (Germany) fluorescent microscope. The proportion of oocytes with one, two, three or four or more hybridization signals for the test probe were recorded.

2.3.d. Statistical Analysis

FISH analysis was performed on disomic controls for each tissue tested, and cut-off values for detection of trisomy were calculated as described by Lomax et al. (1994). To determine the minimum proportion of cells with three

hybridization signals representing a trisomic cell line, an upper reference limit (L) was calculated as follows:

$L = x + t_{(\alpha,n-1)} s \sqrt{(1 + 1/n)}$, where x was the mean proportion of nuclei with three hybridization signals in the disomic control samples, s was the standard deviation and n was the number of specimens studied. The 95% confidence interval (CI) of this upper reference limit was calculated using the following equation:

$$CI = L \quad 1.96 \sqrt{[1 + (t^2_{(\alpha,n-1)}(1+1/n)/2]s^2/n}$$

The upper reference 95% confidence limit was designated as the cut - off value which represents the minimum proportion of nuclei required to diagnose trisomy in a specific tissue.

2.4 FISH analysis of Formalin Fixed - Paraffin Embedded Tissues.

50 μm sections from tissue blocks were cut into narrow slivers and placed into 15 ml polypropylene conical centrifuge tubes (Fisher Scientific, Pittsburgh, PA). The tissues were dewaxed with 10 ml xylene (Fisher Scientific, Fair Lawn, NJ) for 10 min at room temperature. The xylene was removed, and fresh xylene was added a second time for 10 min. The xylene was removed, and the tissues were treated in 10 ml of 100% ethanol four times for 10 min each. the 100% ethanol was removed, and the tissues were rehydrated with 10 ml of 90%, 70% and 50% ethanol twice for 10 min each. The tissues were then washed in distilled water twice

and incubated in 10 ml Hanks balanced salt solution (HBSS) with Ca/Mg (GIBCO, Grand Island, NY) overnight at 4°C.

The HBSS was removed, and the tissues were washed with fresh HBSS twice. A nuclear suspension was then prepared by enzymatic digestion of the tissues. First, the tissues were incubated in 2 ml of collagenase type XI (1.25 mg/ml; Sigma Chemical Company, St. Louis, MO) for 1 h with vigorous agitation every 10 min. The solution was centrifuged at 1500 rpm for 5 min, and the supernatant was discarded and replaced with HBSS without Ca/Mg (Gibco BRL, Grand Island, NY). The tissues were washed in HBSS without Ca/Mg twice, and resuspended in 4 ml trypsin/EDTA (Gibco BRL, Grand Island, NY). This was incubated for 1 h at 37°C and examined under the Nikon (Japan) dissection microscope to determine the state of digestion. If the digestion was not complete, then the tissues were incubated in trypsin/EDTA for another 1 to 2 h. When large numbers of single cells could be seen in solution, the tubes were agitated vigorously, allowed to sit on the bench top for 3 min and the supernatant was decanted into another conical centrifuge tube. The supernatant was centrifuged, and the cells were resuspended in 4 to 10 drops of HBSS without Ca/Mg to make slides. The cell suspension was dropped onto precleaned silanized slides and baked overnight on a Labline Instruments (Melrose Park, IL) slide warmer at 37°C. The next day the slides were dehydrated in the ethanol series and allowed to air dry. They were then treated with 30%

sodium bisulfite (w/v)p(Aldrich Chemical Company, Milwaukee, WI) at 43°C, washed three times in 2X SSC for 1 min, and dehydrated in ethanol series. This was followed by incubation with proteinase K (250 µg/ml to 1 mg/ml; Sigma Chemical Company, St. Louis MI) at 37°C for 15 min to 1 h. The enzyme was washed off with 2X SSC three times for 1 min and the slides were then dehydrated in ethanol series. The most successful pretreatments for each tissue are summarized in Table I.

Table 2.1: Suggested pretreatments for formalin fixed - paraffin embedded tissues.

Tissue	30% Sodium Bisulfite at 43°C	Proteinase K at 37°C (concentration)
kidney, lung, placenta	15 to 30 min	15 to 30 min (250 µg/ml)
oocytes	30 min	15 min to 1 h (250 µg/ml to 1mg/ml)

The slides were then hybridized with a digoxigenin labeled centromeric DNA probe. The hybridization mixture consisted of 10 µl Hybrysol VIII (Oncor, Gaithersburg, MD) + 2 µl probe mixture and were applied to the slide. This was covered with a 22 X 22 mm glass coverslip, sealed with rubber cement and baked at 45°C until the cement was

completely dry. The slide preparation was placed in a 90°C Blue M oven (Blue Island, IL) for 12 min to denature the probe and genomic DNA. The slides were then hybridized overnight at 37°C.

The next day the coverslips were removed as described above in section 2.3.b. Post-hybridization washes consisted of a 50% formamide in 2X SSC wash at 37°C for 5 to 7 min, followed by a 0.1 X SSC wash at 37°C for 5 to 7 min. The immunodetection steps and evaluation of hybridization signals were performed as described in section 2.3.b. The placenta, lung and kidney slide preparations were counterstained with 2 μg/ml propidium iodide in vectashield mounting solution and analyzed using a Nikon fluorescent microscope at 600X magnification. The oocytes were counterstained with DAPI as described in section 2.3.c. and analyzed under a Zeiss fluorescent microscope at 1000X magnification. Approximately 100 to 500 nuclei were analyzed per sample, and the proportion of nuclei with one, two, three or four or more hybridization signals were recorded. Statistical analysis for detection of trisomy was performed as described in section 2.3.d.

2.5 Conventional Cytogenetic Analysis

Cytogenetic analysis of metaphases using trypsin G-banding was performed on fetal and placental tissues by standard cytogenetic methods (Rooney and Czepulkowski,

1992). Analysis of cultured amnion and chorion of case 1 was performed by the B.C. Children's Hospital Cytogenetic Laboratory. Conventional cytogenetic analysis of cultured amnion, villus stroma and chorionic plate for case 2 was performed by Irene Barrett in the Cytogenetic Laboratory of the B.C. Research Institute for Child and Family Health. Results from cytogenetic analysis of prenatal amniotic fluid culture, cytotrophoblast, villus stroma, amnion, skin, fetal blood, kidney, lung and spleen for case 3 were obtained from Kalousek et al. (1993).

2.6 Microsatellite Analysis

Molecular analysis of parental origin of chromosome 16 for case 2 was performed on parental blood and fetal tissues by Fabiana Bernasconi in the B.C. Research Institute for Child and Family Health. The highly polymorphic microsatellite markers, D16S398 and D16S423 of chromosome 16, were amplified using polymerase chain reaction and analyzed by gel electrophoresis as previously described by Robinson et al. (1995). For case 3, the polymorphic marker D16S83 of chromosome 16, was analyzed by restriction enzyme digestion and Southern blot analysis by standard techniques as described by Kalousek et al. (1993).

3 Results

3.1 Case 1

3.1.a. Patient History and Conventional Cytogenetic Analysis

This pregnancy was terminated at 21 weeks of gestation after a prenatal diagnosis of fetal anomalies. The mother was a 29 year old, Gravida 2, Para 1 and Aborta 1 woman with a healthy child from a previous pregnancy. Ultrasound showed a hyperechogenic bowel along with ventriculomegaly. Gross examination of damaged fetal parts after termination failed to reveal any developmental abnormalities. A normal male karyotype was found by cytogenetic analysis of cultured amnion subsequent to termination (Table 3.1). Two metaphases of cultured chorionic plate showed a male karyotype with trisomy of chromosome 7, while two metaphases were normal.

Table 3.1: Conventional Cytogenetic Analysis of Case 1.

Tissue	Number of Trisomic Cells/Total Number of Cells Analyzed
Chorionic Plate	2/4
Amnion	0/5

Note: Cytogenetic analysis was performed by the Cytogenetic Laboratory of the B.C. Children's Hospital.

3.1.b. FISH Analysis of Case 1

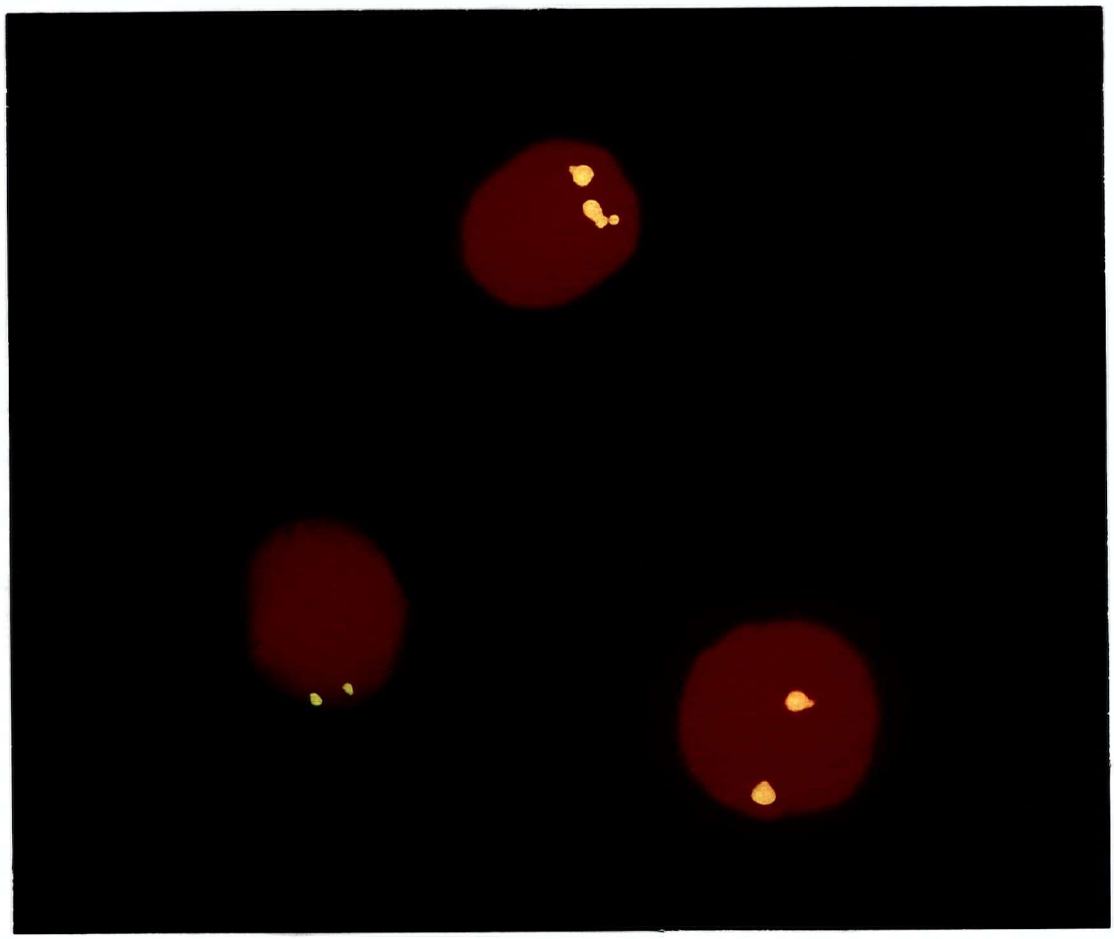
Results from the molecular cytogenetic analysis of formalin fixed - paraffin embedded fetal tissues are summarized in Table 3.2. The proportion of nuclei with three hybridization signals for the chromosome 7 specific probe in the lung and kidney is 7.2 and 2.2%, respectively. These are less than the cut-off values, 10.91 and 11.78%, established by the disomic control samples. FISH analysis of three sites from the placenta, which includes cells derived from both stroma and trophoblast, indicates mosaicism for trisomy 7. The proportion of cells with three signals representing chromosome 7 are significantly greater than the mean of disomic control samples with 95% confidence (cut - off value = 7.89%). The level of trisomy detected by FISH in placental sites 1, 2 and 3 are 11.4, 12.2 and 23.5%, respectively.

The nuclear preparation of the testis represents a population of somatic and germ cells. FISH analysis was performed on all cell types of the testis since the germ cells could not be morphologically distinguished from somatic cells (Figure 3.1). No evidence of trisomy for chromosome 7 has been detected since the proportion of nuclei with three signals in the testis of the CPM gestation is less than the cut-off values set by the control samples (7.69%).

Table 3.2: FISH analysis of tissues from Case 1.

Tissue	% Nuclei with Three Signals (Number of Nuclei Scored)	Cut-Off Values (Number of Controls)
Placenta Site 1	11.4 (501)	7.89 (8)
Placenta Site 2	12.2 (501)	7.89 (8)
Placenta Site 3	23.5 (601)	7.89 (8)
Lung	7.2 (501)	9.92(9)
Kidney	2.2 (501)	11.78 (10)
Testis	2.5 (708)	7.69 (5)

Figure 3.1: FISH analysis of formalin fixed - paraffin embedded testis from Case 1 with a chromosome 7 specific centromeric probe. The germ cells are indistinguishable from the somatic cells in the testis as observed with the propidium iodide DNA counterstain.



3.2 Case 2

3.2.a. Patient History and Conventional Cytogenetic Analysis of Case 2.

This conceptus was terminated at 12 weeks of gestation and was the third pregnancy of a 37 year old woman, Gravida 3, Para 1 and Aborta 2. She has a healthy daughter and had a spontaneous abortion in a previous pregnancy. Ultrasound examination of this pregnancy did not show developmental abnormalities. Prenatal diagnosis for advanced maternal age by culture of chorionic villus sampling revealed trisomy for chromosome 16 in all 21 metaphases scored (Table 3.3). Post-termination conventional cytogenetic analysis of cultured chorionic plate revealed that all five metaphases analyzed were trisomic for chromosome 16; in cultured villus stroma eight out of ten cells exhibited trisomy 16. Culture of amnion was discordant from the results obtained from the placenta. No trisomic cells were detected in five metaphases scored from the amnion.

Table 3.3: Conventional Cytogenetics of Case 2.

Tissue	Number of Trisomic Metaphases/Total Number of Metaphases Analyzed
Culture of CVS#	21/21
Chorionic Plate*	5/5
Villus Stroma*	8/10
Amnion*	0/5

Analysis performed at the Genetics and IVF Centre, Fairfax, VA.

* Analysis performed by Irene Barrett, B.C. Research Institute for Child and Family Health.

3.2.b. FISH Analysis of Tissues from Case 2.

FISH analysis was performed on trophoblast and villus stroma from two sites of the placenta. The villus stroma from sites 1 and 2 showed significant proportions of nuclei with three hybridization signals, consistent with 50.4% and 68.2% trisomy for chromosome 16 (Table 3.4). The results obtained from the trophoblast lineage of the placenta also indicated that there were high levels of trisomy for chromosome 16 (site 1 = 72.5% and site 2 = 79.6%). Conversely, the proportion of cells with three hybridization signals from cord blood (5.8%), brain (6.6%), lung (1.2%) and kidney (2.2%) were not greater than the cut-off values

established from the disomic controls (5.92, 7.67, 8.82 and 6.17%, respectively).

For molecular cytogenetic analysis of the gonad, only oocytes which were morphologically distinguishable from the surrounding somatic cells were scored. As illustrated in Figure 3.2, oocytes in prophase of meiosis I can be identified by their large size and diffuse chromatin. The oocyte shown in this figure was obtained from a diploid fetus at 19 weeks gestational age which was used as a disomic control. The two distinct hybridization signals representing chromosomes 16 and 18 can be seen by the FITC and rhodamine fluorochromes respectively. The lack of pairing between homologous chromosomes in this oocyte suggests that it is in the leptotene stage. The smaller cell below the oocyte may either be a somatic cell or an oogonium which has not yet entered into prophase of meiosis I. Figure 3.3 illustrates an oocyte from the same disomic control ovary. This oocyte is in the pachytene stage of meiosis I and condensed chromatin which forms thick chromosomes can be observed. The single hybridization signal for chromosome 16 and 18 indicates that homologous chromosomes are paired. Oocytes from both Figures 3.2 and 3.3 where harvested from the same fetal gonad at approximately 19 weeks gestational age. The finding of oocytes at various stages of meiosis in any one ovary is consistent with the results published by Monotaya and Potter (1963), Speed (1985) and Cheng and Gartler (1994).

Progression of oocytes through the stages of prophase I is not synchronized i.e. all oocytes within the gonad are not at the same stage of development. Rather, oocytes characteristic of leptotene, zygotene and pachytene stages can be identified at this gestational age.

In order to enrich for oocytes with chromosomes that were unpaired, only those oocytes in which the internal control (chromosome 18 in Figure 3.2) showed two hybridization signals were scored during the cytogenetic analysis. As reported in Table 3.4, 26.0% of oocytes from Case 2 exhibited three hybridization signals for chromosome 16. This number was greater than the cut-off value derived from the disomic controls (7.06%) indicating a significant level of trisomic oocytes in this ovary. Figures 3.4 and 3.5 illustrate the appearance of oocytes identified in this gestation. No oocytes characteristic of pachytene stage were identified. Previous studies also did not identify pachytene stage oocytes in ovaries from fetuses at 11 to 12 weeks gestational age (Monotaya and Potter, 1963; Speed, 1984).

Figure 3.2: FISH analysis of an oocyte prepared by the squash preparation, obtained from a diploid fetus at 19 weeks gestational age. Two colour FISH with chromosome 16 (green) and chromosome 18 (red) specific DNA probes labeled with FITC and rhodamine fluorochromes respectively, is shown. The chromosome 18 specific probe was used as an internal control to select oocytes for scoring, which have unpaired homologous chromosomes. This chromatin is counterstained with DAPI. Notice the large size of the oocyte with diffuse chromatin. The smaller cell is either a somatic cell or an oogonium which has not entered into meiosis.

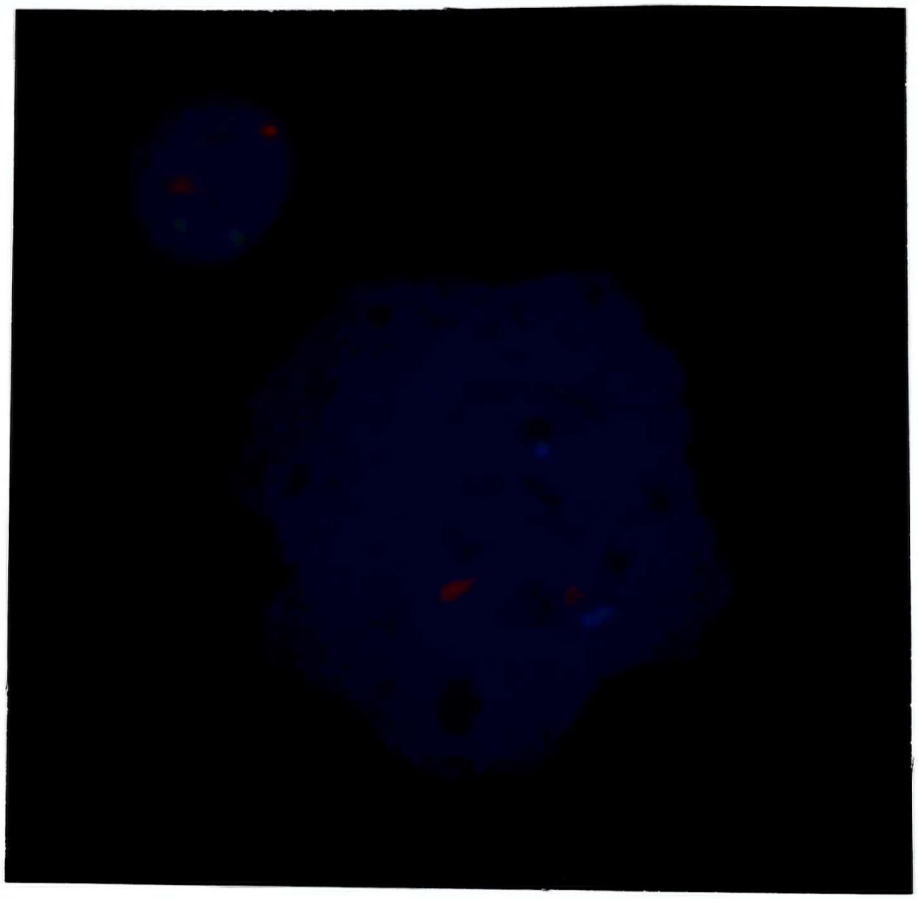


Figure 3.3: Squash preparation and FISH analysis of a diploid oocyte exhibiting one signal for the chromosome 16 (green) and chromosome 18 (red) specific probes. Oocytes similar to this which only exhibit one signal for the internal control were not scored in the FISH analysis. Notice the thick condensed chromatin and chromosome formation. One signal for the chromosome 16 and 18 specific probes suggest that this oocyte is in the pachytene stage of meiosis in which the homologous chromosomes have paired.

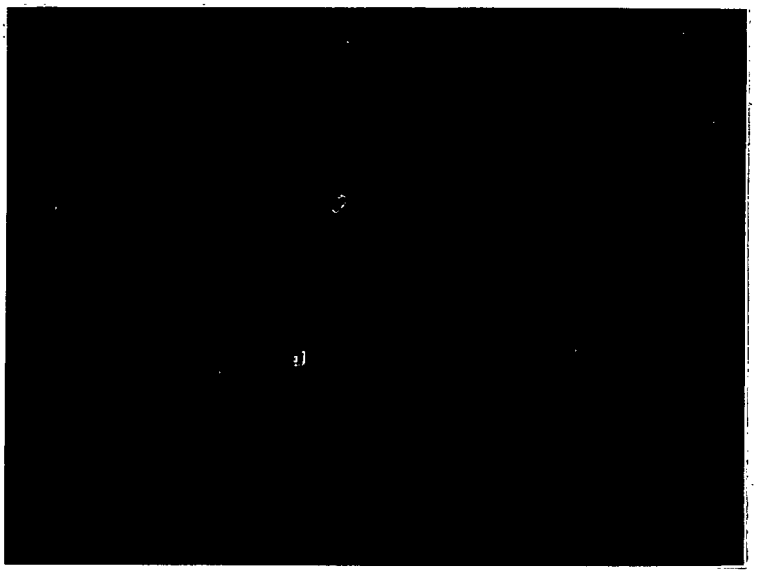


Figure 3.4: Squash preparation and FISH analysis of an oocyte obtained from Case 2. Notice the large size of this oocyte and diffuse appearing chromatin. Two signals for the chromosome 18 (red) specific and chromosome 16 (green) specific probes indicate that this oocyte is diploid.

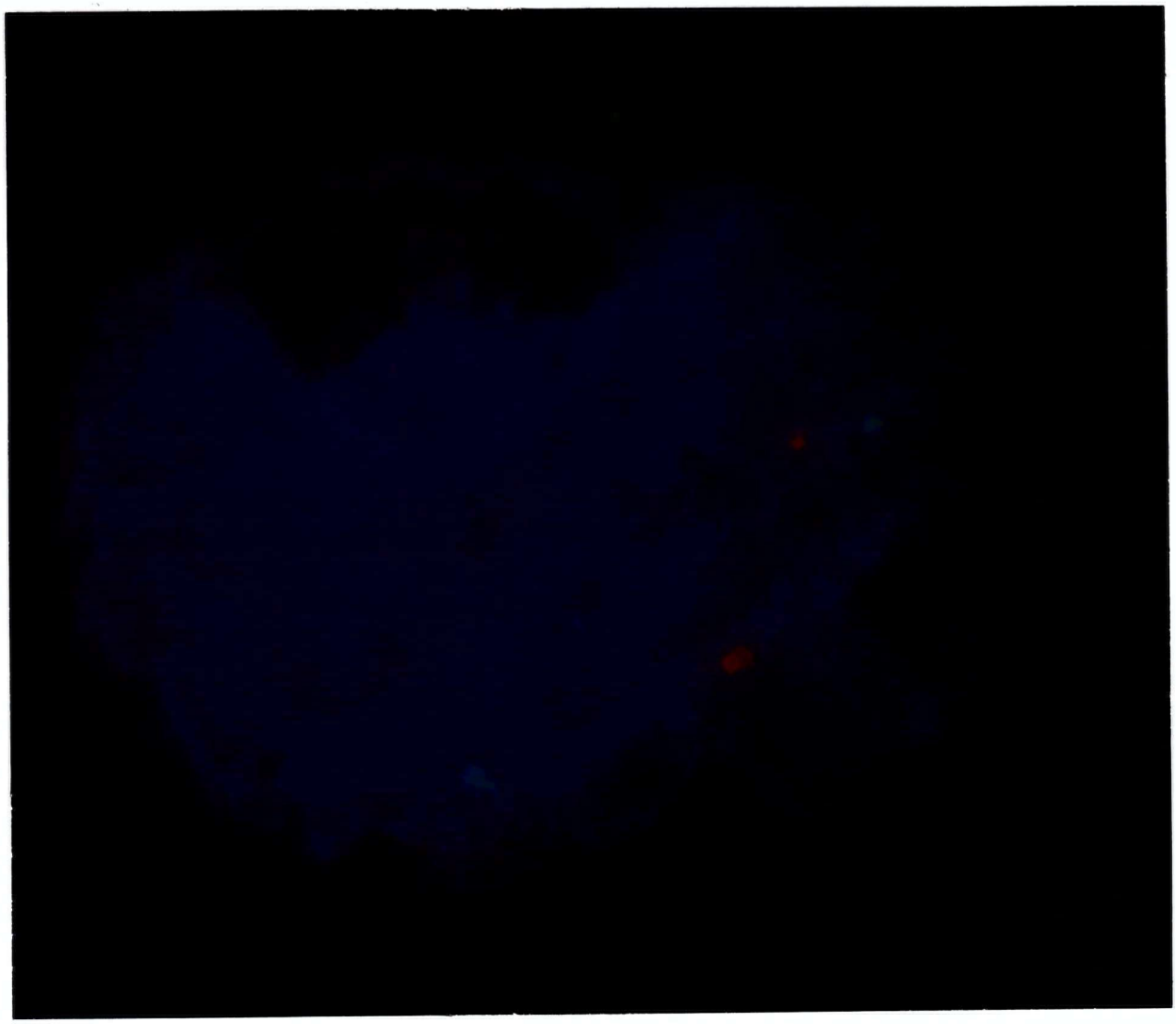


Figure 3.5: FISH analysis of a squash preparation of a trisomic oocyte from Case 2. Notice the larger nucleus with diffuse chromatin which has been identified as an oocyte. The three signals for the chromosome 16 (green) specific probe indicate that this oocyte is trisomic for chromosome 16. The smaller diploid nucleus is either a somatic cell or oogonium which has not entered into meiosis.

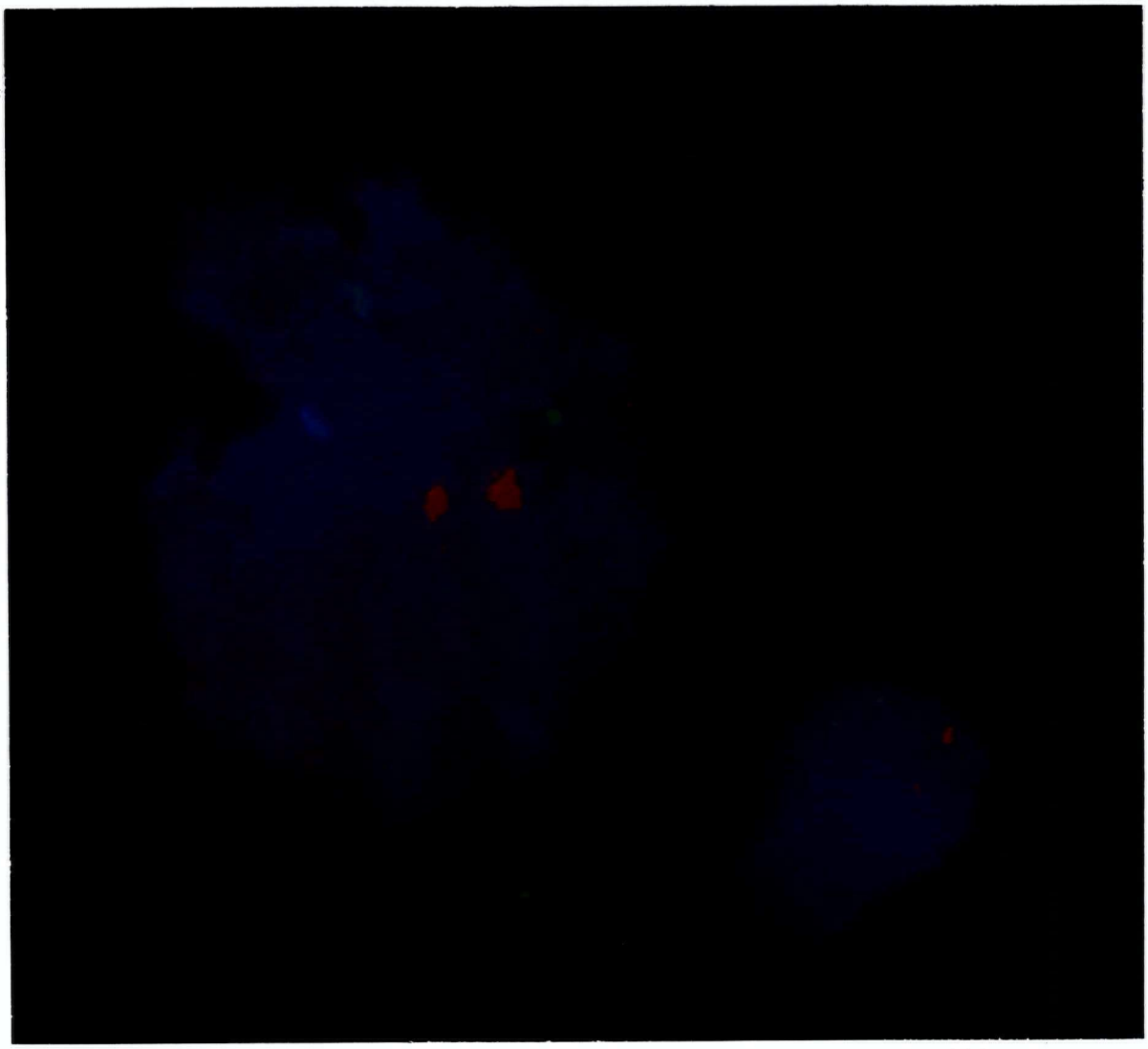


Table 3.4: FISH analysis of tissues from Case 2.

Tissue	% Nuclei with Three Signals (Number of Nuclei Scored)	Cut-Off Values (Number of Controls)
Stroma Site 1	50.4 (1148)	9.66 (8)
Stroma Site 2	68.2 (500)	9.66 (8)
Trophoblast Site 1	72.4 (500)	6.59 (6)
Trophoblast Site 2	79.6 (504)	6.59 (6)
Cord Blood	5.8 (501)	5.92 (9)
Brain	6.6 (502)	7.67 (8)
Lung	1.2 (500)	8.82 (5)
Kidney	2.2 (500)	6.17 (4)
Oocytes	26.0 (227)	7.06 (8)

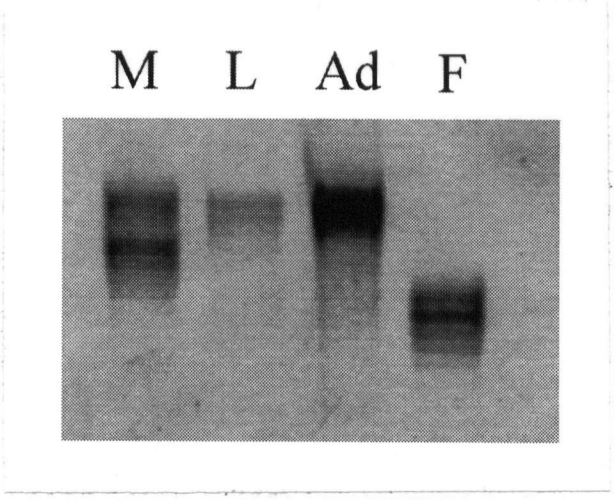
3.2.c. Microsatellite Analysis of Case 2.

Microsatellite analysis was performed with polymorphic markers specific for chromosome 16 (Figure 3.6). The PCR amplification products for marker D16S423 show that there was maternal contribution to the fetal lung and adrenal while neither of the father's alleles were found in these

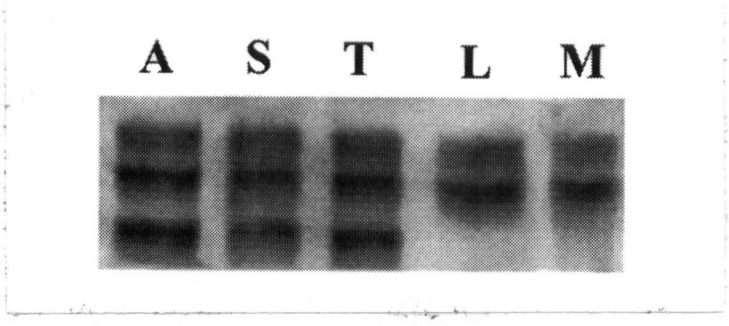
fetal tissues. Results from marker D16S398 showed three different alleles for the trophoblast, stroma and amnion while the fetal lung has two alleles which are the same size as the maternal alleles.

Figure 3.6: Microsatellite analysis of DNA from parental blood and tissues from Case 2. (Abbreviations: M = mother, F = father, A = amnion, T = trophoblast, S = villus stroma, L = fetal lung and Ad = fetal adrenal gland)

A) PCR amplification of marker D16S423, which is from the short arm of chromosome 16, shows no paternal contribution to the fetal tissues.



B) PCR amplification of marker D16S398, from the long arm of chromosome 16, shows three different alleles in the amnion, stroma and trophoblast, while the fetal lung has two alleles.



Note: Molecular analysis was performed by the laboratory of Dr. Wendy Robinson at the B.C. Research Institute for Child and Family Health.

3.3 Case 3

3.3.a. Patient History and Conventional Cytogenetic Analysis

This pregnancy was terminated at 25 weeks gestation. The mother was a 27 year old Gravida 2, Para 1 and Aborta 1. At 21 weeks gestation the mother developed severe hypertension and proteinuria. An appropriately grown fetus with a thick placenta containing echogenic regions was shown on ultrasound. Prenatal diagnosis by CVS showed trisomy 16 in all metaphases examined by the direct and culture method while amniocentesis revealed a normal female karyotype (Table 3.5). The pregnancy was terminated at 25 weeks gestation due to severe maternal hypertension, persistent vomiting and elevated hCG. Postmortem examination revealed a growth retarded fetus with no congenital defects. Cytogenetic analysis of fetal kidney, spleen, skin, amnion and blood showed a normal female karyotype, while cultured chorionic plate and stroma of villi were trisomic for chromosome 16.

Table 3.5: Conventional cytogenetic analysis of Case 3.

Tissue	Number of Trisomic Nuclei/Total Number Scored
Amniotic Fluid	0/36
CVS - Direct Method	9/9
CVS - Culture Method	20/20
Chorionic Plate	3/3
Villus Stroma	24/24
Amnion	0/15
Skin	0/15
Kidney	0/15
Spleen	0/15
Blood	0/63

Note: Previously published results from Kalousek et al., 1993.

3.3.b. Molecular Cytogenetic Analysis of Case 3.

FISH analysis was attempted on formalin fixed-paraffin embedded placenta, kidney, lung and oocytes.
The nuclear preparations of kidney and placental tissues failed to hybridize with the chromosome 16 specific probe, regardless of several attempts with varying enzyme pretreatments. A successful hybridization was achieved with

the lung which showed no significant levels of trisomy (Table 3.6).

For analysis of formalin fixed-paraffin embedded oocytes, a nuclear preparation was hybridized with a chromosome 18 specific centromeric probe (internal control) and a chromosome 16 specific centromeric probe. However, attempts to amplify the signals with antibodies for two-colour FISH resulted in unacceptable levels of background fluorescence. Therefore, only one-colour FISH with a chromosome 16 specific probe was used in the analysis of formalin fixed-paraffin embedded oocytes, since this resulted in lower levels of background fluorescence.

The distinguishing features of oocytes prepared by this method were not as dramatic as in fresh oocytes prepared by the squash method. As seen in Figures 3.7 and 3.8, the appearance of diffuse chromatin is not as obvious in paraffin embedded-formalin fixed oocytes (Figures 3.2 to 3.5). However, oocytes were identified by the appearance of string-like chromatin.

In this analysis it was not possible to enrich for oocytes with unpaired chromosomes since an internal control was not utilized when selecting cells to be scored (Figure 3.7). This resulted in levels of apparent monosomy as high as 36.6% in the disomic controls (Table 6.8 in Appendix). The single hybridization signal of the chromosome 16 specific probe illustrates this point in Figure 3.8. It is also apparent that the pairing characteristics of oocytes

resulted in only 39% trisomy 18 in a formalin fixed - paraffin embedded ovary which was obtained from a trisomy 18 syndrome fetus (Table 6.9 in Appendix). When fresh oocytes from a trisomy 18 syndrome fetus were analyzed with an internal control, 71% of the oocytes analyzed showed three signals (Table 6.11 in Appendix).

Similar to the disomic control samples, the level of monosomy in the patient was found to be 39% as a result of paired homologues as illustrated in Figure 3.9. Figure 3.10 shows an example of a disomic oocyte from the mosaic conceptus. Two signals for chromosome 16 were observed in 56% of the oocytes analyzed from the mosaic conceptus (Table 6.3 in Appendix). Also, 5.0% of the oocytes from this fetus exhibited three hybridization signals while the cut-off value for a diagnosis of trisomy is 6.87% (Table 3.6).

Figure 3.7: FISH analysis with a chromosome 16 specific centromeric DNA probe (green signals) of a formalin fixed - paraffin embedded oocyte obtained from a diploid gestation. Close examination of the chromatin structure of this nucleus reveals the string - like chromatin which facilitated the identification of this nucleus as an oocyte. Two green signals representing the chromosome 16 centromeric probe confirm that this oocyte is disomic for chromosome 16.

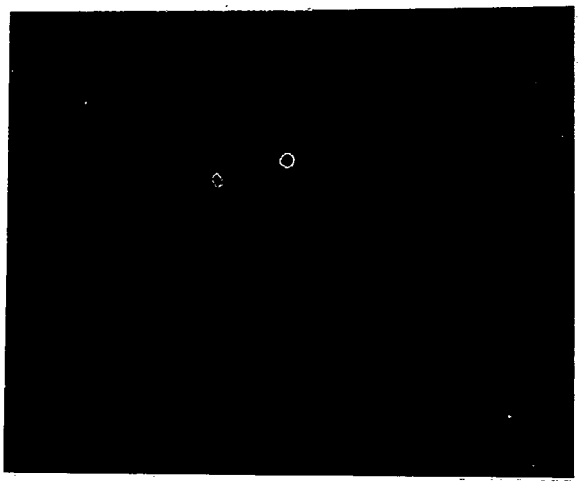


Figure 3.8: FISH analysis with a chromosome 16 specific probe of a formalin fixed - paraffin embedded oocyte obtained from a diploid conceptus. Notice the diffuse chromatin and string - like chromosome structures which are characteristic of an oocyte in meiosis. The single green signal represents chromosome 16 and may be a result of paired homologous chromosomes.

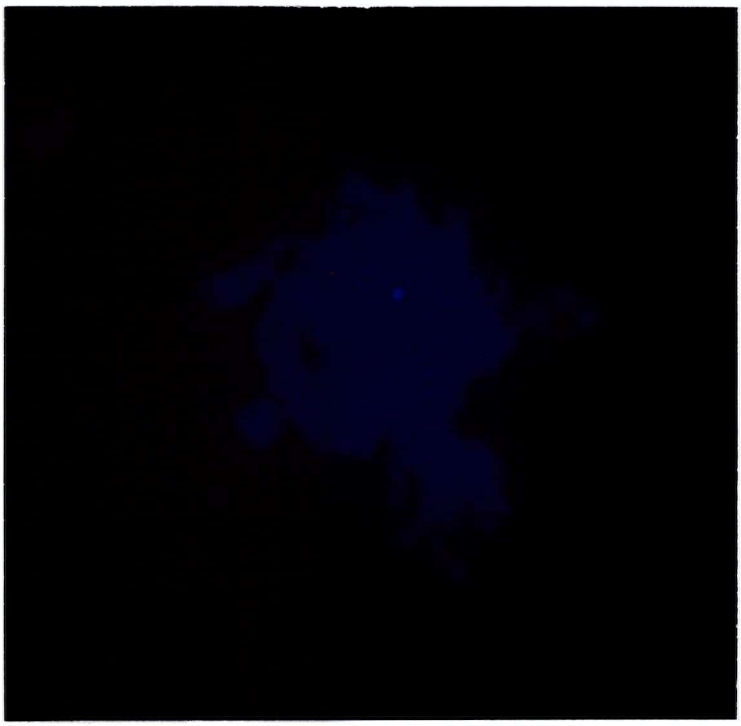


Figure 3.9: FISH analysis of a formalin fixed - paraffin embedded oocyte form Case 3. One signal was observed for the chromosome 16 specific centromeric DNA probe in 39% of the oocytes examined as shown in this figure. This is presumed to be a result of paired chromosome 16 homologues in the meiotic oocytes.

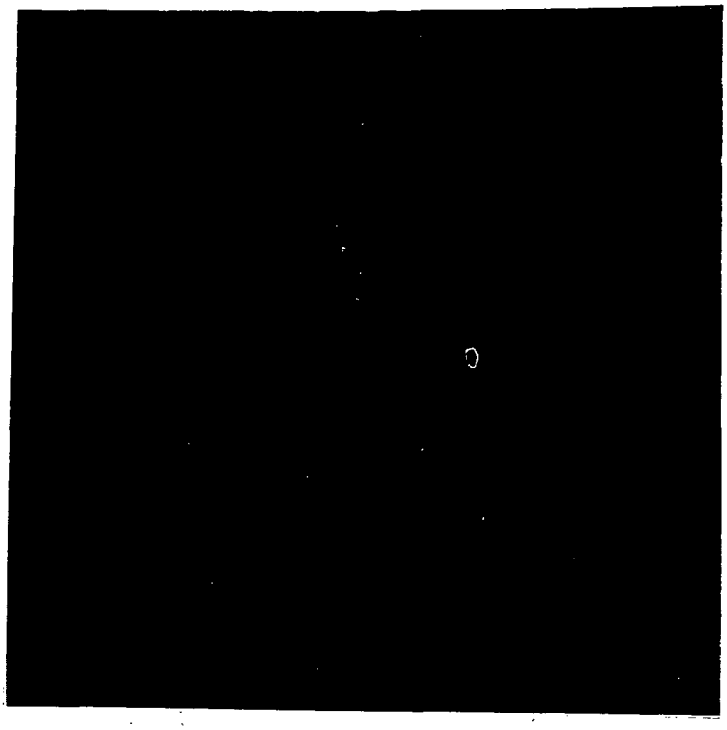


Figure 3.10: FISH analysis of a formalin fixed - paraffin embedded oocyte from Case 3. The two green signals represent the centromeric region of chromosome 16 and indicate that this oocyte is disomic for this chromosome.

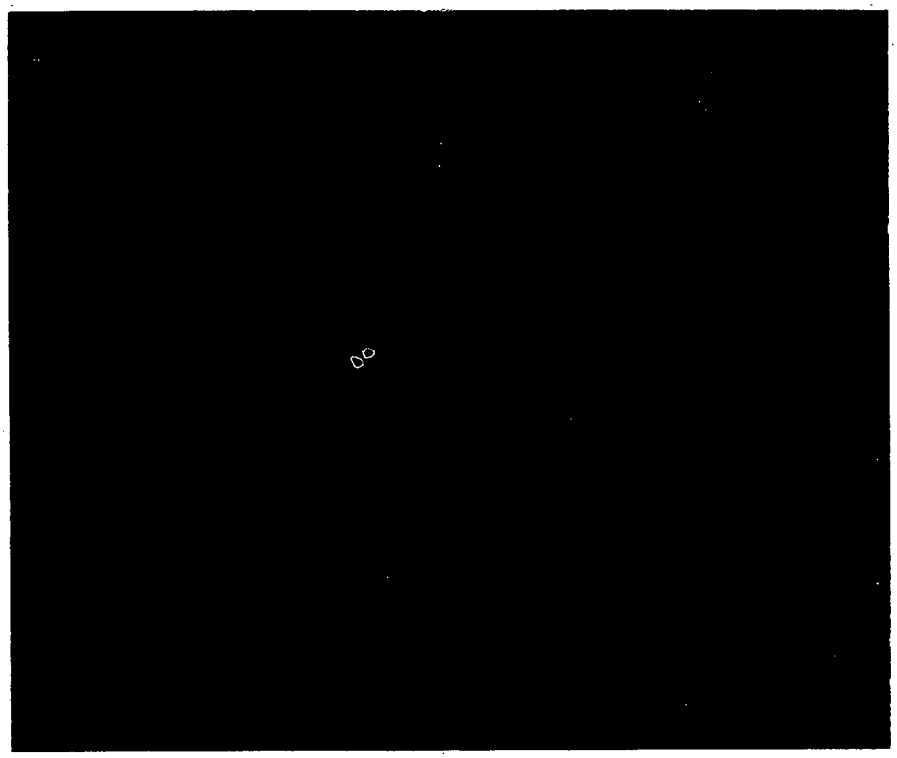


Table 3.6: Molecular cytogenetic analysis of Case 3.

Tissue	Proportion of Trisomic Nuclei (Total Number of Nuclei)	Cut-Off Values (Number of Samples)
Lung	3.2 (500)	9.92 (9)
Oocytes	5.0 (100)	6.87 (10)

3.3.c. Parental Origin of Chromosome 16

Molecular analysis of DNA from parental blood, fetal and placental tissues was performed to determine parental origin of chromosome 16. As reported in Table 3.7, there is maternal origin of the extra chromosome in the trisomic placenta. Further the diploid fetus has maternal uniparental disomy for chromosome 16.

Table 3.7: Molecular analysis of the parental origin of chromosome 16.

Probe	Mother	Father	Placental Villi	Fetus
D16S83	a,c	b,b	a,b,c	a,c

Note: These are previously published results from Kalousek et al., 1993

4 Discussion

4.1 Embryonic Origin of Tissues and Chromosomal Mosaicism

4.1.a. General Comments

In order to understand the concept of confined versus generalized mosaicism, several factors affecting the distribution of the mutant cell line in the developing conceptus must be considered. Tracing the cell lineage of specific tissues affected with chromosomal mosaicism helps one understand how the mutant cell line contributes to specific tissues of the conceptus during the process of differentiation. During early embryogenesis cell fate or differentiation is thought to proceed through a number of developmental mechanisms including: cell polarization, cleavage, compaction, position effects, cell adhesion and cell death (Vogler, 1987). Allocation of a cell or group of cells by these processes and the genetic changes within the cells and their mitotic progeny give rise to a specific pattern of development (Carlson, 1994).

The distribution of a mutant cell line throughout the conceptus is determined by the effects of a mutational event such as nondisjunction, anaphase lag or chromosomal rearrangement, on these developmental processes. The outcome can be affected by the timing of the mutational

event, the viability of the mutant cell line and the cell lineage affected.

Much of what we know of cell lineage in human embryology is based on observations of histological preparations of embryos recovered at successive stages of pregnancy. The resulting fate maps are based on the interpretations of the investigator as to what occurs between each of the stages which have been sampled. As Gardner (1983) states "Clearly, this approach is an inherently unreliable one, and it is hardly surprising to find opinion divided about one or more cell lineage in virtually every species whose early development has been looked at closely in this way. This is especially so in man, in whom specimens are limited both in number and quality and often of uncertain gestational age." A more reliable method has been to mark cells or tissues in early mammalian embryos and follow the cell lineage. These types of experiments were performed in rodent embryos and have contributed a great deal towards the understanding of mammalian embryology. This knowledge has been used to gain insight into human cell lineage, however such comparisons must be considered with caution since human development differs from murine development. (Gardner, 1983).

4.1.b. Embryo/Fetus Proper

The precursor cells which give rise to the embryo/fetus proper are found within the inner cell mass of the early blastocyst. The inner cell mass also contributes to the development of extraembryonic tissues such as the yolk sac and extraembryonic mesoderm (Luckett, 1975, 1978). The proportion of cells of the inner cell mass which contribute to the embryo/fetus proper is not known, but experiments in the mouse suggest that three cells are involved. Markert and Petters (1978) produced chimeric mice by aggregating three cleavage stage embryos genetically distinct with respect to their coat colour. The coat of some mice had contributions from all three colours, suggesting that at least three cells of the blastocyst give rise to the embryo. Aggregation of four genetically distinct embryos gave rise to mice with no more than three contributions to their coat colour. These results have been interpreted to suggest that three to five cells from the inner cell mass in the developing human blastocyst give rise to the embryo/fetus proper (Kalousek and Dill, 1983; Simoni and Serchia, 1994).

Since so few cells give rise to the fetus as compared to the extraembryonic tissues, Kalousek and Dill (1983) predicted that there would be many more instances of mosaicism in the placenta than the embryo/fetus proper. It has been since reported that the incidence of generalized mosaicism affecting both the fetus and placental tissues is

0.1 to 0.3% of viable pregnancies tested by amniocentesis at 16 to 20 weeks of gestation (Hsu et al., 1984; Worton and Stern, 1984; Bui et al., 1984). While the incidence of chromosomal mosaicism confined to the placenta is approximately 1 to 2% in viable pregnancies tested by CVS at 9 to 12 weeks of gestation followed by cytogenetic analysis of fetal tissues (Ledbetter et al., 1991; Wang et al., 1994).

Another hypothesis explaining the higher incidence of CPM as compared to generalized mosaicism is that the embryo has control mechanisms to limit the replication of aneuploid cells, i.e. diploid cells may have a growth advantage and outgrow the abnormal cells. In the extraembryonic tissues, the abnormal cells may proliferate clonally and are simply eliminated with the placenta at delivery (Simoni and Serchia, 1994).

4.1.c. Placental Tissues

The chorionic villi of the placenta are composed of an outer layer of hormonally active and invasive syncytiotrophoblast, a middle layer of cytotrophoblast, from which the syncytiotrophoblast is derived, and an inner mesodermal core containing blood capillaries. The cytotrophoblast and syncytiotrophoblast are derived from the trophoblast cell lineage. In the developing human, the trophoblast is the first lineage to differentiate from the

multipotential cells of the morula. Differentiation begins as the outer cells of the 16 cell morula develop a polar shape and eventually form the trophoblast cell lineage in the blastocyst stage (Vogler, 1987). The remaining inner cell mass will constitute the embryo/fetus proper, amnion, yolk sac and the chorionic stroma. Therefore it is assumed that Type I CPM arises as a result of a mitotic error in the progenitor cells of the trophoblast soon after they have been determined to form the trophoblast cell lineage (Crane and Cheung, 1988).

The developmental history of the chorionic stroma is more complex, and thus the mechanism of CPM Type II and III formation is less understood. Examination of the Carnigie collection by Luckett (1978) suggests that the extraembryonic mesoderm first arises at 12 days of development in a thickening of the caudal pole of the epiblast. This modification of the epiblast is interpreted as the caudal margin of the primitive streak. In 13 day human embryos the caudal margin of the primitive streak is observed to give rise to extraembryonic mesoderm which spreads peripherally over the amnion and primary yolk sac and eventually gives rise to the stroma of the chorionic villi.

Luckett's interpretations of the Carnigie sections are in agreement with fate mapping experiments performed in the mouse. Parameswaran and Tam (1995) utilized *lac* Z expressing epiblast cells from transgenic mice grafted to

nontransgenic early primitive streak stage mouse embryos to assess the fate of epiblast cells. They found that cells in the proximal region of the epiblast differentiated into extraembryonic mesoderm.

When considering the cell lineage map established by Luckett et al. (1978), it is difficult to explain how a mosaic blastocyst may contribute to the formation of CPM Types II and III. Since the epiblast is thought to give rise to both the villus stroma and embryonic tissues, the chromosomal constitution of the embyro/fetus is expected to be concordant with that of the villus stroma. However, the experiments by Markert and Petters (1978) suggest that three to four cells of the epiblast give rise to the embryo/fetus proper. A mutational event occurring in epiblast cells which are allocated for extraembryonic mesoderm or during the proliferation of the extraembryonic mesoderm itself can result in CPM type II. Type III CPM, in which both the trophoblast and chorionic stroma are trisomic, probably arises from a trisomic zygote rescue (Kalousek et al., 1993). A meiotic non-disjunction produces a trisomic zygote, and post-zygotic loss of one of the three chromosomes results in a diploid cell line. When the diploid cells in the mosaic gestation contribute to all the embryonic/fetal tissues while chorionic stroma and trophoblast have significant levels of trisomy, type III CPM results. When considering Luckett's (1978) observations, it is expected that the epiblast of the developing gestation

would be mosaic in type III CPM. The cells which give rise to the embryo/fetus proper would be diploid, and the caudal region which differentiates into extraembryonic mesoderm would contain trisomic cells.

Enders and King (1988) suggest that the hypoblast contributes cells to the extraembryonic mesoderm. Histological examination of sectioned 10.5 day rhesus monkey embryos showed branching processes from the primary yolk sac extending across the chorionic cavity toward the trophoblast. Ultrastructural studies of these cells revealed expanded endoplasmic reticulum cisternae which are characteristic of the extraembryonic mesodermal cells in slightly later stages. Between day 13 and 16 the network of cells between the yolk sac and the trophoblast seemed to differentiate into mesenchymal cells in a relatively uniform fashion. The authors interpreted these results to suggest that extraembryonic mesoderm is derived from the hypoblast before the formation the primitive streak.

Bianchi et al. (1993) incorporated the observations by Enders and King and reviewed the cytogenetic data obtained by the U.S. collaborative study on CVS (Ledbetter et al., 1992) and the Munster CVS program (Miny et al., 1991) to assess cell lineage in humans. Bianchi et al. (1993) interpret the discordance observed between the chromosomal constitution of the chorionic villus and embryo/fetus proper in CPM type II as evidence that the origin of the extraembryonic mesoderm is not the epiblast but rather the

hypoblast. Since the inner cell mass is known to split into the hypoblast and epiblast during embryologic development, CPM type II would result when the mosaic cells of the inner cell mass contribute to the formation of the hypoblast but not the epiblast. Similarly, it is expected that type III CPM results when a trisomic zygote rescue occurs in diploid progenitors of the epiblast.

However, the hypothesis presented by Bianchi et al. (1993) by no means has been established as correct. Firstly, Enders and King (1988) were not able to dismiss the observations by Luckett (1978) in which the caudal margin of the epiblast seems to be involved in contributing to the formation of the extraembryonic mesoderm. Further, Luckett (1978) described a different fate for the network of cells derived from the hypoblast which are observed between the primary yolk sac endoderm and trophoblast. Luckett suggested that this tissue develops an endodermal meshwork, the inner layer of which eventually constitutes the primary yolk sac endoderm. As the blastocyst grows, the meshlike tissue unfolds until only a few single strings are recognizable on day 12. Luckett (1978) also pointed out that the primordium of mesodermal villi develop initially in a localized region overlying the precociously differentiated primitive streak. The close temporal and spatial relationship between primitive streak differentiation and the initiation of the chorionic villus formation in the 12 to 14 day human embryo suggests that the sheet of

extraembryonic mesoderm spreading from the primitive streak and the overlying trophoblast have an inductive interaction in the formation of the chorionic villi (ibid.).

Another hypothesis is that the hypoblast contributes to the formation of the extraembryonic mesoderm in the chorionic cavity, which is later joined by extraembryonic mesoderm derived from the epiblast (Carlson, 1994). In this scenario the extraembryonic mesoderm from the hypoblast is expected to be trisomic while that from the epiblast is diploid resulting in a mosaic placenta in gestations with CPM types II and III.

Kalousek (1993) has suggested that the complete dichotomy between the aneuploid placenta and diploid fetus in conceptuses diagnosed with CPM type III arises from an initiation of the twinning process. A meiotic nondisjunction event gives rise to a trisomic zygote and develops into a nonmosaic trisomic blastocyst. The trisomic zygote rescue occurs in a fraction of the cells of the inner cell mass of the blastocyst destined to differentiate into the embryo. The diploid embryonic progenitors aggregate to form one embryo and the trisomic embryonic progenitors form a second embryo. The trisomic nonviable twin "vanishes" and the diploid twin develops as a singleton supported by a trisomic placenta. There are two lines of evidence found in the literature which support this hypothesis. Firstly, there have been reports of monozygotic twins who differ in their chromosomal constitution. Nielsen (1967) summarized

twin studies which indicate that one twin of monozygotic pairs has trisomy 21 or monosomy for the X chromosome while the other has a normal karyotype. Secondly, Landy et al. (1986) performed sonographic analysis of 1000 pregnancies with viable gestations in the first trimester and showed that 3.29% were twins and 21.2% of theses gestations demonstrated the "vanishing twin" phenomenon with a good prognosis for the remaining fetus.

Regardless of several efforts to establish the embryological origin of the extraembryonic mesoderm, a conclusive result has not been reached to this day. Clinical material obtained from gestations with CPM provides an opportunity to assess the embryologic relatedness between different tissues, but the unknown embryologic stage at which the mutational event occurs during development undermines the reliability of making conclusive statements from such studies. Further, possible forces of cell selection which likely take place in chromosomally mosaic gestations complicate the interpretations of the developmental processes leading to CPM.

4.1.d. Formation of the Definitive Yolk Sac

Luckett (1978) was the first to hold the now generally accepted view that the hypoblast in humans is derived from the inner cell mass. Also, it is now clear that the hypoblast gives rise to cells which line the trophoblast of the blastocyst cavity to form the endoderm of the primary yolk sac.

Formation of the secondary yolk sac is a developmental process unique to the anthropoid primates including monkeys, apes and humans. In the rhesus monkey, the secondary yolk sac is formed by a folding and pinching off of the endoderm of the primary yolk sac (Luckett, 1978). The development of the secondary yolk sac in the human is poorly understood. Luckett (1978) describes the collapse of the primary yolk sac in the human between 12 and 13 days gestation. Analysis of day 12 to 14 human embryonic serial sections prompted Luckett (1978) to hypothesize that the intact remnants of the primary yolk sac which come to lie immediately beneath the embryonic disc are pinched off to form the secondary yolk sac. However, at this stage of human development the ruptured secondary yolk sac is surrounded by multipotential cells of the extraembryonic mesoderm. It is difficult to conclude with certainty that cells of the primary endoderm contribute exclusively to the development of the secondary yolk sac. Thus, it is also possible that cells of the

extraembryonic mesoderm are involved in the formation of the secondary yolk sac.

At 16 days of gestation the endoderm of the secondary yolk sac is completely covered with extraembryonic mesoderm which forms the definitive yolk sac splanchnopleure (Luckett, 1978).

The chromosomal constitution of the secondary yolk sac in gestations with CPM has not been documented. The amnion and villus stroma are diploid in gestations affected with CPM type I. Since the yolk sac endoderm like the amnion and villus stroma, is derived from the inner cell mass, it is also expected to be diploid.

We hypothesize that the extraembryonic mesoderm contributes to the formation of the secondary yolk sac. Therefore we expect that in conceptuses with CPM types II and III in which there is chromosomal mosaicism or complete aneuploidy in the extraembryonic mesoderm, the endoderm of the secondary yolk sac will be mosaic or completely aneuploid.

4.1.e. Primordial Germ Cells

It is well established that human PGCs are first identifiable in the secondary yolk sac endoderm at four weeks of development (Fujimoto et al., 1977). This is followed by a separation of the PGCs from the endoderm and migration into the embryo by amoeboid movements (Fujimoto et

al., 1977). The PGCs continue their migration until they reach the gonadal ridge where they invade the primitive sex cords. In female development, the PGCs undergo a series of mitotic divisions until the number of oogonia in each ovary reaches approximately 7 000 000 at 18 weeks of development. The oogonia enter into prophase of meiosis I at eight weeks and arrest in dictyotene of meiosis at seven months. The majority of oocytes undergo apoptosis so that the number declines to 400 000 by the seventh month of development and only 30 000 in the seven year old child.

In the fetal testis the germ cells proliferate from week 8 until week 11, at which time they enter a resting stage. Then at puberty, subpopulations of spermatogonia undergo waves of mitosis, and the progeny of these divisions enter meiosis as synchronous groups to form spermatozoa (Carlson, 1994).

Although the PGCs are first identifiable by their alkaline phosphatase activity in the endoderm of the secondary yolk sac, the cell lineage from which they evolve is not understood in the human (Fujimoto et al., 1977). It is possible that the hypoblast contributes to their formation since this cell lineage is involved in the development of the secondary yolk sac endoderm. However, since the extraembryonic mesoderm may also contribute to the formation of the secondary yolk sac, it is possible that the PGCs arise from the differentiation of the extraembryonic mesoderm.

Finally, there is evidence in mouse embryological studies that the PGCs arise from the same cells of the epiblast that give rise to the extraembryonic mesoderm. Epiblast cells in the proximal region give rise to the extraembryonic mesoderm as well as the PGCs (Lawson and Hage, 1994). This suggests that the extraembryonic mesoderm and the PGCs have a common embryological origin in mammals.

The chromosomal constitution of the gametes has not previously been studied in cases diagnosed with CPM. Since the PGCs seem to have a common embryological origin with the extraembryonic mesoderm, we expect that conceptuses diagnosed with type II and III CPM may show chromosomal mosaicism of the germ cells.

4.1.f. Hematopoiesis

It has been generally accepted that the progenitor blood cells which contribute to hematopoiesis throughout the embryonic, fetal and adult periods of life arise from the mesoderm of the secondary yolk sac and migrate to the liver, spleen and bone marrow during the embryonic/fetal period (Schwartz et al., 1991). The yolk sac mesoderm is the first site of hematopoiesis during the third week of development. Blood cell production is then taken over by the fetal liver at six weeks, followed by the spleen and bone marrow (Dzierzak and Medvinsky, 1995). If the origin of hematopoiesis is the mesoderm of the yolk sac, then it is

difficult to explain the finding of diploid blood in cases diagnosed with confined placental mosaicism types II and III in which the extraembryonic mesoderm is mosaic (Kalousek and Dill, 1983; Ledbetter et al., 1992). It has been suggested that this discrepancy is due to selective pressures during hematopoietic differentiation, during which only diploid stem cells survive (Bianchi et al., 1993). Another possibility is that yolk sac hematopoiesis is transient and definitive stem cell production occurs in the embryonic mesoderm. There is very persuasive evidence emerging from studies of hematopoiesis in the mouse which support the hypothesis that definitive blood cell production occurs in the AGM region. Dickson et al. (1995) have shown that mutant mouse embryos with a defficiency of yolk sac hematopoiesis contain erythrocytes in the AGM region. Medvinsky and Dzierzak (1996) demonstrated that only the AGM region and neither the yolk sac nor the liver of 10 day post coitum mouse embryos was able to contribute to definitive hematopoiesis of lethally irradiated mouse recipients.

4.1.g. Amnion

In his investigation, Luckett (1975) describes the process of amniogenesis as cavitation of the dorsal epiblast. This cavity enlarges and the roof becomes thinner until it opens up. The edges of the epiblast grow towards each other until they join to form the ectoderm of the

amnion. The thin amniotic ectoderm is later joined by a layer of extraembryonic mesoderm which proliferates from the caudal margin of the epiblast to form the outer mesoblastic layer of the amnion (Luckett, 1978).

In mosaic gestations affected with CPM, amniocentesis is assumed to represent the karyotype of the fetus and not the placenta. While chromosomal mosaicism is found in the placenta in 1 to 2% (Ledbetter et al., 1992; Wang et al., 1994) of chorionic villus samplings, the incidence in pregnancies tested by amniocentesis is 0.1 to 0.3% (Hsu et al., 1984; Worton and Stern, 1984; Bui et al., 1984). Since amniocentesis involves the culture of fibroblasts from amniotic ectoderm as well as fetal tissues including skin cells and epithelial cells of the gastrointestinal, respiratory and urinary tracts, it has been established in the literature that the chromosomal constitution of the amniotic ectoderm is most often concordant with the fetal tissues in cases diagnosed with CPM. Also, chromosomal mosaicism detected in amniotic fluid has been confirmed in the fetus in 70% of the cases (Hsu et al., 1984). Failure to confirm mosaicism in the fetus has been explained by contamination of amniotic fluid by chorionic cells during amniocentesis in gestations with CPM. It is also possible that there are low levels of mosaicism in the fetus which are not confirmed at term because tissues sampled are not representative of all fetal cell lineages, i.e., only fetal skin and/or blood are usually used to confirm mosaicism.

Discrepant CVS and amniocentesis may also be due to the presence of an undetected degenerated dizygotic twin (Reddy et al., 1991; Chitiat et al., 1991).

4.2 Cytogenetic Analysis of Mosaicism

Fluorescence *in situ* hybridization has allowed the detection of specific numerical chromosome aberrations in interphase nuclei. This was accomplished with a labeled DNA probe specific for the repetitive alpha-satellite sequences found in the centromeric region of the chromosome of interest. Subsequent immunochemical detection with conjugated fluorochromes allowed direct visualization of the number of copies of specific chromosomal regions. In this way hundreds of nuclei were scored for numerical abnormalities of a specific chromosome relatively quickly. At the same time the power of the statistical analysis was increased compared to conventional cytogenetic analysis since hundreds of interphase nuclei were analyzed. Only 15 metaphases are routinely analyzed by conventional cytogenetic analysis. Assay sensitivity was enhanced because the probability of detecting low levels of mosaicism was increased by the analysis of hundreds of nuclei.

Another advantage of FISH was that non-dividing cells were amenable to analysis including archival tissues fixed in formalin and paraffin embedded. Further, since conventional cytogenetic analysis most often requires tissue

culture to obtain analyzable metaphases the possible growth advantage of one cell line over another can result in tissue culture bias (Lomax et al., 1994). By using FISH analysis to detect aneuploidy this tissue culture bias was eliminated.

Although FISH analysis is a powerful technique it is limited by the probe used to target a specific sequence whereas conventional cytogenetic analysis can provide information on numerical and structural abnormalities of the entire genome (Eastmond et al., 1995). Thus analysis of the mosaic gestations first required conventional cytogenetic techniques to identify the chromosome involved in the trisomy before molecular cytogenetics could be employed. For example, culture of villus stroma followed by conventional cytogenetic analysis of Case 1 revealed the trisomy of chromosome 7. The distribution of trisomy in fetal and placental tissues could then be explored by the utilization of FISH analysis with a chromosome 7 specific probe.

In addition, technical artifacts can potentially result in diploid controls with false positive findings of trisomy or monosomy. Thus statistical analysis of control data to set cut-off values for the detection of trisomy was performed as described in the Materials and Methods.

Proportions of false trisomy in the disomic control tissues analyzed in this study ranged from 1 to 8.4% (see Appendix). Greater than two hybridization signals can appear

in diploid nuclei by the splitting of one signal to make it appear as a doublet. This is thought to arise from either premature chromatid separation during fixation or cells in G2 phase of the cell cycle (Eastmond et al., 1995). Also, insufficient stringency of the post-hybridization wash can result in hybridization of the DNA probe to non-specific sites in the genome (Lomax et al., 1994). Extra signals may also appear as background fluorescence due to non-specific binding of fluorochrome-conjugated antibodies to nuclear or cellular proteins fixed to the slide. The proportion of somatic cells with one signal for the chromosome of interest in disomic controls ranged from 1.2 to 11.2% (see Appendix). These results may be due to inefficient penetration of the probe into the nucleus, high stringency post-hybridization wash, overlapping signals from two chromosomes and suboptimal hybridization conditions (Lomax et al., 1994; Eastmond et al., 1995).

4.3 Cytogenetic Analysis of Mosaic Conceptuses

4.3.a. Case 1

Conventional cytogenetic analysis of cultured chorionic plate showed that two out of four metaphases were 47,XY,+7 while cultured amnion showed five diploid metaphases. These results suggest that this conceptus was affected with confined placental mosaicism since the amniotic ectoderm is

derived from the same cell lineage as the fetal skin. From these results alone, it was not possible to determine if the fetus was indeed diploid since only amnion was analyzed. Therefore, molecular cytogenetic analysis was performed on formalin fixed - paraffin embedded fetal tissues. FISH analysis of fetal lung and kidney with the chromosome 7 specific probe showed that 7.2% and 2.2% of the nuclei exhibited three signals respectively. The upper range of the 95% confidence limit for a diagnosis of trisomy was calculated to be 9.92 and 11.78% for the lung and kidney, respectively. Therefore the proportion of nuclei with three signals in these tissues was not considered to be significant.

In order to confirm that the placenta was mosaic for trisomy 7, FISH analysis was performed on formalin fixed - paraffin embedded chorionic villi from three sites of the placenta. The proportion of nuclei with three signals exceeded the 95% confidence limit of the upper reference limit in all three sites. The level of trisomy in the placenta was 11.45% for site 1, 12.2% for site 2 and 23.5% for site 3. As these values are greater than the cut - off value for trisomy calculated to be 7.89%, significant levels of mosaicism for trisomy 7 in the placenta were confirmed.

The distribution of trisomic cells in the placenta was not determined since the trophoblast and villus stroma of the placenta could not be analyzed separately. The stroma of the placenta has been shown to be mosaic since 2/4

trisomic cells were identified in the cultured stroma by conventional cytogenetics. However, the exact level of trisomic cells in the villus stroma could not be determined since the FISH analysis also included nuclei derived from the trophoblast cell lineage. If the trophoblast cell lineage was nonmosaic diploid, then high levels of trisomy in the villus stroma would have been diluted in the FISH analysis.

Since the embryological origin of the PGCs is closely related to the villus stroma, FISH analysis was performed on the testis of this gestation. The population of nuclei analyzed from this testis included both somatic and germ cells. The proportion of nuclei with three signals was 2.5% in the mosaic conceptus which was well below the cut - off value for trisomy, calculated to be 7.69%. These results suggest that the germ cells of the testis were nonmosaic diploid. However, the possibility that there was a low level of germline mosaicism can not be ruled out because germ cells constitute a small proportion of the number of cells in the fetal testis. Mendez and Emery (1979) found that the germ cells constitute 5 to 15% of the seminiferous tubules in the fetal gonad. There is also a population of interstitial cells surrounding the tubules in the fetal gonad which proliferate and occupy almost half of the testicular volume at 14 to 18 weeks of development. After 18 weeks, the interstitial cells degenerate until they occupy approximately 12% of the testis at birth (Orvis et

al., 1988; Mancini et al., 1965). Therefore, due to the low proportion of germ cells expected in a fetus of 19 weeks development, such as Case 1, the ability to detect trisomy in the germ cell population by this molecular cytogenetic analysis is compromised.

Ideally, only germ cells should be scored to detect chromosomal mosaicism in the germline. However, in this case the fetal germ cells can not be distinguished from the surrounding somatic cells by the cytogenetic method utilized. An alternative method to investigate the presence of trisomy in male fetal germ cells is to perform FISH analysis on sections of paraffin embedded - formalin fixed testis mounted on a microscope slide. The advantage to this technique when compared to preparing a cell suspension as in the present study, is that the morphology of the testis is maintained and germ cells can be stained with hematoxalin and eosin and identified . Then the position of the germ cells on the microscope slide can be recorded before the cells are processed for the FISH analysis. However, this technique requires thin sectioning of the gonad (5 to 7 microns) and can result in truncation of the nuclei under investigation and an underestimation of the number of chromosomes in the nuclei (Kuchinka et al., 1995).

4.3.b. Case 2

Prenatal diagnosis performed on this pregnancy showed that 21/21 cells were 47,XX,+16 from cultured villi. Following termination, conventional cytogenetic analysis showed trisomy for 5/5 cells in cultured chorionic plate and 8/10 cells for villus stroma, while the amnion was diploid in the 5 metaphases analyzed.

The findings from the conventional cytogenetic analyses suggested that this mosaic conceptus was affected with confined placental mosaicism. In order to explore the extent of chromosomal mosaicism in the placenta, FISH analysis was performed on the villus stroma and trophoblast cell lineages from two sites. Following enzymatic separation of the stroma from the chorionic villi, FISH analysis revealed trisomy for chromosome 16 in 50.4% (site 1) and 68.2% (site 2) of the nuclei. High levels of trisomy were also found in both sites of the trophoblast (79.6% and 72.4%).

FISH analysis with a chromosome 16 specific probe was performed on the lung, kidney and brain since they represent the fetal endoderm, mesoderm and ectoderm, respectively. The proportion of nuclei with three signals for these tissues were 1.2%, 2.2% and 6.6%, respectively, which were less than the cut-off values for trisomy (8.82%, 6.17% and 7.06%). The cord blood showed 5.8% of the nuclei with three signals while the upper reference limit of the 95%

confidence interval for detection of trisomy was 5.92%. Therefore no significant levels of trisomy were observed in any fetal somatic tissues analyzed.

High levels of trisomy 16 in the placenta of a diploid fetus has been associated with a meiotic origin of the extra chromosome (Kalousek et al., 1993; Robinson et al., 1997). Molecular analysis of marker D16S398 on chromosome 16 showed three alleles for the villus stroma, trophoblast and amniotic membrane. This indicated that the extra chromosome in the trisomic tissues arose from a meiotic nondisjunction event. PCR amplification of marker D16S423 indicated maternal UPD for this case since fetal tissues showed maternal contribution for the chromosome 16 marker while there was no contribution from either of the paternal alleles. The single allele for the fetal tissues indicated that the fetus was homozygous for this microsatellite marker even though the mother was heterozygous. Since there was a meiotic origin of the extra chromosome, the reduction to homozygosity of this allele in the fetus most likely arose from meiotic recombination occurring between this marker and the centromere of the chromosome. Meiotic recombination in this region of the chromosome is likely since marker D16S423 is approximately 48.7 centimorgans away from the centromere (Buetow et al., 1994).

The meiotic origin of the trisomic cell line and maternal UPD in fetal tissues suggest that the mosaic gestation arose from a trisomic zygote rescue. Further, the

finding of diploid cells in the chorionic stroma suggests that the formation of the diploid cell line occurred early in development before the extraembryonic mesoderm fully differentiated from the fetal tissues. Alternatively, the trisomic extraembryonic mesoderm may have arisen from the hypoblast while the diploid extraembryonic mesoderm differentiated from diploid epiblast cells.

The molecular result from the amniotic membrane is not in agreement with the conventional cytogenetic analysis. The sample of amnion for molecular analysis and conventional cytogenetics was taken from the amniotic sac which consists of ectoderm and mesoderm. The amniotic ectoderm is derived from the same cell lineage which gives rise to fetal ectoderm, and the amniotic mesoderm is derived from the extraembryonic mesoderm (Vogler, 1987). Therefore the third allele in the microsatellite analysis is probably a result of trisomy in the amniotic mesoderm. The diploid result obtained from the conventional cytogenetic analysis of the amnion sampled from the amniotic sac suggests that there was selective growth of diploid cells in culture. Another possibility is that trisomic cells grew in culture but were missed in the analysis since only 5 metaphases were analyzed.

Identification of oocytes for FISH analysis was possible in this conceptus of 12 weeks gestational age because female meiosis is initiated at 8 weeks of gestation. The meiotic oocytes have diffuse chromatin and are much

larger than somatic cells. The molecular cytogenetic analysis of these oocytes showed that 26.0% were trisomic for chromosome 16. This is in agreement with our hypothesis that the extraembryonic mesoderm contributes to the formation of the secondary yolk sac endoderm and in doing so is involved in the formation of the PGCs. This is also in agreement with the twinning hypothesis proposed by Kalousek (1993). In this scenario, the trisomic zygote rescue occurred in a fraction of the embryonic progenitor cells at the blastocyst stage. The trisomic embryonic progenitors gave rise to a nonviable trisomic twin and diploid cells contributed to the chromosomally normal embryo. The extraembryonic mesoderm is formed from trisomic cells of hypoblast origin and diploid cells from the primitive streak. Thus, since the extraembryonic mesoderm was mosaic, it contributed trisomic cells to the development of the secondary yolk sac and subsequently the PGCs.

Another possibility is that the hypoblast cell lineage was trisomic in this gestation and contributed to the formation of the primary and secondary yolk sac as well as to the extraembryonic mesoderm, as suggested by Enders and King (1988) and Bianchi et al. (1993). If we consider that the PGCs differentiate from the endoderm of the secondary yolk sac, then it is expected that they are at risk of being trisomic as well.

It is also possible that the human PGCs are derived from the epiblast cells, as is the case in the rodent

(Bianchi et al., 1993). Clonal analysis of epiblast cells by injection of a fixable fluorescent dye followed by fate mapping has shown that the clones which gave rise to the PGCs were derived from the same region of the epiblast which contributed to the formation of the extraembryonic mesoderm (Lawson and Hage, 1994). In the mouse, the PGCs migrate into the endoderm of the yolk sac and then migrate back into the germinal ridge (ibid.). Perhaps the epiblast of case 2 became mosaic with a diploid and trisomic cell line subsequent to the trisomic zygote rescue. The cells of the epiblast which gave rise to the fetal somatic tissues were all diploid due to selection forces or by chance, and both diploid and trisomic cells contributed to the formation of the extraembryonic mesoderm and PGCs. Such a hypothesis suggests that the precursor populations for major tissue types are localized to specific regions in the epiblast. Embryological studies by manipulation of mouse embryos have constructed fate maps of the epiblast to depict the geographical organization of the progenitors of various embryonic tissues. For example, cells in the distal part of the epiblast eventually give rise to the neuroectoderm and surface ectoderm of the early somite stage embryo (Quinlan et al, 1995; Parameswaran and Tam, 1995). The cells in the lateral epiblast midway along the proximal distal axis give rise mostly to embryonic mesoderm while the proximal epiblast cells contribute to the extraembryonic mesoderm and PGCs (Parameswaran and Tam, 1995). These results are

consistent with the observations made by Lawson and Hage in which proximal epiblast cells in the mouse gave rise to both extraembryonic mesoderm and PGCs.

The trisomic result found in oocytes of this mosaic gestation suggests that there is a similar origin of the extraembryonic mesoderm and PGCs. However, since the exact stage of development during which the trisomic zygote rescue took place in this mosaic gestation is not known, these results do not support one of the above hypotheses over another.

4.3.c. Case 3

Conventional cytogenetic analysis was previously performed on this gestation and published by Kalousek et al. (1993). Direct preparation from the trophoblast cell lineage showed that 9/9 cells were trisomic for chromosome 16 as were 47/47 cells from cultured chorionic stroma. Conversely, 15 cells from cultured amnion, skin, kidney and spleen as well as 63 cells from blood were found to be diploid by conventional cytogenetic analysis. Molecular analysis on blood of the fetus revealed that it was UPD for the maternal copies of chromosome 16. Therefore Kalousek et al. (1993) classified this gestation with CPM type III and suggested that it arose from trisomic zygote rescue.

FISH analysis with a chromosome 16 specific probe was performed on formalin fixed - paraffin embedded archival

placenta, kidney, lung and oocytes. The FISH analysis failed on several attempts for the kidney and three sites from the placenta while it was successful for the lung and oocytes. Although analysis of formalin fixed - paraffin embedded tissues is essential for the study of cases several years old, FISH analysis on such tissues can become problematic. For example, the tissues may not have been fixed in formalin for several days after the pregnancy was terminated or the tissues may not have been fixed in formalin for a sufficient period of time. As a result, the genomic DNA may have degenerated, making the FISH analysis difficult.

The lung and oocytes showed 3.2% and 5.0% of nuclei with three signals while the cut-off values from the disomic controls were 9.92% and 6.87% respectively. The data provided by Kalousek et al. (1993), indicated that cultured amniotic fluid, post-termination amniotic membrane, skin, kidney, spleen and blood showed only diploid cells while all cells from direct analysis of chorionic villi, culture of chorionic plate and villi showed trisomy in all cells analyzed. Together these results provide strong evidence that the placenta has high levels of trisomy 16 in trophoblast and stroma while the fetus is diploid. Unlike case 2, the oocytes of this gestation had no significant levels of trisomy 16. However, in this FISH analysis, the ability to detect trisomy in the oocytes was compromised by the paired chromosomes in zygotene and pachytene stages of

meiosis. Since technical difficulties prevented the ability to perform two - colour FISH with an internal control on the paraffin embedded - formalin fixed oocytes, one - colour FISH with a chromosome 16 specific probe was used. Thus it was not possible to select oocytes which had unpaired chromosomes for FISH analysis. In order to explore the effects that paired homologues may have had on the ability to detect trisomy in oocytes, FISH analysis was performed on formalin fixed - paraffin embedded oocytes from a trisomy 18 fetus. As shown in Table 6.9 in the Appendix, the chromosome 18 specific DNA probe showed one signal in 29%, two signals in 36% and three signals in 39% of the oocytes analyzed, even though it is expected that 100% of the oocytes are trisomic for chromosome 18. The results from this trisomic fetus are consistent with those obtained by Cheng et al. (1995). They studied the pairing characteristics of homologous chromosomes in oocytes from trisomy 18 fetuses. FISH analysis with a chromosome 18 specific paint indicated that the homologues in the pachytene stage of meiosis were arranged in three possible configurations: 1) all three chromosomes synapsed to form one thick hybridization signal; 2) two of the chromosomes synapsed while one remained unpaired; and 3) all three chromosomes remained unpaired and were visualized as three separate univalents. Thus, the pairing characteristics of homologous chromosomes decreased the ability to detect trisomy in oocytes from Case 3, and there is a possibility

that low levels of trisomy may have been undetectable using this analysis.

In order to illustrate the advantage of using an internal control to select for oocytes in which the homologous chromosomes were not paired, FISH analysis was performed on fresh oocytes from a trisomy 18 fetus. Since the oocytes were not formalin fixed - paraffin embedded, two colour FISH was used successfully to visualize centromeric specific probes for chromosomes 18 and 16. In this analysis the oocytes were scored for the number of signals displayed by the chromosome 18 specific probe only if the internal control, chromosome 16, showed two hybridization signals. As shown in Table 6.11 in the Appendix, 4% of the oocytes showed one signal, 17% showed two signals and 71% showed three signals for chromosome 18. A comparison of these results with those obtained from the formalin fixed - paraffin embedded oocytes above, shows the advantage of performing FISH analysis on fresh oocytes.

One approach which may be useful for future studies of aneuploidy in oocytes would be to use a cosmid DNA probe specific for DNA sequences close to the centromere. Since cosmid probes are typically smaller than the commercial alpha satellite centromeric probes, smaller hybridization signals may allow for accurate identification of the number of homologous chromosomes which are paired.

The lack of trisomic oocytes in Case 3 suggest that not all cases diagnosed with CPM type III will show germline

mosaicism. This can occur for several different reasons. The position of the trisomic cells in the mosaic gestation may be the most important factor. For example, the trisomic extraembryonic mesoderm may have contributed to the formation of the PGCs in case 2 but not case 3. Developmental age of the fetus may also play a role. It is possible that case 3 showed no evidence of trisomic oocytes as a result of selection due to a developmental disadvantage of oocytes with an extra chromosome 16. The pregnancy from case 2 was terminated at 12 weeks while case 3 was terminated at 25 weeks gestation. During development, the majority of oocytes undergo apoptosis so that the number declines from 7 000 000 at 18 weeks to 400 000 by 28 weeks of development. It is possible that trisomic oocytes from case 3 were selected against during this developmental process.

4.4 Clinical Implications of this Study

The trisomic oocytes identified in case 2 of this study suggests that individuals who are classified with CPM type III will also have chromosomal mosaicism in their germ cells. Chromosomal mosaicism identified in the germ cells may affect the fecundity of an individual in several ways. Firstly, the aneuploid germ cells may not be capable of developing into fertile ova/spermatozoa. Depending on the proportion of aneuploid germ cells in the gonad, this may

lead to insufficient ova/spermatozoa production and thus infertility. For example, the majority of males affected with trisomy 21 are infertile and are unable to produce sperm or have a marked decrease in spermatogenesis. (Stearns et al., 1960). Another possibility is that the germ cells are capable of completing development and fertilization; as is the case in females affected with Down's syndrome. These individuals are able to conceive children with a normal karyotype, but are at a greater risk of producing aneuploid conceptuses which are expected to abort (Neri, 1984). In individuals affected with germ cell chromosomal mosaicism, the proportion of trisomic germ cells will influence the individuals fecundity in the reproductive years. A high proportion of aneuploid germ cells is expected to decrease an individual's ability to conceive normal children, while a low proportion of aneuploid germ cells may have little or no detrimental effects.

Although this study suggests that a diagnosis of CPM type III is associated with germ cell mosaicism, it is limited in the ability to predict the incidence of germ cell mosaicism in cases diagnosed with CPM type III. The failure to find a significant level of trisomy in case 3 indicates that germ cell mosaicism may not occur in all cases diagnosed with CPM type III. Further studies must be performed in order to determine the empirical risk of germline mosaicism in individuals diagnosed with CPM types II and III. This study has shown that trisomy for

chromosome 16 can persist in the germ cells until at least 12 weeks of gestation. However, older fetuses and individuals of reproductive age must be studied to determine the effects of chromosomal mosaicism on the developmental dynamics of the germ cells.

4.5. Concluding Remarks

This study was undertaken to explore the possibility that mosaic conceptuses diagnosed with CPM types II and/or III are associated with germline mosaicism. Three mosaic gestations which were ascertained as a result of suspected CPM were analyzed using interphase FISH in order to determine the tissue specific distribution of chromosomal mosaicism. The molecular cytogenetic analysis proved to be a useful technique since nondividing tissues including formalin fixed - paraffin embedded archival tissues were amenable to cytogenetic analysis. This method allowed for analysis of hundreds of nuclei which increased the ability to detect mosaicism and avoided the risk of tissue culture bias which can be experienced with conventional cytogenetic analysis.

For case 1, the distribution of trisomy 7 in the placenta was not determined since the villus stroma and cytotrophoblast of the placenta could not be analyzed separately. The FISH analysis showed significant (11 to 23%) but low levels of trisomy 7 in the placenta; however,

the specific level of mosaicism in the villus stroma could not be determined. The ability to detect trisomy in the germ cells of this fetus was compromised by the inability to distinguish gonocytes from somatic cells in the testis. Since the proportion of germ cells in the prepubertal testis is significantly less than 15% then levels lower than 100% of trisomy in the germ cells would be difficult to detect.

Cases 2 and 3 of this study were more informative than case 1 for two reasons: 1) the fetuses were female and thus the oocytes were distinguishable from somatic cells of the ovary; and 2) high levels of trisomy for chromosome 16 were observed in the stroma of the placenta by FISH and/or conventional cytogenetic analysis. Molecular cytogenetic analysis of case 2 showed significant levels of trisomy in both placental cell lineages and the oocytes while significant levels were not detected in the fetal somatic cells. This finding is in agreement with the hypothesis that mosaicism detected in the stroma of the placenta is associated with germ cell mosaicism. However, the failure to detect germl cell mosaicism in case 3 indicates that this observation does not hold true for every individual diagnosed with CPM type III.

To further explore the clinical implications of this study, many more mosaic conceptuses diagnosed with confined placental mosaicism must be analysed. Cytogenetic analysis of germ cells from fetuses of several gestational ages as well as adults with a history of CPM type III will provide

information about the emperical risk of germ cell mosaicism and on the developmental potential of aneuploid germ cells.

The results from case 2 also suggest that the developmental origin of the PGCs is the same as the villus stroma. We propose that the extraembryonic mesoderm is involved in the repair of the ruptured yolk sac at 14 days of human development to form the secondary yolk sac. The extraembryonic mesoderm may therefore, contribute to the formation of the PGCs which are first observed in the endoderm of the secondary yolk sac at 4 weeks of development. As a result of the lack of current knowledge about the developmental origin of the PGCs and the extraembryonic mesoderm in humans, several hypotheses concerning early embryologic development can not be distinguished. The results from this study do not favor one postulate over another to explain the developmental events involved in the formation of the PGCs for several reasons: 1) the possibility of selection against mutant cell lines during human development; 2) the exact timing of the mutational event which gave rise to the diploid cell line in cases 2 and 3 is not known; and 3) the exact cell which first gave rise to the diploid cell line in early embryogenesis can not be traced.

A more informative method to establish the origin of the PGCs in humans would be to construct fate maps by labeling precursor cells of early embryos and following their developmental patterns. However, since such

experiments are unethical we must gain insight into the developmental origin of the human PGCs using more indirect approaches. One method would be to develop fate maps in embryos of monkeys by labeling precursor cells and following their developmental patterns. Insight into the developmental origin of the PGCs can also come from the study of human conceptions which are affected with chromosomal mosaicism. Many more conceptuses diagnosed with types II and III CPM must be studied to document the presence of aneuploid cells in the different embryologic cell lineages and correlate that information to the presence of germ cell mosaicism.

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6. Appendix

6.1. Raw Data for Mosaic Conceptuses

Table 6.1: FISH analysis of tissues from Case 1 using a centromeric DNA probe specific for chromosome 7.

Tissue	Proportion of Nuclei with Indicated Number of Signals				
	1	2	3	4	N
Lung	4.2	88.0	7.2	0.6	501
Kidney	4.6	93.2	2.2	0	501
Placenta Site 1	5.2	82.8	11.4	0.6	500
Placenta Site 2	3.6	83.6	12.2	0.6	500
Placenta Site 3	4.0	70.7	23.5	1.8	601
Testis	6.1	91.2	2.5	2.7	708

N = total number of nuclei scored

Table 6.2: FISH analysis of tissues from Case 2 using a centromeric DNA probe specific for chromosome 16.

Tissue	Proportion of Nuclei with Indicated Number of Signals				
	1	2	3	4	N
Brain	7	85.6	6.6	0.8	501
Kidney	5.2	93.6	1.2	0	500
Lung	8.2	89.4	2.2	1.0	500
Blood	6	87.4	5.8	0.8	501
Trophoblast Site 1	2	24	72.4	1	500
Trophoblast Site 2	1.4	18.0	79.6	1.2	504
Stroma Site 1	0.6	29.8	68.2	1.4	500
Stroma Site 2	1.0	48.2	50.4	0.4	1148
Oocytes	7.5	64.8	26	1.7	227

N = total number of nuclei scored

Table 6.3: FISH analysis of tissues from Case 3 using a centromeric DNA probe specific for chromosome 16.

Tissue	Proportion of Nuclei with Indicated Number of Signals				
	1	2	3	4	N
Brain	7	85.6	6.6	0.8	501
Kidney	5.2	93.6	1.2	0	500

N = total number of nuclei scored

6.2 Raw data of FISH analysis performed on control samples for formalin fixed - paraffin embedded tissues.

Table 6.4: FISH analysis of disomic kidney control samples using a centromeric DNA probe.

Patient Number	Proportion of Nuclei with Indicated Number of Signals				N	probe
	1	2	3	4		
91335	5.1	92.3	2.6	0	507	16
96100	7.8	90.2	2.0	0	511	16
96126	6.4	92.0	1.4	0.2	502	16
9628	3.5	94.2	2.3	0	517	16
96144	10.4	80.6	8.4	0.6	500	16
96182	11.2	92.0	1.8	0	500	7
96178	9.8	79.6	7.3	0.8	510	7
96210	3.6	78.6	7.4	1.0	501	7
91111	3.2	87.0	3.2	0.4	500	7
93326	2.2	95.0	2.4	0	500	7

N = total number of nuclei scored

Cut - off value for trisomy = 11.78

Table 6.5: FISH analysis of disomic lung control samples using a centromeric DNA probe.

Patient Number	Proportion of Nuclei with Indicated Number of Signals					
	1	2	3	4	N	probe
96139	11.2	81.6	6	1.2	500	7
96182	9.8	82.4	7.4	0.4	500	7
93187	3.6	94.2	2.2	0	500	7
9628	3.2	94.6	2.2	0	500	7
96100	2.2	95.8	2.0	0	500	7
91335	3.8	92.6	2.8	0.2	503	16
96126	4.4	91.4	2.4	0.8	501	16
93187	5.4	88.8	5.2	0.4	501	16
9673	8.8	89.2	2.0	0	501	16

N = total number of nuclei scored

Cut - off value for trisomy = 9.92

Table 6.6: FISH analysis of disomic controls for placental samples using a centromeric DNA probe.

Patient Number	Proportion of Nuclei with Indicated Number of Signals					
	1	2	3	4	N	probe
93173	11.4	82.8	5.4	0.4	500	7
96182	10.2	85.4	3.8	0.6	500	7
96193	9.2	86.8	3.5	0.5	197	7
96151	8.0	88.1	3.6	0.3	327	7
96173	2.1	95.4	2.1	0.4	512	16
96182	3.8	90.6	4.8	0.8	501	16
96151	6.4	87.4	6.0	0.2	500	16
96144	5.8	90.3	3.7	0.2	513	16

N = total number of nuclei scored

Cut - off value for trisomy = 7.89

Table 6.7: FISH analysis of formalin fixed - paraffin embeded disomic testes using a centromeric DNA probe specific for chromosome 7.

Patient Number	Proportion of Nuclei with Indicated Number of Signals				
	1	2	3	4	N
9628	4.6	92.6	2.6	0.2	500
96126	7.1	90.6	2.3	0	512
93187	6.4	90.6	2.8	0.2	501
93326	6.1	90.8	2.9	0.2	511
93140	6.2	88.6	5.2	0	501

N = total number of nuclei scored

Cut - off value for trisomy = 7.69

Table 6.8: FISH analysis of formalin fixed - paraffin embeded disomic oocytes using a centromeric DNA probe specific for chromosome 16.

Patient Number	Proportion of Nuclei with Indicated Number of Signals				
	1	2	3	4	N
9660	31.4	67.0	2.9	0	102
95399	22	71.6	5.0	1.4	141
96151	26.7	66.3	5.0	2.0	101
95400	36.6	57.4	5.0	1.0	101
9661	20.0	77.3	2.7	0	75
9639	23.0	71.0	5.0	1.0	100
95317	33.0	63.0	3.0	1.0	100
9196	34.0	61.0	4.0	1.0	100
95272	22.0	74.0	3.0	1.0	100
95252	31.0	65.0	4.0	0	100

N = total number of nuclei scored

Cut - off value for trisomy = 6.87

Table 6.9: FISH analysis of formalin fixed - paraffin embeded oocytes from a trisomic 18 conceptus using a centromeric DNA probe specific for chromosome 18.

Patient Number	Proportion of Nuclei with Indicated Number of Signals					
	1	2	3	4	N	Probe
9790	29	36	39	6	100	18

N = total number of nuclei scored

6.3 Raw data for FISH analysis performed on fresh control samples prepared by the squash preparation.

Table 6.10: FISH analysis of disomc fresh oocytes using a chromosome specific centromeric DNA probe.

Patient Number	Proportion of Nuclei with Indicated Number of Signals				N	Probe (Internal control)
	1	2	3	4		
9722	7	89.0	4.0	0	200	16(18)
9650	13.5	83.0	3.5	0	200	16(18)
9694	14.5	80.5	4.5	0.5	200	16(18)
96112	7.5	90.0	2.5	0	200	16(18)
976	8.3	90.7	0.5	0.5	193	16(18)
9689	6.2	89.7	3.9	0.2	517	X(8)
9659	4.0	94.2	1.8	0	502	X(8)
9683	5.4	92.4	2.2	0	501	X(8)

N= total number of oocytes scored

Cut - off value for trisomy = 7.06

Table 6.11: FISH analysis of a squash preparation of an oocyte from a trisomy 18 syndrome fetus.

Patient Number	Proportion of Nuclei with Indicated Number of Signals				N	Probe (Internal control)
	1	2	3	4		
9733	4	17	71	8	100	18(16)

N= total number of nuclei scored

6.4 Raw data for FISH analysis of touch preparations of disomic fresh control tissues.

Table 6.12: FISH analysis of disomic fresh kidney using a chromomsome specific DNA centromeric probe.

Patient Number	Proportion of Nuclei with Indicated Number of Singnals				N	Probe
	1	2	3	4		
9662	3.9	94.3	1.8	0	441	16
9522	2.7	93.8	3.3	0.2	600	18
9643	4.0	92.0	3.0	0	505	18
9645	12	86.8	1.2	0	500	2

N = total number of nuclei scored

Cut - off value for trisomy = 6.17

Table 6.12: FISH analysis of disomic lung touch preparations using a chromosome specific DNA centromeric probe.

Patient Number	Proportion of Nuclei with Indicated Number of Signals					
	1	2	3	4	N	Probe
96115	4.2	94.8	1.0	0	500	16
9643	5.1	89.5	5.4	0	600	18
9612	5.0	93.0	2.0	0	500	2
9659	7.2	93.8	3.2	0	500	18
9645	9.4	84.6	5.8	0.2	500	7

N= total number of nuclei analyzed

Cut - off value for trisomy = 8.82

Table 6.13: FISH analysis of disomic fresh brain using a centromeric chromosome specific DNA probe.

Patient Number	Proportion of Nuclei with Indicated Number of Signals					
	1	2	3	4	N	Probe
9659	8.8	89.0	2.2	0	504	18
9685	3.0	92.4	4.4	0.2	502	16
96115	3.4	94.4	2.2	0	502	16
96106	3.3	95.2	1.5	0	522	16
9694	2.0	91.6	5.8	0.6	500	16
9657	6.0	93.0	1.0	0	501	16
9682	5.2	92.4	2.4	0	500	16
9676	5.2	92.8	1.8	0.2	500	16

N = total number of nuclei scored

Cut - off value for trisomy = 7.57

Table 6.14: FISH analysis of disomic cord blood using a chromosome specific centromeric DNA probe.

Patient Number	Proportion of Nuclei with Indicated Number of Signals					
	1	2	3	4	N	Probe
96106	2.0	93.2	4.6	0.2	504	16
9712	3.6	93.6	2.2	0.6	502	16
9721	6.8	89.8	3.4	0.2	503	16
96112	3.0	94.2	2.8	0	500	16
9655	5.2	92.6	1.8	0.4	502	16
9722	6.3	90.4	3.3	0	511	16
9657	7.0	89.6	3.0	0.4	503	18
9658	5.0	90.7	4.0	0.4	505	18
976	5.8	92.6	1.6	0	501	16

N = total number of nuclei scored

Cut - off value for trisomy = 5.92

6.5 FISH analyis of fresh enzymatically digested disomic placenta.

Table 6.15: FISH analysis using a centromeric DNA probe specific for chromosome 16, of disomic trophoblast isolated from chorionic villi.

Patient Number	Proportion of Nuclei with Indicated Number of Signals					
	1	2	3	4	N	Probe
9694	3.1	95.1	1.8	0	512	16
9722	2.5	94.7	2.1	0.6	512	16
96115	3.5	94.9	1.2	0.4	492	16
9662	1.2	85.0	3.8	0	503	16
9612	3.5	95.1	1.2	0.2	511	16
9645	3.2	89.4	7.2	0.2	500	18
9650	8.8	89.0	2.0	0.2	500	18
9659	3	92.0	5.0	0	504	18

N = total number of nuclei scored

Cut - off value for trisomy = 6.59

Table 6.16: FISH analysis using a chromosome specific centromeric DNA probe, of fresh stromal cells isolated from chorionic villi.

Patient Number	Proportion of Nuclei					
	1	2	3	4	N	Probe
9645	1.6	89.5	3.7	0	600	18
9486*	3	95.8	1.2	0	500	16
94107*	10.8	87.0	1.0	0.4	500	16
9259*	9.6	87.6	1.8	1.0	500	18
9454*	6.2	91.6	1.6	0.4	500	16
94105*	2.0	93.4	3.8	0	500	7

* Laboratory established control data.

N = total number of nuclei scored

Cut - off value for trisomy = 6.59