TRANSMISSION AND DISTRIBUTION PATTERN OF DISEASE-RELATED GENES IN EARLY MAMMALIAN EMBRYOS

By

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PREFACE

Presentation of the current thesis

This Ph.D. thesis was prepared in accordance with the "*Guidelines for Thesis Preparation*" issued by the Faculty of Graduate Studies and Research at McGill University. I have elected to present my research in a manuscript-based format. The *Contributions of Authors* is clearly indicated in the Preface. The thesis includes a *Table of Contents* and an *Abstract* in English and in French. Chapter 1 contains a *General Introduction*, which provides a comprehensive review of the relevant literature and states the rationale and the objectives of the Ph.D. research. This is followed by Chapters 2 to 5, which are based on series of manuscripts of which I was an author. This thesis contains work from four publications of which I was the first author and one of which I was the second author (see list below). In order to ensure continuity, each of these chapters includes a foreword, which bridges the specific topics addressed. A summary of the work and the final conclusions are presented in Chapter 6, followed by a complete list of the references cited in Chapters 1 to 6. Finally Appendices I and II include a statement on the *Contribution to Original Knowledge*, and a list of all scientific communications, comprised of the abstracts, posters, oral presentations and manuscripts that have resulted from the research work described in the thesis. All relevant ethics certificates and copyright waivers have also been submitted.

Published manuscripts included in the current thesis

1. Deborah L Blake, Nicola L Dean, Casey Knight, Seang Lin Tan, Asangla Ao (2001) Direct comparison of detection systems used for the development of

single-cell genetic tests in preimplantation genetic diagnosis. *Journal of Assisted Reproduction and Genetics* **18**(10):557-565.

2. Nicola L Dean, Seang Lin Tan, Asangla Ao (2001) The development of preimplantation genetic diagnosis for myotonic dystrophy using multiplex fluorescent polymerase chain reaction and its clinical application. *Molecular Human Reproduction* **7**(9):895-901.

3. Nicola L Dean, J Concepción Loredo-Osti, T Mary Fujiwara, Kenneth Morgan, Seang Lin Tan, Anna K Naumova, Asangla Ao (2006) Transmission ratio distortion in the myotonic dystrophy locus in human preimplantation embryos. *European Journal of Human Genetics* **14**(3):299-306.

4. Nicola L Dean, Seang Lin Tan, Asangla Ao (2006) Instability in the transmission of the myotonic dystrophy CTG repeat in human oocytes and preimplantation embryos. *Fertility and Sterility* **86**(1):98-105.

5. Nicola L Dean, Brendan J Battersby, Asangla Ao, Roger G Gosden, Seang Lin Tan, Eric A Shoubridge, Maria J Molnar (2003) Prospect of preimplantation genetic diagnosis for heritable mitochondrial DNA diseases. *Molecular Human Reproduction* **9**(10):631-638.

Contribution of authors

The data presented in Chapters 2 to 5 of the current thesis contains text adapted from these published papers. The candidate performed the majority of experiments, data analysis and preparation of manuscripts for these publications. All the work was directly supervised by Dr. Asangla Ao and co-supervised by Dr. Seang Lin Tan. The following individuals appeared as co-authors on some of these manuscripts and their contribution was as follows:

Chapter 2

Deborah Blake, Casey Knight and Dr. Dewen Kong provided technical assistance for some of the cystic fibrosis experiments. Deborah Blake co-wrote the manuscript.

Chapter 3

Drs. Anna Naumova and Kenneth Morgan advised on the design of this study. Assistance with the statistical analysis for the manuscript was provided by Concepción Loredo-Osti and Mary Fujiwara.

Chapter 5

Dr. Brendan Battersby provided technical assistance in caring and preparing the mice and in the addition of the radioactive isotope. The experiments were partly carried out in Dr. Eric Shoubridge's laboratory and he assisted in the experimental design. Dr. Roger Gosden also assisted in the design of the experiment. Dr. Maria Molnar carried out some preliminary work in but none of her data were incorporated in this thesis.

ABBREVIATIONS

AD	Autosomal dominant
ADO	Allele drop-out
AME	Amelogenin gene
AR	Autosomal recessive
AS	Angelman syndrome
ATP	Adenosine triphosphate
BWS	Beckwith-Wiedemann syndrome
CAG	Cytosine-adenine-guanine
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary deoxyribonucleic acid
CDM1	Congenital dystrophia myotonica type 1
CEPH	Centre d' Etude du Polymorphisme Humain
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator gene
CGH	Comparative genome hybridisation
CTG	Cytosine-thymine-guanine
CUG	Cytosine-uracil-guanine
CUG-BP	Cytosine-uracil-guanine binding protein
CV	Coefficient of variation
dGTP	Deoxyguanosine triphosphate
DM1, 2 and 3	Dystrophia myotonica type 1/type 2/type 3
DMPK	Human dystrophia myotonica-protein kinase gene
DMSO	Dimethylsulphoxide
DMWD	Myotonic dystrophy tryptophan–aspartate repeat gene
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DOP PCR	Degenerate oligonucleotide primed polymerase chain reaction
ESHRE	European Society of Human Reproduction and Embryology
FISH	Fluorescent *in-situ* hybridisation
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hES	Human embryonic stem cells
HLA	Human leukocyte antigen
KSOM	Potassium simplex optimised media
ICM	Inner cell mass
IVF	*In-vitro* fertilisation

LB	Lysis buffer system
LHON	Leber hereditary optic neuropathy
MDA	Multiple displacement amplification
mRNA	Messenger ribonucleic acid
mtDNA	Mitochondrial deoxyribonucleic acid
NARP	Neuropathy, ataxia and retinitis pigmentosa
OMIM	Online Mendelian inheritance in man
OXPHOS	Oxidative phosphorylation
PB	Polar body
PBS	Phosphate buffered saline
PEP PCR	Primer extension pre-amplification polymerase chain reaction
PCR	Polymerase chain reaction
PGD	Preimplantation genetic diagnosis
PK	Proteinase K
POLG	Mitochondrial DNA polymerase γ
PWS	Prader-Willi syndrome
RLFP	Restriction length fragment polymorphism
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
ROS	Reactive oxygen species
RT-PCR	Reverse transcriptase polymerase chain reaction
SCA1	Spino-cerebellar ataxia type 1
SDS	Sodium dodecyl sulphate
SIX5	Homologue of *Drosophila* sine oculis homeobox 5 gene
SLSJ	Saguenay-Lac-St-Jean
SNP	Single nucleotide polymorphism
SSCP	Single-strand conformational polymorphism analysis
STR	Short tandem repeat
TP-PCR	Triplet primed polymerase chain reaction
TRD	Transmission ratio distortion
tRNA	Transfer ribonucleic acid
UPD	Uniparental disomy
UTR	Untranslated region

ABSTRACT

Knowledge of the mechanisms involved in the transmission of pathogenic mutations during early development is paramount to understanding the inheritance of human disease. The current thesis investigated the transmission to early embryos of the $(CTG)_n$ repeat in the human dystrophia myotonica-protein kinase (*DMPK*) gene, which is expanded in dystrophia myotonica type 1 (DM1) and of heteroplasmic mitochondrial DNA (mtDNA). For each of these studies, single *in-vitro* cultured human or *in-vivo* generated mouse oocytes and embryos were genotyped for the specific DNA sequences of interest.

Initially, genotyping was optimised at the single-cell level using different protocols and methods of product detection. Fluorescent-based PCR analysis of the mutation of interest multiplexed with a linked polymorphic marker was found to provide maximal efficiency and accuracy. Once standardised, these tests were applied to clinical preimplantation genetic diagnosis (PGD) and served as the experimental basis for further work presented in this thesis. This included two studies investigating the transmission of the *DMPK* repeat. In the first study, the objective was to determine whether transmission ratio distortion (TRD) in favour of larger normal-sized alleles ($(CTG)_{19-37}$) was evident during early human embryogenesis. A statistically significant TRD in favour of *DMPK* $(CTG)_{19-37}$ repeats was observed in embryos, which remained significant when female embryos were considered separately. This TRD was shown to be specifically due to the transmission of repeats in the $(CTG)_{19-37}$ range. The developmental time-point of TRD of these alleles was suggested to occur around the time of fertilisation. In addition, instability in this repeat length was demonstrated during paternal transmission. The

second objective was to further elucidate the timing and variability of the intergenerational enlargements observed in expanded *DMPK* alleles in early development. From our analysis, repeat expansion was found to occur in oogenesis, most likely before the completion of meiosis I. Furthermore, variable degrees of expansion were observed in each cohort of embryos, which are comparable to the phenotypic variability seen between DM1 siblings.

In the final chapter, the segregation pattern of murine heteroplasmic mtDNA in oocytes and early embryos was examined in order to determine the feasibility of PGD for mtDNA mutations. This study demonstrated that the mean level of heteroplasmy in the polar body of an oocyte or a single blastomere of an embryo was representative of the level in the embryo as a whole and, therefore, PGD would be a viable option for women heteroplasmic for mtDNA point mutations.

RÉSUMÉ

La connaissance des mécanismes impliqués dans la transmission de mutations pathogènes en début de développement est d'une extrême importance pour comprendre l'hérédité des maladies chez l'humain. La présente thèse a examiné la transmission à des embryons au stade précoce de la répétition de $(CTG)_n$ dans le gène de la protéine kinase de la dystrophie myotonique humaine (*DMPK*), qui est amplifiée dans la dystrophie myotonique de type 1 (DM1), et de l'ADN mitochondrial (ADNmt) à l'état hétéroplasmique. Dans chacune de ces études, des ovocytes humains cultivés *in vitro* et des ovocytes de souris produits *in vivo* ainsi que des embryons ont été génotypes pour les séquences d'ADN spécifiques.

Initialement, le génotypage a été optimisé au stade unicellulaire à l'aide de divers protocoles et procédés de détection de produits. L'analyse PCR en fluorescence de la mutation en question a été réalisée en multiplex avec un marqueur polymorphe lié et s'est révélée d'une efficacité et d'une exactitude maximales. Une fois standardisés, ces tests ont été appliqués au diagnostic génétique de préimplantatoire clinique (DPI) et ont servi de base expérimentale aux autres travaux présentés dans cette thèse, dont deux études portant sur la transmission de la répétition du *DMPK*. L'objectif de la première étude était de déterminer si la distorsion de transmission allélique (DTA) en faveur de plus grands allèles de taille normale $(CTG)_{19\text{-}37}$ était apparente pendant l'embryogenèse humaine au stade précoce. Une DTA significative sur le plan statistique en faveur de répétitions du *DMPK* $(CTG)_{19\text{-}37}$ a été observée dans des embryons, et elle est demeurée significative lorsque des embryons femelles ont été étudiés séparément. Cette DTA était spécifiquement due à la transmission de répétitions dans la palette de $(CTG)_{19\text{-}37}$. Le

temps ponctuel de développement de la DTA de ces allèles a été présumé se produire au moment de la fécondation. De plus, une instabilité dans la longueur de cette répétition a été démontrée dans la transmission paternelle. Le deuxième objectif était de comprendre davantage le timing et la variabilité des croissances intergénérationnelles observées dans les allèles *DMPK* amplifiés en début de développement. Notre analyse a démontré que l'expansion de la répétition se produisait pendant l'ovogenèse, fort probablement avant la fin de la méiose I. En outre, des degrés variables d'expansion ont été observés dans chaque cohorte d'embryons et ils sont comparables à la variabilité phénotypique rencontrée dans les fratries atteintes de DM1.

Dans le dernier chapitre, le processus de ségrégation de l'ADNmt à l'état hétéroplasmique, a été examiné chez la souris, dans les ovocytes et embryons au stade précoce, afin de déterminer la fonctionnalité du DPI pour détecter des mutations d'ADNmt. Cette étude a démontré que le taux moyen d'hétéroplasmie dans le globule polaire d'un ovocyte ou dans un seul blastomère d'un embryon était représentatif du taux contenu dans l'embryon entier et que, par conséquent, le DPI serait une solution envisageable pour déceler les mutations ponctuelles de l'ADNmt dans le cas de femmes hétéroplasmiques.

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CHAPTER ONE

Introduction, literature review and objectives

1.1 INHERITED GENETIC DISEASE IN THE POPULATION

Inherited genetic diseases are identifiable in 1 - 3% of the general population at birth (Weatheral 1991) and it has been estimated, based on a population study, that before 25 years of age more than one in twenty (5.3%) live-born individuals will suffer from a disease with an important genetic component (Baird et al 1988). In terms of the effects of genetic disease on human health, approximately 25% of disadaptive phenotypes will be apparent at birth and over 90% by the end of puberty (Costa et al 1985). These diseases are composed of single gene defects, including autosomal dominant, autosomal recessive and X-linked disorders and chromosomal abnormalities, multifactorial disorders, and those diseases in which a precise etiology is not identified.

1.1.1 Chromosomal abnormalities

The identification of the 46 human chromosomes (Ford and Hamerton 1956, Tijo and Levan 1956) and the ability to characterise changes in their structure have led to the development of techniques allowing detailed analysis of these chromosomes in order to detect abnormalities. Chromosomal abnormalities may be inherited from a parent or, as in the majority of numerical chromosomal imbalances, arise as a sporadic error in gametogenesis (especially maternal meiosis), at fertilisation or during postzygotic cleavage divisions.

In the human, numerical or structural chromosome changes are a major cause of congenital anomalies, stillbirths and spontaneous abortions. An estimated 20% of human conceptions are chromosomally abnormal (Jacobs 1992). Chromosome anomalies include aneuploidies, an abnormal chromosome number due to the presence of an extra (trisomy) or missing (monosomy) chromosome, triploidy or tetraploidy (3 or 4 sets of

chromosomes), and structural abnormalities. The overall incidence of aneuploidy is directly associated with advanced maternal age (Hassold and Chiu 1985, Hassold and Jacobs 1984). Among the different classes of aneuploidy, Trisomy 21 is the most common and is found in 0.13% of newborns (Hassold and Jacobs 1984, Hook and Hamerton 1977) although trisomies for the sex chromosomes, trisomy 18, trisomy 13 and monosomy for chromosome X are also compatible with postnatal survival (Hassold and Jacobs 1984, Hassold 1986). The phenotypic features of each chromosome syndrome are distinct however, most are associated with developmental delay and mental retardation, alterations in facial morphogenesis, growth delay and congenital malformations.

Structural defects such as translocations, deletions, duplications, inversions, ring chromosomes and isochromosomes are often associated with mental retardation, growth retardation and infertility (Jacobs 1977, Jalbert 1988, Polani 1969). Individuals with balanced abnormalities, such as reciprocal or Robertsonian translocations, can have a normal phenotype but are at risk of having abnormal offspring as they can produce unbalanced gametes (Jalbert 1988).

1.1.2 Single gene defects

Gregor Mendel's fundamental principles of heredity (Mendel 1866) still form the foundation of modern genetics, even though the significance of his work was only understood after his death (Correns 1900, de Vries 1900, Tschermak 1900). In the first half of the twentieth century, several genetic breakthroughs were made including the discoveries that chromosomes are the carriers of genes (Morgan et al 1915) and that genes are composed of deoxyribonucleic acid (DNA) (Avery et al 1944), which is organised in a double helical structure (Watson and Crick 1953). Recently, it has been determined that

the human genome comprises almost 3 billion base pairs of DNA (International Human Genome Sequencing Consortium, 2004). However, the 20,000-25,000 genes estimated to be present make up less than 5% of the total DNA, while the remainder consists of non-coding regions and repeated sequences (International Human Genome Sequencing Consortium, 2004). Currently, this ranges from nearly 3,000 genes on chromosome 1 down to 231 genes on the Y chromosome.

Mutations, which together with recombination lead to the evolution of a species, are changes in the DNA sequence that can arise spontaneously or can be induced by mutagens. The majority, but not all, of these changes will be recognised and corrected by DNA repair enzymes. A mutation in a somatic cell will be present in all future copies of that cell but will not be transmitted beyond the life of the individual. A germ-cell mutation in a spermatozoa- or oocyte-producing cell will be transmitted to the succeeding generation. It is estimated that the number of new mutations in the human zygote is in the order of 100 (Crow 1995) and increases linearly with paternal age, giving a calculated male/female mutation ratio of 5.06 (Huang et al 1997). Some DNA changes are neutral and are called polymorphisms if they occur in at least 1% of the population. If a change, or mutation, is in gene coding DNA, then a single gene (monogenic) defect can result due to either the formation of an abnormal protein or the reduced synthesis of a normal protein.

Every individual is thought to be a carrier of 5-10 deleterious mutations, although most of these are recessive and therefore do not have any serious phenotypic effect. About 80-85% of these are inherited and the remainder represent *de-novo* mutations. As of July 17, 2006, the Online Mendelian Inheritance in Man (OMIM) database comprises 11,251 entries describing mutations in genes that cause genetic disorders with known

sequence and 5,671 entries describing phenotypes. Overall, nearly 17,000 entries were present over 9,825 loci, however, the actual number of distinct diseases represented is less because some genes encode untranslated ribonucleic acids (RNA), some proteins are encoded by more than one gene, and dissimilar phenotypes can be caused by a single mutation.

The consequences of single gene mutations are varied and depend on the type of mutation and the genomic site affected by it. Mutations can occur within introns or exons of protein coding genes. If a mutation occurs within a promoter or enhancer of an intron it can interfere with the binding of transcription factors, often leading to decreased messenger RNA (mRNA) and, consequently, decreased protein. Other mutations within introns, such as splice-site mutations, can also alter the processing of RNA and can lead to abnormally spliced mature mRNA.

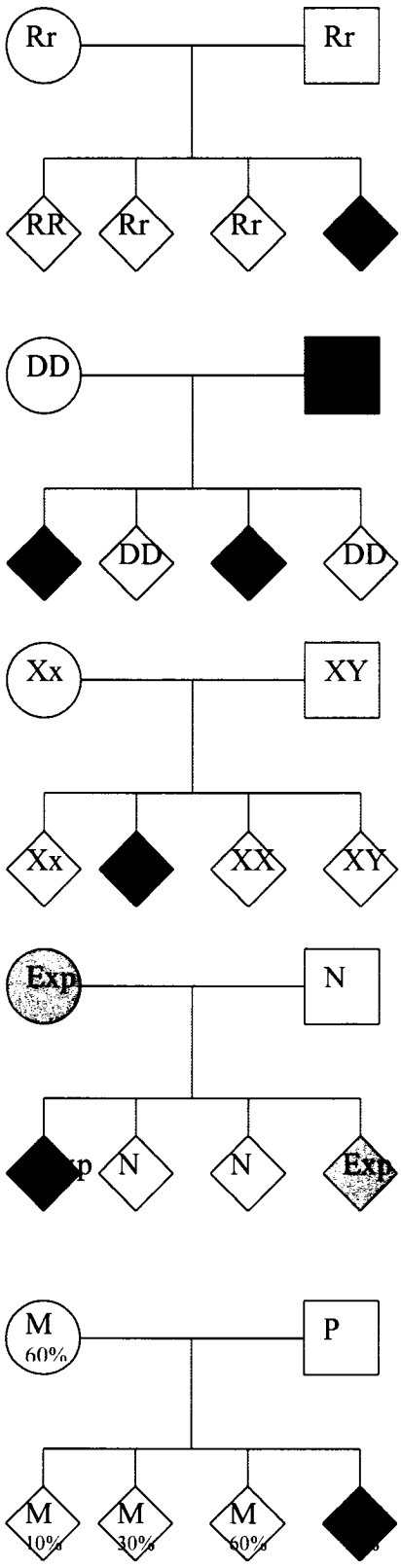
When one base pair is replaced by a different base pair, a point mutation arises. If a point mutation occurs in an exon, it can have no effect (a silent mutation) or result in a change in a single amino acid (a missense mutation) or cause premature termination (a nonsense mutation) or abnormal elongation of the polypeptide. Another major type of mutation is the deletion or insertion of base pairs as they can result in a protein containing extra or missing amino acids. This is most detrimental when the change is not a multiple of three, the number of amino acids in a codon, and results in a frameshift mutation.

Single gene defects are classified according to the way in which they are inherited and as to whether they involve the autosomes or the X chromosome (Figure 1.1).

1.1.2.1 Mendelian autosomal conditions

Genetic diseases involving the autosomes (chromosomes 1-22) can be described as having dominant or recessive inheritance and affect males and females equally.

Figure 1.1: Examples of the modes of inheritance of human genetic disease



A: Mendelian autosomal recessive inheritance

The inheritance of two copies of the mutant gene, one from each parent, will lead to the disease phenotype.

R: normal gene, r: gene with mutation

B: Mendelian autosomal dominant inheritance

The inheritance of one copy of the mutant gene, from either parent, can produce a disease phenotype.

D: normal gene, d: gene with mutation

C: Mendelian X-linked recessive inheritance

The inheritance of the maternal mutant gene on the X chromosome will produce a phenotype in sons.

X: normal X gene, x: mutant X gene

D: Dynamic mutation caused by unstable repeat

An expanded repeat in the parental gene can expand further upon transmission leading to expression of a more severe disease phenotype.

N: normal repeat, Exp: expanded repeat

E: Maternal mitochondrial inheritance

Different levels of mutant mitochondrial DNA (mtDNA) can be inherited from the mother and a phenotype will be seen once the threshold level is crossed.

M: maternal mtDNA, P: paternal mtDNA

Phenotypically affected individuals are shaded in red

Recessive disorders

Autosomal recessive (AR) diseases affect between 1:300 and 1:200,000 of individuals (McKusick 1998) and, in most cases, cause severe symptoms in postnatal life. An individual with one normal copy of the gene and one copy carrying a mutation will either be a heterozygous disease carrier without himself being affected or will express a less severe phenotype. However, if his partner is also a carrier of a mutation in the same gene, there is one in four chance that their offspring will inherit the mutated copy of the gene from each parent and thus will be affected by the disease (Figure 1.1A). On average, half of their children will be heterozygous carriers while one quarter will inherit two normal genes. Common AR diseases include cystic fibrosis, β-thalassemia and sickle-cell anemia.

Dominant disorders

Each autosomal dominant (AD) disorder has a gene frequency of around 1:200 to 1:25,000 (McKusick 1998). In AD disorders, a single copy of the mutated gene will lead to the disease and half of all offspring from the affected parent will also be affected (Figure 1.1B). Generally, AD disorders are not as severe as AR disorders, are not always fully penetrant, and can have variable phenotypic expression. As a result of this or because the disease itself can be late onset, AD gene carriers are often able to have children. Examples of AD disorders include familial hypercholesterolemia, Marfan syndrome, neurofibromatosis, polycystic kidney disease and achondroplasia.

1.1.2.2 Mendelian X-linked inheritance

The X chromosome is estimated to carry more than 1,000 genes (International Human Genome Sequencing Consortium 2004). Mutations carried on the X chromosome can be either recessively or dominantly inherited. Almost all severe examples are

recessive and are carried by females who are themselves unaffected or mildly affected because they carry a normal copy of the gene on their second X chromosome. However, half of their sons who inherit the copy of the X chromosome carrying the mutation will be affected, while the other half will be unaffected as they inherit the normal maternal X chromosome (Figure 1.1C). Common X-linked recessive disorders have an occurrence of 1:10 to 1:100,000 (McKusick 1998). Examples of X-linked recessive disorders are Duchenne muscular dystrophy, haemophilia A and red-green colour blindness. X-linked dominant disorders have the least common inheritance apart from Y-linked disorders, which so far have only been found linked to male sexual function. For X-linked dominant disorders, there is no male-to-male transmission, however, both genders can acquire these disorders and transmit the affected gene, as seen in incontinentia pigmenti and hypophosphatemic rickets.

1.1.2.3 Mechanisms of non-Mendelian inheritance in genetic disease

A disease might have a strong genetic component and some familial recurrence but might not follow classic Mendelian inheritance. This includes the groups of diseases described below as well as those occurring through recurrent *de-novo* mutations and those with incomplete penetrance or variable expressivity.

Contiguous gene syndromes

Chromosomal rearrangements involving large genetic regions can cause interstitial or terminal deletions or duplications, and unbalanced translocations, leading to differential gene expression in a number of genes and ultimately imbalanced gene dosage. The clinical expression of these contiguous gene syndromes are correlated with the extent of the DNA changes but most conditions are associated with congenital malformations and mental retardation, such as DiGeorge and cri-du-chat syndromes. Each of these

rearrangements occurs sporadically as a *de-novo* mutation and may arise in specific regions of the genome, suggesting specific mechanisms. Although each of these disorders is rare, when combined, they are responsible for 0.7% of birth defects (Borgaonker 1997).

Multifactorial disorders and inheritance

Multifactorial disorders are probably due to multiple genetic and environmental factors and run in families but do not follow any particular familial pedigree pattern. The genetic determination of these disorders may involve a small number of loci (oligogenic) or many loci, each with a small individual effect (polygenic), or even a single major locus with a multifactorial background. In a multifactorial disorder, when a threshold based on genetic and environmental factors is exceeded, the affected gene or genes are turned on and a phenotype manifested. Often one gender is more frequently affected due to a different threshold of expression between sexes, as in hip dysplasia, which is nine times more common in females. Other examples include congenital malformations (cleft lip and palate and spina bifida) and common adult disorders (asthma, diabetes, cancer, schizophrenia, hypertension and coronary heart disease).

Dynamic mutations

These unstable repeat disorders generally manifest as late-onset diseases and are AD, AR or X-linked, although they show many non-Mendelian features in their inheritance (Figure 1.1D). These repeats are highly heterozygous in the normal population but, once beyond a critical size, become unstable upon intergenerational transmission, leading to an expansion of the repeat sequence. The presence of an expanded repeat sequence causes a progressive neuro-degenerative phenotype. Generally, these disorders display genetic anticipation, which is the tendency for an

increased severity of phenotype and a progressively earlier onset with transmission to successive generations. They also show variable penetrance and parental-origin effects upon repeat transmission.

Currently, close to 20 trinucleotide repeat disorders have been identified which can be classified as type I and type II diseases (Gatchel and Zoghbi 2005). Molecularly, the expansions cause the respective RNA or protein to lose or alter its function. Type I diseases, such as Huntington disease, have a $(CAG)_n$ repeat in the coding region of the gene and a progressive disease-specific loss of particular groups of neurons. In type I diseases, the abnormal protein contains expanded glutamine tracts, which cause cellular dysfunction and eventually cell death. Generally, in type I disorders, a 30 - 40% increase in repeat size can cause disease pathology and very large expansions are not seen. In type II diseases such as Fragile X syndrome, none of the repeats are located in the coding region and can undergo huge expansions. Transmission of a $(CTG)_n$ repeat in a type II disease, dystrophia myotonica type 1 (DM1) is discussed in Section 1.5.2 and in Chapters 3 and 4.

Mitochondrial DNA disorders

In addition to nuclear DNA, all mammalian nucleated cells contain mitochondria, which are the principal generators of cellular adenosine triphosphate (ATP) by oxidative phosphorylation (OXPHOS). Mitochondria contain mitochondrial DNA (mtDNA), which is inherited maternally through the cytoplasm (Giles et al 1980) (Figure 1.1E). Mutations occurring in the mitochondrial genome can result in the phenotypic expression of mtDNA disease. The most recent publications estimate that the prevalence of either having an mtDNA disease or being at risk of developing one could be as high as 1 in 3,500 individuals (Schaefer et al 2004), making these the most common inherited forms

of metabolic disease. Mitochondrial DNA, mtDNA diseases and their modes of inheritance are described in Section 1.5.3.

Epigenetic DNA modifications

Mutations not associated with DNA sequence changes can be implicated in human disease. Epigenetic DNA modification is seen in imprinting disorders. Monoallelic expression or the expression of an allele inherited from one parent with inactivation of its counterpart from the other parent, has been seen in approximately 60 mammalian protein-coding genes (Morison et al 2005) and may be regulated by DNA methylation and chromatin modifications. Disruption of this monoallelic expression can lead to disease. Imprinted genes tend to be clustered in the mammalian genome and, so far, most have been seen to affect the regulation of growth of the fetus and placenta as well as influence behaviour (Reik and Walter 2001, Tycko and Morison 2002).

One AD inherited imprinting disorder is Beckwith-Wiedemann syndrome (BWS), which results from dysregulation of imprinted genes on chromosome 11p15 when maternally inherited. Prader-Willi syndrome (PWS) and Angelmans syndrome (AS) are also imprinting disorders associated with preferential loss of chromosome region 15q11-q13 of the paternal and maternal allele, respectively, and can be caused by *de-novo* deletions or by uniparental disomy (UPD). In UPD, two chromosomes of a pair are inherited from only one parent (isodisomy or hetereodisomy). Whether UPD exerts a phenotypic effect depends on whether a given chromosome displays monoallelic expression for any of its genes, in which case imprinting effects may be seen.

Another epigenetic modification that can lead to disease is skewed X-inactivation. The random inactivation of the majority of genes occurs on one of the two parental X chromosomes in each cell that will give rise to a female fetus (Lyon 1961). Once an X

chromosome is inactivated in a cell, it usually remains inactive in all progeny cells. Females are, therefore, mosaics for two cell lines: one expressing genes from the maternal and the other from the paternal X chromosome. In young females, these two cell lines are expected to approximate a normal distribution. However, different mechanisms can lead to extremely skewed ratios of X-inactivation, resulting in more than 90% of cells having the same X chromosome active. Such skewed X-inactivation can result in female expression of an X-linked recessive disorder, depending on the percentage of cells in which the mutant gene is expressed (Jorgensen et al 1992), and in variable expressivity for women with X-linked dominant mutations.

Transmission ratio distortion

A fundamental principle of Mendelian genetics is the equal transmission to offspring of the two homologues from a heterozygous pair of alleles. However, significant preferential transmission of one allele at the expense of its partner, known as transmission ratio distortion (TRD), has been shown to exist, leading to the survival of offspring carrying chromosomes that skew away from Mendelian predictions (reviewed by Lyttle 1993). Various biological processes can cause TRD, including meiotic drive, which usually occurs in females, where a property of the structure or size of a chromosome gives it an advantage on the meiotic spindle, leading to preferential recovery of the drive chromosome in the functional gametes (Rhoades and Dempsey 1966, Sandler and Novitski 1957). Gametic selection can also occur, causing the differential survival of an individual's gametes, as seen in the Segregation Distorter system in *Drosophila* (Sandler et al 1959) and the mouse *t*-haplotype (Schimenti 2000), or selection involving a preferential fertilisation of a particular oocyte or by a particular spermatozoa carrying a specific allele. Postzygotic viability or preferential survival in favour of or against an

embryo carrying a particular allele can also result in TRD (Pardo-Manuel de Villena et al 2000).

The repeated finding of an excess number of affected offspring, significantly exceeding the percentage expected from Mendelian segregation, could be due to TRD of disease alleles. In humans, TRD has been claimed in the transmission of the cystic fibrosis transmembrane regulator (*CFTR*) gene mutation ΔF508 (Williams et al 1993), in retinoblastoma susceptibility (Munier et al 1992), in the cone rod retinal dystrophy gene (Evans et al 1994) and in split-hand/split-foot malformation (Ozen et al 1999), although it is difficult to prove conclusively. TRD has also been suggested for genes containing repeat regions and this is discussed further in Section 1.5.2.5 and Chapter 3.

1.2 DIAGNOSIS OF HUMAN SINGLE GENE DISORDERS

Single gene disorders can be caused by diverse mutations in different regions of different genes leading to different phenotypes.

1.2.1 Direct and indirect testing for human diseases

Once the gene containing a mutation is cloned, direct molecular diagnosis can be performed which is based on the principle that the gene sequence in affected individuals will differ from the same sequence found in those with normal phenotypes. There are a number of standard molecular techniques used to detect mutations such as Southern blot, dot blot and DNA sequencing (Sambrook and Russell 2001). The introduction of the polymerase chain reaction (PCR) has allowed more rapid and precise amplification of specific DNA sequences (Mullis and Faloona 1987, Saiki et al 1985, 1988) for the direct detection of single mutations, even from very small starting samples.

For many single gene defects with unknown gene sequence, diagnosis can often be made through identifying the phenotype or the protein. This can be performed through the protein truncation test (Sambrook and Russell 2001) or through biochemical testing whereby the quantity of specific amino acids, enzymes or proteins in a particular biochemical pathway can be used as an indicator of genetic mutation.

Linkage analysis, which is used in Chapter 2 of this thesis, can also be useful in the indirect diagnosis of a genetic mutation. Linkage analysis can be used when a gene can be located to within a specific region of a chromosome and linked polymorphic microsatellite markers can be determined (Ott 1991). Genotyping can be performed on family members over three generations, including an affected individual, to establish the phase of the markers with respect to the mutation. The affected haplotype can then be determined and individuals with that haplotype can be diagnosed with the disease.

1.2.2 Model systems for the study of human disease

Model systems, especially the mouse, can aid in the genetic analysis of human diseases. Murine physiology, development and cell biology have many similarities to those of the human, as do the genome size, number of genes and genomic organisation. Mice also have a short lifespan and generation time and produce many offspring, so the effects of transmitting a pathogenic mutation can be monitored fairly rapidly.

Mouse disease models originated spontaneously or were induced by random mutagenesis, but now gene targeting and transgenic technologies allow modification in a chosen target gene to create models of specific human diseases. Most recessive and many dominantly inherited disorders result from the loss of gene function, which can be modelled using a knockout mouse with complete absence of expression for the targeted

gene. Mutations causing a protein gain-of-function, as with many dynamic mutations can be modelled by inserting the mutant human or mouse gene as a transgene. A mouse model is homologous if the symptoms and course of the condition are identical to those in humans. Most mouse models do not fulfil these requirements and may be termed as isomorphic or partial. In an isomorphic mouse, the symptoms are similar but their cause differs from those in the human. Partial mouse models cannot replicate the entire human disease, but they can be used to study certain aspects of it. Mouse models for the specific mutations of interest to this thesis are discussed in Sections 1.5.1.3, 1.5.2.4 and 1.5.3.6.

1.3 PREVENTION OF HUMAN DISEASES

Medical intervention is available to treat just a few genetic disorders, with variable success rates. Gene therapy is one possibility and was first reported to treat severe combined immunodeficiency (Blaese et al 1995). A promising new disease therapy involves the use of pluripotent human embryonic stem (hES) cells, which if directed to differentiate into specific cell types, offer the possibility of a renewable source of replacement cells and tissues. These hES cells could potentially be used to treat diseases such as Parkinson and Alzheimer disease, Duchenne muscular dystrophy, cancer, stroke, heart disease and diabetes although research is in the early stages of development.

Genetic screening is an alternative strategy to prevent the transmission of diseases and involves identifying individuals affected with a disease, at risk of developing one or at risk of having a child affected with a disease before the onset of symptoms.

1.3.1 Population screening

Population screening is the testing of at-risk groups before the appearance of symptoms. Screening of newborns began with a test to detect phenylketonuria (Guthrie and Susi 1963) in which irreversible brain damage can be prevented if an affected infant is put on a restrictive diet soon after birth. Other newborn programs include screening for congenital adrenal hyperplasia, cystic fibrosis, galactosaemia, haemoglobinopathies and hypothyroidism, as well as other diseases prevalent in specific populations.

Screening of adult carriers has been effective for several AR disorders including Tay-Sachs disease, thalassemia, cystic fibrosis, hereditary hemochromatosis and Factor V Leiden (reviewed by Khoury et al 2003). Screening for heterozygous disease carriers before pregnancy can identify individuals who are at risk of having an affected child if the other parent is also a carrier and allows them to consider their reproductive options. Population-based carrier screening of Tay-Sachs disease has proven to be very successful for Ashkenazi Jewish couples and has led to a greater than 90% reduction in the birth of Tay-Sachs affected children in this population (Kaback et al 1993).

1.3.2 Prenatal diagnosis

A fetus can be genetically tested during pregnancy for the presence or absence of a chromosomal abnormality or specific genetic defect. If the mutation is detected then the couple will need to decide whether to terminate the affected fetus. Diagnosis is generally based on fetal tissue collected from the placental chorionic villi or from amniocytes. Non-invasive methods of prenatal diagnosis, such as testing transcervical trophoblast cells (Adinolfi et al 1993) or fetal DNA extracted from maternal plasma (Elias 1992), have been proposed. Prenatal genomic DNA is tested either by PCR or by Southern blot

analysis for genetic mutations and fluorescent *in-situ* hybridisation (FISH) to detect chromosome abnormalities.

1.3.3 Preimplantation genetic diagnosis

Preimplantation genetic diagnosis (PGD) is designed for couples at high genetic risk and can diagnose specific genetic disorders using a single cell from a preimplantation embryo, thereby giving a result before a pregnancy is established. The first report of PGD was in rabbit blastocysts (Edwards and Gardner 1967). After the technique was found to be successful in human embryos (Handyside et al 1989), PGD was applied clinically for gender selection and for mutation analysis (Handyside et al 1990, 1992).

The approach used for genetic analysis of single blastomeres depends on the type of disease under investigation. For chromosomal abnormalities and gender determination for X-linked diseases, FISH is used. For single gene defects, where direct detection of the mutation is not possible from the DNA of a single cell, most diagnoses rely on PCR to amplify the relevant sequence, although FISH has been used to detect large mutations in the dystrophin gene (Malmgren et al 2006). If the exact disease-causing mutation is not identified, then PGD can be carried out through linkage analysis (Verlinsky et al 2004a).

To date, over 10,000 PGD cycles have been performed worldwide, including those documented by ESHRE (Table 1.1) with over 1,500 babies born and many more ongoing pregnancies (Harper et al 2006, Kuliev and Verlinsky 2004). All PGD treatment has an associated risk of misdiagnosis, currently reported at around 2% for FISH and 8% for PCR (Harper et al 2006). These rates are lower than previously reported which is probably due to the advances made in single-cell molecular technologies and these will be discussed further in Chapter 2.

Table 1.1: Number of cycles performed and results for PGD for single gene defects and sexing as reported by the ESHRE PGD Consortium: data collection V

Indication	Number of cycles	Number of successfully biopsied embryos (%)	Number of diagnosed embryos (%)	Number of transferable embryos (%)	Pregnancies (rate per embryo transfer (%))	Clinical pregnancy (rate per embryo transfer (%))
Autosomal recessive single gene disorder using PCR	596	3148 (99%)	2633 (84%)	1565 (59%)	149 (33%)	117 (26%)
Autosomal dominant single gene disorder using PCR	398	1949 (99%)	1642 (84%)	732 (45%)	71 (26%)	55 (20%)
Specific diagnosis for X-linked disease using PCR	95	530 (99%)	432 (82%)	252 (58%)	26 (35%)	23 (31%)
Sexing for X-linked diseases using FISH	545	3195 (97%)	2905 (91%)	1023 (35%)	121 (31%)	93 (24%)
Sexing for X-linked disease using PCR	68	395 (92%)	305 (77%)	160 (52%)	22 (43%)	16 (31%)
Social sexing using PCR or FISH	254	1505 (95%)	1367 (91%)	652 (48%)	78 (41%)	59 (31%)

Adapted from Harper et al (2006)

The disorders for which PGD for specific mutation analysis has been applied are listed in Table 1.2. Traditionally, PGD has been performed for chromosome abnormalities and for Mendelian diseases with a severe phenotype presenting at birth or in early childhood. However, PGD may now be performed for mutations that result in heritable congenital malformations such as Crouzon syndrome, Currarino triad, familial holoprosencephaly and Holt-Oram syndrome (Abou-Sleiman et al 2002a, He et al 2004, Verlinsky et al 2003a, 2005a). Other, more controversial applications of PGD involve testing an embryo for a late-onset disease or a disease with incomplete penetrance. These include cancer predisposition genes: ataxia telangiectasia, breast cancer, familial adenomatous polyposis coli, familial posterior fossa brain tumour, Gorlin syndrome, Li-Fraumeni syndrome, hereditary retinoblastoma, neurofibromatosis types I and II, von Hippel-Lindau syndrome and Wiskott-Aldrich syndrome (Ao et al 1998, Abou-Sleiman et al 2002b, Harper et al 2006, Hellani et al 2002, Rechitsky et al 2002, 2004a, Verlinsky et al 2001a, 2002a), and the following adult onset neuro-degenerative disorders: early-onset Alzheimer disease, familial amyloid polyneuropathy and Huntington disease (Carvalho et al 2001, Sermon et al 1998a, Verlinsky et al 2002b) (Table 1.2). Another application of PGD has been the testing for blood group incompatibility to permit the transfer of compatible embryos and avoid isoimmunisation during pregnancy (Avner et al 1996, Verlinsky et al 2003b). Most controversially, PGD has been used in human leukocyte antigen (HLA) typing, either combined with a causative gene to allow a conceived child to be a compatible stem cell donor for an affected sibling or for HLA typing alone for bone-marrow disorders (Fiorentino et al 2004, Van deVelde et al 2004, Verlinsky et al 2001b, 2004b).

Table 1.2: Reported clinical PGD for specific diagnosis of single gene defects (certain mutations) by PCR

Disorder and mode of inheritance	Mutated gene	Reference
Autosomal recessive		
α-1-antitrypsin deficiency	*AAT*	Verlinksy et al 1990
α-thalassemia	*α-globin*	Kuliev et al 2005
Ataxia telangiectasia	*ATM*	Hellani et al 2002
β-thalassemia	*β-globin*	Kuliev et al 1998
Canavan disease	*ASPA*	Yaron et al 2005
Glucose-6-phosphatase deficiency	*G6P*	Harper et al 2006
Congenital disorder of glycosylation type 1C	*ALG6*	ESHRE PGD consortium 2002
Congenital adrenal hyperplasia	*CYP21*	Van de Velde et al 1999
Cystic fibrosis	*CFTR*	Handyside et al 1992
Familial dysautonomia	*IKBKAP*	Rechitsky et al 2003
Fanconi anemia	*FANCC/A*	Verlinsky et al 2001b
Gaucher disease	*GBA*	Rechitsky et al 1999
Herlitz junctional epidermolysis bullosa	*LAMB3*	Cserhalmi-Friedman et al 2000
Hyperinsulinaemic hypoglycaemia (PHHI)	*SUR*	ESHRE PGD consortium 2002
Infantile neuronal ceroid lipofuscinosis	*PPT*	Harper et al 2006
Kell genotype	*KEL*	Verlinsky et al 2003b
Leukocyte adhesion deficiency type 1	*ITGB2*	Lorusso et al 2006
Long-chain acyl-CoA dehydrogenase deficiency	*LCHAD*	Rechitsky et al 1999
Medium-chain acyl-CoA dehydrogenase deficiency	*MCAD*	Sermon et al 2000 Ioulianos et al 2000
Metachromatic leukodystrophy	*ARSA*	Harper et al 2006
Mucopolysaccharidosis type I	*IDUA*	Tomi et al 2006
Mucopolysaccharidosis type IIIA (Sanfilippo syndrome)	*SGSH*	Fiorentino et al 2006
Mucopolysaccharidosis type IV (Maroteaux-Lamy syndrome)	*ARSB*	Fiorentino et al 2006
Niemann-Pick disease type B	*SMPD1*	Hellani et al 2004a
Phenylketonuria	*PAH*	Verlinksy et al 2001c
Rhesus isoimmunization	*RhD*	Avner et al 1996
Rhizomelic chondro dysplasia punctata	*PEX7*	Harper et al 2006
Sandhoff disease	*HEXB*	Kuliev et al 2006
Sanjad-Sakati syndrome	*TBCE*	Hellani et al 2004b
Skin fragility ectodermal dysplasia syndrome	*PKP1*	Thornhill et al 2000
Sickle-cell anemia	*β-globin*	Xu et al 1999

Spinal muscular atrophy type 1	*SMN*	Dreesen et al 1998
Tay-Sachs disease	*HEXA*	Gibbons et al 1995
Autosomal dominant		
Achondroplasia	*FGFR3*	Moutou et al 2003
Angelman syndrome	*UBE3A*	Girardet et al 2005
Autosomal dominant retinitis pigmentosum	*Rhodopsin*	Strom et al 1998
Breast cancer	*BRCA1*	Harper et al 2006
Central core disease	*RYR1*	ESHRE PGD consortium 2002
Charcot-Marie-Tooth disease type 1A	*PMP22*	De Vos et al 1998
Charcot-Marie-Tooth disease type 2A	*KIF1B*	ESHRE PGD consortium 2002
Crouzon syndrome	*FGFR2*	Abou-Sleiman et al 2002a
Currarino triad syndrome	*HLXB9*	Verlinsky et al 2005a
Early onset Alzheimer disease	*APP*	Verlinsky et al 2002b
Early-onset torsion dystonia	*DYT1*	Rechitsky et al 2004b
Epidermolysis bullosa simplex	*KRT5/14*	Harper et al 2006
Facioscapulohumeral muscular dystrophy	*FSHD*	Marshall et al 2003
Familial adenomatous polyposis coli	*APC*	Ao et al 1998
Familial amyloid polyneuropathy	*TTR*	Carvalho et al 2001
Familial holoprosencephaly	*SHH*	Verlinsky et al 2003a
Familial posterior fossa brain tumour	*hSNF5*	Rechitsky et al 2002
Gorlin syndrome	*PTCH*	Harper et al 2006
Hereditary retinoblastoma	*RB1*	Rechitsky et al 2002
Holt-Oram syndrome	*TBX5*	He et al 2004
Li-Fraumeni syndrome	*p53*	Verlinsky et al 2001a
Marfan syndrome	*FBN1*	Blaszczyk 1998
Multiple exostoses	*EXT1*	Harper et al 2006
Neurofibromatosis type 1	*NF1*	Verlinsky et al 2002a
Neurofibromatosis type 2	*NF2*	Abou-Sleiman et al 2002b
Osteogenesis Imperfecta type 1 & 4	*COL1A1/2*	De Vos et al 2000
Polycystic kidney disease	*PKD1/2*	Verlinsky et al 2004a
Spastic paraplegia 3	*SPG3A*	Fiorentino et al 2006
Stickler syndrome	*COL2A1*	ESHRE PGD consortium 2002
Tuberous sclerosis	*TSC*	ESHRE PGD consortium 2002
von Hippel-Lindau syndrome	*VHL*	Rechitsky et al 2002
X –linked recessive diseases		
Adrenoleukodystrophy	*ABCD1*	Vandervorst et al 2000
Agammaglobulinaemia	*BTK*	ESHRE PGD consortium 2002
Alport syndrome	*COL4A5*	Verlinsky et al 1990
Becker muscular dystrophy	*Dystrophin*	Harper et al 2006

Duchenne muscular dystrophy	*Dystrophin*	Liu et al 1995
Glucose-6-phosphate dehydrogenase deficiency	*G6PD*	Fiorentino et al 2006
Haemophilia A	*Factor VIII*	Fiorentino et al 2003
Haemophilia B	*Factor IX*	Verlinsky et al 2002c
Mucopolysaccharidosis type II (Hunter syndrome)	*IDS*	ESHRE PGD consortium 2002
Hyper-Immunoglobulin M syndrome	*CD40*	Rechitsky et al 2004a
Hypohidrotic ectodermal dysplasia with immune deficiency	*NEMO-IKBKG*	Rechitsky et al 2004a
Lesch-Nyhan syndrome	*HPRT*	Ray et al 1999
Myotubular myopathy	*MTM1*	Verlinsky et al 2002c
Norries disease	*NDP*	Harper et al 2006
Pelizaeus-Merzbacher disease	*PLP1*	Verlinsky et al 2006
Wiskott-Aldrich syndrome	*WAS*	Rechitsky et al 2004a
X-linked hydrocephalus	*L1CAM*	Verlinsky et al 2002c
X-linked mental retardation	*XMR1*	Verlinsky and Kuliev 2003
X –linked dominant diseases		
Charcot-Marie-Tooth disease type X	*GJB1*	Iacobelli et al 2003
Ornithine transcarbamylase deficiency	*OTC*	Ray et al 2000
Oro-facial-digital syndrome type 1	*CXORF5*	ESHRE PGD consortium 2002
Triplet repeat disorders		
Dystrophia myotonica type 1 (Dominant)	*DMPK*	Sermon et al 1997
Fragile X syndrome (FRAXA) (X-linked)	*FMR1*	Sermon et al 1999
Huntington disease (Dominant)	*IT15*	Sermon et al 1998a
Spinal and bulbar muscular atrophy (X-linked)	*AR*	Georgiou et al 2001
Spinocerebellar ataxia 3 (Dominant)	*MJD1*	Drusedau et al 2004
Spinocerebellar ataxia 7 (Dominant)	*ATXN7*	Harper et al 2006
Mitochondrial DNA diseases		
Leber hereditary optic neuropathy	*ND1*	Harper et al 2006
Mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes	$tRNA^{leu}$	ESHRE PGD consortium 2002
Neurogenic ataxia retinitis pigmentosa	*ATPase 6*	Steffann et al 2006

1.4 PREIMPLANTATION GENETIC DIAGNOSIS PROTOCOLS

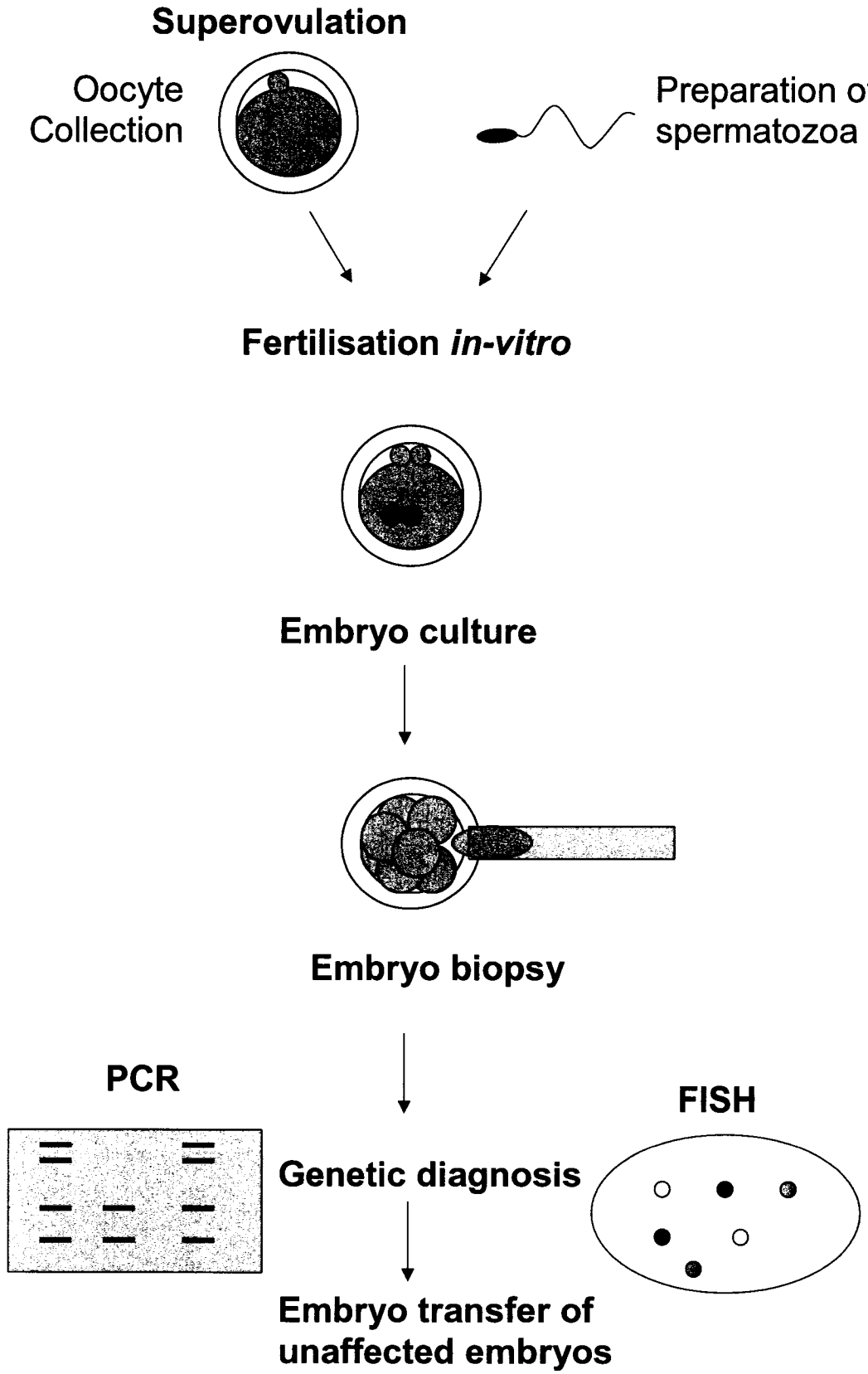
1.4.1 *In-vitro* fertilisation

PGD is carried out in conjunction with *in-vitro* fertilisation (IVF) (Figure 1.2). In IVF, oocytes are collected from an infertile woman's ovaries and inseminated with the partner's prepared spermatozoa. Sixteen to eighteen hours later, each normally fertilised zygote is further cultured *in-vitro* in specially prepared media where it continues to divide until day 2, 3 or 5 of development, at which time the embryo(s) chosen for replacement are loaded into a catheter and transferred to the woman's uterus.

Since the birth of the first IVF baby (Steptoe and Edwards 1978), the technique has become a well-recognised treatment for infertility. It was recently estimated that over one million children have been born as a result of IVF (Bonduelle et al 2005) and in Europe in 2002, IVF births ranged from 1.3 to 4.2% of the total live-births in each country (The European IVF-monitoring programme for ESHRE 2006). Initially, IVF was performed in a natural menstrual cycle with, on average, one oocyte being collected. Now with ovarian stimulation, to recruit multiple follicles, multiple oocytes can be collected in a single cycle (Trounson et al 1981) and multiple embryos generated, thereby allowing the selection of the most morphologically normal embryos for transfer, which can result in a higher pregnancy rate.

Currently, pregnancy rates after PGD are similar to those for IVF but, unlike IVF patients, PGD patients are generally fertile so their chances of success should theoretically be higher. However, in PGD, a percentage of embryos will be diagnosed as being affected which will reduce the overall number of embryos available for transfer. This is even more evident for an autosomal dominant condition where, on average, 50% of embryos will be affected.

Figure 1.2: Schematic diagram of the process of PGD in conjunction with IVF



Advancements in IVF technologies, including the use of improved media to culture embryos *in-vitro* to the blastocyst stage (Gardner et al 1998), have led to a gradual increase in worldwide IVF pregnancy and implantation rates (Society for Assisted Reproductive Technology and American Society for Reproductive Medicine 2004, The European IVF-monitoring programme for ESHRE 2006). With continued advances in the understanding of preimplantation embryo development and uterine receptivity, IVF and therefore PGD pregnancy rates will likely continue to rise.

1.4.2 Cell biopsy

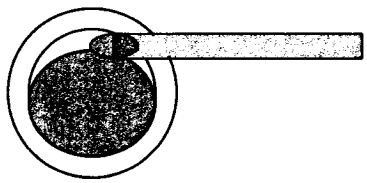
It has been postulated that it may be possible to diagnose some inherited diseases in the early embryo by non-invasive methods, such as assessing their uptake of metabolites or for gender selection through labelling X- and Y-bearing spermatozoa (Edwards and Hollands 1988, Hadjantonakis et al 1998). However, currently, and likely in the future, PGD for most genetic diseases and chromosomal abnormalities will involve the removal of an embryonic cell or cells. The biopsy technique can be undertaken using mechanical dissection, an acid Tyrode solution or by infrared diode laser to breach an area of the zona pellucida (Boada et al 1998, Cohen et al 1992, Germond et al 1995, Grifo et al 1994). Genetic analysis for PGD can be performed at three stages of cell development (Figure 1.3).

1.4.2.1 Polar body biopsy

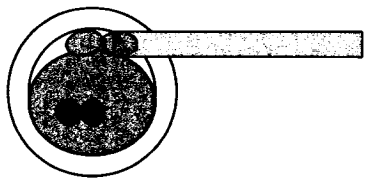
Preconception diagnosis of the first polar body (PB) formed after the first meiotic division is usually followed by analysis of the second PB, which is extruded during fertilisation, to verify the diagnosis (Verlinsky et al 1990, 1996) (Figures 1.3A and 1.3B).

Figure 1.3: Feasible stages of oocyte and embryo biopsy

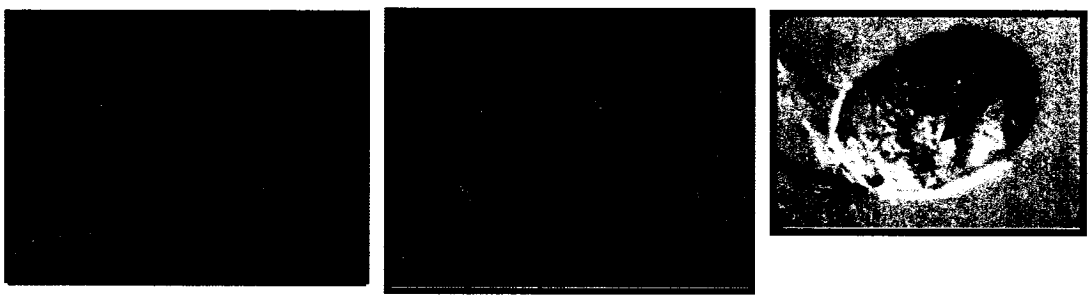
A. First polar body of mature oocyte



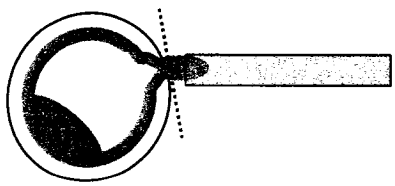
B. Second polar body of fertilised zygote



C. Blastomere of cleaved embryo



D. Trophectoderm of blastocyst



Preimplantation genetic diagnosis can be performed by analysis of A) the first polar body extruded from a metaphase II unfertilised oocyte; B) the second polar body extruded after fertilisation and completion of meiosis II; C) the blastomere from a six- to eight- cell cleaved embryo (the arrow indicates the cell nucleus); or D) trophectoderm cells extruded from an expanded blastocyst.

Polar body biopsy has advantages in that it circumvents issues concerned with the manipulation of human embryos and allows an extended period of time for genetic analysis before the cleavage-stage transfer. Aneuploidies have been successfully diagnosed using PB biopsy. The most common cause of aneuploidy in women of advanced maternal age is due to errors in meiosis I during oogenesis (Dailey et al 1996) resulting in the failure of homologous chromosome pairs to segregate normally so that one pair remains in the oocyte (resulting in trisomy in the subsequent embryo) or the pair is extruded to the polar body (resulting in monosomy). However, PB biopsy cannot be used for paternally-transmitted diseases or for gender determination for X-linked diseases and it is less efficient for telomeric genes. Another limitation of PB stage biopsy is that only one or two cells are available for genetic analysis.

1.4.2.2 Cleavage-stage embryo biopsy

The method preferred for PGD by most groups is the biopsy and genetic diagnosis of cleavage-stage embryos (Ao and Handyside 1995) (Figure 1.3C) but this also has the limitation that only one or two blastomeres are available for genetic analysis.

For cleavage-stage biopsy, fertilised oocytes are allowed to divide *in-vitro* until the 6-8-cell stage. In some cases, cleaved embryos may compact prior to biopsy, in which case the embryo can be pre-incubated for 2-3 minutes in Ca^{2+}-and Mg^{2+}- free media (Dumoulin 1998, Santalo 1996) to allow blastomere decompaction.

The testing of two blastomeres from each embryo is preferred, if the cell number permits (Ao and Handyside 1995, Ray et al 1998). Removal of two blastomeres from a 7-8-cell embryo does not adversely affect preimplantation development, as assessed by comparing biopsied and non-biopsied embryos developing to the blastocyst stage *in-vitro* (Hardy et al 1990, Krzyminska et al 1990). The individual blastomeres of an early

cleavage-stage mammalian embryo are still considered totipotent and can, therefore, contribute to all the tissues of the conceptus (Mottla et al 1995, Pedersen et al 1986). However, embryo metabolism, as well as the number of cells at the blastocyst stage, was lower in biopsed than in non-biopsied embryos, but the reduction was proportional to the number of cells removed, and the ratio of the inner cell mass (ICM) to trophectoderm was not affected (Hardy et al 1990). Live-births after PGD and after the transfer of cryopreserved embryos in which some cells were destroyed on thawing, with so far no increase in reported fetal abnormalities suggests that removal of up to two cells from an early cleavage-stage embryo does not prohibit its continued normal development (Harper et al 2006, Van den Abbeel 1997). However, the removal of two blastomeres from an embryo with 6 or less cells has been shown to cause reduced future viability (Krzyminska et al 1990, Tarin et al 1992).

1.4.2.3 Blastocyst biopsy

Embryos can be cultured until day 5 or 6 of development, when they are made up of over one hundred cells. At this stage, the embryo has differentiated into an outer epithelial layer of trophectoderm cells that will give rise to the placenta and extraembryonic membranes and the ICM from which the fetus is derived. Biopsy of 10-30 trophectoderm cells (Figure 1.3D) does not appear to impair the hatching rate of human blastocysts (Dokras et al 1991). However, it is possible that the biopsied cells from the trophectoderm may have diverged genetically from the ICM so that any results obtained from this material may not accurately represent the situation within the future fetus. The ability of blastocysts to implant after trophectoderm biopsy was initially reported in animal studies (Gardner and Edwards 1968, Gardner 1971, Monk et al 1988, Summers et al 1988). Blastocyst biopsy has now been reported with PGD leading to

pregnancies and live-births (de Boer et al 2004, Kokklali et al 2005, McArthur et al 2005). However, some embryos may not develop to the blastocyst stage *in vitro* and therefore, a reduced number of diagnosable embryos may be available.

1.4.3 PGD for chromosome abnormalities

Fluorescence *in-situ* hybridisation has been employed for PGD in aneuploidy screening for women of advanced maternal age, for translocation carriers and contiguous gene syndromes such as for DiGeorge syndrome (Iwarsson et al 1998) and for cases of recurrent spontaneous abortions, repeated IVF failure and sex-linked diseases. This technique permits chromosome analysis in both interphase- and metaphase-stage nuclei and interphase FISH is the most common technique used for PGD of chromosomal abnormalities (Delhanty et al 1997, Liu et al 1998a, 1998b, Munne and Weier 1996).

A new approach to the diagnosis of chromosomal abnormalities is metaphase comparative genome hybridisation (CGH), which gives the relative copy number of each chromosomal region and detects imbalances such as deletions and duplications. For single blastomeres, CGH requires preamplification of the cell DNA using degenerate oligonucleotide primed (DOP) PCR (Voullaire et al 2000, Wells et al 1999, Wells and Delhanty 2000) and takes over 72 hours. Therefore, although successfully applied for a few PGD cycles, this approach is not routinely used for clinical PGD (Wells et al 2002, Wilton et al 2001, 2003).

Another technique is spectral karyotyping (Schrock et al 1996, Speicher et al 1996) to produce a computerised karyotype. This technique has been applied to oocytes, first polar bodies and blastomeres (Clyde et al 2001, Marquez et al 1998) but since it requires a metaphase spread, only limited cells have been analysed (Marquez et al 1998).

Chromosomes in embryonic cells can also be analysed using nuclear conversion, in which biopsied blastomere nuclei are transformed into metaphase chromosomes by blastomere electrofusion with an enucleated zygote (Evsikov and Verlinsky 1999, Verlinsky and Evsikov 1999, Willadsen et al 1999). The chromosomes are then analysed using spectral karyotyping. This technique has been applied clinically to test embryos from translocation carriers (Verlinsky et al 2002d, Willadsen et al 1999).

1.4.4 PGD for single gene defects

Theoretically, PGD can be developed for any single gene defect, provided the causative mutation is well characterised. However, fewer than 100 disorders have been clinically applied to PGD (Table 1.2). One reason for this is that each PCR protocol must be developed specifically for the mutation of interest at the single-cell level and must be rigorously tested prior to use, which involves the intensive use of time and resources. The test must also be conducted quickly to allow embryo transfer within the limited window of time available for successful implantation. Optimal efficiency is necessary to ensure amplification of the two copies of target DNA present in the nucleus of a single blastomere, which in the context of conventional PCR, is developed in Chapter 2. Other approaches for the amplification and detection of products that are appropriate for use at the single-cell level are also available, some of which are briefly described below.

1.4.4.1 Amplification of products

Whole genome amplification

Primer extension preamplification (PEP) PCR is a technique that involves amplifying the whole genome using a mixture of random primers (Zhang et al 1992) resulting in approximately 90% of a cell's genome being amplified to a minimum of 30

copies (Wells et al 1999, Zhang et al 1992). Small fractions of this reaction mix are then used in separate reactions to amplify locus-specific DNA sequences and allow analysis of multiple separate genetic loci. The possibilities of using PEP at the single oocyte, PB and blastomere levels have been shown (Kristjansson et al 1994, Sanchez-Garcia 2005, Sermon et al 1996, Snabes et al 1994, Xu et al 1993), and PEP has been used in clinical PGD for single gene defects (Ao et al 1998, Jiao et al 2003).

Degenerate oligonucleotide primed (DOP) PCR is an alternative method that results in whole genome amplification using a partially degenerate oligonucleotide primer that binds at multiple sites in the genome during several low-temperature annealing cycles (Telenius et al 1992). This is followed by PCR at a higher temperature to allow only those initial fragments that are tagged with the specific DOP sequences at both ends to be amplified. This technique has not been used in PGD for single gene defects but has been used for CGH at the single nucleus level (see Section 1.4.3).

A more recent technique that has been shown to be an efficient method of whole genome amplification without repeat cycling is isothermal multiple displacement amplification (MDA), using bacteriophage ϕ29 DNA polymerase in conjunction with modified random primers (Dean et al 2002). A short denaturation step is followed by an enzyme-specific incubation step during which time the primers anneal at multiple sites to the denatured DNA and then, initiated by the polymerase, are extended by thousands of bases using the single-stranded DNA as a template. As synthesis progresses, the 5' end of any extending strand is displaced by another upstream strand extending in the same direction. The strand displacement reactions generate a hyperbranching mechanism that can produce thousands of copies of the genome in just a few hours before the enzyme is inactivated (Dean et al 2002). Multiple displacement amplification, followed by PCR to

analyse multiple loci, has been tested on single blastomeres (Handyside et al 2004) and has recently been applied to clinical PGD (Hellani et al 2005). The efficiency and accuracy of MDA need to be tested further before incorporating this approach for routine clinical application.

Reverse transcriptase PCR

Reverse transcriptase (RT) PCR is a highly sensitive technique for mRNA detection and quantification. Initially, complementary DNA (cDNA) is reverse transcribed from single-stranded mRNA, providing the gene of interest is expressed during the developmental stage tested. The cDNA then serves as a template for standard PCR amplification. Potentially, RT-PCR may prove to be more reliable for PGD due to the higher number of target gene copies that are available from the starting material versus the two copies of genomic DNA in a single cell. Real-time RT-PCR further increases the sensitivity and specificity of the assay while making it more rapid.

The use of RT-PCR and real-time RT-PCR for analysis of gene expression in mammalian oocytes and single embryos and blastomeres has been explored (Daniels et al 1998, Lindeberg et al 2004, Steuerwald et al 1999, 2000a). Although a general activation of the embryonic genome occurs between the 4-and 8-cell stages of human preimplantation embryonic development (Braude 1988), overall knowledge about human embryonic gene expression is still limited, thereby restricting the current application of RT-PCR in PGD.

Real-time PCR

A method for diagnosing single gene defects, using quantitative PCR measurements is real-time PCR. In real-time PCR, the accumulation of the PCR product is measured during each cycle of the reaction when PCR is in its exponential phase,

instead of after the process is complete. In one type of real-time PCR, molecular beacons are used to differentiate between distinct PCR products, such as a gene with a mutation, and a wild-type gene. These beacons consist of short oligonucleotides complementary to the region of interest within the PCR fragment with a fluorochrome on one end so that fluorescence is detected if they anneal to the template. However, if they remain in solution, they fold up upon themselves and do not fluoresce. Quantification is achieved though comparison with the amplification of an internal reference target, such as another gene locus. Real-time PCR has been used for assessment of the mitochondrial DNA content of human blastomeres (Lin et al 2004), for diagnosis of single gene defects in blastomeres (Pierce et al 2003, Rice et al 2002, Vrettou et al 2004) and, recently, for diagnosis in clinical PGD cycles of a single gene defect (Almeida et al 2005). This technique is also at the early stages of development, and more research is required before adapting it to routine diagnosis for PGD.

Minisequencing

Minisequencing is based on a primer extension technique whereby a detection primer anneals to the template one nucleotide upstream of a known polymorphic site. A DNA polymerase then specifically extends the 3' end of the primer with a single labelled nucleotide that is complementary to the nucleotide at the variable site, and this labelled nucleotide reveals the identity of the base at that site. This permits quick, accurate and simultaneous detection of single nucleotide polymorphisms (SNP), point mutations and compound genotypes, which with the exception of PCR products containing repeat sequences can be applied to virtually any sequence. It also allows the detection of sequence variants in extremely short stretches of amplified DNA that can be amplified more efficiently from a single cell than from larger fragments. Minisequencing has been

used more successfully in clinical PGD practice than whole genome amplification, RT-PCR and real-time PCR (Cserhalmi-Friedman et al 2000, Fiorentino et al 2003, Hussey et al 2002, Thornhill et al 2000).

1.4.4.2 Genotyping amplified products

Genetic analysis of the PCR products obtained by following one of the afore-mentioned approaches can be performed using several techniques. Some of the most common methods are amplification refractory mutation system, chemical mismatch cleavage, denaturing gradient gel electrophoresis and automated DNA sequencing (Sambrook and Russell 2001). Four other techniques, namely heteroduplex analysis, single-strand conformation polymorphism analysis (SSCP), the detection of fluorescently-labelled products in conjunction with various computerised software programs and restriction length fragment polymorphism (RLFP) are applied in this thesis.

Single-strand conformation polymorphism analysis (SSCP)

For SSCP, PCR products are denatured, rapidly cooled and electrophoresed whilst single stranded. Strands of DNA of different lengths, and even those differing by a single nucleotide pair, will have unique three-dimensional conformations and therefore different rates of migration. Unknown DNA can be genotyped by comparing its migration with that of unaffected and affected DNA (Sambrook and Russell 2001).

Heteroduplex analysis

Heteroduplex analysis can distinguish between PCR products with small insertions or deletions. Test product is mixed separately with previously amplified homozygous-normal and homozygous-affected DNA. These two mixtures are denatured and then allowed to reanneal. Homoduplexes are formed if the mixture contains just full-length or just mutant fragments. Heteroduplexes (2 strands similar enough to form

double-stranded DNA along the majority of the sequence) are formed in addition to homoduplexes if a mixture has both full-length and mutant DNA and these have significantly retarded migration on the gel. If the heteroduplex band appears in only one of the mixtures, the unknown DNA is either homozygous normal or affected, depending on the added DNA genotype, whereas if the heteroduplex band appears in both mixtures, heterozygous (carrier) DNA was tested (Sambrook and Russell 2001).

Analysis of fluorescently-labelled PCR products

Fluorescent PCR is a modification of PCR technology using primers end-labelled with fluorescent molecules and an automated DNA sequencer, such as a fragment analyser. Fluorescently-labelled amplified DNA fragments can be detected as excitation peaks using a laser system, which has improved the efficiency of detection at the single-cell level (Findlay et al 1995a, 1995b, Hattori et al 1992, Sermon et al 1998a, 1998b).

Restriction length fragment polymorphism

Restriction enzymes recognise specific DNA sequences and cleave the DNA strand at or near this site. An endonuclease can be selected to cleave the normal DNA strand while the mutant remains undigested or vice versa. The two digested products can then be distinguished from the undigested product (Sambrook and Russell 2001).

1.5 DISEASE-RELATED GENES STUDIED IN THIS THESIS

There is a high prevalence of several autosomal recessive and dominant disorders in the Saguenay-Lac-St-Jean (SLSJ) region of Quebec (De Braekeleer et al 1991). The diseases studied in this thesis, cystic fibrosis (CF) and dystrophia myotonica type 1 (DM1), have an increased incidence in this population (Laberge et al 2005, Rozen et al 1990) and for mitochondrial diseases, French-Canadian type Leigh syndrome has a high carrier frequency in the same population (Morin et al 1993).

1.5.1 Cystic fibrosis

Cystic fibrosis is the most common severe autosomal recessive disease affecting 1 in 2,500 live-births in Caucasians with a carrier frequency of 5% (Boat et al 1989).

1.5.1.1 Genotype

Cystic fibrosis ([MIM 219700] OMIM) is caused by mutations in the *CFTR* gene on chromosome region 7q31.3 (Kerem et al, 1989, Riordan et al 1989, Rommens et al 1989). The *CFTR* gene comprises 27 exons and encodes a 1,480-amino acid protein (Kerem et al 1989, Riordan et al 1989). It is a member of the ATP Binding Cassette transporter supergene family and is responsible for the ATP- and protein kinase A-dependent bidirectional conductance of chloride across the apical membrane of secretory epithelial cells in the lungs, pancreas, colon, genitourinary tract and sweat glands.

Over 1,400 *CFTR* mutations and nearly 200 polymorphisms (Welsh and Smith 1993) are known, although 32 mutations account for 90% of CF cases (Rosenstein and Cutting 1998, http:genet.sickkids.on.ca). The most common CF mutation is a 3-base pair deletion of phenylalanine in exon 10 of the *CFTR* gene, ΔF508. The ΔF508 mutation accounts for 66% of CF mutations worldwide (Kerem et al 1989, The Cystic Fibrosis

Genetic Analysis Consortium 1994). In Quebec, the ΔF508 mutation is responsible for 71% of CF mutations occurring in urban French-Canadian families, 55% in those from the SLSJ region and 70% in those of Louisiana Acadian descent (Rozen et al 1990).

1.5.1.2 Phenotype and pathogenesis

Mutations in the *CFTR* gene cause decreased cyclic adenosine monophosphate (cAMP)-dependent chloride transport across the apical membranes of secretory epithelia accompanied by increased cellular sodium uptake water absorption (Stutts et al 1995, Widdicombe et al 1985). This leads to an increased protein concentration and results in the accumulation and impaired clearance of thick mucus in the lungs and intestines (Anderson et al 1991, Riordan et al 1989). Inflammation and altered pH also occur in the lung airways with increased salt levels and decreased volume on the airway surface, which inactivates antimicrobial peptides and causes an increase in mucus viscosity (Khan et al 1995, Imundo et al 1995, Matsui et al 1998, Smith et al 1996, Zabner et al 1998). These conditions trigger *Pseudomonas aeruginosa* to switch to mucoid strains and cause the chronic bacterial infections that lead to the death of most CF-affected individuals (Boat et al 1989). In addition, *CFTR* mutations often cause pancreatic insufficiency, elevated sweat chloride concentration and can cause liver disease and obstructive azoospermia (Boat et al 1989, Chillon et al 1995).

Severity of the CF phenotype will depend on the type of mutation inherited (Welsh and Smith 1993). An individual who inherits two different mutations will be a CF compound heterozygote. Mutations in the *CFTR* gene can affect the biosynthesis of the full-length polypeptide, protein processing, chloride channel regulation, chloride conductance or channel gating and regulation of other channels or lead to reduced

synthesis of the protein. The ΔF508 mutation disrupts the processing of *CFTR*, resulting in the protein being retained and degraded in the endoplasmic reticulum (Cheng et al 1990, Ward and Kopito 1994).

1.5.1.3 Murine models of cystic fibrosis

The murine *Cftr* gene, located on mouse chromosome 6, has over 78% homology to the human *CFTR* gene (Ellsworth et al 2000, Siegel et al 1992, Tata et al 1991).

The first CF mice models had disrupted *Cftr* expression with either complete loss of function or with low levels of *Cftr* mRNA (Dorin et al 1992, Hasty et al 1995, O'Neal et al 1993, Ratcliff et al 1993, Rozmahel et al 1996, Snouwaert et al 1992). The survival to maturity ranged from less than 5% in null *Cftr* mice to 90% in the residual function mice, with death due mostly to intestinal obstruction. To replicate the effects of specific human CF mutations, ΔF508 mice and mice with other common CF mutations were created (Colledge et al 1995, Delaney et al 1996, Dickinson et al 2002, French et al 1996, van Doorninck et al 1995, Zeiher et al 1995). However, none of these mice manifested the gross human CF lung pathology. A mouse generated with lung pathology similar to that of human CF, via the airway-specific overexpression of epithelial sodium channels (Mall et al 2004), should help further the understanding of the human CF lung phenotype.

Also, inbred *Cftr* knockout mice on different genetic backgrounds have been used to identify several genetic loci that may act as modifiers of the CF disease severity and lung phenotype in mice, many of which are specific to the sex of the animal (Haston et al 2002, Kent et al 1997, Rozmahel et al 1996). Through these models, any variable genetic and environmental factors that could control the severity of CF could be elucidated, thereby improving the prospects of treatment for CF patients.

1.5.2 Dystrophia myotonica type 1

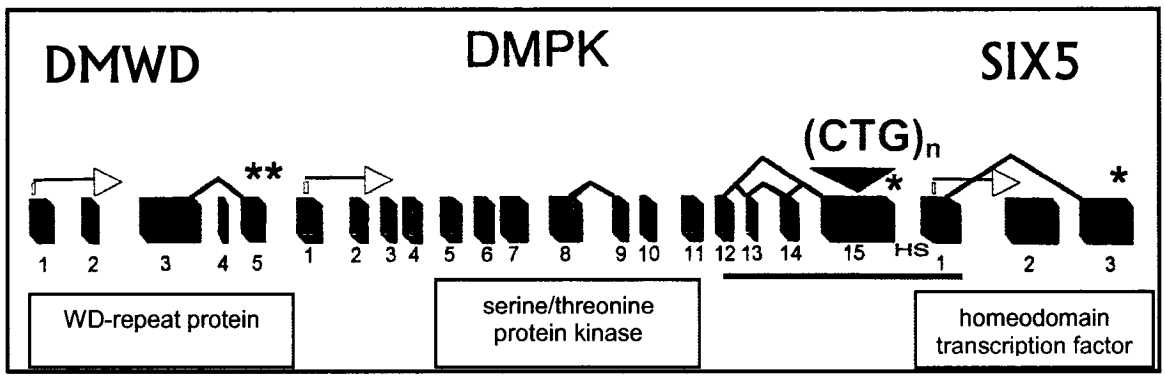
Dystrophia myotonica type 1 (DM1), or Steinert disease, is an autosomal dominant disorder that was first described in 1909 (Batten and Gibb 1909, Steinert 1909). It is the most common form of adult-onset muscular dystrophy, with an incidence of approximately 1 in 8,000 adults of Western and North American populations (Harper 1997) however, a higher incidence of about 1 in 530 people has been reported in the SLSJ region of Quebec (Laberge et al 2005).

1.5.2.1 Genotype

The human dystrophia myotonica-protein kinase (*DMPK*) gene locus is located on chromosome region 19q13.3 (Harley et al 1991) and was cloned in 1992 (Aslanidis et al 1992, Brook et al 1992, Buxton et al 1992, Fu et al 1992, Harley et al 1992, Mahadevan et al 1992). It comprises 15 exons over 14 kb and encodes a 624-amino acid protein (Mahadevan et al 1993, Shaw et al 1993) (Figure 1.4). The underlying genetic mutation causing DM1 ([MIM 160900] OMIM) is the expansion of an unstable uninterrupted $(CTG)_n$ repeat in the 3' untranslated region (UTR) of the gene (Brook et al 1992, Buxton et al 1992, Fu et al 1992, Mahadevan et al 1992). Repeat expansion could result from the formation of hairpin and slipped-strand DNA structures, facilitating DNA replication and repair errors (Gordenin et al 1997, Kunkel et al 1997, Parsons et al 1998, Pearson and Sinden 1996, Seroz et al 1995, Tishkoff et al 1997).

Seven alternatively spliced *DMPK* mRNAs exist, each of which is expressed at different developmental stages and each of which has its own distinct substrate specificity and subcellular location in the endoplasmic reticulum, mitochondria and cytosol (Groenen et al 2000, Jansen et al 1993, Tiscornia and Mahadevan 2000, Wansink et al 2003).

Figure 1.4: Diagrammatic representation of the human DM1 locus



Transcription initiation sites are represented by arrows and exons with boxes. Alternative splicing variants are identified by connecting lines between exons and polyadenylation sites are indicated by asterisks. The blue line indicates a DNase I hypersensitive site, which overlaps the 3' end of *DMPK* and the 5' end of *SIX5*. The unstable $(CTG)_n$ repeat is located in exon 15 of the *DMPK* gene.

Adapted from: Wansink and Wieringa 2003

The *DMPK* protein belongs to the cAMP-dependent serine-threonine subfamily of protein kinases (Brook et al 1992, Buxton et al 1992, Fu et al 1992, Mahadevan et al 1992, Wansink et al 2003). It is expressed predominantly in the smooth, skeletal and heart muscles but low levels are also found in brain and endocrine tissues (Jansen et al 1992). Functionally, *DMPK* may play an important role in Ca^{2+} homeostatis and the signal transduction system in these muscles (Chahine and George 1997, Timchenko et al 1995).

Amplification of the *DMPK* CTG repeat has been found in approximately 98% of the global dystrophia myotonica population however, a small group of patients has been clinically diagnosed with DM type 2 (DM2) caused by the expansion of a $(CCTG)_n$ repeat in the zinc finger protein 9 on chromosome 3q with similar, but not identical, phenotypes to those found in DM1 (Liquori et al 2001, Ranum et al 1998). Recently a family suggested to have DM type 3 (DM3) was also described (Le Ber et al 2004).

1.5.2.2 Phenotype

Clinically, DM1 causes general muscular degeneration, including myotonia, progressive weakness and wasting of muscles and cardio-respiratory problems. The symptoms usually present in the third or fourth decade of life and are progressive, often leading to significant disability. Severe cases of adult-onset DM1 show a high incidence of presenile cataracts, testicular atrophy, diabetes, kidney failure, early frontal male balding and occasionally mental retardation. A more severe congenital form of DM1 (CDM1) is characterised by general hypotonia, neonatal respiratory distress, increased perinatal mortality, delayed motor development and mental retardation. The variable phenotypic expression and age of onset seen in DM1 are correlated with the length of repeat expansion (Table 1.3) (Buxton et al 1992, Harley et al 1993, Harper 1997).

Table 1.3: Correlation of phenotype with number of repeats in the *DMPK* gene

DM1 phenotype	Number of CTG repeats
Unaffected	5-37
Pre-mutation carrier	38-49
Proto-mutation carrier	50-80
Mildly affected/very late onset	50-150
Adult onset	100-1,000
Congenital	>1,000-6,000

Note that in the proto-mutation, mildly affected and adult onset phenotypes there are some overlaps in the range of CTG repeats due to the variable expression of symptoms

Adapted from: Barcelo et al 1993, Brook et al 1992, Harper 1997

1.5.2.3 Pathogenesis

Expression of *DMPK* has been shown during oogenesis, is not present in mature spermatozoa and *de-novo* transcription is detected in the 1-cell embryo (Daniels et al 1995). Therefore, embryos with large repeat expansions could be affected from a very early stage and, providing that the *DMPK* gene is developmentally important, this may indicate why the congenital phenotype is so severe, or it may just represent a general de-repression of gene activity at this stage.

Three models have been proposed to suggest why expanded *DMPK* repeats lead to the DM1 phenotypes, and it may be that the multisystemic features of DM1 are the result of one mechanism or a cumulative effect of two or more mechanisms. The first model suggests that the expanded repeat causes *DMPK* haploinsufficiency. A negative effect of the repeat on gene transcription and translation and decreased amounts of *DMPK* mRNA and protein has been shown (Carango et al 1993, Fu et al 1993) but it has also been demonstrated that far less than 50% of normal *DMPK* mRNA and protein are present in DM1 patient muscle (Hofmann-Radvanyi et al 1993, Novelli et al 1993).

The second mechanism proposes that the mutation interferes with the expression of multiple genes in the DM1 region (Figure 1.4), causing their haploinsufficiency. Expanded CTG repeats can form strong natural nucleosome positioning elements that have the ability to interfere with the local chromatin structure (Otten and Tapscott 1995, Wang et al 1994). This could affect the expression of the gene encoding the myotonic dystrophy tryptophan-aspartate repeat protein (*DMWD*), which is expressed in the testis and is proposed to have a role in infertility (Alwazzan et al 1999, Shaw et al 1993); the homeodomain gene, sine oculis homeobox 5 (*SIX5*), which encodes a transcription factor critical for eye, muscle and gonad development (Boucher et al 1995, Kirby et al 2001,

Klesert et al 1997); and Fc fragment of IgG receptor transporter gene which encodes an IgG receptor (Junghans et al 2001). However, the discovery of DM2, on a separate locus, but with similar phenotypes to DM1, brings the involvement of other genes into question.

Finally, an RNA gain-of-function effect that disrupts splicing and possibly other cellular functions may be responsible for DM1. Expanded CUG repeats form stem-loop structures, which accumulate and are retained in the nuclei of DM1 cells, where they are thought to sequester specific RNA binding proteins, including CUG binding protein (CUG-BP) and three different forms of muscleblind (Davis et al 1997, Fardaei et al 2002, Hamshere et al 1997, Krahe et al 1995, Miller et al 2000, Philips et al 1998, Sobczak et al 2003, Taneja et al 1995, Timchenko et al 1996, 2001, Wang et al 1995). This could have a *trans*-dominant effect leading to aberrant splicing of many pre-mRNAs, sometimes to favour forms that are normally produced during fetal development, which could be relevant to the clinical features of DM1 (Buj-Bello et al 2002, Charlet-B et al 2002, Philips et al 1998, Savkur et al 2001, Sergeant et al 2001).

1.5.2.4 Murine models of dystrophia myotonica type 1

The mouse homolog to the *DMPK* gene, *Dm15,* is found on mouse proximal 7, (Cavanna et al 1990) and the chromosomal segment in which the unstable $(CTG)_n$ repeat is located is relatively well conserved between the two species. This has permitted the generation of DM1 transgenic animals with different gene constructs and knockouts.

Models of DM1 pathogenesis

The first class of DM1 mouse model investigated the mechanism(s) by which expanded *DMPK* repeats exert their pathogenic effects. The consequences of *DMPK* haploinsufficiency (Berul et al 1999, Jansen et al 1996, Mounsey et al 2000, Reddy et al 1996), effects on neighbouring genes (Klesert et al 2000, Sarkar et al 2000), and possible

gain-of-function toxicity of expanded repeat mRNA (Ho et al 2005, Kanadia et al 2003, Mankodi et al 2000, 2002, Seznec et al 2001, Storbeck et al 2004) were studied, but no model faithfully mimicked the human DM1 phenotype.

Models of DM1 repeat instability

Mouse models have also been used to study the repeat instability seen in DM1. Transgenic mice with the 3'-UTR of the *DMPK* gene and $(CTG)_{162}$ showed mild intergenerational instability with greater variation upon paternal transmission, and some evidence of somatic instability between tissues but no DM1 phenotype (Monckton et al 1997). There was also transmission in favour of the transgene containing the expanded allele, which was inherited by 66% of the offspring (Monckton et al 1997).

Another group of transgenic mice with the complete human *DMPK* locus with the two surrounding genes carried expanded repeats of either $(CTG)_{55}$ or $(CTG)_{>300}$ (Gourdon et al 1997, Seznec et al 2000). Meiotic instability was noted with increases of up to 60 repeats in the descendents of the mice carrying the larger expansion (Seznec et al 2000). Similarly, somatic instability, which was greater in the mice with larger expanded repeats, was both age and tissue dependent with more pronounced instability in the liver, pancreas and kidney when compared to heart, muscle and eye, making the instability independent of tissue proliferation capacity (Lia et al 1998, Seznec et al 2000). Somatic instability was seen in the ovary by 13 months of age but was not noted in 4-month-old mice (Lia et al 1998). More dramatic somatic expansions, representing a tripling of allele length, were detected in the kidney of another transgenic mouse model (Fortune et al 2000).

A third series of transgenic mouse models with an expanded *DMPK* CTG repeat introduced onto different mismatch repair protein knockout backgrounds have been investigated (Foiry et al 2006, Savouret et al 2003, van de Broek et al 2002) and have

implicated different mismatch repair proteins in intergenerational and somatic repeat instability.

1.5.2.5 Instability in transmission of human *DMPK* CTG repeats

Although meiotic and mitotic instabilities were observed in transgenic mice carrying expanded *DMPK* repeats, the instabilities were much less dramatic than seen in human DM1 patients. These differences could be a consequence of the differing physiology of mouse and human in that different pathways could be involved in CTG repeat instability in the two species or that mouse muscles may respond differently in degeneration-regeneration processes. Alternatively, it may reflect an incorrect construction of mouse models as molecular replicas of human DM1 mutations. This could be due to an absence of various *cis*- or *trans*- acting factors that affect repeat instability or that the repeat copy number threshold for instability, and thus large increases, might be higher in mice. Therefore, until suitable mice models are found, it is vital that investigations into the inheritance of human DM1 repeats are studied using the human system.

Transmission of CTG repeats within the normal, pre- and proto-mutation range

A trimodal distribution of normal CTG repeat lengths in the *DMPK* gene is found in the population of European ancestry reflecting $(CTG)_5$, $(CTG)_{11\text{-}17}$ and $(CTG)_{19\text{-}37}$ repeats (Deka et al 1996, Zerylnick et al 1995). All repeat lengths from $(CTG)_{3\text{-}37}$ have been observed, except for $(CTG)_4$ and $(CTG)_9$, with a predominance of $(CTG)_5$ on normal Caucasian chromosomes (Imbert et al 1993). The $(CTG)_5$, $(CTG)_{19\text{-}37}$ and expanded DM1 alleles are in linkage disequilibrium with an *Alu*-insertion polymorphism in Caucasians, while $(CTG)_{11\text{-}13}$ and $(CTG)_{15}$ are associated with an *Alu*-deletion polymorphism, suggesting a founder haplotype for the DM1 phenotype (Harley et al 1992, Mahadevan et

al 1993) in this population. It has been concluded that certain $(CTG)_5$ alleles were the predecessors of the $(CTG)_{19-37}$ alleles (Imbert et al 1993, Neville et al 1994).

The transmission of *DMPK* $(CTG)_{5-18}$ repeats within a large number of pedigrees and also in human spermatozoa has been shown to segregate stably (Martorell et al 2001, Zhang et al 1994). However, for $(CTG)_{19-37}$ repeats, intergenerational instability has been observed in offspring during maternal (Weber and Wong 1993), and especially during paternal transmissions (Dow et al 1997, Martorell et al 2001, Meiner et al 1998). Instability of $(CTG)_{19-37}$ repeats has also been detected in the male germline (Zhang et al 1994), however, the timing of repeat instability during early development has not been fully determined. The timing of instability in the transmission of *DMPK* $(CTG)_{19-37}$ repeats in early embryonic development was investigated in Chapter 4 of this thesis.

Instability has also been reported in the transmission of pre-mutation repeats. Changes in repeat length, usually in the form of increases of a few repeat units, have been documented for maternal transmissions (Martorell et al 2001, Yamagata et al 1994) and also for the transmission of paternal pre-mutations, although larger expansions could be observed through paternal transmission (Martorell et al 1996, 2001, Yamagata et al 1994, 1998). A similar tendency for expansion of proto-mutation repeats, especially of large increases upon paternal transmission, has been reported (Abbruzzese et al 2002, Barcelo et al 1993, Martorell et al 2001, Monckton et al 1995, Simmons et al 1998). This larger paternal intergenerational expansion could result from the greater number of mitotic cell divisions occurring over the effective fertile lifespan during spermatogenesis when compared to oogenesis (Edwards 1989). Occasional contractions in repeat length that appear to be clustered within sibships have also been observed (Ashizawa et al 1994, Monckton et al 1995).

Instability of expanded DMPK repeats

In DM1, as with most other trinucleotide repeat diseases, genetic anticipation is observed in the transmission of expanded *DMPK* repeats (Harper et al 1992). Both meiotic and somatic increases can be observed in the transmission of expanded *DMPK* repeats. Expansion-biased somatic instability in DM1 is tissue specific and can occur during human fetal development (Hecht et al 1993, Jansen et al 1994, Lavedan et al 1993, Martorell et al 1997, Wohrle et al 1995) with continued expansion, occurring in the cells, throughout the life of a DM1-affected individual (Anvret et al 1993, Ashizawa et al 1994, Martorell et al 1995, 1998, Monckton et al 1995, Wong et al 1995).

The degree of germline instability in expanded CTG repeats is dependent on the gender of the transmitting parent and on the initial number of repeats. With repeats of $(CTG)_{<200}$, a positive correlation has been found between the size of the repeat and inter-generational enlargement equally in female and male meioses, however, above this size range, anticipation was more evident through maternal meiosis (Ashizawa et al 1992, Brunner et al 1993a, Lavedan et al 1993, O'Hoy et al 1993, Shelbourne et al 1992). For DM1 males with $(CTG)_{<500}$ in their lymphocytes, larger repeats were detected in their mature spermatozoa and even larger expansions were detected in the blood of affected offspring (Jansen et al 1994, Lavedan et al 1993).

Congenital DM1 is usually transmitted from an affected mother (Hoffman-Radvanyi et al 1993, Lavedan et al 1993), although rare cases of paternally inherited CDM1 have been described (Nakagawa et al 1994, Zeesman et al 2002). When an expanded repeat is passed through the maternal germline, it can undergo up to a twenty-fold increase in repeat length when compared with the repeat length in the transmitting mother's lymphocytes (Harley et al 1993, Redman et al 1993). Females with $(CTG)_{>300}$

appear at higher risk of having CDM1 offspring than females with smaller expansions (Cobo et al 1995, Harley et al 1993, Tsilfidis et al 1992). Conversely, for DM1 males who have $(CTG)_{>700}$ in their lymphocytes, there appears to be a selection against very large repeats in their spermatozoa, with no spermatozoa carrying repeats of $(CTG)_{>1000}$ (Jansen et al 1994, Lavedan et al 1993). Paternal contractions of repeats during meiosis could provide an explanation for the absence of spermatozoa with very large expansions and the rarity of paternally transmitted CDM1. Interestingly, in the *Drosophila* Segregation Distorter system, allele-specific male gamete dysfunction is dependent on an expanded repeat sequence (Lyttle 1993).

Meiotic instability at the DM1 locus most likely occurs before day 10.5 of embryonic development (Hecht et al 1993, Jansen et al 1994, Martorell et al 1997, Phillips 1993), which is discussed further in Section 4.3. However, a more precise timing of meiotic *DMPK* CTG repeat expansion could not be given at the initiation of this doctorate project therefore, the experiments described in Chapter 4 were designed to better define the timing of expansion in *DMPK* CTG repeats.

Maintenance of the DM1 mutation in the population

DM1 is a dominant disease and, as such, expanded alleles would be expected to be eliminated from the population over successive transmissions (de Die-Smulders et al 1994). Loss of expanded alleles can occur from maternal anticipation leading to CDM1 individuals with greatly reduced reproductive fitness (Harper 1992, Hofmann-Radvanyi et al 1993), and from the occasional contraction of paternally transmitted expanded repeats back to within the normal range (Brunner et al 1993b, O'Hoy et al 1993, Shelbourne et al 1992). To counteract the elimination of expanded repeats on disease alleles, a mechanism to generate new expanded repeats has been proposed (Imbert et al 1993). If CTG repeats

in the sub-clinical range underwent small intergenerational expansions, the rate of which increased with increasing repeat size this could, over many generations, serve as a mechanism to maintain the population disease frequency of DM1 (Imbert et al 1993).

Although pre-and proto-mutations can be inherited in a relatively stable fashion during maternal transmission, large expansions can result from paternal transmission, leading to DM1 phenotypic expression in the subsequent generation. Therefore, it seems unlikely that the DM1 mutation could be stably maintained in the population for any significant length of time in the proto-mutation state. Furthermore, $(CTG)_{5-18}$ repeats are not prone to instability are therefore unlikely to provide future disease alleles. However, larger normal *DMPK* $(CTG)_{19-37}$ repeats could act as a reservoir of future expanded *DMPK* alleles (Chakraborty et al 1996, Imbert et al 1993) since the modest expansions that occur upon transmission could, over many generations, result in repeat lengths responsible for the expression of a DM1 phenotype.

To ensure constant replenishment of the *DMPK* $(CTG)_{19-37}$ pool, a preferential transmission of alleles in this size range, from unaffected individuals, has been proposed (Carey et al 1994, Chakraborty et al 1996). This TRD hypothesis is supported by the observation that, in certain populations, there is both a lower prevalence of these larger normal-sized repeats and a lower incidence of DM1 when compared to Caucasian populations (Ashizawa and Epstein 1991, Deka et al 1996, Gennarelli et al 1999, Zerylnick et al 1995, Zhang et al 2000). Research into whether TRD of *DMPK* repeats exists in offspring and in spermatozoa is discussed in Chapter 3. The experiments carried out in Chapter 3 were designed to determine whether TRD of *DMPK* $(CTG)_{19-37}$ repeats occurred at the level of the human embryo.

1.5.3 Mitochondrial DNA transmission

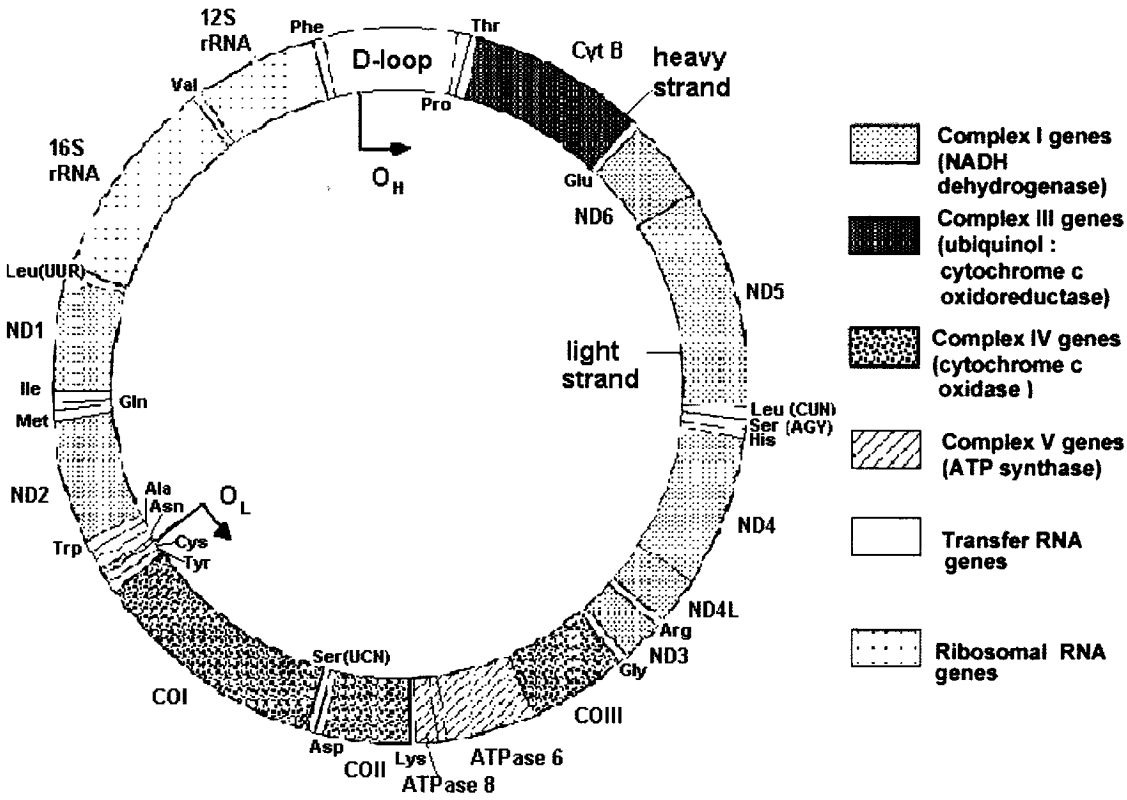
Most human somatic cells contain hundreds of mitochondria in dynamic networks, with each mitochondrion containing from 2 to 10 copies of mtDNA (Bogenhagen and Clayton 1974, Nass et al 1969, Satoh and Kuroiwa 1991, Westermann 2002). Dependent on tissue and energy demands, each cell contains between 500 and 10,000 mtDNA molecules, except for mature oocytes which have a higher mtDNA copy number but probably a single copy of mtDNA per organelle (Chen et al 1995, Piko and Taylor 1987).

In humans, mtDNA consists of a 16.6 kb double-stranded circular genome (Anderson et al 1981, Andrews et al 1999, Nass 1966) (Figure 1.5). The mitochondrial genetic code is unique and encodes for 37 genes, including 13 polypeptides involved in oxidative phosphorylation (OXPHOS) and of the RNA machinery (2 ribosomal RNAs (rRNA) and 22 transfer RNAs (tRNA)) for mitochondrial protein synthesis (Anderson et al 1981). The remaining subunits of the OXPHOS complexes and other mitochondrial proteins are encoded in the nucleus and transported to the mitochondria (Neupert 1997). The mitochondrial genome is compact, consisting of 94% coding DNA with no introns, several overlapping genes, and one non-coding D-loop region (Lightowlers et al 1997).

1.5.3.1 Mitochondrial DNA replication

Mitochondrial replication starts in the mitochondria located in the perinuclear cytoplasm and occurs mainly by a strand displacement mechanism (Clayton 1982) involving unidirectional initiation from the origin of replication of the heavy strand (O_H) (Figure 1.5). Alternatively, for cells in need of accelerated mtDNA synthesis, coordinated leading-lagging strand-coupled replication, similar to that occurring in nuclear DNA, has been proposed but is still debated (Bowmaker et al 2003, Brown et al 2005, Holt et al 2000, Yang et al 2002).

Figure 1.5: Diagrammatic representation of the human mitochondrial genome



The 16.6kb human mitochondrial genome showing the protein coding genes for the seven subunits of complex I (*ND1-ND6*), the three subunits of cytochrome *c* oxidase (*COI-COIII*), cytochrome *b* (*cyt b*) and the two subunits of ATP synthase (*ATPase 6* and *8*); the *12S* and *16S* ribosomal RNA; and the 22 transfer RNAs (tRNAs) identified by the three letter code for the corresponding amino acids. The displacement loop (D-loop), or non-coding region, contains sequences that are vital for the initiation of both mtDNA replication and transcription including the proposed origin of heavy strand replication (O_H). The origin of light strand replication (O_L) is also indicated.

Adapted from: http://www.mitomap.org/MITOMAP/mitomapgenome.pdf

Mitochondrial DNA is synthesised by a mitochondrial-specific polymerase, DNA polymerase γ (POLG), but many additional factors are required (Gaspari et al 2004, Jacobs et al 2006).

Replication in mitochondria is independent from the S phase of the cell cycle (Davis and Clayton 1996, Shadel and Clayton 1997). During mitosis, the overall number of mtDNA within each daughter cell is regulated but there is no apparent accounting of mtDNA origins, permitting some copies to replicate many times and others not at all, a process known as replicative segregation (Clayton 1982).

1.5.3.2 Inheritance of mitochondrial DNA

The mtDNA of most individuals will have the same nucleotide sequence, mtDNA homoplasmy (Monnat and Loeb 1985). However, an mtDNA mutation can cause heteroplasmy, the presence of two or more mtDNA genotypes (DiMauro 1993, Holt et al 1988). During random meiotic segregation, a cell heteroplasmic for a pathogenic mtDNA mutation will produce daughter cells with a potentially wide range of mtDNA genotypes (Wallace 1986). The rapid nature of this segregation can cause mtDNA mutations to become fixed in the female germline, within one or two generations (Blok et al 1997, Jenuth et al 1996, Koehler et al 1991).

This rapid segregation can occur as a result of random genetic drift, as shown by the wide variation in levels of mtDNA heteroplasmy in mouse oocytes (Jenuth et al 1996) and in the proportional amounts of an mtDNA point mutation in human primary oocytes (Brown et al 2001). The mechanism most favoured to explain this stochastic process is the presence of a mitochondrial genetic "bottleneck" occurring during fetal life between the differentiation of the early primordial germ cell and the oogonium stage of oogenesis (Ashley et al 1989, Hauswirth and Laipis 1982, Howell et al 1992, Jenuth et al 1996). By

some estimates, less than 10 mitochondrial genomes (Jansen and de Boer 1998) are present in the early human primordial germ cell, some of which undergo rapid replication to give rise to the nearly 200 mtDNA present in the oogonium and the eventual mean reported mtDNA numbers of between 10,000 up to 795,000 in metaphase II oocytes (Barritt et al 2002, Chen et al 1995, Jansen and de Boer 1998, Reynier et al 2001, Santos et al 2006).

1.5.3.3 Mitochondrial mutations in oocytes

For women who are heteroplasmic for pathogenic mtDNA point mutations, there appears to be no preferential loss of high mutant load during oogenesis or in early embryogenesis, suggesting that random genetic drift is the predominant mechanism in these transmissions (Brown et al 2001, Chinnery et al 2000). This is supported by reports that some mature human oocytes contain mtDNA with near homoplasmic levels of the T8993G Leber hereditary optic neuropathy mutation (LHON) (Blok et al 1997) and that oocytes that are homoplasmic for mutant mtDNA have occasionally continued development (McFarland et al 2002).

Mitochondrial DNA deletions are thought to arise sporadically during early embryo development (Chen et al 1995). A selection mechanism appears to exist in the human for the general elimination of oocytes and/or destruction of early cleavage embryos with higher levels of mtDNA rearrangements, as demonstrated by a reduced percentage of deleted mtDNA in embryos in comparison to oocytes and primordial follicles (Brenner et al 1998, Perez et al 2000). The selection to remove oocytes and embryos with high mutant load could be related to their severely reduced ATP-producing ability causing impaired fertilisation and increased embryonic arrest (Van Blerkom et al 1995, 2000) or due to a loss of membrane potential leading to the release of

mitochondria-associated factors that trigger the oocyte into the apoptotic pathway (Kroemer et al 1997, Zamzami et al 1995). These mechanisms together with the mitochondrial "bottleneck", could counteract Müller's ratchet (Muller 1964), the susceptibility of the mitochondrial genome to extinction owing to the accumulation of deleterious mutations by genetic drift.

1.5.3.4 Mitochondrial function in gametes and preimplantation embryos

The energy requirements for the completion of meiosis, fertilisation and preimplantation embryo development are dependent, at least in the mouse, on the mitochondrial complement in a mature oocyte (Piko and Taylor 1987). Until the resumption of mtDNA synthesis at the late blastocyst stage (Dvorak and Tesarik 1985), the mitochondrial number in each blastomere is diluted with each round of cell division. Mitochondrial DNA and ATP content vary considerably between mature human oocytes, even from the same individual, and were found to be significantly lower in cohorts suffering from unexplained fertilisation failure (Reynier et al 2001, Van Blerkom et al 1995). This was also found in poorer quality bovine oocytes (Stojkovic et al 2001), suggesting that low mtDNA numbers may reduce fertilisability or oocyte quality.

The mitochondria in mature mammalian oocytes are structurally undifferentiated organelles that generate ATP at lower levels than the blastocyst (Van Blerkom 1989). In the immature mouse oocyte, these mitochondria are uniformly distributed in the cytoplasm and migrate to the perinuclear region during the formation of the first metaphase spindle, which may allow increased ATP generation in regions where there are higher energy demands (Van Blerkom and Runner 1984).

After fertilisation, a similar transient mitochondrial accumulation surrounds the pronuclei and this accumulation is also seen in each blastomere during early cleavage

stages (Van Blerkom et al 2000). From the pronuclear to two-cell stage, a short period of mtDNA replication has been suggested to occur when the rate of synthesis is equivalent to the rate of destruction (McConnell and Petrie 2004). This mtDNA turnover may assist in the elimination of paternal mtDNA from the spermatozoon mid-piece, which may occur through an ubiquitin-mediated proteolysis (Kaneda et al 1995, Sutovsky et al 1999).

Transformation to the active elongated mitochondrial form occurs gradually at the later cleavage and early morula stages in the human embryo, coinciding with the activation of the embryonic genome (Sathananthan and Trouson 2000). Shortly after this, there is an increased dependence on glycolysis in most mammalian species (Dvorak and Tesarik 1985). If this transition fails to occur or involves only a fraction of the cellular mitochondria, it may lead to developmental arrest of the embryo (Van Blerkom 1989).

1.5.3.5 Mitochondrial DNA mutations in human disease

In addition to causing many mtDNA disease phenotypes (Table 1.4A and 1.4B), mtDNA mutations and/or mitochondrial respiratory chain dysfunction, may have a role in the pathogenesis of other human diseases and processes. Mutations in several nuclear genes are also indirectly related to mitochondrial disease (Table 1.5). Mitochondrial dysfunction has been suggested in many late-onset neurodegenerative disorders such as idiopathic Alzheimer disease and Parkinson and Huntington diseases, although mtDNA mutation may be a consequence of the disorder rather than the cause (reviewed by Howell et al 2005). Somatic mtDNA mutations could be involved in human ageing, in cancer and in age-related diseases, including diabetes and heart disease (reviewed by Beal 2005, Wallace 2005). Furthermore, mtDNA mutations are speculated to be involved in male infertility (reviewed by St John et al 2005) and the age-related decline in human female fertility (reviewed by Jansen and Burton 2004).

Table 1.4A: Examples of mitochondrial DNA diseases resulting from some common maternally transmitted mitochondrial point mutations

Disease	Homo/ Hetero Plasmy	Type of mutation	Location of mutation	Reference
CPEO	Hetero	A3243G	$tRNA^{Leu(UUR)}$	Moraes et al 1993
LHON	Homo/ Hetero	G3460A G11778A T14484C	ND1 ND4 ND6	Huoponen et al 1991 Wallace et al 1988 Johns et al 1992
Leigh syndrome	Hetero	T8993G/C	ATPase 6	Tatuch et al 1992
MELAS	Hetero	A3243G	$tRNA^{Leu(UUR)}$	Goto et al 1990
MERFF	Hetero	A8344G	$tRNA^{Lys}$	Shoffner et al 1990
NARP	Hetero	T8993G/C	ATPase 6	Holt et al 1990
Maternally inherited diabetes and deafness	Hetero	A3243G	$tRNA^{Leu(UUR)}$	van den Ouweland et al 1992
Aminoglycoside induced hearing loss	Homo	A1555G	12S rRNA	Prezant et al 1993

Table 1.4B: Examples of mitochondrial DNA diseases resulting from some common sporadic mutations of the mitochondrial genome

Disease	Homo/ Hetero	Type of mutation	Location of mutation	Reference
CPEO	Hetero	Large-scale deletion	Several deleted genes	Holt et al 1988 Moraes et al 1989
KSS	Hetero	Large-scale deletion	Several deleted genes	Moraes et al 1989 Zeviani et al 1988
Pearson Syndrome	Hetero	Large-scale deletion	Several deleted genes	Rotig et al 1989
KSS	Hetero	Large-scale duplication	Several duplicated genes	Poulton et al 1989

CPEO - chronic progressive external ophthalmoplegia; KSS - Kearns-Sayre Syndrome; LHON - Leber hereditary optic neuropathy; MELAS - mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes; MERFF - myoclonic epilepsy with ragged-red fibers; NARP - neuropathy, ataxia and retinitis pigmentosa

Table 1.5: Examples of nuclear genome mutations leading to mitochondrial disease

Disease	Transmi-ssion	Mutant gene	Reference
Alpers syndrome	AR	*POLG1*	Naviaux et al 1999, 2004
Barth syndrome	X-linked	*TAZ*	Bione et al 1996
Charcot-Marie-Tooth neuropathy 2A	AD	*Mitofusin2*	Zuchner et al 2004
Congenital microcephaly of Amish	AR	*SLC25A19*	Rosenberg et al 2002
Familial paraganglioma type 1	AD	*SDHD*	Baysal et al 2000
Familial paraganglioma type 3	AD	*SDHC*	Niemann and Muller 2000
Familial pheochromocytoma, cervical paraganglioma and renal cell carcinoma	AD	*SDHB*	Astuti et al 2001 Vanharanta et al 2004
Fatal infantile hypertrophic cardiomyopathy	AR	*SCO2* *COX15*	Papadopoulou et al 1999 Antonicka et al 2003
Fatal multisystemic complex I deficiency	AR	*NDUFS4*	van den Heuvel et al 1998
Friedreich ataxia	AR	*FRDA*	Rotig et al 1997
Fumarase deficiency	AR	*FH*	Bourgeron et al 1994
GRACILE		*BCS1L*	Visapaa et al 2002
HSP type 7	AR	*paraplegin*	Casari et al 1998
HSP type 13	AD	*HSP60*	Hansen et al 2002
HSP type 31	AD	*REEP1*	Zuchner et al 2006
Hypertrophic cardiomyopathy and encephalomyopathy	AR	*NDUFS2*	Loeffen et al 2001
	AR	*NDUFV2*	Benit et al 2003
Infantile onset spinocerebellar ataxia	AR	*C10orf2*	Nikali et al 2005
Ketoacidotic coma and hepatopathy	AR	*SCO1*	Valnot et al 2000a
Lactic acidosis, mitochondrial complex I deficiency	AR	*NDUFS1*	Benit et al 2001
Leigh syndrome	X-linked	*PDH* E1α	Matthews et al 1993
	AR	*SDHA*	Bourgeron et al 1995
	AR	*SURF1*	Tiranti et al 1998
	AR	*SURF1*	Zhu et al 1998
	AR	*NDUFS8*	Loeffen et al 1998
	AR	*NDUFS7*	Triepels et al 1999
	AR	*NDUFS3*	Benit et al 2004

Leigh syndrome, French-Canadian type	AR	*LRPPRC*	Mootha et al 2003
Lethal neonatal mitochondrial complex I deficiency	AR	*NDUFS6*	Kirby et al 2004
Leukodystrophy and myoclonic epilepsy	AR	*NDUFV1*	Schuelke et al 1999
mtDNA depletion syndrome, encephalomyopathic form	AR	*SUCLA2*	Elpeleg et al 2005
mtDNA depletion syndrome, hepatocerebral form	AR	*DGUOK*	Mandel et al 2001
	AR	*MPV17*	Spinazzola et al 2006
mtDNA depletion syndrome, myopathic form	AR	*TK2*	Saada et al 2001
Mitochondrial translation defects	AR	*MRPS16*	Miller et al 2004
	AR	*EFG1*	Coenen et al 2004
Mohr-Tranebjaerg syndrome	X-linked	*DDP*	Koehler et al 1999
MNGIE	AR	*ECGF1*	Nishino et al 1999
	AR	*POLG1*	Van Goethem et al 2003
Multiple cutaneous and uterine leiomyomatosis and renal cell cancer	AD	*FH*	Tomlinson et al 2002
Optic atrophy	AD	*OPA1*	Alexander et al 2000 Delettre et al 2000
Parkinson disease (early onset)	AR	*PINK1*	Valente et al 2004
Parkinsonism and premature menopause	AD	*POLG1*	Luoma et al 2004
Progressive encephalopathy	AR	*B17.2L*	Ogilvie et al 2005
Progressive external ophthalmoplegia	AD	*SLC25A4*	Kaukonen et al 2000
	AD	*C10orf2*	Spelbrink et al 2001
	AD/AR	*POLG1*	Van Goethem et al 2001
	AD	*POLG2*	Longley et al 2006
Sideroblastic anemia and ataxia	X-linked	*ABC7*	Allikmets et al 1999
Tubulopathy and leukodystrophy	AR	*COX10*	Valnot et al 2000b
Tubulopathy, encephalopathy and liver failure	AR	*BCS1L*	de Lonlay et al 2001

AD - autosomal dominant; AR - autosomal recessive; GRACILE - growth retardation, amino aciduria, cholestasis, iron overload, lactic acidosis, and early death syndrome; HSP - hereditary spastic paraplegia; MNGIE - mitochondrial neurogastrointestinal encephalomyopathy; mtDNA - mitochondrial DNA

Pathological mtDNA mutations in the germline

Expression of an mtDNA disease is seen once the mutant load (percentage of mutant mtDNA forms) reaches a critical threshold affecting energy production, which is commonly between 85 and 90% of the total mitochondrial population (Boulet et al 1992, Dubeau et al 2000, Hayashi et al 1991, Shoffner et al 1990, Thorburn and Dahl 2001). Long-lived post-mitotic tissues with high oxidative energy requirements are the most likely to be affected.

Since the first germline human mtDNA mutations were identified (Wallace et al 1988, Holt et al 1988), more than 250 point mutations and a similar number of mtDNA rearrangements have been associated with disease (MITOMAP http://www.mitomap.org). Mutations are usually heteroplasmic at the level of the mitochondrion, the tissue or the organ, although a few are homoplasmic (Huoponen et al 1991, Ozawa et al 1998, Prezant et al 1993, Wallace et al 1988). Also, males and females are equally affected, except in LHON where males are 5-10 times more likely to be affected (Hudson et al 2005).

Mitochondrial DNA diseases can be caused by mutations in the mitochondrial genome, nuclear genome and can also be sporadic. These mutations can impair overall mitochondrial DNA synthesis, including deletions, duplications and mutations in tRNA and rRNA genes, or affect individual protein coding genes, impairing the activity of a particular OXPHOS complex.

Maternally inherited mtDNA diseases usually occur as a result of point mutations (Table 1.4A). Deletions are not generally inherited or, in the case of an affected woman, not transmitted to her offspring (Brown et al 2001, Larsson et al 1992) (Table 1.4B). The sporadic rate of mtDNA mutation is estimated to be at least ten-fold higher than that of nuclear DNA (Brown et al 1979), possibly due to oxidative damage from ROS (Chance et

al 1979, Richter et al 1988). Mutations in the nuclear DNA that affect mitochondrial function generally show autosomal recessive inheritance and are usually severe with infantile onset (Rubio-Gozalbo et al 2000). Many nuclear genes encoding for mitochondrial proteins have currently been associated with mitochondrial disease (Table 1.5). Nuclear gene mutations often manifest as multiple mtDNA deletions or depletions. Reversible mtDNA depletion can be induced by HIV therapy drugs (Arnaudo et al 1991).

1.5.3.6 Murine models for mitochondrial DNA inheritance

The effects of mtDNA and nuclear DNA mutations in mitochondrial diseases have been studied through mouse models. A natural mtDNA disease mouse with hearing loss exists (Johnson et al 2001), and two models segregating mutant mtDNA (Inoue et al 2000, Sligh et al 2000) as well as knockout mouse models for Mendelian OXPHOS disease (Agostino et al 2003, Arsenijevic et al 2000, Enerback et al 1997, Graham et al 1997, Larsson et al 1998, Vidal-Puig et al 2000, Wang et al 1999) have been generated. In addition, mouse models have shown a direct relationship between somatic mtDNA mutations and ageing (Kujoth et al 2005, Trifunovic et al 2004). Conversely, mice overexpressing mitochondrial enzymes have shown an increased lifespan (Schriner et al 2005). Other mice have be used to study the mitochondrial component in other diseases, mainly neurodegenerative disorders (Bae et al 2005, Ellis et al 2005, Ferreirinha et al 2004, Gurney et al 1994, Panov et al 2002, Perier et al 2005, Silva et al 2000) and one mouse model even has an increased tumour burden with age (Van Remmen et al 2003).

Heteroplasmic murine models have also been constructed by the fusion of a cytoplast (peripheral-origin mitochondria) or karyoplast (perinuclear-origin mitochondria) from one strain with a fertilised oocyte from another strain, to look at the segregation of mtDNA to the tissues and organs of subsequent generations (Jenuth et al 1996, Laipis

1996, Meirelles and Smith 1997, 1998). Significant variation in the segregation of different mitochondrial genomes was found in selected tissues (liver, kidney, and haematopoietic) but remained neutral in other tissues in these mice. Furthermore, tissue-specific and age-related variations in mtDNA genotype survival were noted that were not primarily based on respiratory chain function or efficiency of replication (Battersby and Shoubridge 2001). Nuclear factors could be involved in mtDNA maintenance and turnover as a predominant cause for the tissue-specific selection of different mtDNA haplotypes, and several quantitative trait loci for tissue-specific nuclear control of mtDNA segregation have been mapped (Battersby et al 2003, 2005). However, the segregation of these mtDNA variants in the oocyte and preimplantation embryo, which is important for the understanding of disease transmission and essential to be able to perform PGD for mtDNA diseases, had not been previously studied in these heteroplasmic animals and was the subject of Chapter 5 of this thesis.

1.6 RATIONALE AND OBJECTIVES OF THE PH.D. STUDY

1.6.1 Rationale of the study

Pathogenic genetic mutations are a major cause of human disease and, therefore, determining the mode of inheritance of these mutations is paramount to our understanding, diagnosing and, hopefully, eventually treating such disorders. Some diseases follow classic Mendelian segregation and present with generally straightforward modes of inheritance, but many diseases display more complex transmission patterns. Certain mutations can be regulated through epigenetic interactions, by sex-specific effects related to the gender of the parent or embryo, or through modifications from other genetic elements. These processes usually manifest during gametogenesis or early embryogenesis and can influence the phenotypic outcome of a particular mutation.

To determine the mode of inheritance of a human disease that is regulated during development, it is essential to study the transmission of the mutated gene during early development: in spermatozoa, oocytes or preimplantation embryos. Most of the research in this area has occurred through reproducing the transmission of the human mutation in an appropriate animal model. Now, with limited access to human IVF material, the transmission of genetic mutations can be investigated during early human development.

In this thesis, we chose to investigate, at the level of the mammalian oocyte and embryo, the transmission patterns of two different types of developmentally regulated disease-causing genes, those that are responsible for dystrophia myotonica type 1 and mitochondrial DNA diseases. It was necessary to study the transmission of these genes in gametes and zygotes, in order to explore the effects of the various processes that could be involved in the regulation of these diseases. This could lead to a better understanding of how the manifestations of diseases are caused by mutations in these and similar genes.

1.6.2 Source of material for the study

Our unique situation of having affiliation with an IVF centre allowed us to obtain limited numbers of donated embryos for these studies. Research presented in this thesis was carried out on "supernumerary" human preimplantation embryos donated by IVF patients of the McGill Reproductive Centre. All studies involving human embryos were reviewed and approved by the Royal Victoria Hospital Research Ethics Review Board.

The embryos became available for research after transfer of the sibling embryos to the patient had been performed. Embryos were defined as "supernumerary" to the patients treatment under the following conditions: 1) the couple had signed a written consent to donate these embryos to research, 2) patients had embryos in excess of those which were transferred, and 3) the couple had chosen not to have these embryos cryopreserved, or the embryos did not qualify for cryopreservation based on poor morphology or retarded growth. For the couples that donated embryos from PGD cycles, the embryos had been diagnosed as "affected" or had not been suitable to biopsy at the time of PGD. The oocytes that were tested in Chapter 4 were either immature or had failed to fertilise during the patient's treatment.

The McGill University Animal Care Committee approved the study analysing the oocytes and embryos of heteroplasmic mice. The treatment of the mice and the collection of gametes and zygotes were carried out in accordance with the regulations set by the McGill University Animal Care Committee.

1.6.3 Choice of methodology for the study

Due to the limited number of human embryos donated to research the methodology chosen for these studies had to be extremely efficient to ensure that the

experimental findings would be reproducible and would not give inconclusive results. For this reason, the first set of experiments was aimed at determining the optimal technique to detect the amplified DNA sequence of interest at the single-cell level. Initially, each PCR assay was developed using single somatic cells, and thorough standardisation was performed before any donated gamete or zygote was tested.

For the heteroplasmic mice, the oocytes and embryos were collected after the mice had been superovulated to maximise the number of samples that could be collected from each mouse, which in turn minimised the number of mice that were needed for the study.

1.6.4 Objectives of the study

The overall objective of this Ph.D. research was to study in detail the mode of inheritance of specific disease or disease related genes that do not follow classical Mendelian transmission, in early mammalian embryos in order to i) better understand the processes involved in the transmission of these or similarly transmitted genes and their distribution patterns during preimplantation development and ii) to generate knowledge that could permit individuals, who are at risk for transmitting similar mutations, better counselling as to their reproductive options. The more detailed objectives of this research work were as follows:

1) To develop PCR protocols to genotype specific mutations at the single-cell level, either from the adaptation of existing approaches which require much higher starting DNA concentrations, or by designing and developing new protocols.
2) To generate the most efficient amplification and accurate detection of each specific mutation in order to maximise the number of embryos diagnosed through PGD and to reduce the chance of misdiagnosis, thereby hopefully increasing the success rates

and, in the context of the research presented in this thesis, to ensure reliable and optimal results from the limited research material available.

Objectives 1 and 2 are addressed in the studies presented in Chapter 2. The DM1 PCR was adapted to perform the study presented in Chapter 3, and the knowledge gained from Chapter 2 was used to adapt existing protocols for Chapters 4 and 5.

3) To conduct an analysis of the transmission of larger normal-sized human *DMPK* alleles, from parents to preimplantation embryos, in order to determine whether transmission ratio distortion exists in the inheritance of such alleles and whether there are any parent-of-origin and gender-of-embryo effects during this transmission. This study is presented in Chapter 3.

4) To determine whether intergenerational instability occurs in the transmission of larger normal-sized *DMPK* alleles at the level of the embryo.

5) To elucidate the timing and examine the variability of *DMPK* repeat expansion and its instability in early human development.

Objectives 4 and 5 are addressed the study presented in Chapter 4.

6) To investigate the segregation of heteroplasmic mtDNA in murine oocytes and embryos in order determine the distribution patterns of the different mtDNA genotypes during development and to evaluate the potential of PGD for human mtDNA disease. This study is the subject of Chapter 5.

CHAPTER TWO

Detection of mutations based on single-cell analysis

Some of the work described in this chapter has been published in the following papers:

Nicola L Dean, Seang Lin Tan, Asangla Ao (2001) The development of preimplantation genetic diagnosis for myotonic dystrophy using multiplex fluorescent polymerase chain reaction and its clinical application. *Mol Hum Reprod* 7:895-901.

Deborah L Blake, Nicola L. Dean, Casey Knight, Seang Lin Tan, Asangla Ao (2001) Direct comparison of detection systems used for the development of single-cell genetic tests in preimplantation genetic diagnosis. *J Assist Reprod Genet* **18**:557-565.

2.1 FOREWORD

Studying early human embryonic development is a research challenge due to the limited availability of suitable biological material. For gene expression studies or for the analysis of genetic mutations, precise tests have to be designed and developed, which is currently achieved almost exclusively through PCR-based technology. Each protocol developed must be thoroughly standardised in order to optimise the PCR conditions and methods of detection at the single-cell level, thereby allowing the genotyping of a single oocyte or an individual blastomere from an early cleavage-stage embryo. Once maximal sensitivity in amplifying the low concentration of DNA found in a single cell is achieved, a protocol can be easily adapted for analysis of embryos at later stages of development.

In the clinical genotyping for specific mutations from human embryos it is crucial to attain maximal accuracy and efficiency of amplification. This permits the genetic diagnosis of the highest number of embryos combined with the lowest risk of misdiagnosis. For research work on early embryonic cells, both in the mouse and particularly when donated human material is utilised, the PCR protocol must be standardised as rigorously as in clinical testing; otherwise the experimental findings may not be reproducible or may generate inconclusive results.

The aim of this chapter was to design PCR protocols at the single-cell level to permit the accurate and efficient genotyping of single diploid cells. Once developed and standardised, these tests were applied to clinical preimplantation genetic diagnosis for single-gene mutations and adapted to perform the research presented in this thesis, much of which was based on the analysis of single embryonic cells.

2.2 ABSTRACT

When performing genetic analysis using PCR, particularly at the single-cell level, the rate and accuracy of amplification and therefore the overall genotyping can be influenced by many factors, both intrinsic and extrinsic to the PCR assay. Adaptations to the PCR assay can be performed to enhance efficiency and sensitivity at the single-cell level and to counteract any of these factors that may complicate analysis. These adaptations include alterations to the PCR protocol itself and the use of different techniques to detect the amplified products of PCR.

The method of product detection used may affect the ability to visualise the amplified sequence of the single cell, thereby impacting the efficiency of amplification. In the first part of this chapter, the accuracy of heteroduplex, fluorescent fragment and fluorescent single-strand conformational polymorphism (F-SSCP) analysis as detection systems for the analysis of amplified PCR products from single cells were compared for the first time. A single-cell fluorescent multiplex PCR assay was developed for the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene exon 10 region deleted in cystic fibrosis (CF) F508 and for the short tandem repeat, D21S11. The amplification rates for cystic fibrosis were 89% by heteroduplex and 91% by fragment analysis, and 72% for D21S11 by fragment analysis. No difference in the rate of allele drop out was detected for cystic fibrosis by any method (2%). The total accuracy of each method was high, greater than 97%, although SSCP was the least accurate. Overall, however, fragment analysis gave the highest amplification rate with maximal accuracy of detection as well as the highest rate of correct diagnosis.

In addition, fragment analysis permitted simultaneous amplification of a second locus, in this case, a polymorphic marker. To assess the accuracy of detection of the CF Δ508 mutation, different protocols were compared. These assays were designed to amplify the *CFTR* exon 10 region alone, the intron 6 polymorphism alone and the *CFTR* exon 10 region multiplexed with either D21S11 or intron 6. The accuracy of diagnosis achieved using each method ranged from 94% - 98%, and it was concluded that the *CFTR* exon 10 region multiplexed with intron 6 was the approach offering the highest efficiency while minimising the risk of a serious misdiagnosis. This protocol was then applied to clinical preimplantation genetic diagnosis.

A further multiplex PCR was developed for dominantly transmitted dystrophia myotonica type 1 (DM1). The clinical application of single blastomere multiplex PCR for DM1 with a linked polymorphic marker had not been previously reported. In this work, primers were designed to amplify the dystrophia myotonica-protein kinase (*DMPK*) gene repeat region together with one of two closely mapped, highly heterozygous, short tandem repeats. After development and standardisation, this multiplex approach was found to give a diagnosis that was 99% - 100% correct. This protocol was applied clinically and adapted for the embryo genotyping performed in Chapter 3.

2.3 INTRODUCTION

Genotyping at the single-cell level

The amplification of purified DNA using PCR technologies (Mullis and Faloona 1987) has become the method of choice in medical and biological research laboratories for a variety of tasks, such as the detection of hereditary diseases, identification of genetic fingerprints, diagnosis of infectious diseases and cancer, cloning of genes, paternity testing and DNA computing (Benenson et al 2004). In recent years, PCR protocols have been adapted to permit the amplification and detection of the 5-10pg of DNA (Vendrely 1955) found in a single cell. Single-cell PCR has a wide spectrum of applications in areas ranging from genetic analysis, such as in preimplantation genetic diagnosis (PGD) and forensic science, to addressing research questions in immunology, oncology, virology, neurology and developmental biology.

Complications of PCR at the single-cell level

With genotyping at the single-cell level, many of the problems encountered with PCR using purified DNA are even more pronounced, because a large number of PCR cycles are required for sufficient amplification. Therefore, amplification of contaminating DNA sequences, which may mask or overwhelm the signal from the targeted cell, is particularly relevant at this level. Also, there are size limitations in the ability of the polymerase to amplify longer DNA fragments, which is more obvious at smaller template concentrations. Other complications affecting single-cell amplification are: allele drop-out (ADO), which is the random failure of amplification of one of the two alleles in a heterozygous cell (Findlay et al 1995a, Ray and Handyside 1996), an

increased rate of amplification failure due to the limited quantity of the starting DNA template, and the preferential amplification of one allele over the other (Walsh et al 1992).

Analysis of single blastomeres from preimplantation embryos

The utilisation of suitable mammalian embryos or somatic cells facilitates investigations into the transmission of human genes because the availability of donated human embryonic material is generally restricted to "supernumerary" embryos generated from *in-vitro* fertilisation (IVF) cycles that are not of sufficient quality to survive freezing. Testing of the human preimplantation embryo is essential in order to diagnose the inheritance of a genetic mutation before a pregnancy occurs, such as in PGD, although initial standardisation of the test can be performed using a suitable mammalian model. In order to determine the distribution pattern of a human gene during transmission it is sometimes possible to test embryos from an appropriate animal model and then to confirm the results with human embryos. To study genes or modes of transmission where no comparable animal embryo exists, a specific PCR protocol can be developed using single somatic cells such as lymphocytes, buccal cells or fibroblasts prior to testing human blastomeres.

In addition to their limited supply, it has also been suggested that blastomeres can have lower rates of amplification and higher rates of ADO than somatic cells (Cui et al 1996, Rechitsky et al 1998). Intrinsic factors that cannot be corrected, such as the absence of the target sequence caused by the accidental sampling of an anucleate blastomere or of a nucleus carrying a chromosomal mosaicism or the sampling of a

nucleus in which the DNA is partially degenerated, may be partly responsible for this observation. However, sub-optimal PCR conditions may also affect the sensitivity of the reaction, and these conditions can be modified to maximise DNA amplification from the nucleus of a single blastomere.

Therefore, if a PCR is intended for testing single human blastomeres, extensive adaptations and rigorous testing must be performed to maximise the efficiency of amplification, to obtain the highest accuracy of diagnosis and to minimise the complications of single-cell PCR. This will ensure that the greatest number of embryos can be genotyped so that the most complete information can be obtained from this limited research material.

Adaptations of PCR at the single-cell level

Technical modifications to the PCR protocol itself in order to maximise cell lysis and to promote denaturation of the DNA, annealing of the primers and elongation of the target strand, can be achieved by varying the concentrations of PCR reagents and adjusting the cycling conditions. In addition to direct adaptations to the PCR protocol, the method used to resolve the products can play a role in optimising single-cell PCR.

Maximising product detection

Conventional methods of analysing the amplified products of single cells for the presence or absence of specific gene sequences involved either gel electrophoresis followed by detection with ethidium bromide or silver staining, or the labelling of the product with radioactive primers or nucleotides. These methods had a low detection sensitivity, which could be interpreted as failure of amplification or ADO (Findlay et al

1995a), and thus required a large number of PCR cycles. Such methods were also not able to distinguish small size differences between products. Fluorescent fragment analysis was reported to be a thousand times more sensitive than standard gel electrophoresis (Hattori et al 1992), enabling lower copy numbers of product to be detected. Subsequently, fewer amplification cycles are necessary and the time for diagnosis is reduced, which is paramount in a clinical setting. Furthermore, small differences in product size, even of one base pair, can be detected.

Comparison of detection systems

At the time of initiating the experiments described in this chapter, there were reports promoting the advantages of fluorescent fragment analysis (Sermon et al 1998a,1998b) and single-strand conformational polymorphism (SSCP) (Ao et al 1998, El-Hashemite and Delhanty 1997) in the detection of genetic disorders at the single-blastomere level from preimplantation embryos. These papers described the standardisation of PCR protocols for vastly different disease mutations but did not make a direct comparison between detection systems. Therefore, the initial part of this study was designed to compare directly the reliability and accuracy of the analysis of amplified fluorescently-labelled products of single cells in the detection of the *CFTR* exon 10 region deleted in CF F508 using 3 techniques: fragment analysis, SSCP and standard gel electrophoresis.

In blinded experiments, normal and CF carrier single lymphocytes and also CF affected genomic DNA underwent a multiplex PCR assay to detect the *CFTR* exon 10 region and the highly heterozygous polymorphic tetranucleotide short tandem repeat (STR), D21S11. The amplified fragments were subjected to heteroduplex analysis using

standard gel electrophoresis or were visualised directly on an ALFexpress DNA sequencer for fragment analysis or fluorescent-SSCP (F-SSCP). The results are analysed in terms of the efficiency of PCR (amplification rate), the accuracy of detection which was defined as how truly the detection system reflected the amplified product, and also of correct diagnosis which was defined as the percentage of samples that produced the expected genotype. In this experiment, ADO did not affect the accuracy, as it is related to the efficiency of PCR, but was considered as a misdiagnosis.

Improving the accuracy of single-blastomere genotyping

In addition to optimal amplification and detection of the PCR product, it is vital, especially for mutation analysis, to correctly genotype the tested blastomere. Fluorescent PCR with fragment analysis, as well as being more sensitive, also has the advantage over heteroduplex analysis and F-SSCP of being able to detect the presence of more than one DNA sequence from any single PCR assay. The addition of multiple pairs of primers to one reaction, known as multiplex PCR (Chamberlain et al 1988), permits the simultaneous amplification of many target sequences of interest. In diagnosing genetic mutations at the single-cell level, linked polymorphic markers, for example, STRs which are highly polymorphic and usually composed of differing numbers of a 2-5 base pair repeated unit, can be used in combination with the primers for the mutation of interest (Findlay et al 1995b). The amplification of these markers can act as additional controls for ADO and DNA contamination by identifying other specific parental alleles and ensuring that the amplified product is of embryonic origin, which should reduce the risk of incorrect genotyping.

Comparison of protocols to detect CF Δ508 mutation

The second part of this study was designed to assess the accuracy of diagnosis in the detection of the CF ΔF508 mutation. This included a protocol that was developed for amplification of the *CFTR* exon 10 region multiplexed with an intron 6 polymorphism in the *CFTR* gene (Chehab et al 1991). This tetranucleotide repeat, located at the junction of intron 6 and exon 6B, exists in two forms, a hexameric form that is in absolute linkage disequilibrium with the CF ΔF508 mutation (Chehab et al 1991) and a heptameric form that is found on the majority of normal alleles. The accuracy of this assay was compared to other singleplex and the previously developed multiplex reaction for the exon 10 region and D21S11. The superior approach to detecting the CF ΔF508 mutation was determined as the one that, in addition to efficient amplification, gave the lowest risk for serious misdiagnosis.

Application of single-cell PCR to clinical PGD

The optimal PCR approach was determined from the results of the comparison experiments and was applied to clinical PGD. Such an approach would permit the maximum number of embryos to be genotyped, allowing a greater selection of the best, unaffected embryos for transfer and a higher chance of a successful pregnancy.

The two genetic disorders, CF ΔF508 and DM1, were chosen as representative tests for which direct mutation analysis on single blastomeres could be clinically applied. There is a relatively high frequency of both diseases in the Quebec population (Laberge et al 2005, Rozen et al 1990), but the mutations that cause the two diseases are very different. The transmission of CF ΔF508 is autosomal recessive with a 3 base pair

deletion in exon 10 of the *CFTR* gene being responsible for the disease. Transmission of DM1 is autosomal dominant with an expansion of a $(CTG)_n$ repeat in the *DMPK* gene causing the phenotype. Therefore, mutation detection is different for each disease but technically the protocols could be developed based on a similar approach.

Preimplantation genetic diagnosis for DM1

PGD for dominant disorders

The amplification of additional markers in the genotyping of single cells for a specific mutation is particularly relevant for dominantly transmitted disorders. A model for controlling the rate of misdiagnosis in PGD (Lewis et al 2001) has estimated that the chance of an affected embryo being classified as unaffected, when diagnosing a dominant disorder from the disease allele alone, is 10.9%. However, additional information gained from genotyping a linked marker reduces the possibility of replacing an affected embryo to 0.1%. In addition, the use of multiplex PCR can result in an increase in the number of samples that can be genotyped, which is particularly significant for DM1 or other dominant disorders, since only 50% of embryos would be expected to be unaffected.

Approaches to PGD for DM1

Most PCR protocols for DM1 are unable to amplify the expanded $(CTG)_n$ repeat region of the *DMPK* gene at the single-cell level. The high CG content of the repeat makes it refractory to PCR amplification due to secondary structures forming to stabilise the double-stranded DNA, making them more difficult to denature (Fu et al 1991). However, the $(CTG)_n$ repeat carried on non-expanded alleles is highly heterogeneous in the general population (Fu et al 1992, Lavedan et al 1993) and, therefore, preimplantation diagnosis has depended on the detection of the healthy allele from the affected parent

(Sermon et al 1997, 1998b). In this case, candidate couples for PGD must be informative for their healthy alleles i.e., the non-expanded allele of the affected parent should be different from the alleles of the unaffected parent. This indirect approach leaves open the possibility of misdiagnosis due to contamination from exogenous DNA that carries the same number of repeats.

Development of multiplex PCR for DM1 PGD

In this study, a PCR protocol was designed, based on the conclusions of our earlier experiments, and developed to perform PGD for DM1. Also, for the first time clinically, a second set of primers were multiplexed with the $(CTG)_n$ repeat primers in order to amplify a linked polymorphic marker. Primers were designed to amplify the *DMPK* repeat region and one of two closely mapped, highly heterozygous STRs on chromosome 19, D19S219 and D19S559 (Gyapay et al 1994). This novel protocol for DM1 was aimed at reducing the risk of incorrect genotyping.

2.4 MATERIALS AND METHODS

Isolation of single research cells

Human blood samples were collected and lymphocytes isolated by passing samples through a Ficoll-Paque gradient (Amersham Pharmacia Biotech, Baie d'Urfe, QC, Canada), as described by the manufacturer. Extracted lymphocytes were then washed in phosphate buffered saline (PBS). Each single lymphocyte was collected under an inverted microscope, using a sterile pulled-glass pipette, washed through 3 drops of PBS containing 4mg/ml bovine serum albumin (Sigma, Oakville, ON, Canada) and individually pipetted into a 0.2ml tube containing 5µl of alkaline lysis buffer (LB) (Cui et

al 1989) which was overlaid with sterile oil. For the study to compare methods of analysis in the detection of the CF mutation, in addition to the LB system, single lymphocytes were collected in 2μl proteinase K (125μg/ml) (PK) and 1μl sodium dodecyl sulphate (17μM) (SDS) buffer (Holding et al 1993) and again overlaid with sterile oil. Previous work and personal experiments indicated there was no significant advantage of using LB over PK/SDS buffer system (Blake et al 1999).

Human embryos were donated to research by couples undergoing IVF at the McGill Reproductive Centre (Montreal, QC, Canada). For the CF studies, embryos from couples undergoing PGD for CFΔF508 detection that were not suitable for transfer to the female were utilised. Each research embryo was incubated for 5 min in Ca^{2+}- and Mg^{2+}-free media (Cook Canada, Stouffville, ON, Canada) to allow the decompaction of blastomeres. The zona pellucida was removed using acid Tyrode solution (pH 2.5) (Sigma, Oakville, ON, Canada) and each embryo was disaggregated using a sterile pulled-glass pipette. Individual blastomeres were collected in the same manner as previously for lymphocytes, using the LB system.

Every cell sample was kept at -80°C for at least 20 minutes before processing. Prior to PCR amplification, each cell in LB was lysed by incubating at 65°C for 15 minutes whilst each cell in PK/SDS buffer was lysed by incubating at 37°C for one hour followed by inactivation of the enzyme at 95°C for 15 minutes.

Patient description

All couples presented to the McGill Reproductive Centre for PGD. Once counselled, each individual gave a sample of peripheral blood from which DNA was

extracted for mutation confirmation and a sample of buccal cells that were washed and individually tubed for the preliminary stages of each protocol development. Once the exact mutation was confirmed in each case and the appropriate test standardised, all couples underwent one cycle (two cycles for couple 1) of IVF with PGD.

DM1 couples

Couple 1: The female partner of couple 1 was a 32-year-old asymptomatic DM1 woman who had been diagnosed at the age of 18 and had previously undergone a pregnancy termination after the fetus was found to be carrying an expanded *DMPK* allele in the congenitally affected range. Both partners were confirmed to be informative for their normal alleles. The affected female carried *DMPK* alleles with $(CTG)_5$ and $(CTG)_{444}$ repeats whilst her husband was homozygous for $(CTG)_{13}$ repeats. From the two chromosome 19 STRs available (Table 2.1), the patients were found to be informative as a couple for D19S219 dinucleotide STR, and this was used for the PGD.

Couple 2: For couple 2, the male partner was affected with DM1. He had been diagnosed at the age of 27 with muscle weakness. His partner had undergone one pregnancy termination due to the fetus inheriting an expanded allele. Genetic testing confirmed that the couple was informative for their normal *DMPK* alleles. The affected male carried $(CTG)_{10}$ and $(CTG)_{500}$ repeats whilst his wife carried $(CTG)_{12}$ and $(CTG)_{20}$ repeats. This couple were also uninformative for the D19S559 STR but informative for the D19S219 STR, which was used in the PGD. Furthermore, the affected male's two siblings and both parents submitted DNA samples for linkage analysis so that the haplotype of the expanded DM1 allele could be determined.

Table 2.1: Description of primers designed for single-cell mutation analysis

Loci	Primers (5' to 3')	Expected product size (base pairs)	No. of alleles	Heteroz-ygosity	Type of repeat unit	Chromosome number (genetic location (cM))
D21S11	D21S11F: GTGAGTCAATTCCCCAAGTG D21S11R: GTTGTATTAGTCAATGTTCTCC	172-264	12	0.90	TCTA	21
DMPK	DMF: AAGGGTCCTTGTAGCCGGGAATG DMOR: CTTTGCGAACCAACGATAGGTGG DMR: CAGCCTGGCCGAAAGAAAGAAAT	70 –172	normal 33	0.85	CTG	19 (70.14)
D19S219	219F: CAGGAAGCGGAGGTTGCAGTGAG 219OR: TCCTAACCCCTTCACCGCAAGC 219R: GTGGAATTGCTGGGTGGACTGGT	152 – 182	9	0.77	CA	19 (70.14)
D19S559	559OF: GTCTTGATTGCACCACTGCACTCC 559F: CACCACTGCACTCCAGTCTGTGTG 559R: CGATTTGGGACATAATAGGTTTGAGG	191-215	6	0.88	GATA	19 (68.08)
CFTR intron 6	CF6F: GCCCATCTGTTGAATAAAA CF6R: TCTGTTAAGGCATACTGCTG	196-200	2	-	GATT	7

CFΔF508 carrier couples

Couple 3: The female partner, who was 33 years old at the time of treatment, was found to be a carrier of the ΔF508 mutation whilst the male was a carrier of the *CFTR* G551D mutation. This couple had a previous CF affected birth followed by a pregnancy that ended in a miscarriage.

Couple 4: This couple, with the female partner being 34 years of age at the time of treatment, were both carriers of the ΔF508 mutation, which was discovered after the birth of an affected child. Subsequently, the next pregnancy was terminated after the fetus was found to be CF affected.

IVF and embryo biopsy procedure

In-vitro fertilisation treatment was performed according to standard protocol at the McGill Reproductive Centre (Biljan et al 1997). On the morning of biopsy (day 3 of embryo development), the number of cells in each embryo and its grade (Dean et al 2000) were assessed. Prior to biopsy, each embryo was incubated for 5 minutes in Ca^{2+}- and Mg^{2+}- free media to allow decompaction of blastomeres. After this, the embryo was placed in a drop of HEPES buffered media (Gamete-20, Scandinavian IVF Science, Gothenburg, Sweden) and biopsied, as previously described (Ao and Handyside 1995). If an embryo had 7 or more cells, 2 blastomeres were biopsied and if it had less than 7 cells, only one blastomere was removed. After removal, each blastomere was washed through two drops of media, the presence/absence of a nucleus noted and was it transferred to a 0.2 ml PCR tube containing 5μl LB. For each embryo biopsied, a sample of media from the drop in which the blastomere(s) had been washed was placed in a PCR tube with 5μl

LB to act as a "blank" for that embryo. All samples were processed in the same manner as the research cells described earlier. After biopsy, the embryo was washed through three 40μl drops of G2.2 media (Scandinavian IVF Science), and was incubated in a separate dish in a clean labelled 40μl drop under oil.

Comparison of detection system for single-cell PCR

Multiplex PCR for the detection of CFΔF508 mutation and chromosome 21 STR

A nested PCR was designed to amplify the exon 10 region of the *CFTR* gene in conjunction with the polymorphic D21S11 locus on chromosome 21. The CF primers were as previously described (Handyside et al 1992) and new DNA primers were developed to amplify D21S11 (Table 2.1). Both CF inner forward and D21S11 forward primers were labelled with CY5 fluorescent dye.

Reaction mixes for the first round of PCR were carried out in a total volume of 50μl (lymphocytes) or 30μl (blastomeres) and included either: 1) neutralisation buffer (900mM Tris-HCl [pH 8.3], 300mM KCl, 200mM HCl), and potassium-free PCR buffer (25mM MgCl2, 100mM Tris-HCl [pH 8.3]) for lymphocytes or 2) Qiagen PCR buffer (200mM Tris-HCL [pH 8.4], 500mM KCl, 15mM MgCl2) for blastomeres; and 100μM of each dNTP, 1 unit of Taq polymerase (Qiagen, Mississauga, ON, Canada), 0.2μM of CF primers, outer forward (CFO1) and outer reverse (CFO2) (Intergrated DNA Technology, Coralville IA, USA) and 0.4μM of D21S11 primers, forward (D21S11F) and reverse (D21S11R) (Intergrated DNA Technology). The PTC-200 DNA engine thermocycler (MJ Research, Watertown, MA, USA) was programmed to denature the

DNA for 4 minutes (min) at 96 °C; followed by 18 cycles at 96°C for 60 seconds (sec), 40°C for 45 sec, 72°C for 45 sec and a final elongation step of 72 °C for 10 min.

For the second round, *CFTR* exon 10 and D21S11 were amplified separately. Two μl of the first-round products were added to reaction mixes in a total volume of 30μl with Qiagen PCR buffer, 1 unit of Taq polymerase (Qiagen) and either 200μM dNTPs and 0.8μM of CF primers, inner forward (CFI3) and inner reverse (CFI4) or 100μM dNTPs and 0.4μM of D21S11 primers, D21S11F and D21S11R. The cycling conditions for both *CFTR* exon 10 and D21S11 were the same as those described for the primary round, with the following exceptions: 1) CF PCR products were initially denatured at 96°C for 10 cycles with an annealing temperature of 50 °C for 60 sec and an elongation step of 72 °C for 90 sec, followed by an additional 20 cycles with the denaturing temperature at 94 °C and 2) D21S11 primers were annealed at 58 °C for 28 cycles.

Analysis of PCR products

To accurately assess the reliability of these detection techniques, all experiments were performed blind. Samples were coded such that individuals assigning diagnoses were not aware of the expected number of single cells or the relative ratio of genotypes included. Single lymphocytes from tested individuals who were "normal" or "carrier"; i.e., homozygous for F508 non-deleted alleles or heterozygous for the ΔF508 mutation, respectively, were tested along with 100pg "affected" genomic DNA aliquots (equivalent to 16 - 20 single cells) from individuals homozygous for CFΔ508.

Every amplified sample was initially analysed using heteroduplex and fragment analysis and subsequently, previously amplified products that gave results with fragment

analysis were blindly analysed using F-SSCP (F-SSCP had previously shown the same level of detection of fluorescent products as fragment analysis).

Heteroduplex analysis

PCR aliquots of 8μl were mixed with either 6μl of previously amplified known homozygous normal or homozygous CF affected DNA. The samples were denatured at 95 °C for 4 min and then allowed to cool to room temperature. Homo- and heteroduplex double-stranded DNA samples were resolved on a 10 % polyacrylamide gel and visualised after ethidium bromide staining (Figure 2.1A).

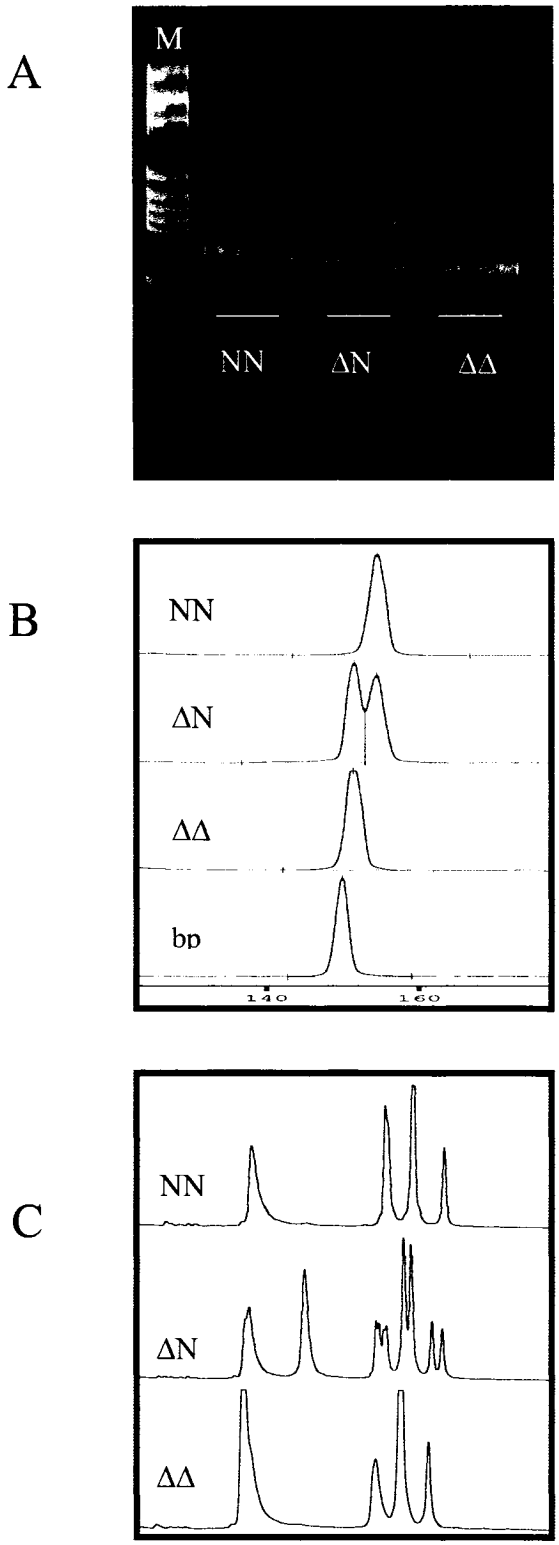
Fragment analysis

The fluorescently-labelled amplified DNA was visualised by running products on an ALFexpress sequence analyser (Amersham Pharmacia Biotech, Montreal, QC, Canada). The equivalent of 1μl of CF (Figure 2.1B) and 5μl of D21S11 PCR products with 4 μl loading dye were denatured for 3 min at 90°C prior to the samples being loaded onto a 9% polyacrylamide denaturing gel. DNA was visualised as fluorescent peaks using the instrument's packaged computer software. A fluorescently-labelled 50 - 500 base pair marker (Amersham Pharmacia Biotech) was loaded onto each gel and used to size peak fragments.

Fluorescent single-strand conformational polymorphism

After standardisation of running conditions, CF normal, carrier and affected samples could be easily distinguished after F-SSCP. PCR aliquots (2μl) of amplified CF DNA were mixed with 4.5 μl of both formamide and loading dye, denatured at 95 °C for 4 min and loaded onto an ALFexpress 9% non-denaturing polyacrylamide gel. Single-stranded DNA was resolved over 200 min at 800 V at 15 °C (Figure 2.1C).

Figure 2.1: CF diagnosis by conventional and fluorescent detection systems



Normal (NN) and carrier (ΔN) single cells, as well as CF affected (ΔΔ) DNA, were screened for the CF ΔF508 mutation using our multiplex PCR assay. Aliquots of each amplified sample were analysed by standard gel electrophoresis/heteroduplex (A), fragment (B) and F-SSCP (C) analysis. (M, Hae III marker; bp, base pair marker).

Outcome measures of study

For the products of the *CFTR* exon 10 PCR, the number of amplified samples detected was determined by heteroduplex and fragment analysis and the rates of detected ADO, accuracy of detection and correct diagnosis were calculated for each method of analysis. For the D21S11 PCR, the products were detected using fragment analysis and the rate of amplification, ADO and correct diagnosis calculated.

For our purpose, "accuracy of detection" was defined as to how faithfully the detection system profiled each amplified product and was independent of PCR efficiency. "Correct diagnosis" was defined as the percentage of samples that produced the expected genotype, which was related to the efficiency of PCR and therefore did not include the cells that failed to amplify but did take into account ADO.

Comparison of protocols for accuracy of diagnosis at single-cell level

Four PCR protocols, designed to detect the presence or absence of the CF ΔF508 mutation, were compared to determine the accuracy of diagnosis for each test. These assays were designed to detect the *CFTR* exon 10 region alone, an intron 6 polymorphism alone and the *CFTR* exon 10 region multiplexed with either the intron 6 polymorphism or, the previously developed D21S11 STR.

Multiplex PCR for the detection of the CFΔF508 mutation and intron 6 polymorphism

The primers used to detect the *CFTR* exon 10 region were the same as described earlier in this chapter. New DNA primers were designed to detect the intron 6 sequence (Table 2.1), in order to ensure annealing temperature compatibility with the CF primers. Both CF inner forward and CF6F primers were labelled with CY5 fluorescent dye.

Reactions for the first round of PCR were carried out in a total volume of 50μl and included neutralisation buffer (as above), potassium-free PCR buffer (as above), 100μM of each dNTP, 1 unit of Taq polymerase (Qiagen), 0.2μM of CF outer primers, CFO1 and CFO2 (Intergrated DNA Technology), and 0.4μM of intron 6 polymorphism primers, forward (CF6F) and reverse (CF6R) (Intergrated DNA Technology). For the first round of amplification, the thermocycler (as above) was programmed to denature the DNA for 4 min at 96 °C followed by 18 cycles at 96°C for 60 sec, 48°C for 45 sec, 72°C for 90 sec and a final elongation step of 72 °C for 8 min.

For the second round of DNA amplification, *CFTR* exon 10 region and intron 6 STR were amplified separately. Two μl of the first-round products were added to reaction mixes in a total volume of 30μl with Qiagen PCR buffer (as above), 100μM dNTPs, 1 unit of Taq polymerase (Qiagen) and either 0.4μM of CF inner primers, CFI3 and CFI4 or 0.67μM of intron 6 STR primers, CF6F and CF6R. The cycling conditions for the CF PCR were: 4 min denaturation at 96°C followed by 10 cycles at 96°C for 60 sec, 54.4°C for 45 sec and 72°C for 1 min at, then 20 cycles at 94°C for 60 sec, 54.4°C for 45 sec and 72°C for 60 sec, and finally 6 min at 72°C. For the intron 6 STR, the cycling conditions were similar except that the annealing temperature was 50.3°C, the elongation time was 70 sec, there were 10 and then 22 cycles, and the final elongation step was for 10 min.

Singleplex PCRs for *CFTR* exon 10 region and for intron 6

For the *CFTR* exon 10 region alone, the PCR conditions were the same as for multiplexing with D21S11. The conditions for the intron 6 amplified alone differed from the *CFTR* exon 10 region multiplexed with intron 6 only in that the first round annealing temperature was 50.3°C.

Analysis of PCR products

All the experiments were performed blind with the sample being coded as above. The fluorescently-labelled amplified DNA from each sample was run on the ALF Automated DNA Sequencer as described above. For the amplified products of both singleplex and multiplex *CFTR* exon 10 region, 3μl was mixed with the loading dye, whilst 5μl was used for the products of the amplified markers.

Application of single-cell protocols to clinical PGD

CFTR exon 10 region multiplexed with intron 6 polymorphism

This protocol was used after preclinical testing in further single somatic cells and research blastomeres to perform clinical PGD. This PCR protocol was later further adapted to include a set of primers to allow the detection of the CF G551D mutation.

DMPK multiplexed with chromosome 19 markers

Genetic analysis for DMPK alleles in single cells

A hemi-nested approach was used to amplify the $(CTG)_n$ repeat in the *DMPK* 3'UTR using 3 primer sequences (Table 2.1). DNA primers were also designed in a hemi-nested PCR to amplify D19S219 and D19S559 STRs (Table 2.1) from polymorphic loci on chromosome 19. The forward primers (inner forward for D19S559) in each set were labelled with CY5 fluorescent dye.

Reaction mastermixes for the first round of PCR were in a total volume of 50 μl including 5% dimethylsulphoxide (DMSO), neutralisation buffer (as above), potassium-free PCR buffer (as above), 100μM of each dNTP, 1 unit of Taq polymerase (Qiagen), 0.4μM of *DMPK* primers, forward (DMF) and outer reverse (DMOR) and 0.4μM of

either D19S219 primers, forward (219F) and outer reverse (219OR) or D19S559 primers, outer forward (559OF) and reverse (559R). For the first round of amplification, the thermocycler was programmed to denature the DNA at 96°C for 5 min, followed by 15 cycles of 60 sec at 96°C, 45 sec at 60°C and 60 sec at 72°C, and finally 5 min at 72°C.

For the second round of DNA amplification, *DMPK* and chromosome 19 marker were amplified separately. Two μl of the first-round products were added to reaction mixes in a total volume of 30μl with Qiagen PCR buffer (as above), 100μM of each dNTP, 1 unit of Taq polymerase (Qiagen) and either 0.4μM of *DMPK* primers, DMF and reverse (DMR), 0.4μM of D19S219 primers, 219F and 219R or 0.4μM of D19S559 primers, 559F and 559R. The PCR cycling conditions were as follows: 5 min denaturation at 96°C, followed by 10 cycles at 96°C for 30 sec, 62°C for 45 sec and 70°C for 60 sec, then 15 cycles at 94°C for 60 sec, 62°C for 45 sec and 70°C for 60 sec, and finally 5 min at 72°C. Three μl of the fluorescently-labelled amplified DNA was mixed with loading dye, denatured and loaded onto a denaturing gel as described above.

Once developed, this protocol was tested prior to clinical application using single lymphocytes and research blastomeres.

2.5 RESULTS

Comparison of detection systems study

CF mutational analysis and D21S11 allele detection in single cells

Initially, PCR conditions were standardised using fluorescent PCR and fragment analysis. Amplification for both the *CFTR* exon 10 region and D21S11 was attempted using singleplex, hemi-nested, two-round and nested PCR protocols. The highest

amplification was achieved using a two-round PCR with nested *CFTR* exon 10 region primers as described in the methods. Using this protocol, the amplification and ADO rates for 55 CFΔF508 carrier single lymphocytes were 95% and 2% for *CFTR* exon 10 region and 92% and 5% for D21S11, with a correct diagnosis rate of 100%. This assay was subsequently used for all blinded PCR experiments.

Blinded study results

A total of 120 single lymphocytes, both normal and carrier for CFΔ508, along with 20 aliquots containing 100pg of affected DNA were genotyped (Table 2.2).

Amplification rates for CF

The data showed that fragment analysis was slightly more sensitive than standard gel electrophoresis. Two CF normal cells scored as failed amplification by heteroduplex analysis were correctly genotyped by fragment analysis, therefore increasing the overall amplification rate for single cells from 89% - 91%.

Accuracy of detection and correct diagnosis for CF

For single-cell diagnosis, heteroduplex and fragment analysis were highly accurate in the detection of amplified product (99% and 100%, respectively) but showed one misdiagnosis in the detection of a normal cell with heteroduplex analysis. In addition, in calculating the rate of correct diagnosis, one carrier cell that amplified with ADO of the deleted allele was misdiagnosed by both detection methods, giving correct diagnosis rates of 98% for heteroduplex analysis and 99% for fragment analysis. The affected genomic CF DNA was accurately detected and correctly diagnosed in all 20 samples by both techniques.

Table 2.2:Amplification efficiency and accuracy of blinded CF diagnosis by heteroduplex analysis, fragment analysis, and SSCP

						Fluorescent genotyping						
		Heteroduplex analysis				Fragment analysis				SSCP		
Sample Type	Genotype	Amplification	ADO	Accuracy of detection	Correct diagnosis	Amplification	ADO	Accuracy of detection	Correct diagnosis	ADO	Accuracy of detection	Correct diagnosis
Lymphocyte	NN	52/60 (87%)	N/A	51/52 (98%)	51/52 (98%)	54/60 (90%)	N/A	54/54 (100%)	54/54 (100%)	N/A	51/54 (94%)	51/54 (94%)
Lymphocyte	ΔN	55/60 (92%)	1/55 (2%)	55/55 (100%)	54/55 (98%)	55/60 (92%)	1/55 (2%)	55/55 (100%)	54/55 (98%)	1/55 (2%)	55/55 (100%)	54/55 (98%)
Total		**107/120 (89%)**	**2%**	**106/107 (99%)**	**105/107 (98%)**	**109/120 (91%)**	**2%**	**109/109 (100%)**	**108/109 (99%)**	**0%**	**106/109 (97%)**	**105/109 (96%)**
DNA (100pg)	ΔΔ	20/20 (100%)	N/A	20/20 (100%)	20/20 (100%)	20/20 (100%)	N/A	20/20 (100%)	20/20 (100%)	N/A	20/20 (100%)	20/20 (100%)

"accuracy" is defined as how faithfully the detection system profiles each amplified product and is independent of PCR efficiency. ADO would not be an inaccuracy of detection as is related to the efficiency of PCR but would be considered a misdiagnosis.

"correct diagnosis" is defined as the percentage of samples that produce the expected genotype and is related to the efficiency of PCR

For CF diagnosis by F-SSCP, the results proved to be less accurate. Out of 129 samples analysed, 126 (98%) were accurately detected and 125 (97%) were correctly diagnosed. One of the four samples showing misdiagnosis by F-SSCP was confirmed as displaying ADO by fragment analysis. Of the remaining three samples, one normal cell was scored as affected while two other normal cells were identified as carriers. These results were discordant with the genotypes assigned by both heteroduplex and fragment analysis and therefore affected the accuracy and correct diagnosis rate of F-SSCP.

Fluorescent fragment analysis for D21S11

Fragment analysis was used to detect D21S11 alleles in 72% of the single cells tested with 10% ADO and in 100% of CF affected DNA with 5% ADO (Table 2.3). Even though all experiments were performed under identical conditions, there was a variation in the ADO rate between experiments. A single-cell population produced 5 of the 7 samples showing ADO for D21S11. Two of the samples exhibiting ADO for D21S11 could be genotyped due to amplification of an allele unique to these samples and therefore 93% of amplified samples were correctly diagnosed.

Genotyping of blastomeres

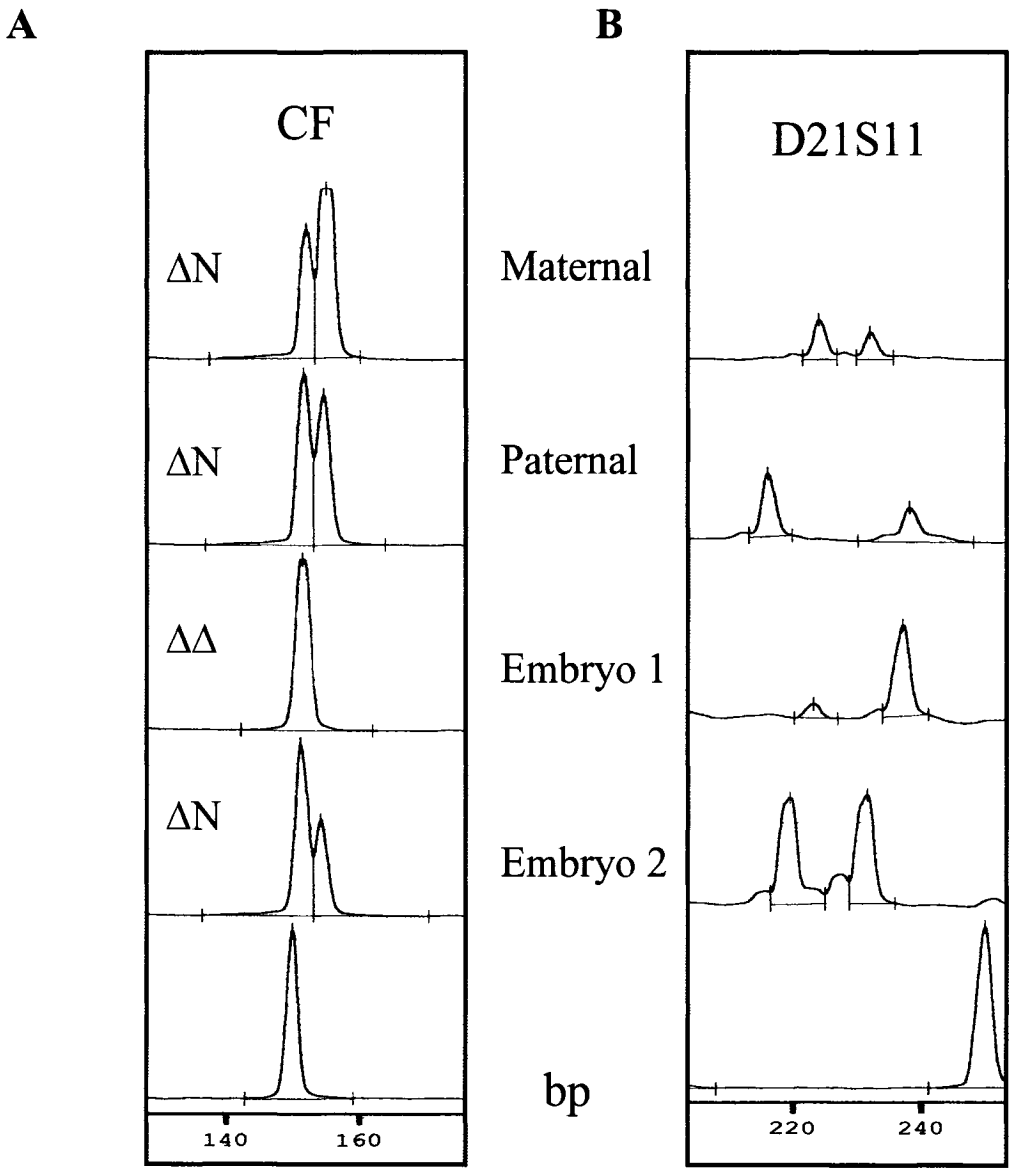
Blastomeres from spare embryos donated by a couple undergoing PGD for CF were screened for the ΔF508 mutation and D21S11 profiles. In this case, all CF normal embryos were used for embryo transfer. Figure 2.2A illustrates the CFΔF508 carrier profiles of each parent and the examples of carrier and affected embryos. The genotypes of the spare embryos can be distinguished from each other and shown to be related to the parents by the analysis of the inherited STR alleles (Figure 2.2B).

Table 2.3: Amplification efficiency and correct diagnosis rates in blinded study for D21S11 using fluorescent fragment analysis

Sample Type	Sample Size	Genotype	D21S11		
			Amplification	ADO	Correct diagnosis
Lymphocyte	60	NN	39/60 (65%)	7/39 (18%)	33/39* (85%)
Lymphocyte	60	ΔN	48/60 (80%)	2/48 (4%)	46/48 (96%)
Total	120		87/120 (72%)	9/87 (10%)	79/87 (91%)
DNA (100pg)	20	ΔΔ	20/20 (100%)	1/20 (5%)	20/20* (100%)

* Two samples, one normal and another CF affected, exhibited ADO of one D21S11 allele. Nonetheless, due to amplification of an allele unique to these samples, each sample could be correctly genotyped.

Figure 2.2: CF diagnosis and D21S11 alleles in single blastomeres



Parental and embryo profiles were assessed using a multiplex assay to screen single CF carrier lymphocytes (maternal and paternal) and blastomeres for the CF ΔF508 mutation (A) and D21S11 (B). Carrier (ΔN) and CF affected (ΔΔ) single cells were genotyped by running samples on a fluorescent DNA sequencer for fragment analysis
(bp: base pair).

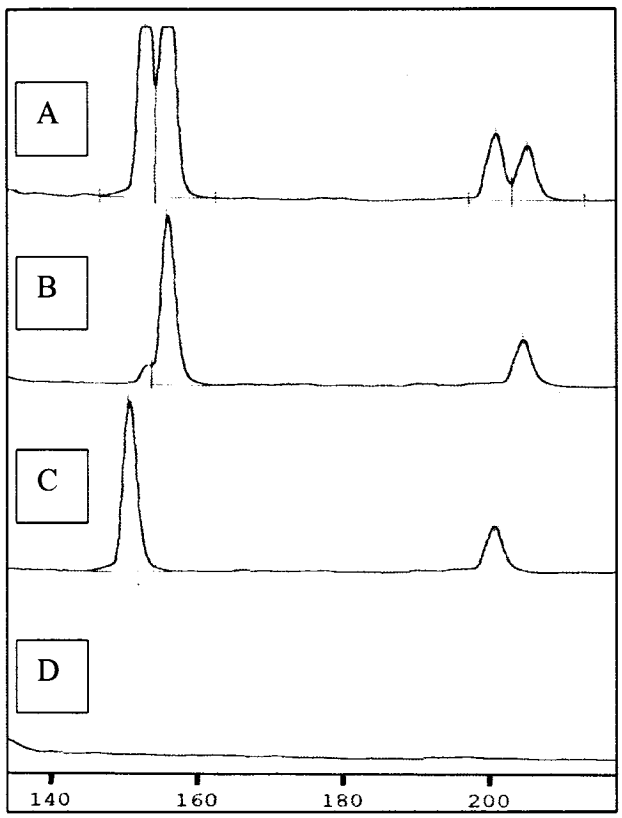
Accuracy of diagnosis at the single-cell level

Standardisation of protocols to detect the CF ΔF508 mutation

Four different protocols were developed to test for the CF ΔF508 mutation. After developing each protocol, standardisation was performed using single lymphocytes and genomic DNA. In the multiplex reaction, for the *CFTR* exon 10 region with intron 6 polymorphism (Figure 2.3), the amplification rate for 27 normal lymphocytes was 100% for *CFTR* exon 10 and 96% for intron 6. For 54 carrier lymphocytes, the rate of *CFTR* exon 10 amplification was 98% and the rate of ADO 2%, whilst the amplification with the intron 6 primers was 96% and the rate of ADO 2%. For the 16 affected samples, the amplification rates for *CFTR* exon 10 and intron 6 were 94% and 88%, respectively. The multiplex PCR for *CFTR* exon 10 region with D21S11 was not further tested since standardisation had recently been performed for the earlier experiments.

In the singleplex protocols, for the 111 normal and carrier single lymphocytes tested for *CFTR* exon 10 amplification alone, the amplification rate was 94% with 9% ADO in carrier cells, and for the 69 affected DNA samples the amplification rate was 97.1%. For intron 6 amplified alone, the amplification rate for 90 single lymphocytes was 97% with 4% ADO in the carrier cells, and for the 25 affected genomic DNA the amplification rate was 92%.

Figure 2.3: Multiplex PCR for CF ΔF508 and intron 6 polymorphism



Multiplex PCR to detect the CF ΔF508 mutation and intron 6 polymorphism in single lymphocytes.
A. Cell from individual heterozygous for CF ΔF508 mutation
B. Cell from unaffected individual
C. Cell from individual affected with CF ΔF508 mutation
D. Reaction blank

Blinded comparison of PCR assays to detect the CF ΔF508 mutation

The results of these experiments are shown in Table 2.4. Briefly, the overall rates of amplification for *CFTR* exon 10 are comparable between the series and range from 95% - 98% with a rate of ADO of between 0% and 10%. For the two markers, the rate of amplification ranged from 82% - 98% for D21S11 and intron 6, respectively, and the rate of ADO was approximately 10% for each assay. This would suggest that the conditions for amplification of the marker were not as optimal as those for the *CFTR* exon 10 region primers.

The proportion of cells that were correctly genotyped ranged from 95% for *CFTR* exon 10 alone to 98% for *CFTR* exon 10 multiplexed with the intron 6 marker. Using this multiplex protocol, one carrier lymphocyte showed ADO of the normal allele and was misdiagnosed due to simultaneous amplification failure of the intron 6 product. However, a second carrier cell with ADO of the affected allele was correctly genotyped due to amplification of the full STR profile.

Clinical preimplantation genetic diagnosis

Multiplex PCR for the *CFTR* exon 10 region and intron 6 polymorphism

Preclinical testing

Before clinical application, to ensure maximum efficiency in blastomeres, further experiments were performed using donated human embryos. In 22 normal blastomeres, from 4 embryos, there was 100% amplification for both *CFTR* exon 10 and intron 6. For the 9 carrier blastomeres there was 89% amplification for both *CFTR* exon 10 and intron 6 with 0% ADO for exon 10 and 13% for intron 6.

Table 2.4: Comparison of blinded PCR protocol for optimum detection of CFΔF508

CF singleplex

Sample	Number of samples	Amplification CF (%)	ADO CF (%)	Correct diagnosis (%)*
NN	24	23 (95.8)	-	23/23(100.0)
ΔN	43	40 (93.0)	4 (10.0)	36/40(92.5)
ΔΔ	13	13 (100.0)	-	13/13(100.0)
Total	80	76 (95.0)	4 (10.0)	72/76 (94.7)

Intron 6 STR singleplex

Sample	Number of samples	Amplification intron 6 (%)	ADO intron 6 (%)	Correct diagnosis (%)*
NN	22	21 (95.5)	-	21/21 (100.0)
ΔN	10	10 (100.0)	1 (10.0)	9/10 (90.0)
Total	32	31 (97.9)	1 (10.0)	30/31 (96.8)

CF multiplexed with D21S11

Sample	Number of samples	Amplific-ation CF (%)	ADO CF (%)	Amplificat-ion D21S11 (%)	ADO D211S11 (%)	Correct diagnosis (%)*
NN	20	19 (95.0)	-	15 (75.0)	3 (20.0)	17/19 (89.5)
ΔN	30	30 (100.0)	0	25 (83.3)	1 (4.0)	30/30 (100.0)
ΔΔ	10	10 (100.0)	-	9 (90.0)	1 (11.1)	10/10 (100.0)
Total	60	59 (98.3)	0 (0)	49 (81.6)	5 (10.2)	57/59 (96.6)

CF multiplexed with intron 6 STR

Sample	Number of samples	Amplific-ation CF (%)	ADO CF (%)	Amplificat-ion intron 6 (%)	ADO intron 6 (%)	Accuracy of diagnosis (%)*
NN	30	28 (93.3)	-	27 (90.0)	-	28/28 (100.0)
ΔN	34	33 (97.1)	2 (6.1)	30 (88.2)	3 (10.0)	32/33 (97.0)
ΔΔ	1	1(100.0)	-	1 (100.0)	-	1/1 (100.0)
Total	65	62(95.4)	2(6.1)	56(89.2)	3(10.0)	61/62 (98.4)

* correct diagnosis from amplified cells

Clinical PGD for CFTR exon 10 region and the intron 6 polymorphism

Clinical PGD was performed for couples 3 and 4. Couple 3 only had 2 embryos available for testing and amplification from the 2 blastomeres biopsied was 100% for both *CFTR* exon 10 and the intron 6 STR. Although both embryos were diagnosed as normal with respect to the CF508 deletion, only 1 embryo was transferred based on embryo quality.

For couple 4, biopsy and diagnosis were performed on 4 embryos. The amplification rates from the 7 blastomeres tested were 86% for *CFTR* exon 10 with 17% ADO and for intron 6, the amplification was 100% and ADO 0%. Based on the products amplified from both the *CFTR* exon 10 region and intron 6 STR primers, and the fact that in most embryos 2 cells were biopsied, all the embryos could be genotyped and a normal and carrier embryo selected for transfer to the patient. One of the 4 blanks tested that was paired to a cell that amplified with an affected profile, showed amplification of carrier DNA. This embryo and the other 2 non-transferred embryos from both cycles were tested post clinically with the post diagnosis genotyping results verifying those obtained on the day of PGD. For the 17 blastomeres tested, the amplification rates for the *CFTR* exon 10 region and intron 6 STR primers were 64% and 71%, respectively.

Multiplex PCR for myotonic dystrophy (DM1)

Development of DM1 protocol

Initially, a singleplex reaction with 36 cycles was designed to detect the *DMPK* $(CTG)_n$ repeat. From 218 single lymphocytes, this gave acceptable rates of amplification (91%) and ADO (9%). This protocol also worked for *DMPK* multiplexed with both D19S219 and D19S559. However, as the analysis print outs did not give completely flat

background peaks in these assays, there was concern that unspecific bands would cause diagnostic problems, so this was not considered suitable for clinical analysis. Therefore, a hemi-nested approach was employed in a two-round PCR protocol.

After standardisation of suitable PCR conditions, the protocol was tested on single lymphocytes. Initially, using *DMPK* alone, the amplification in 112 cells was 93% and ADO was 7%. From 50 lymphocytes used for *DMPK* with D19S559, the amplification rate was 92% for both loci and ADO was 4% for *DMPK* and 7% for D19S559. From the 40 lymphocytes assayed for *DMPK* with D19S219 the amplification rate was 100% for both loci with 5% ADO for *DMPK* and 0% for D19S219.

The final standardisation to verify the accuracy and efficiency of the PCR assay for *DMPK* multiplexed with D19S559 or D19S219 was performed using a blinded approach (Table 2.5), as described above. One hundred and thirty-two tubes to amplify for *DMPK* with D19S219 and 36 for *DMPK* and D19S559 were coded for samples from 6 donors and for blank samples. All donors were heterozygous for their *DMPK* and STR profiles so that ADO could be calculated. The profiles of the donors for *DMPK* and both the STRs can be seen in Figure 2.4. The genuine allele for the D19S219 dinucleotide repeat is sized according to the largest fragment amplified and the smaller peaks seen in the profiles (Figure 2.4A and B) are spurious DNA fragments called "stutter bands". These represent a PCR artefact caused by slippage of the Taq polymerase over the CA repeat unit which generates amplified fragments that differ exactly 2 base pairs in length (Walsh et al 1996).

Table 2.5: Blinded PCR for *DMPK* multiplexed with D19S219 and D19S559

***DMPK* multiplexed with D19S219**					
Number of samples	Amplification *DMPK*	ADO *DMPK*	Amplification STR	ADO STR	Correct diagnosis*
Lymphocytes 132	120 (91.0%)	1 (0.8%)	125 (94.7%)	4 (3.2%)	119 (99.0%)
Blanks 18	1	0	0	0	_
***DMPK* multiplexed with D19S559**					
Number of samples	Amplification *DMPK*	ADO *DMPK*	Amplification STR	ADO STR	Correct diagnosis*
Lymphocytes 36	33 (92.0%)	2 (6.1%)	33 (92.0%)	3 (9.1%)	33 (100.0%)
Blanks 5	0	0	0	0	_

* correct diagnosis from amplified cells

Figure 2.4: Donor genotypes for *DMPK*, D19S219 and D19S559 repeats amplified from single lymphocytes

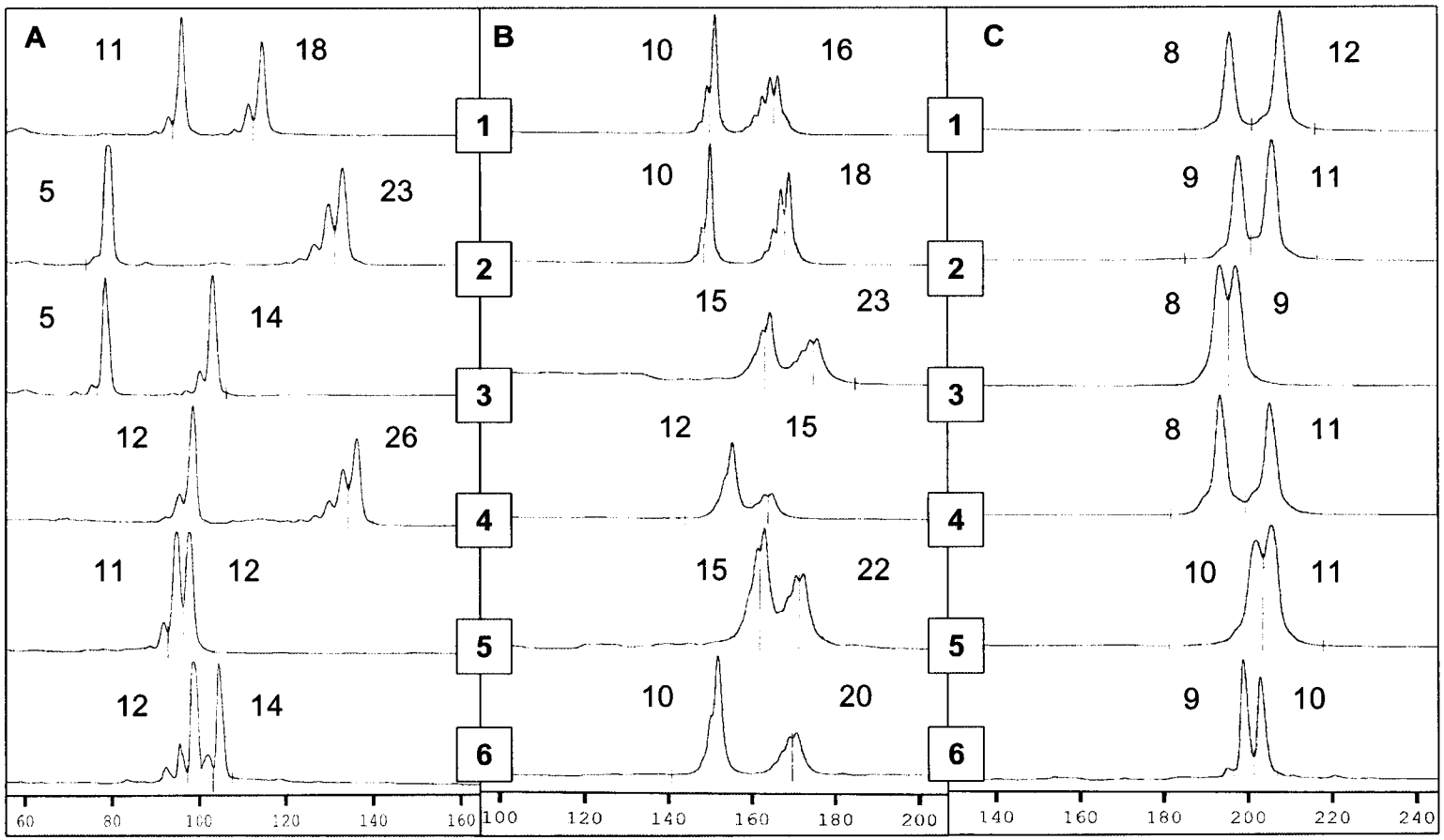


Figure 2.4A: *DMPK* CTG repeats for each of 6 heterozygous donors from single cells.
Figure 2.4B and 2.4C: D19S219 dinucleotide and D19S559 tetranucleotide profiles for the same 6 donors.
The numbers denote the genotype of each individual at that particular locus i.e. the number of repeats. The scale is referring to base pairs and calculated from the inclusion of a fluorescent 50-500 base pair marker on each gel.

The overall amplification rates were over 90% and the rates of ADO were under 10% (Table 2.5). For correct genotyping of cells, for *DMPK* with D19S559 there was 100% accuracy and with D19S219 the rate was 99%. The reason this was not 100% was because of the inability to designate a genotype to one of the lymphocytes as a result of ADO. One of the blanks amplified *DMPK* products of a size that matched the *DMPK* donor genotype from which the blank was collected.

Preclinical testing

Fifty-five blastomeres from 15 embryos donated for research by 3 patients were analysed to test the accuracy and sensitivity of the PCR protocol in embryonic cells. The cells were collected, lysed and analysed the same day to reproduce the situation in a clinical PGD. The amplification rate for *DMPK* was 84% with 7% ADO and for D19S219 the amplification and ADO rates were 82% and 9%, respectively. None of the 14 blanks tested showed amplification of any product.

Clinical PGD for DM1 and D19S219

For couple 1, in the first cycle, 9 embryos were suitable for biopsy on day 3. From the 12 blastomeres tested, the amplification rate for *DMPK* was 92% with ADO of 0% (confirmed in the spares), and amplification for the D19S219 STR was also 92% but with an ADO of 18%. In the second cycle, there were 11 embryos for testing and 16 blastomeres were biopsied. The *DMPK* amplification rate was 75% and ADO was 22%, and for the D19S219 STR the rates were 83% and 18%, respectively.

For couple 2, there were 10 embryos suitable for testing from which 13 blastomeres were biopsied. The rates of amplification for *DMPK* and STR were 85% and 92% respectively and the ADO was 9% for each set of primers.

Overall the rate of amplification in PGD for *DMPK* was 84% whilst the ADO was 10% and for D19S219 the amplification rate was 89% and the ADO was 15%. An example of the print out of the PGD using fluorescent PCR can be seen in Figure 2.5. None of the 32 blanks tested showed amplification of any product. Two embryos diagnosed to be unaffected were transferred in each cycle. After the clinical DM1 cases, 36 blastomeres from non-transferred embryos were tested and the amplification rate for *DMPK* was 67% and ADO 17% whilst for D19S219 the amplification and ADO rates were 64% and 17% respectively, which was lower than for the clinical PCR most likely due further deterioration in embryo quality for the supernumerary embryos after continued culture. The results of the post clinical testing verified those obtained on the day of PGD.

Figure 2.5: Clinical PGD for *DMPK* and D19S219

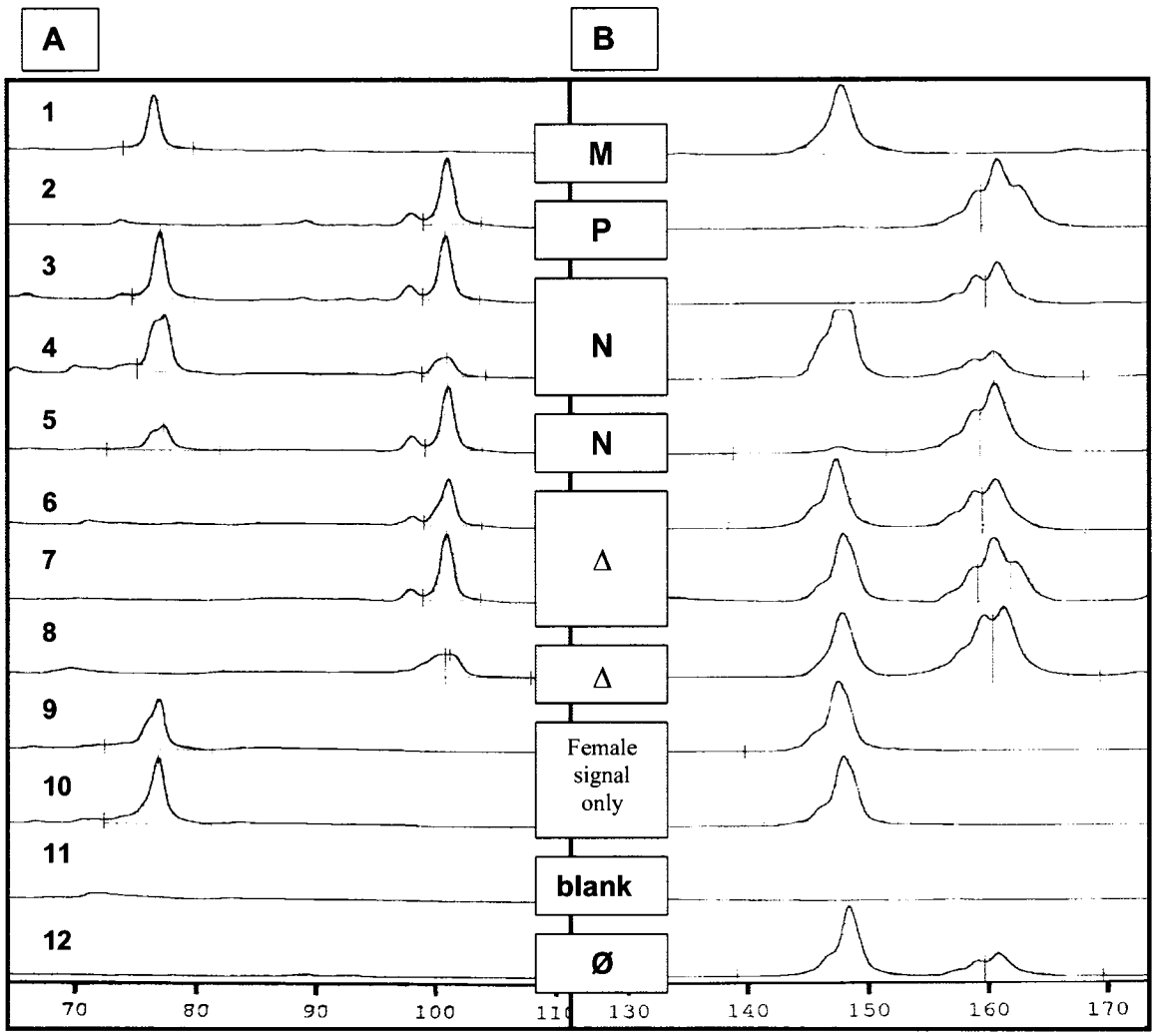


Figure 2.5A Results obtained after PGD for DM1

Figure 2.5B Results obtained for D19S219 STR on the same blastomeres

Lane 1: female profile for *DMPK* with $(CTG)_5$ and expanded allele (not amplified) and D19S219 homozygous for $(CA)_{10}$

Lane 2: male profile for *DMPK* homozygous for $(CTG)_{14}$ and D19S219 homozygous for $(CA)_{16}$

Lanes 3 and 4: two blastomeres from one embryo diagnosed as DM1 unaffected

Lane 5: blastomere from embryo diagnosed as DM1 unaffected

Lanes 6 and 7: two blastomeres from one embryo diagnosed as DM1 affected

Lane 8: cell from blastomere diagnosed as DM1 affected

Lanes 9 and 10: blastomeres with only female signals for both *DMPK* and D19S219

Lane 11: blank sample

Lane 12: blastomere showing amplification failure for *DMPK* repeat

2.6 DISCUSSION

The aim of these experiments was to develop efficient and accurate PCR protocols for the detection of specific single gene mutations, at the single-cell level that could then be applied, both clinically and for research, in the genetic diagnoses of preimplantation human embryos. The applications that directly resulted from the protocols developed included clinical PGD, where optimisation of genetic testing can result in the maximal number of embryos being accurately genotyped, which would hopefully increase the pregnancy rate and also the adaptation of the *DMPK* repeat protocol for application in the study described in Chapter 3.

The tests were developed by designing different nested or hemi-nested PCR protocols (Mullis and Faloona 1987) to increase the sensitivity and specificity of the reaction, each of which underwent rigorous testing at the single-cell level, both for somatic and embryonic cells. Once the results of standardisation gave amplification rates of $\geq 90\%$ and rates of ADO of $\leq 10\%$, comparison experiments were performed. To avoid possible bias of diagnosis, these experiments were run in a blinded fashion. It was concluded from testing the amplified products of the *CFTR* exon 10 region and D21S11 assay that both the reliability and accuracy were greater using fluorescent fragment analysis as the detection system when compared to F-SSCP or heteroduplex analysis. Furthermore, in fragment analysis the simultaneous amplification of a linked polymorphic marker in addition to the mutation of interest, such as the *CFTR* exon 10 and intron 6 protocol further increased the efficiency and accuracy of diagnosis. For each research or non-transferred PGD embryo, all the blastomeres were genotyped. The genotype for each cell within an embryo was concordant throughout all the blastomeres, further demonstrating the accuracy of the developed tests.

Accuracy of PCR

After comparing heteroduplex, fragment and F-SSCP analysis, the accuracy of detection of CF F508 alleles from all amplified samples was 99%, 100% and 98%, respectively. A sample misdiagnosed by fragment analysis due to ADO was similarly genotyped by heteroduplex analysis and F-SSCP.

There was, however, one normal cell misdiagnosed by heteroduplex analysis and several normal samples that were misdiagnosed by F-SSCP, giving total rates of correct diagnosis of 98% for heteroduplex analysis, 99% for fragment analysis and 97% for F-SSCP. One normal sample was genotyped as affected using F-SSCP, which could be explained by possible lane-to-lane variation. A slight shift in the migration of DNA can, in this instance, make a normal sample appear affected. Two other samples diagnosed as carriers had been correctly genotyped by heteroduplex and fragment analysis as normal with no evidence of DNA contamination. Since SSCP profiles are dependent on the secondary structure of DNA, it is most likely that inefficient denaturation of samples prior to gel loading resulted in residual secondary structure, producing a DNA profile for these samples that closely resembled carrier cells.

Therefore, fragment analysis gave the highest overall efficiency of amplification, accuracy of detection and correct diagnosis. In comparison, F-SSCP proved to be unreliable for single-cell analysis.

Advantages of fluorescent fragment analysis

When this study was initiated, the detection of fluorescently-labelled amplified products by automated fragment analysis at the single-cell level was still a fairly new technique (Sermon et al 1998a, 1998b) but today it is a fairly standard procedure in single-cell mutation detection. The results of this study concur with the increased

sensitivity of detection demonstrated previously for fluorescent fragment analysis (Hattori et al 1992). The total amplification rates reported here for mutation detection using fluorescent-based analysis were 94%, 97% and 86% for lymphocytes, genomic DNA and pre-clinical blastomeres, respectively. This agrees with previous reports that blastomeres are prone to lower rates of amplification than somatic cells (Cui et al 1996, Rechitsky et al 1998).

Minimisation of allele drop out

It has also been shown that fluorescent fragment analysis can reduce the rate of ADO (Findlay et al 1995a, Sermon et al 1998b). In our research cells, this ranged from 0 to 13% and averaged 5% for lymphocytes and 7% for pre-clinical blastomeres, which is lower than described in most protocols using conventional analysis (Findlay et al 1996). It has been suggested that a number of cases of ADO are, in fact, preferential amplification of one allele, leading to a lack of detection of the other weakly amplified product using conventional analysis (Findlay et al 1995a). Due to the increased sensitivity of fragment analysis, cases of true ADO and extreme preferential amplification can be distinguished, as long as the X-axis of any fluorescent PCR print out is set to produce flat background lines, thereby removing unspecific background peaks. The background lines were kept flat in these experiments and preferential amplification was noted (Figure 2.4B, donor 4). However, in the CFΔF508 carrier samples tested, there was no difference in the ADO rate (2%) between samples analysed by either heteroduplex analysis or fragment analysis (Table 2.2), suggesting that preferential amplification had not occurred here.

Analysis of multiple products in a single PCR

Another advantage of fluorescent PCR is the ability to detect multiple amplified products. At the outset of the current work, there were only limited publications describing multiplex PCR in single blastomeres (Ao et al 1998, Findlay et al 1998, Kuliev et al 1998, Wu et al 1993), and multiplex PCR had not been described for DM1 single-cell genetic diagnosis. Currently, changes in methodology resulting from an increased understanding of the difficulties of single-cell amplification have resulted in the almost universal inclusion of linked markers in PCR protocols for genetic diagnosis of human embryos (Thornhill et al 2005, Verlinsky and Kuliev 2003).

Advantages of amplifying multiple products simultaneously

Detection of ADO

After linkage analysis in an informative family, the inclusion of linked polymorphic markers can assist in detecting ADO. A few CF PGD misdiagnoses were likely to have been as a result of ADO (Grifo et al 1994, Harper and Handyside 1994, Verlinsky 1996). The two STRs used in our DM1 protocol map very closely to the *DMPK* region of chromosome 19 (with 0% and 2% recombination frequency). Linkage analysis for couple 2 determined the haplotype of the expanded allele with respect to the STRs. Therefore, differentiation between ADO of the normal-sized *DMPK* repeat from the affected parent and an affected embryo (i.e. absence of the normal-sized repeat from the affected parent) could be made based on the embryonic STR profile.

For the CF ΔF508 PGD multiplexed with intron 6 polymorphism since the hexameric repeat is in complete linkage disequilibrium with the mutated CF allele (Chehab et al 1991) it can act as a control for both contamination and ADO monitoring

without linkage analysis. In our experiments, even though the correct diagnosis rates for the *CFTR* exon 10 with D21S11 STR and the *CFTR* exon 10 with intron 6 protocols were comparable, the inclusion of the linked intron 6 polymorphism was deemed the preferential approach to performing PGD for CF ΔF508. This conclusion was reached after an analysis of the type of genotyping errors observed using each protocol. The misdiagnosis in the *CFTR* exon 10 with intron 6 PCR was caused by ADO for both the mutation and the marker, simultaneously, in a carrier cell. However, in the *CFTR* exon 10 with D21S11 STR, a cell was incorrectly genotyped based on its amplified fragment size, in that for two cells of normal genotype after amplification, one was diagnosed as carrier and the other as affected. The likelihood of such a serious misdiagnosis would be reduced with the inclusion of the linked intron 6 polymorphism, since a mis-sized *CFTR* exon 10 fragment would be co-analysed with the incorrect intron 6 fragment allowing detection of this type of error.

Controlling contamination from an unrelated source

When performing mutation analysis, the amplification of a second locus also reduces the possibility of misdiagnosis due to amplification of exogenous contaminating DNA. For a recessive disease such as CFΔF508, contamination of an affected test blastomere with unaffected DNA could lead to this embryo being diagnosed as a heterozygous carrier and potentially being available for transfer to the female. Based on a model for controlling misdiagnosis, 5.8% of affected embryos would be classified as unaffected for a recessive disease when the decision is based on disease alleles alone, compared to 0.44% when the disease alleles are amplified in conjunction with a linked marker (Lewis et al 2001). The two multiplex reactions designed to detect the CF F508

deletion could act as controls for DNA contamination, even though the amplified sequences for the *CFTR* and D21S11 PCR exist on different chromosomes.

In the assay for the detection of dominantly inherited DM1, the presence of exogenous DNA would be identified if a *DMPK* fragment was amplified that was not of a size carried by either parent. However, the incorporation of contaminating DNA containing the same number of repeats as that found on the unaffected allele of the DM1 parent, could lead to a serious misdiagnosis. With the addition of a highly heterozygous marker, the possibility that both the *DMPK* and STR repeats from unrelated DNA would amplify the same profile as that expected from the parent is greatly reduced. The amplification of unexpected STR profiles would be recognised as contamination, thereby avoiding a misdiagnosis.

Controlling contamination from a related source

In PGD, the addition of a polymorphic marker could also reduce the chance of misdiagnosis due to contaminating endogenous DNA such as from maternal cumulus cells or from sperm attached to the zona pellucida. Procedures to minimise these sources of contamination are now implemented in most clinical laboratories after a PGD misdiagnosis for DM1 occurred that was believed to be a consequence of maternal contamination (Sermon 1998c). However, if such contamination were to occur, as long as the affected parent was heterozygous for the marker, the profile amplified from the test cell would have both copies of the parental marker. This would be recognised as contamination as a blastomere should only amplify one copy of the marker from each parent. In our experiments, the amplified PCR products from single lymphocytes, preclinical and clinical blastomeres matched the expected genotype (Tables 2.3, 2.4 and

2.5 and Figures 2.2, 2.3 and 2.4), indicating that the results obtained were the true genotypes and that DNA contamination was unlikely.

Detection of chromosome aneuploidy

Chromosomal mosaicism such as haploidy can be present in a significant proportion (15%) of single blastomeres (Harper et al 1995). Aneuploidy, of the chromosome on which the mutated gene is localised, can be detected with the addition of a polymorphic marker. This will prevent a reduction in the accuracy of single gene defect PGD occurring as a consequence of an incorrect chromosome copy number, which is particularly relevant for patients of advanced reproductive age, for whom the likelihood of aneuploidy is increased (Hassold and Jacobs 1984, Hassold and Chiu 1985). In the scenario where a cell amplifies only one allele of the disease gene, the marker profile will help determine the chromosomal status for the chromosome in question. If a full marker profile is amplified, it is likely that ADO has occurred but if only one marker allele is amplified, the cell could be chromosomally abnormal.

One DM1 PGD embryo amplified only the maternal alleles in each of two blastomeres (Figure 2.5, lines 9 and 10). If *DMPK* had been amplified alone, ADO of the paternal allele would have been diagnosed. However, with simultaneous amplification failure of the paternal STR, a diagnosis of monosomy or mosaic for at least chromosome 19 was given and the selection of an abnormal embryo was avoided. This result was verified in the remaining blastomeres of this embryo.

A polymorphic marker can also be useful to detect chromosomal abnormality if located on a different chromosome from the mutated gene, such as a chromosome prone to age-related aneuploidy. The D21S11 STR was principally designed to detect aneuploidy of chromosome 21 for women heterozygous for this STR, in the proportion of

cases where non-disjunction of maternal meiosis I occurs, that is, when 3 different alleles are present, which is the most prevalent chromosomal error in trisomy 21 live-births (Antonarakis et al 1992, Yoon et al 1996). The use of this protocol for comparing the efficiency of detection was first presented here and, subsequently, this STR design was used for clinical PGD (Lorusso et al 2006). PCR-based PGD for a causative gene in conjunction with aneuploidy screening has now been reported (Rechitsky et al 2006).

Alternatives to multiplex PCR with microsatellite markers

Universally informative markers

One of the limitations of including polymorphic microsatellite markers to aid in the diagnosis of single gene defects for PGD is the necessity of finding an informative marker for each prospective couple. This could involve the testing of many different markers before a suitable one is found. In some populations or ethnic groups, it may be that closely linked informative markers are not available for the mutation of interest. The incorporation of the linked intron 6 repeat in the detection of CF ΔF508 is an approach that is informative in every diagnosis for this mutation and has been described for PGD (Verlinsky et al 1997). This will be beneficial to a PGD laboratory in terms of cost and time, as the incorporation of universally informative markers into mutation analysis will require fewer resources than the development of a specific test for each couple. The cost of performing a genetic test using fluorescent fragment analysis is considerably higher than that for standard gel electrophoresis. However, fragment analysis has been shown to be more accurate and also is amenable to multiplex PCR. Therefore, fluorescent multiplex PGD protocols would be more viable, both in terms of cost and development time, if universally informative markers were available for each mutation of interest.

Single nucleotide polymorphisms

The introduction of single nucleotide polymorphisms (SNP), which are more widespread in the genome than microsatellite markers, is improving considerably the chance of finding informative linked markers to incorporate into single-cell PCR for PGD. Clinical PGD for mutation detection with co-amplification of a linked SNP (Abou-Sleiman et al 2002b) and for indirect mutation detection through the amplification of linked SNPs (Apessos et al 2001) have been described in conjunction with fluorescent fragment analysis. Minisequencing with the detection of the product using microarrays is a new development that permits the quick and accurate detection of SNPs in extremely short stretches of amplified fragment, which is ideally suited for single-cell PCR.

Universal mutation testing

Mutation-specific primers can also be spotted on microarrays (Kurg et al 2000) and, in future, it may not be necessary to develop a specific PGD test for each mutation of interest because microarray technology could permit the development of one genetic test for a spectrum of mutations, such as for the more than 1,000 mutations associated with the *CFTR* gene. This technology could be applied to clinical PGD, once an efficient method of whole genome amplification for single cells is available.

Alternative techniques to amplification by conventional PCR

A limiting factor in the study of human embryos, and even in some instances for forensic analysis, prenatal diagnosis and oncogenetics, is the amount of genomic DNA available in the starting sample. In this work, optimisation of conventional PCR protocols was used to maximise the efficiency in single-cell amplification. Recently, new methods of DNA amplification have been developed that can produce a higher

concentration of the desired sequence from a single cell than standard PCR and therefore could be beneficial in increasing the accuracy of PGD. Real-time PCR and multiple displacement analysis, which are described in Chapter 1, are such techniques.

Applications of real-time PCR and multiple displacement analysis

In addition to increasing the quantity of the product, real-time PCR generates short amplified fragments, thus reducing the likelihood of ADO (Vrettou et al 2004) and the technique has been used in clinical PGD for a single gene defect (Almeida et al 2005).

Multiple displacement analysis (MDA) has proven very efficient in the whole genome amplification of small DNA samples and even single-cell analysis with very little sequence representation bias (Spits et al 2006). However, currently the rates of ADO and preferential amplification tend to be fairly high for MDA compared to other single-cell protocols. In addition, the time generally taken to perform the reaction is prohibitory for use in clinical PGD, since it does not allow the embryo transfer to occur within the defined window for successful implantation.

Direct mutation detection for DM1

Our fluorescent multiplex PGD permits an accurate diagnosis for DM1 and is applicable to many DM1 affected couples but, ideally, if both the expanded and normal *DMPK* alleles can be reliably amplified from a single cell, then direct diagnosis of a DM1 affected embryo will be possible. Recently, a triplet-primed PCR has been developed to identify the presence of the expanded allele thereby allowing PGD to be offered to all DM1 patients (Sermon et al 2001). A protocol similar to this was developed to perform the timing of repeat expansion study presented in chapter 4.

Concluding remarks

To study the transmission of human genes and disease, it is necessary to utilise the human embryo, a limited research material in which the amplification of the low concentration of nuclear DNA present is technically challenging. In this chapter, the efficiency of amplification and accuracy of diagnoses were optimised through the use of fluorescent fragment analysis and of linked polymorphic markers to detect ADO, DNA contamination, preferential amplification and chromosome mosaicism, all of which can complicate single-cell analysis. This approach was then used as the basis of the experimental design in all areas of research throughout this thesis and as the foundation of single gene defect PGD protocol design at the McGill Reproductive Centre.

During the development of the protocols to detect the CFΔF508 mutation and *DMPK* repeat, 1,282 single lymphocytes, 130 genomic DNA samples and 101 pre-clinical research human blastomeres were examined and more than 4,000 samples (single buccal cells, lymphocytes, fibroblasts and blastomeres as well as genomic DNA) were used in the development of the protocols. This averaged over 180 PCR assays per disease, demonstrating the time and testing needed to develop PCR at the single-cell level.

Currently, single-cell genetic analysis is generally performed using conventional PCR protocols similar to those described above. Since this work was completed, new technologies that possibly offer superior genetic diagnosis have been described, although many of these techniques require further development at the single-cell level and can be extremely costly. Therefore, single-cell diagnosis through fluorescent multiplex PCR, which in my hands has proven to be efficient and accurate, will likely retain a place in the molecular genetics laboratory for the foreseeable future.

CHAPTER THREE

Transmission ratio distortion in the myotonic dystrophy locus in human preimplantation embryos

A shortened version of the work described in this chapter has been published in the following paper:

Nicola L Dean, J Concepción Loredo-Osti, T Mary Fujiwara, Kenneth Morgan, Seang Lin Tan, Anna K Naumova, Asangla Ao (2006) Transmission ratio distortion in the myotonic dystrophy locus in human preimplantation embryos. *Eur J Hum Genet* **14**(3):299-306.

3.1 FOREWORD

The mechanism of transmission in the human dystrophia myotonica-protein kinase (*DMPK*) repeat, which is expanded in dystrophia myotonica type 1 (DM1), is not completely understood. The inheritance of the expanded *DMPK* allele essentially follows Mendelian autosomal dominant (AD) transmission, in that if an individual receives one mutant copy of the gene, then a DM1 phenotype will be expressed. Following the rules of Mendelian AD inheritance, one half of all transmissions from a DM1-affected individual should, on average, result in a DM1-affected offspring or at least the inheritance of the parental expanded allele.

This has been reported not to be the situation in DM1. Preferential transmission, or transmission ratio distortion (TRD), has been detected in favour of the inheritance of expanded repeats from DM1-affected parents. Transmission ratio distortion has also been suggested in favour of larger normal-sized *DMPK* repeats in families with no DM1. The existence of TRD in both expanded and normal-sized *DMPK* repeats has been determined through analysing the transmission of parental repeat lengths to live-born offspring. However, it is not known at what developmental time point this TRD could occur as it has not been seen in any earlier stage of development.

We were interested to determine if a TRD was observed in the transmission of *DMPK* alleles at an earlier stage of development in the preimplantation embryo. Our aim was to examine the transmission of larger normal-sized *DMPK* alleles to embryos to investigate whether TRD in favour of inheritance of this larger repeat existed during embryogenesis. Because of our unique situation of having affiliation with a centre performing *in-vitro* fertilisation (IVF), we had limited access to donated embryos, from

IVF patients who had no cause to believe that they were DM1-affected. In order to perform this study it was necessary to genotype *DMPK* alleles in a large number of consenting IVF couples and, when informative, to analyse their supernumerary preimplantation embryos to test whether TRD was apparent in the embryos of parents carrying larger normal-sized *DMPK* repeats.

The PCR protocol developed to detect the *DMPK* $(CTG)_n$ repeat at the single-cell level in the previous chapter was used without modification to genotype the parental samples in this study. This test was modified in a multiplex reaction to detect from single blastomeres the size of the *DMPK* repeat length inherited and the embryo gender in samples donated by IVF couples whose repeat lengths fulfilled the criteria for this study. In this project, we were able to genotype the *DMPK* $(CTG)_n$ repeat length in more than 400 individuals and in almost 350 of their cleavage-stage embryos. This enabled us to investigate whether TRD exists in favour of the transmission of larger *DMPK* repeats at the level of the preimplantation embryo. Elucidation of the timing of TRD acting at the *DMPK* locus is important for understanding the mechanism of transmission of DM1 and may aid in the counselling and reproductive choices available to individuals carrying expanded *DMPK* CTG repeats.

3.2 ABSTRACT

The aim of this study was to determine if TRD of larger normal-sized *DMPK* alleles in the $(CTG)_{19-37}$ repeat range was evident during early embryogenesis and ultimately to better determine the developmental time-point when TRD of *DMPK* alleles in this size range could occur. The transmission of *DMPK* alleles from 61 heterozygous parents with one repeat within the $(CTG)_{5-18}$ range (Group I repeat) and the other within the $(CTG)_{19-37}$ range (Group II repeat) to 335 human preimplantation embryos was analysed. In addition, the gender of each embryo was determined. The transmission from 33 Group I/Group I heterozygous parents was also analysed to determine if the preferential inheritance of a particular range of *DMPK* repeat lengths could be due to a relative size effect, irrespective of the absolute size of the repeat. A statistically significant TRD of 59% (95% confidence interval of 54 - 64, p=0.0005) in favour of Group II repeats from both mothers and fathers was observed in 371 transmissions to preimplantation embryos, which remained significant when transmissions to female embryos were considered separately. In contrast, no significant TRD was detected (46% transmission of longer alleles, p=0.3) in 225 transmissions to preimplantation embryos from informative Group I/Group I parents. Our analysis showed that Group II repeats specifically were preferentially transmitted in human preimplantation embryos. We suggest that, in Group II repeats at the *DMPK* locus, TRD is likely to result from events occurring around the time of fertilisation.

3.3 INTRODUCTION

Transmission ratio distortion (TRD) in human diseases

A fundamental principle of Mendelian genetics is the probability of equal transmission, from parent to offspring, of the two alleles at any given diploid locus. However, deviation from Mendelian 1:1 segregation ratios, or TRD, has been documented for different species and in different genetic loci (reviewed by Lyttle 1993). In humans allele-specific TRD, which refers to preferential transmission of a particular allele(s), has been suggested for the disease genes referred to in Section 1.1.2.3. In addition, TRD has been noted in the transmission of genes containing repeat regions such as the repeat region near the insulin gene (*INS-IGF2* VNTR: Eaves et al 1999), and larger trinucleotide repeats in the genes responsible for Machado-Joseph disease (*ATXN3*: Ikeuchi et al 1996, Riess et al 1997, Iughetti et al 1998), fragile X syndrome (*FMR1*: Drasinover et al 2000), spino-cerebellar ataxia type 1 (*ATXN1*: Riess et al 1997), spino-cerebellar ataxia type 7 (*ATXN7*: Monckton et al 1999) and dentatorubral-pallidoluysian atrophy (*ATN1*: Ikeuchi et al 1996).

Transmission ratio distortion for *DMPK* repeats

Preferential transmission of the larger allele of another trinucleotide repeat, the expanded *DMPK* $(CTG)_n$ repeat, has been reported from DM1-affected parents to live-born offspring in DM1 families (Gennarelli et al 1994, Magee and Hughes 1998, Zatz et al 1997). Moreover, preferential transmission of the larger of the two $(CTG)_n$ repeats in the normal-size range from parents to live-born offspring has been found in families with no DM1 (Carey et al 1994, Chakraborty et al 1996, Shaw et al 1995).

The mechanism of such preferential transmission of larger *DMPK* alleles remains unclear. In principle, TRD may result from events that occur before fertilisation through meiotic drive or preferential survival of gametes, at the time of fertilisation, or after fertilisation through embryonic death, as described in Section 1.1.2.3.

To determine the timing of TRD in the transmission of *DMPK* alleles, individual spermatozoa of three heterozygous males, each with a $(CTG)_5$ and a $(CTG)_{20-37}$ repeat, were studied (Leeflang et al 1996). No segregation distortion of the larger allele was observed, leading to the conclusion that TRD resulted from events following sperm ejaculation. Thus, TRD of larger normal-sized *DMPK* alleles is present among live-born offspring and, at least in the male germ-line, apparently results from events after gametogenesis.

Rationale for studying TRD in larger normal-sized *DMPK* repeats

Large population studies have determined that the $(CTG)_{19-37}$ repeat occurs on 10.8% - 15.0% of Caucasian chromosomes (Imbert et al 1993, Martorell et al 2001). It has been suggested that unlike $(CTG)_{5-18}$ repeats, these larger normal-sized *DMPK* $(CTG)_{19-37}$ repeats, may be susceptible to intergenerational instability and, therefore, preferential transmission of the $(CTG)_{19-37}$ repeats would provide a reservoir for future expanded alleles in the population (Imbert et al 1993) as described in Section 1.5.2.5.

Carey and colleagues selected a cut-off point of $(CTG)_{19}$ repeats for their study of the transmission of *DMPK* alleles in the normal-size range (Carey et al 1994). Based on the functional importance of the $(CTG)_{19-37}$ repeats for the aetiology of DM1 and the evidence for their preferential transmission (Carey et al 1994), the same cut-off point was

selected for this study. To simplify our terminology, the smaller normal-sized *DMPK* alleles with $(CTG)_{5-18}$ will be referred to as Group I repeats, and the larger normal-sized alleles with $(CTG)_{19-37}$ as Group II repeats. To further investigate the timing of the occurrence of TRD of normal-size range repeats in the human *DMPK* locus and as a next step in the elucidation of its mechanism, an analysis of the transmission of larger normal-sized *DMPK* alleles from parents to human preimplantation embryos was performed. The presence or absence of a TRD among preimplantation embryos would bring us closer to understanding what is occurring in the germ-line. Detection of preferential transmission of *DMPK* $(CTG)_{19-37}$ repeats in preimplantation embryos would exclude any mechanism of TRD related to embryo viability, preferential implantation or any further selection during pregnancy. To investigate whether any parent-of-origin and gender-of-offspring effects are occurring at the level of the embryo, as have been previously described in TRD studies both on larger normal-sized and expanded *DMPK* alleles to live-born offspring (Carey et al 1994, Chakraborty et al 1996, Gennarelli et al 1994, Magee and Hughes 1998, Shaw et al 1995, Zatz et al 1997), the results of this study were also analysed according to the sex of the transmitting parent and the sex of the embryo.

3.4 MATERIALS AND METHODS

Meta-analysis of expanded DM1 repeats

In order to aid in the design of our experiment, a literature survey was performed before our study was started to investigate the degree of TRD seen in the population for *DMPK* repeats. Information concerning family pedigrees in the transmission of larger normal-sized alleles was only available from a few studies, but suitable pedigrees were

found for expanded DM1 alleles (Abeliovich et al 1993, Aslanidis et al 1992, Bartlett et al 1987, Brook et al 1992, Buxton et al 1992, Clark et al 1991, Dubel et al 1992, Fu et al 1992, Geifman-Holtzman and Fay 1998, Gennarelli et al 1994, Harley et al 1992, Hecht et al 1993, Jansen et al 1994, Jozefowicz and Griggs 1988, Koch et al 1991, Laberge 1989, Lavedan et al 1991, Magee and Hughes 1998, Mahadevan et al 1993, Neville et al 1994, O'Hoy et al 1993, Redman et al 1993, Sermon et al 1997, Shelbourne et al 1992, Shelbourne et al 1993, Tanaka et al 2000, Taylor et al 1989, Zatz et al 1997, Zuhlke et al 2000). Based on this data, a meta-analysis was carried out using correction for ascertainment bias (Table 3.1). This resulted in a statistically significant TRD in the transmission of expanded *DMPK* repeats to DM1 offspring from maternal transmissions and overall in the inheritance of expanded repeats separately to both daughters and sons. The overall ratio of affected to unaffected offspring was 64% to 36% (p=0.0001).

Patient description

The length of *DMPK* alleles was determined from consenting patients who underwent IVF at the McGill Reproductive Centre (Montreal, QC, Canada) from May 2002 to October 2003. No exclusion was made based on the age of patient, cause of infertility or ethnicity of couple. The research ethics review board of the Royal Victoria Hospital, McGill University Health Centre, approved this study. Couples where at least one of the partners was heterozygous for one Group I and one Group II repeat were approached, after their embryos had been selected for transfer to the female and for embryo freezing, for permission to test the remaining embryos. If they agreed, each couple signed a consent form to permit the use of their embryos in this study.

Table 3.1: Meta-analysis of transmission of DM1 divided by affected parent

	Number of affected/ unaffected daughters	Number of affected/ unaffected sons	% Affected Daughters (p)	% Affected Sons (p)
Maternal transmission	40/25	46/17	62 (.2200)	73(.0100)
Paternal transmission	77/48	78/46	62 (.0750)	63(.0550)
All Transmissions	117/73	124/63	62 (.0029)	66 (.0029)
	241/136		64 (.0001)	

A meta-analysis using publications containing DM1 family pedigrees was performed to give an estimate of the percentage TRD in the transmission of *DMPK* expanded repeats. This analysis was performed excluding the index case in each pedigree to compensate for bias of ascertainment. The ratios of DM1-affected daughters and sons from female and male transmissions were determined. A deviation from the expected 1:1 ratio was found in each category. In most cases, this reached statistically significance and led to an overall statistically significant deviation in the transmission of the expanded allele of 64%.

The number of families included in this meta-analysis could not be given as some of the publications presented pooled data from many families and did not indicate the total number of families included.

All levels of significance were calculated using a 2-tailed T-test.

Determination of the length of maternal and paternal *DMPK* $(CTG)_n$ repeats

The length of the *DMPK* alleles of the mother was determined from PCR on an aliquot of the cumulus cells, surrounding the oocyte. Shortly after oocyte retrieval, cumulus cells (approximating 20% of the total cumulus mass) were removed from two of each consenting female's oocytes using a sterile technique. These cumulus cells were disaggregated in 80IU hyaluronidase (Vitrolife, Goteburg, Sweden) and washed in sterile PBS (Sigma, Oakville, ON, Canada). A 1.5μl aliquot of cumulus cells was added to each of two labelled, sterile, 0.2ml tubes containing 5μl of lysis buffer (LB) (Cui et al 1989) and overlaid with sterile oil. The allele size of the father was determined by PCR on spermatozoa from two 3μl aliquots of the sperm preparation used for IVF, using LB and oil as above. Each aliquot of cumulus cells (from mother) and sperm (from father) was lysed by heating to 65°C for 15 minutes (min).

A fluorescent, hemi-nested PCR was carried out to amplify the $(CTG)_n$ repeat region of the *DMPK* gene (Dean et al 2001 and Section 2.4). Reactions and cycling conditions for the first and second round of PCR and the fluorescent fragment analysis of amplified products were identical to those used for *DMPK* amplification in the Section 2.4 protocol, with the exception that the primer sets to amplify the polymorphic markers were not included.

Collection of embryos

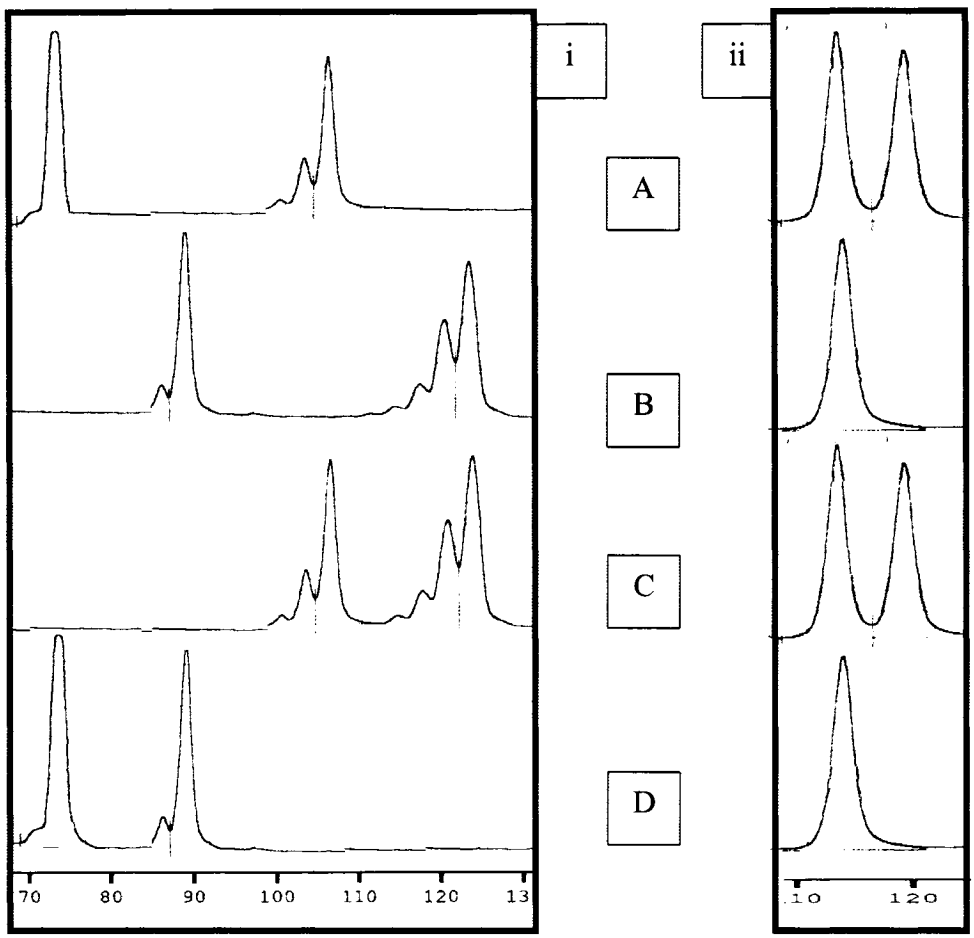
Every donated embryo was tested with no selection being made on the basis of developmental stage. The inclusion criteria for the study were that the fertilised embryo had not been selected to be transferred to the female or to be frozen. Hence,

preimplantation embryos from the two-cell stage to blastocyst were included. Each donated embryo was incubated for 5 min in Ca^{2+}- and Mg^{2+}-free media (Cook Canada Inc, Stouffville, ON, Canada) to allow the decompaction of blastomeres. The zona pellucida was removed using acid Tyrode solution (pH 2.5) (Sigma, Oakville, ON, Canada) and the cells washed through 3 drops of PBS containing 4mg/ml bovine serum albumin (Sigma). The blastomeres were then disaggregated using a sterile pulled-glass pipette and were, depending on the number of cells present, pipetted into 1 to 3 tubes that contained LB and were overlaid with sterile oil. Grouping of cells for the PCR assay ensured that results were obtained from the maximum number of embryos. After collection, all samples were centrifuged and kept at -80°C for at least 20 min before being incubated at 65°C for 15 min to lyse the cells. For each embryo, a sample of media from the last drop in which the blastomeres had been washed was placed in a PCR tube with 5μl LB to act as a negative control for that embryo.

Genotyping of donated embryos

A multiplex, hemi-nested, fluorescent PCR was carried out to amplify the *DMPK* $(CTG)_n$ repeat region along with a sequence of the amelogenin gene (*AME*) to determine gender (Figure 3.1). The primer sequences used to amplify the *AME* region were taken from Ray and colleagues (Ray et al 2001). The *AME* primers allowed the detection of two sequences, one on the X and one on the Y chromosome, that were different in size to distinguish the gender of each embryo by fragment-length analysis. Females have one 115 base pair amplified fragment, whereas males have two fragments of 115 and 121 base pairs.

Figure 3.1: Example of *DMPK* genotyping and gender determination for donated study embryos



The possible genotypes for embryos obtained from a couple in which one individual carried *DMPK* genotypes $(CTG)_5$ and $(CTG)_{23}$ and their partner carried $(CTG)_{12}$ and $(CTG)_{17}$. Four of the 8 possible combinations are shown as for each *DMPK* genotype, the embryo could be male or female.

i. *DMPK* genotyping to determine number of repeats
ii. *AME* genotyping for gender determination

A. Male embryo with *DMPK* $(CTG)_5$ and $(CTG)_{17}$
B. Female embryo with *DMPK* $(CTG)_{12}$ and $(CTG)_{23}$
C. Male embryo with *DMPK* $(CTG)_{17}$ and $(CTG)_{23}$
D. Female embryo with *DMPK* $(CTG)_5$ and $(CTG)_{12}$

The PCR conditions in the first round were similar to those in the *DMPK* parental allele protocol except that 0.05μM of *AME* primers, outer forward (AME-OF) and outer reverse (AME-OR) (Intergrated DNA Technology, Coralville IA, USA), were added. For the second round, *DMPK* and *AME* were amplified separately. The *DMPK* reaction was as described above. For *AME*, 3μl of the first-round products were added to reaction mixes in a total volume of 30μl with PCR buffer, 50μM of each dNTP, 1 unit of Taq polymerase (Qiagen, Mississauga, ON, Canada), and 0.25μM of *AME* primers, inner forward (AME-IF) and AME-OR (Intergrated DNA Technology). The AME-IF primer was labelled with CY5 fluorescent dye. The PCR conditions for both reactions were similar to the second round for the parental alleles, except that 28 cycles were used in total, 10 cycles followed by 18 cycles.

The samples were run as above using 3μl of *DMPK* and 5μl of *AME* products. The *DMPK* genotype and gender of each embryo were recorded. The printouts from all the aliquots amplified from each embryo were compared to ensure that they gave the same result, and each negative control was checked to ensure there was no amplification.

Reliability of genotyping

Amplification rates for *DMPK* and *AME* were 95% and 94%, respectively, and rates of ADO were 12% and 6%, respectively. To confirm the genotypes, duplicate or triplicate samples were run for 86% (293/341) of the embryos, and complete results for the *DMPK* genotype and the gender were obtained for all but six of the embryos tested.

Statistical analysis

Deviation from 1:1 segregation was tested using the exact binomial test in R for Windows, version 1.8.0. (The R Project for Statistical Computing). Reported *P*-values were not corrected for multiple testing. Taking into account for at most nine comparisons, we considered a *P*-value of < 0.0056 to be statistically significant. All other variables were analysed using a two-tailed *t*-test or a two-sided χ^2 test for independent variables. The exact test in GENEPOP, version 3.4 was used to test for Hardy-Weinberg equilibrium (Raymond and Rousset 1995).

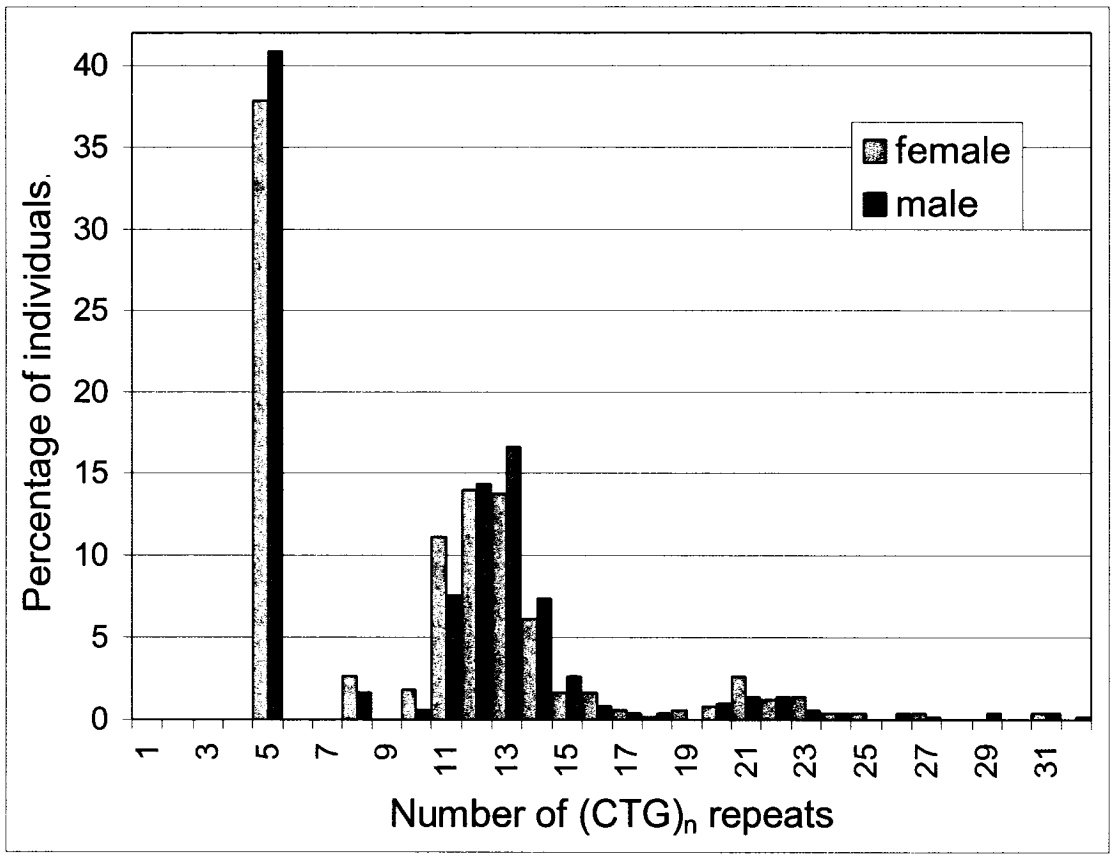
3.5 RESULTS

Inclusion criteria and characteristics of participating couples

In total, 234 IVF couples (468 individuals) consented to participate in the study and will be subsequently referred to as study couples. Analysis of the *DMPK* alleles of these individuals showed a tri-modal distribution of $(CTG)_n$ repeat size similar to that observed in other populations of predominantly European ancestry (Figure 3.2) (Deka et al 1996, Imbert et al 1993, Zerylnick et al 1995). The sample was found to be in Hardy-Weinberg equilibrium ($P = 0.4$). This suggests that although our study group was a selected group that had difficulty in conceiving, it was not different from the general population with respect to the distribution of the *DMPK* alleles. Therefore, our study group could be considered as representing the general population with respect to transmission of *DMPK* alleles.

Of the 234 couples, 62 (26%) had at least one partner with a Group I/Group II genotype with the largest repeat size observed being $(CTG)_{32}$ (Table 3.2A). Fifty-four couples donated embryos (42 donated in one cycle, 9 in two cycles and 3 in three cycles).

Figure 3.2: Distribution of *DMPK* $(CTG)_n$ repeat sizes observed in the 234 study couples (*N*=936 alleles)



Distribution of *DMPK* $(CTG)_n$ repeat sizes observed in the 468 IVF patients (234 males and 234 females) showing a tri-modal distribution of $(CTG)_5$, $(CTG)_{11\text{-}15}$ and $(CTG)_{19\text{-}37}$ repeats.

Table 3.2A: Distribution of study individuals with Group I/Group II genotype[1]

Partner with Group I/Group II genotype	Number of couples	Average Group II repeat size	Median Group II repeat size	Range of Group II repeat size
Only mothers	32	22.3	22	19-31
Only fathers	23	23.8	22	20-32
Both mothers and fathers	7	22.4	21	19-31

Table 3.2B: Cycle parameters for the study couples carrying a Group II repeat compared to those who only carried Group I repeats

Genotypes of couples[2]	Number of couples	Average female age (range)	Average male age (range)	Fertilis-ation rate	Blastocyst formation rate	Implant-ation rate[3]	Clinical pregnancy rate[4]
At least one parent with Group I/ II genotype	62	34.9 (22-43)	37.4 (23-51)	61%	34%	26%	46%
Both parents with Group I repeats	171	34.9 (20-43)	37.6 (22-51)	60%	35%	20%	43%

[1] Group I repeat = *DMPK* $(CTG)_{5-18}$; Group II repeat = *DMPK* $(CTG)_{19-37}$.

[2] N=233 couples; one couple in which the paternal genotype was Group I homozygous and the maternal genotype was Group II/Group II was not included in this table. Group I repeat = *DMPK* $(CTG)_{5-18}$; Group II repeat =*DMPK* $(CTG)_{19-37}$.

[3] Implantation rate is defined as the total number of sacs seen at six-week ultrasound divided by the total number of embryos transferred to the uterus of the female patient at embryo transfer.

[4] Clinical pregnancy rate is defined as ultrasound evidence of pregnancy seen at six weeks of pregnancy.

The age and IVF parameters of the study couples were analysed. The characteristics of couples in which there was at least one partner with Group I/Group II genotype were compared to those of couples carrying two Group I repeats (Table 3.2B). No significant differences were found between the two groups with respect to the age of the male or female partner, fertilisation rate, *in-vitro* blastocyst formation rate, implantation rate or clinical pregnancy rate.

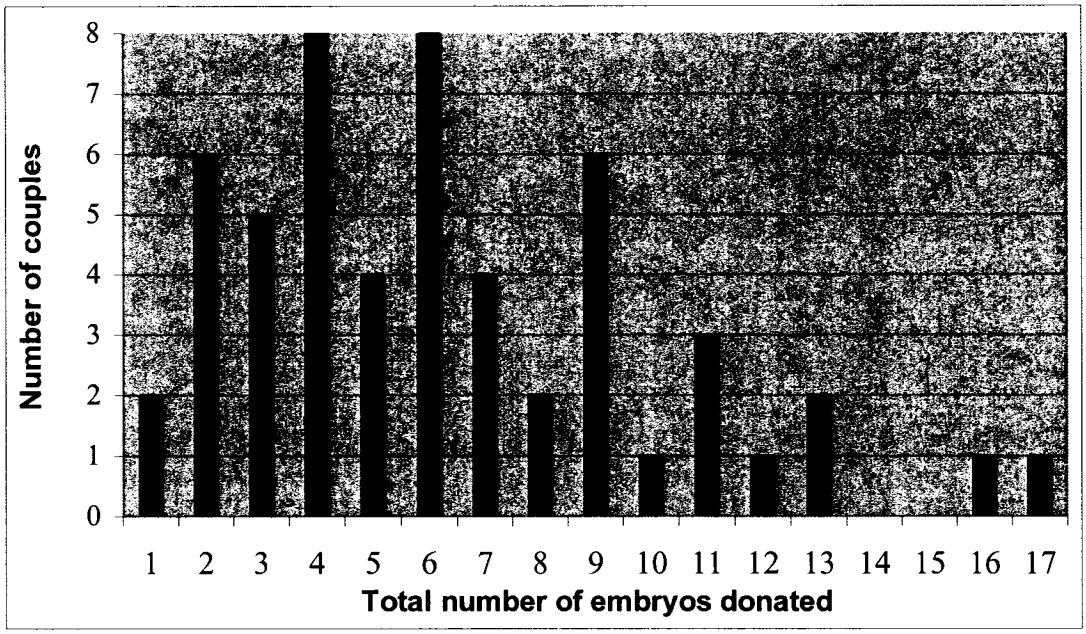
In total, 341 embryos were donated and genotyping results were obtained for 335. The number of genotyped embryos per couple ranged from 1 to 16 with a mean and a median of 6 (Figure 3.3). Each embryo provided information for two transmissions, one maternal and one paternal and, therefore, 670 transmissions were recorded (Table 3.3).

Transmission ratios of *DMPK* $(CTG)_n$ repeats

Transmission ratios from individuals with Group I/Group II genotype

Among the 335 embryos genotyped, 59% (95% confidence interval of 54-64) inherited the Group II repeat from parents with Group I/Group II genotype (Table 3.4). Therefore, we detected a statistically significant deviation from Mendelian 1:1 segregation in the embryos ($P = 0.0004$, exact binomial test). TRD in favour of the Group II repeat was observed following both maternal and paternal transmissions (60%, $P = 0.0055$ and 59%, $P = 0.03$, respectively), although with correction for multiple testing, the significance level ($P < 0.0056$) was reached only in maternal transmissions. When sex of the embryo was considered, a greater magnitude of TRD was observed in female than in male embryos (65%, $P = 0.0001$ and 55%, $P = 0.2$, respectively).

Figure 3.3: Histogram of the number of embryos donated by individuals with a Group I/Group II genotype



Distribution of number of embryos donated from each IVF couple in which at least one partner had a Group I/Group II genotype. These embryos were donated during the course of one to three IVF attempts over the period of the study (range of embryos 1-17, average per couple = 6.3 and median per couple = 6.0)

Table 3.3: Number of transmissions observed in our study by parental genotype

DMPK genotype of the parent[1]	Number of parents (Mothers/Fathers)	Number of transmissions observed
Group I/Group II	61 (34/27)	371[2]
Group I/Group I heterozygous	33 (16/17)	225[3]
Group I/Group I homozygous	13 (4/9)	68[4]
Group II/Group II Heterozygous	1 (0/1)	6[5]
Total	108 (54/54)	670

1. Group I repeat = *DMPK* $(CTG)_{5-18}$; Group II repeat = *DMPK* $(CTG)_{19-37}$.

2. Includes 36 embryos (72 transmissions) where both parents had a Group I/Group II genotype. These 371 transmissions were used to examine TRD in Group I versus Group II repeats (Table 3.4).

3. These 225 transmissions were used to examine if TRD could be due a relative repeat size effect, irrespective of the allele type, i.e., transmission of "Short" versus "Long" allele.

4. 68 transmissions from Group I/Group I homozygous parents were uninformative because they could only transmit one particular $(CTG)_n$ repeat.

5. 6 transmissions in which the father had two different sized Group II repeats were excluded from the analysis due to the small sample size.

Table 3.4: Transmission of *DMPK* $(CTG)_n$ repeats to embryos

	DMPK $(CTG)_n$ repeat transmitted[1]											
	Female embryos				Male embryos				Embryos of both sexes			
Parental origin of the alleles	Group I	Group II	T_{GII}	95% CI	Group I	Group II	T_{GII}	95% CI	Group I	Group II	T_{GII}	95% CI
Maternal	33	64	66%[3]	56 - 75	51	61	54%	45 - 64	84	125	60%[3]	53 - 67
Paternal	25	44	64%[2]	51 - 75	42	51	55%	41 - 65	67	95	59%[2]	51 - 66
Total	58	108	65%[4]	57 - 72	93	112	55%	48 - 62	151	220	59%[4]	54 - 64

[1] Group I repeat = *DMPK* $(CTG)_{5\text{-}18}$; Group II repeat = *DMPK* $(CTG)_{19\text{-}37}$; T_{GII} = percent of embryos inheriting the Group II repeat; 95% CI = 95% confidence interval.

[2,3,4] Indicates significant transmission ratio distortion from 1:1 at $P < 0.05$, $P < 0.01$, or $P < 0.0005$, respectively

Transmission ratios from individuals with Group I/Group I genotype

To determine if the preferential transmission of the longer *DMPK* repeat in individuals with a Group I/Group II genotype was due to a relative repeat size effect (as proposed by Chakraborty et al 1996) or whether it was due to preferential transmission of the Group II repeat, the 225 transmissions from informative Group I/Group I parents, who were heterozygous for $(CTG)_{5\text{-}18}$ repeats, were analysed. The transmission of the Group I repeats to the embryos were recorded, with the smaller repeat present being designated as "Short" and the larger as "Long", irrespective of the actual size of the repeat. That is, the same allele could be designated as "Short" or "Long" dependent upon the length of the other parental repeat. For example, in an individual with a $(CTG)_5$ /$(CTG)_{11}$ genotype, the $(CTG)_{11}$ was considered as "Long". However, in an individual with a $(CTG)_{11}$ /$(CTG)_{14}$ genotype, the $(CTG)_{11}$ was considered as "Short". The "Long" allele was transmitted to the embryo in 46% (104/225, $P = 0.3$) of transmissions (Table 3.5). Thus, in our study, we observed TRD in favour of the larger allele only from individuals with Group I/Group II genotype.

Table 3.5: Transmission from heterozygous individuals with Group I/Group I genotype

	Short Group I	Long Group I	% Long	*P*-value[1]
Maternal origin	50	48	49%	
Paternal origin	71	56	44%	
Total	121	104	46%	0.3

[1] Statistical significance of the exact binomial test was not computed on subsets of the data if the overall *P*-value was > 0.05

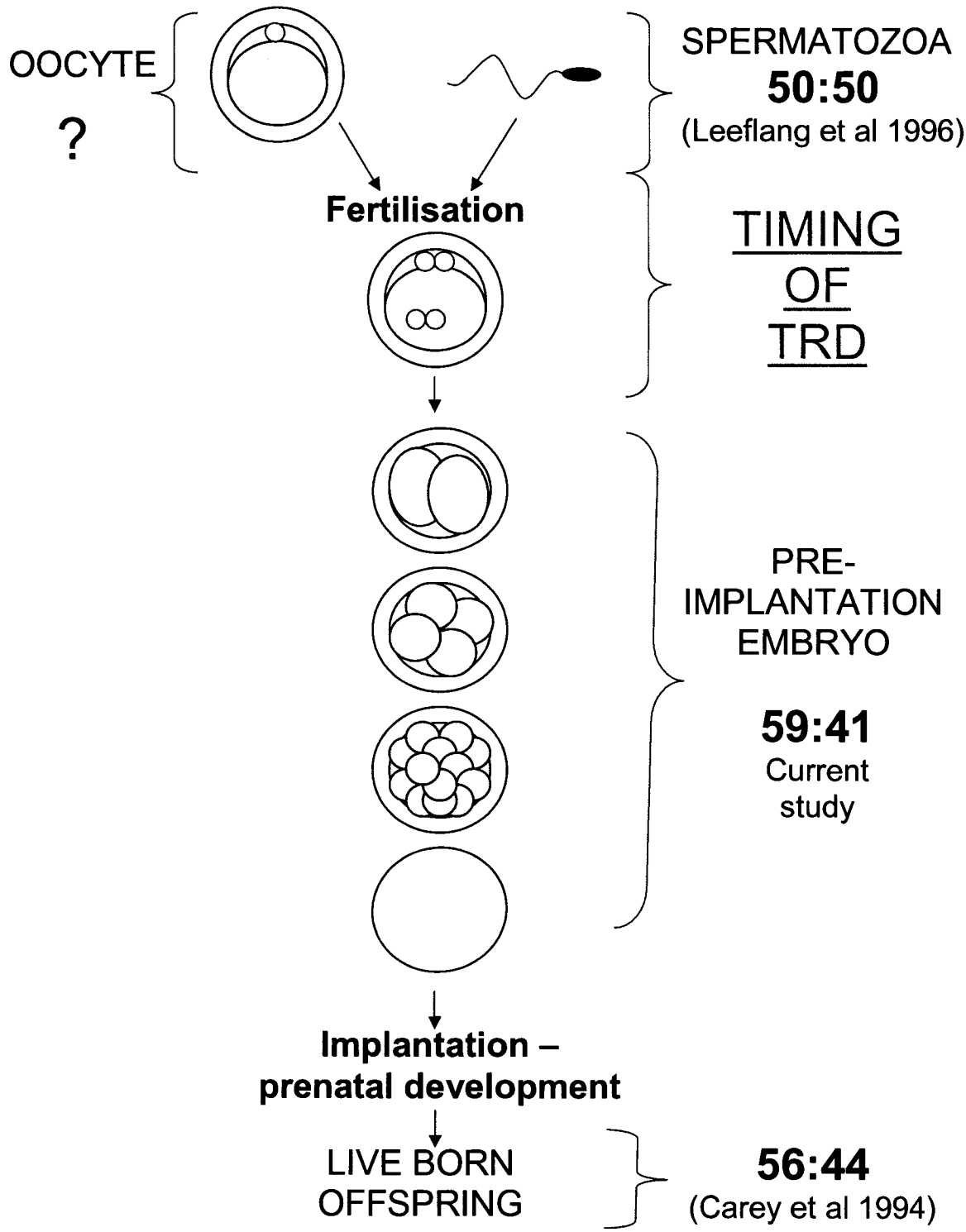
3.6 DISCUSSION

We report here, for the first time, preferential transmission (59%) of larger *DMPK* alleles (Group II repeats, $(CTG)_{19-37}$) compared to smaller alleles (Group I repeats, $(CTG)_{5-18}$) to preimplantation embryos and showed that this was specifically due to the presence of the Group II repeat. These results support the observation of TRD in favour of Group II repeats in the live-born offspring of couples with at least one parent with Group I/Group II genotype (56% Group II, N = 150/266 transmissions; $P = 0.04$, exact binomial test) (Carey et al 1994) (Appendix I). The low frequencies of individual Group II alleles and insufficient number of observations for each particular Group II allele do not allow us to determine if a particular subgroup of alleles drives the TRD.

Timing of TRD in larger normal-sized *DMPK* repeats

This finding advances our knowledge about the timing of TRD in Group II repeats. Arnheim's group had previously excluded segregation distortion for Group I versus Group II repeats in the male germline as a possible mechanism of TRD and hypothesised it resulted from events following sperm ejaculation (Leeflang et al 1996). Our results further narrow the time period when the TRD is generated and suggest that TRD results from events preceding preimplantation development (Figure 3.4).
In principle, these events could be preferential fertilisation of oocytes by Group II repeat-bearing sperm, preferential retention of Group II repeats in the oocytes, preferential survival of Group II repeat-bearing gametes, or meiotic drive in the female germline at the time of the second meiotic division favouring generation of heterozygous embryos with a Group I/Group II genotype.

Figure 3.4: Transmission ratios for *DMPK* Group II repeats versus Group I repeats



Transmission ratios for Group II repeats ($(CTG)_{19-37}$) versus Group I repeats ($(CTG)_{5-18}$) at the *DMPK* locus at different stages of human development including the findings of the current study. TRD in favour of Group II repeats at the *DMPK* locus is likely to occur around the time of fertilisation as indicated in the diagram.

Meiotic drive at the second meiotic division has been reported for maternal transmissions at the mouse *Om* locus (Wu et al 2005). We think that preimplantation embryo loss and post-implantation lethality are unlikely causes of TRD in our study group, as all donated embryos were included, even those arrested in their development, and a magnitude of TRD (59%) in preimplantation embryos similar to the one reported for live-born offspring (56%) (Carey et al 1994) was demonstrated.

Parent-of-origin effects on observed TRD

For larger normal-sized *DMPK* alleles

Our results showed TRD from both maternal and paternal transmissions (60% and 59%, respectively), which, for this work, reached the threshold for level of significance only in maternal transmissions. Carey and colleagues also found TRD in Group II repeats to live-born offspring, in families with no known history of myotonic dystrophy, although it was stronger from paternal (60%) than from maternal transmission (55%) (Carey et al 1994). Upon reanalysis of this data by another group, no evidence was found to support a male-specific TRD but, similar to our results, a statistically significant TRD in favour of Group II repeats acting in both sexes was demonstrated (Hurst et al 1995). In another study examining TRD in live-born offspring from CEPH pedigrees, which represent families selected for high fertility and longevity, a preferential transmission of $(CTG)_{>29}$ repeats was observed in female transmissions but no TRD was found in the small subset of transmissions with our Group I/Group II genotype (49% Group II, N = 46/94 transmissions) (Chakraborty et al 1996) (Appendix II). However, this could be due to the sample size.

For expanded DM1 alleles

Three studies reported TRD in myotonic dystrophy pedigrees (251 Italian and Spanish pedigrees, 69 Brazilian families, and 59 pedigrees in Northern Ireland), which for purposes of comparison we consider as DM1 (Gennarelli et al 1994, Magee and Hughes 1998, Zatz et al 1997) (Appendix II). These studies reported the number of affected and unaffected offspring born to parents with DM1. Individual studies report statistically significant TRD in favour of maternal transmissions (Magee and Hughes 1998) and conversely paternal transmissions (Gennarelli et al 1994). However, in the combined data TRD in favour of the *DMPK* allele was similar from affected fathers as compared to affected mothers (56% and 54%, respectively). Our results also demonstrate a similar degree of TRD for Group II repeats from both paternal and maternal transmissions, although the mechanism of TRD of Group II repeats may not be the same as for TRD of highly expanded repeats causing DM1.

Sex-of-offspring effects on TRD

When the male and female embryos in our study were considered separately, a statistically significant TRD in favour of Group II repeats was seen in female embryos following both maternal and paternal transmissions. These findings are in contrast to the greater degree of TRD in sons compared to daughters in the DM pedigrees (Gennarelli et al 1994, Magee and Hughes 1998, Zatz et al 1997) (AppendixII) but, as previously mentioned, the mechanism of TRD of Group II repeats may not be the same as in highly expanded repeats. Unfortunately, none of the studies looking at TRD of larger normal-

sized *DMPK* alleles reported their data by gender of offspring, so we were unable to compare our results with the ratios occurring in live-born offspring.

The absence of statistically significant TRD in our male embryos could result from the limited numbers of embryos tested or, alternatively, could be due to a difference in the mode of transmission of Group II repeats to males compared to females.

One possible explanation for differences in the magnitude of TRD dependent on the sex of the offspring is the presence of modifiers on the X and/or Y chromosomes affecting embryo survival or causing preferential transmission of a particular parental allele (de la Casa-Esperon et al 2000, Naumova et al 1998, Zollner et al 2004). Another possibility is the genetic heterogeneity of individuals with respect to *cis*- or *trans*-acting factors involved in the genesis of TRD (Ikeuchi et al 1996, Riess et al 1997). This is unlikely to involve the *DMPK* repeat on the other chromosome because in the case of DM1 patients, the $(CTG)_n$ repeat number of the non-expanded allele has no *trans*-acting effect on allele segregation at the gamete stage (Cipollaro et al 1997). It is interesting to note that CORD2, which maps 0.8-2.4Mb distal to DM1 (Gordon et al 1995), shows an increased transmission from affected mothers to their offspring (Evans et al 1994), supporting an effect of linked genes or gene interactions being responsible for the distortion in this region.

A preferential distortion of $(CTG)_{19\text{-}37}$ repeats either through maternal inheritance or to female embryos as was observed in this study, would be consistent with the theory that this repeat was responsible for maintaining the frequency of DM1, since transmission of this repeat range through the female is more stable than through the male. Therefore, $(CTG)_{19\text{-}37}$ repeats could be maternally transmitted through a greater number of

generations with more lateral branching before a CDM1-affected individual was generated and the potential for further transmission ended.

Transmission of Group I repeats

The TRD in our embryos was due to the Group II repeat specifically rather than an overall relative allele size effect, as demonstrated by no significant TRD in transmissions from informative Group I/Group I parents. In contrast to our results, two other studies found preferential transmission of the "Long" allele (compared to the "Short" allele, irrespective of the absolute size) in maternal transmission (Chakraborty et al 1996, Shaw et al 1995) (Appendix I). However, the repeat size in these studies ranged from $(CTG)_{5\text{-}30}$, which was different from the $(CTG)_{5\text{-}18}$ in our analysis.

Selection within the Group I repeat range

Is there a preferential selection for $(CTG)_5$ alleles?

A selection in favour of the transmission of $(CTG)_5$ repeats could provide a means of offsetting the loss of this allele size seen in transmissions from individuals with $(CTG)_5$/Group II genotype. The $(CTG)_5$ repeat is the most common allele in the majority of populations (Deka et al 1996). Therefore a preferential selection against this repeat in favour of Group II repeats should be counteracted by a selection in favour of $(CTG)_5$ repeats in particular transmissions to allow maintenance of genetic equilibrium.

However in our study, there was no overall significant TRD of $(CTG)_5$ in the "Short" versus the "Long" allele analysis (Table 3.5), that is, among the 174 transmissions from Group I/Group I parents who carried one $(CTG)_5$ allele, 96 embryos (55%) inherited the $(CTG)_5$ allele (P=0.2). The proportion was the same for both parents.

This is in agreement with Carey and colleagues who found no enhanced transmission of a $(CTG)_5$ allele from parent to child in preference to $(CTG)_{11\text{-}13}$ or $(CTG)_{15}$ alleles (Carey et al 1994).

Is there a preferential selection in particular genotypes?

There may, however, be a possible selection in favour of or against particular combinations of Group I/Group I genotypes. Inspection of the transmission of specific alleles from these individuals indicated a trend for preferential transmission of $(CTG)_5$ from parents with $(CTG)_5/(CTG)_{13}$ genotype (67% $(CTG)_5$, N = 31/46 transmissions; P = 0.03) (Table 3.6) that was more extreme for paternal transmissions to male embryos (94% $(CTG)_5$, N = 16/17 transmissions; $P = 0.0003$). Due to the limited sample size used in this study we cannot speculate about these observations until they have been confirmed by a larger sample. However, if proven true, they would provide a means of offsetting the loss of $(CTG)_5$ alleles in the preferential inheritance of Group II repeats in transmissions from individuals with $(CTG)_5$/Group II genotype.

Thirteen percent of the partners among the 234 couples in our study had $(CTG)_5/(CTG)_{13}$ genotype compared to 7% with $(CTG)_5$/Group II genotype. Therefore a preferential selection against $(CTG)_5$ in favour of Group II repeats could be hypothesised to be counteracted by a selection in favour of $(CTG)_5$ repeats in particular transmissions, such as may be from $(CTG)_5/(CTG)_{13}$ genotypes, to allow maintenance of genetic equilibrium.

Table 3.6: Transmission from heterozygous individuals with Group I/Group I genotype and for the subset with $(CTG)_5/(CTG)_{13}$ genotype

	Shorter Group I	**Longer Group I**	**% Shorter**	***P*-value[a]**
Maternal origin	50	48	51%	
Paternal origin	71	56	56%	
Total	121	104	54%	0.3
	$(CTG)_5$	**$(CTG)_{13}$**	**% $(CTG)_5$**	***P*-value**
Maternal origin to male embryos	4	5	44%	
Maternal origin to female embryos	1	4	20%	
Subtotal: maternal origin	5	9	36%	0.4
Paternal origin to male embryos	16	1	94%	0.0003
Paternal origin to female embryos	10	5	67%	0.3
Subtotal: paternal origin[b]	26	6	81%	0.0005
Total: maternal & paternal origin	31	15	67%	0.03

[a] Statistical significance of the exact binomial test was not computed on subsets of the data if the overall *P*-value was > 0.05.

[b] There were 3 different fathers with $(CTG)_5/(CTG)_{13}$ genotype. Following is the number of transmissions of each allele to male and female embryos by father.

	Male Embryos		Female Embryos	
	$(CTG)_5$	$(CTG)_{13}$	$(CTG)_5$	$(CTG)_{13}$
Father #1	8	0	5	0
Father #2	3	1	3	1
Father #3	5	0	2	4
Subtotal	16	1	10	5

Possible influence on TRD from testing infertile individuals

It could be suggested that the TRD in favour of Group II repeats, as demonstrated in embryos donated by IVF patients, was an effect of testing couples selected through an infertility clinic rather than the general population. However, the distribution of the *DMPK* alleles within our study, which was not selected for ethnicity, but was largely French Canadian, showed a similar tri-modal pattern and prevalence of $(CTG)_5$ repeats as previously observed in other populations of predominantly European ancestry (Figure 3.2) (Deka et al 1996, Imbert et al 1993, Zerylnick et al 1995). If infertility were associated with a change in transmission patterns one would expect this to be reflected in the distribution of allele frequencies.

Furthermore, we did not find any differences in the embryos produced by couples where at least one partner had Group I/Group II genotype and couples where both partners had Group I/Group I genotype with respect to age or measurable IVF outcomes (Table 3.2B). In particular, the quality of donated embryos, as assessed by the percentage that developed to the blastocyst stage on day six of *in-vitro* culture, was similar in both genotypic groups demonstrating that the presence of a Group II repeat did not influence preimplantation embryo development (Table 3.2B). From this finding, we assume that the *DMPK* allele distribution in the donated embryos was similar to the distribution of embryos transferred to the female patient. Therefore, it is unlikely that the TRD observed in our IVF embryos was caused by the infertility of the parents or that the presence of a Group II repeat was linked to infertility. It is also unlikely that the IVF hormonal treatment undertaken by the women influenced the allelic transmission. However, it is not possible to exclude the effects of additional genes downstream of

DMPK, which could influence the transmission process of *DMPK* alleles. A lack of association of *DMPK* genotype with fertility parameters is consistent with normal fertility in women carrying *DMPK* $(CTG)_{50-150}$ repeats (Dao et al 1992, Lavedan et al 1993). Although fertility can be compromised in males with mild or severe DM1 (Harper 1997, Hortas et al 2000, Jansen et al 1994), the fathers in our study carried repeat sizes within the normal range. Therefore, no reduction in fertility would be expected in our study group due to $(CTG)_n$ repeat size.

Concluding remarks

In conclusion, TRD in favour of Group II repeats from both mothers and fathers with two normal-sized *DMPK* $(CTG)_n$ repeats, one Group I and one Group II, was observed in human preimplantation embryos. The TRD was stronger in female compared to male embryos. This TRD was due to the presence of the Group II repeats specifically and not the relative size of the $(CTG)_n$ repeats. It is likely to have occurred around the time of fertilisation. Further studies of the transmission of *DMPK* $(CTG)_n$ repeats are needed to identify the factors responsible for the TRD observed at the *DMPK* locus and the compensatory factors to maintain the normal-sized alleles in the population.

3.7 APPENDIX I

Data from studies of transmission ratio distortion of *DMPK* $(CTG)_n$ repeats in the normal-size range (data were not reported by gender of offspring).

Carey et al 1994 (Transmissions from parents heterozygous for $(CTG)_{\geq 19}$ and $(CTG)_{<19}$ repeats from selected families with no known history of DM from genetic counselling centres in the UK, Finland, and Sweden.)

	≥19	<19	% ≥19	*P*-value[1]
Maternal origin	65	53	55%	0.3
Paternal origin	74	50	60%	0.04
Subtotal	139	103	57%	0.02
Total including transmissions of unknown parental origin	150	116	56%	0.04

Shaw et al 1995 (Transmissions from parents who were heterozygous for $(CTG)_{5\text{-}30}$)

	Longer	Shorter	% Longer	*P*-value
Maternal origin	146	107	58%	0.02
Paternal origin	126	116	52%	0.6
Total	272	223	55%	0.03

Chakraborty et al 1996 (Transmissions from parents in 78 nuclear families from 40 CEPH pedigrees in which both parents were typed and were not heterozygous for the same two alleles. Allele size ranged from $(CTG)_{5\text{-}39}$; subset of data from Table 2.)

	Longer	Shorter	% Longer	*P*-value[1]
Maternal origin	141	120	54%	
Paternal origin	112	129	46%	
Total	253	249	50%	0.9

Chakraborty et al 1996 (Transmission from parents heterozygous for $(CTG)_{\geq 19}$ and $(CTG)_{<19}$ repeats; subset of data from Table 3)

	≥19	<19	% ≥19	*P*-value[1]
Maternal origin	30	28	52%	
Paternal origin	16	20	44%	
Total	46	48	49%	0.9

[1] Statistical significance of the exact binomial test was not computed on subsets of the data if the overall *P*-value was > 0.05.

APPENDIX II

Combined data from studies of transmission ratio distortion in myotonic dystrophy (DM1) pedigrees (Note: no adjustment for ascertainment except in footnote 2)

	DAUGHTERS			SONS		
	DM1	Normal	%DM1	DM1	Normal	%DM1
Gennarelli et al 1994 (251 Italian and Spanish DM1 pedigrees)[1]						
Maternal origin	95	94	50%	112	71	61%
Paternal origin	148	106	58%	166	105	61%
Zatz et al 1997 (69 Brazilian families with an affected DM1 parent)						
Maternal origin	45	50	47%	57	57	50%
Paternal origin	89	103	46%	117	97	55%
Magee & Hughes 1998 (59 DM1 pedigrees from a survey in Northern Ireland)[2]						
Maternal origin	15	10	60%	18	5	78%
Paternal origin	17	15	53%	18	10	64%
COMBINED DATA						
Maternal origin	155	154	50%	187	133	58%
Paternal origin	254	224	53%	301	212	59%
Total	409	378	52%	488	345	58%

[1] Clinical and molecular analyses were done to substantiate differential transmission of the DM1 allele. The range of the repeats was $(CTG)_{54-1100}$ in the fathers and $(CTG)_{90-2000}$ in the mothers. The repeat size in DM1 offspring ranged from $(CTG)_{100-1900}$ for those who inherited the DM1 allele from their affected fathers, and from $(CTG)_{70-2200}$ for those who inherited the DM1 allele from their affected mothers. Information on the repeat size of normal alleles was not reported.

[2] The study by Magee & Hughes was the only one among the three studies that provided counts excluding the index cases to adjust for ascertainment (22 index cases). Adjusted counts are given in the table below.

	DAUGHTERS			SONS		
	DM1	Normal	%DM1	DM1	Normal	%DM1
Maternal origin	14	9	61%	8	5	62%
Paternal origin	13	15	46%	12	10	55%

Overall, there were 47 DM1 offspring and 39 unaffected offspring; P-value = 0.5, binomial exact test

CHAPTER FOUR

Instability in the transmission of the myotonic dystrophy CTG repeat in human oocytes and preimplantation embryos

These results were initially orally presented as:

Nicola L Dean, Seang Lin Tan, Asangla Ao (2004) Transmission of the myotonic dystrophy CTG repeat in early human embryos. 18th World Congress on Fertility and Sterility, Montreal, Canada, May 23-28, 2004. Oral abstract 208.1.

The condensed version of this chapter was published in the following paper:

Nicola L Dean, Seang Lin Tan, and Asangla Ao (2006) Instability in the transmission of the myotonic dystrophy CTG repeat in human oocytes and preimplantation embryos. *Fertil Steril* **86**:98-105.

4.1 FOREWORD

The trinucleotide repeat expansions in dystrophia myotonica type 1 (DM1) are among the largest known in this novel class of disorders and are important for studies of genome instability in development. Somatic expansion of repeat length in the human dystrophia myotonica-protein kinase (*DMPK*) gene has been demonstrated in DM1-affected individuals. It has also been determined that genetic anticipation can occur during the transmission of expanded *DMPK* alleles to a fetus. The degree of these intergenerational increases are dependent on the gender of the transmitting parent and the number of repeats present on the expanded allele but could also involve other genetic factors. Therefore, a DM1 offspring has a strong likelihood of carrying a larger repeat than their transmitting parent.

These pronounced expansions of *DMPK* repeat lengths are suggested to take place before day 10.5 of development. However, the precise developmental stage at which this repeat instability occurs was unknown. This project was initiated to further investigate the timing of the initial *DMPK* repeat expansion during preimplantation embryo development. We proposed three models for this initial increase in repeat length: I) expansion occurs during oogenesis and then the repeat is stably transmitted until after embryonic implantation, II) the initial expansion occurs during oogenesis but the repeat continues to increase in length through early embryogenesis or III) expansion only occurs post-fertilisation either before or after embryo implantation. In order to carry out this research, embryonic samples are needed that would be expected to have a further increase in *DMPK* repeat expansion when compared to the repeat size of the DM1 parent.

Through preimplantation genetic diagnosis (PGD) we have been able to offer *DMPK* genotyping of embryos, using the protocol developed in Section 2.4, for couples

in which one partner had an expanded *DMPK* repeat. The PGD genotyping of DM1-affected embryos was based on negative selection, i.e., the absence of the normal-sized *DMPK* repeat from the affected parent. In two of these three couples, it was the female partner who carried the expanded *DMPK* repeat and both women had undergone a previous pregnancy termination for a congenitally affected DM1 (CDM1) fetus. From the literature it is known that after conceiving a CDM1 fetus, the risk of having a subsequent CDM1 pregnancy is much higher and, therefore, a good proportion of the DM1-affected embryos from these women should show genetic anticipation and have further expansion in the maternal *DMPK* repeat length. The embryos diagnosed as DM1-affected during 3 cycles of PGD for these two women and any immature or "failed to fertilise" oocytes were collected and tested. Ideally we would have preferred to have a greater number of research oocytes and embryos from DM1-affected women but this depended on more couples requesting PGD for DM1, which did not occur within the time frame of this Ph.D. work.

The primary findings from this study were presented in the spring of 2004 at which time there were no other reports on the timing of *DMPK* expansion in early development. Later in 2004, another group reported similar results at the oocyte level, although their methods of detection and analysis differed from those used in our study.

Instabilities in the form of small changes in repeat number have also been documented during the transmission of *DMPK* $(CTG)_{19\text{-}37}$ repeats to offspring and in male gametogenesis but not at other stages of development. Therefore, we also decided to re-analyse the data generated in Chapter 3 to investigate whether any of the embryos that inherited the $(CTG)_{19\text{-}37}$ repeat underwent any change in repeat number upon transmission when compared to the parental repeat length.

4.2 ABSTRACT

In the current study, the timing of the expansion seen in the transmission of *DMPK* repeats was investigated in early human embryos. To further define the timing and variability of the large changes in repeat length that can occur during the transmission of expanded *DMPK* alleles in early development, one germinal vesicle and 4 metaphase II stage oocytes were analysed along with DM1-affected embryos donated from two DM1 females carrying different $(CTG)_n$ repeat lengths. In addition, the exact size of the normal *DMPK* repeat was re-analysed for each of the 220 embryos genotyped in Chapter 3 that were found to have inherited the parental $(CTG)_{19\text{-}37}$ repeat.

Our findings showed that the *DMPK* repeat length was larger, than the size detected in the DM1 maternal lymphocytes, for 2 of the 4 oocytes tested, including the immature oocyte, and in 17 of the 20 three-cell to blastocyst stage embryos. We concluded that, as the maternal *DMPK* repeat was already further expanded in the immature oocyte, this initial expansion most likely occurs during oogenesis, which is in agreement with the report published after presentation of our results. Variable degrees of embryonic *DMPK* expansion were seen upon the transmission of expanded repeats from different mothers. Also, for *DMPK* repeats in the normal-sized $(CTG)_{19\text{-}37}$ range, instability was seen in 7% of paternal transmissions but not in maternal transmissions.

In addition, we suggest for the first time that repeat expansion might be a continuous process and could occur during mitotic division of early cleavage-stage embryos. It can be further concluded that instability in larger normal-sized *DMPK* repeats also occurs early in development either during male gametogenesis or early embryogenesis.

4.3 INTRODUCTION

In general, there is genetic anticipation, or an increase in repeat length, as expanded *DMPK* $(CTG)_n$ repeats are transmitted to successive generations of a family, resulting in a progressively earlier onset and increased severity of DM1 (Harper et al 1992). The extent of the intergenerational change in *DMPK* repeat length is dependent on whether the repeat is inherited from the mother or from the father, and also upon the length of the repeat in the transmitting parent (Lavedan et al 1993). Small expansions $(CTG)_{<100}$ are most unstable when transmitted by males (Brunner et al 1993a, Martorell et al 2001) but the largest expansions, such as those seen in congenital DM1 (CDM1) individuals, are almost exclusively transmitted by an affected mother (Hofmann-Radvanyi et al 1993).

Meiotic and mitotic instability in the transmission of expanded repeats

The intergenerational increases that lead to changes in the phenotypic expression of DM1 are thought to result from an initial expansion of the repeat followed by two waves of somatic instability (Jansen et al 1994).

Timing of mitotic instability

The first pronounced wave of somatic instability has been suggested to occur between 13 and 16 weeks' gestation, leading to major heterogeneity between the tissues as shown in a series of congenitally affected fetuses and neonates (Hecht et al 1993, Jansen et al 1994, Lavedan et al 1993, Martorell et al 1997, Wohrle et al 1995). The second wave of instability, which is to a lesser degree than the first, could continue throughout adulthood (Anvret et al 1993, Brook et al 1992, Jansen et al 1994, Martorell et al 1995, Wang et al 1995). This could explain why the repeat length in an individual

patient increases with age and the progression of symptoms. This mitotic instability can lead to different repeat lengths in the blood compared to the tissues affected in CDM1 individuals, namely, the muscle and brain, and could explain why the size of the mutation in lymphocytes does not necessarily correlate with the severity of the phenotype (Mahadevan et al 1992, Martorell et al 1995).

Timing of meiotic instability

The precise timing of the initial expansion of the $(CTG)_n$ repeat in the *DMPK* gene has long been speculated. The youngest fetuses in previous studies were at 10 to 12 weeks' gestation and already had dramatically larger repeat lengths than the transmitting parent but showed no length variation between tissues (Hecht et al 1993, Jansen et al 1994, Martorell et al 1997), indicating that the initial expansion had occurred prior to the tenth week of embryogenesis. To define this further, some monozygotic twin pairs have shown identical patterns of expansion in the same tissues (Dubel et al 1992, Jansen et al 1994, Lopez de Munain et al 1994), suggesting that the timing of the initial $(CTG)_n$ expansion was most pronounced during early embryonic stages, either before or around the time of monozygotic twinning, which is at the latest day 10.5 of development (Phillips et al 1993).

Studies examining repeat expansion at earlier stages of development are limited. In the male germline, the repeat was found to be unstable during spermatogenesis, as shown in DM1-affected males who carried larger repeat expansions in their mature spermatozoa than seen in their lymphocytes and who could transmit even larger expansions to their DM1-affected offspring (Jansen et al 1994, Lavedan et al 1993). In the female germline, a report published after this work had been presented suggested that the CTG repeat had already expanded in DM1-affected oocytes, leading to the conclusion

that the initial CTG expansion occurs before completion of meiosis I of oogenesis (De Temmerman et al 2004).

Meiotic instability in the transmission of *DMPK* $(CTG)_{19-37}$ repeats

In addition to TRD shown in the transmission of normal-sized *DMPK* $(CTG)_{19-37}$ repeats as determined in the previous chapter, intergenerational instability at the level of offspring has also been shown in the transmission of this repeat range (Dow et al 1997, Imbert et al 1993, Meiner et al 1998, Weber and Wong 1993). A maximum increase of 11 repeats was described during a paternal transmission (Meiner et al 1998). It has been suggested that the $(CTG)_{19-37}$ repeat is the point where repeat instability begins which, in conjunction with a preferential transmission of *DMPK* repeats in this size range, could provide a reservoir for future DM1-affected alleles in the population (Chakraborty et al 1996, Imbert et al 1993) as addressed in Section 1.5.2.5.

Further defining the timing of meiotic instability in DMPK repeat

The aim of the current study was to further elucidate the timing and examine the variability of *DMPK* $(CTG)_n$ expansion and instability in early human development. Research in this area is only possible in institutions carrying out *in-vitro* fertilisation (IVF) with preimplantation genetic diagnosis (PGD) (Dean et al 2001, Sermon et al 2001). Using oocytes and embryos donated from two couples undergoing PGD for DM1, in which both the female partners were the DM1-affected individuals and carried different degrees of CTG repeat expansion, a detailed analysis was performed to determine the transmission of expanded CTG repeats in early development. In addition, data from the TRD in larger normal-sized *DMPK* alleles study were re-analysed to determine if

intergenerational instability occurred in the transmission of normal-sized $(CTG)_{19-37}$ repeats at the level of the embryo.

4.4 MATERIALS AND METHODS

Patient description

The research ethics review board of the Royal Victoria Hospital approved the studies on both the expanded and larger normal-sized *DMPK* repeats. All couples signed consent forms to donate to this study any embryos diagnosed as unsuitable for transfer.

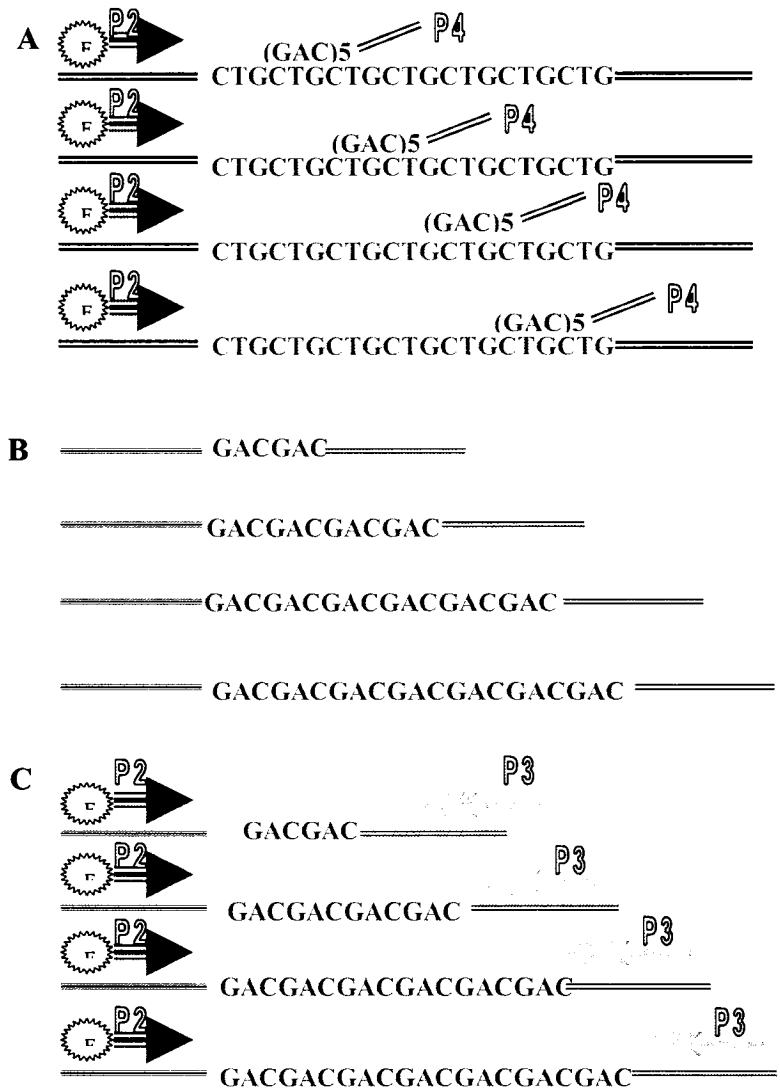
Patient A: For the study in expanded *DMPK* repeats, patient A was the female partner of couple 1 (see Section 2.4) who had previously undergone a pregnancy termination because the fetus had inherited the expanded maternal *DMPK* allele.

Patient B: This female partner was 35 years old and showed slight facial weakness. She had also undergone a termination of pregnancy for a CDM1-affected fetus. The affected female carried $(CTG)_5$ and $(CTG)_{230}$ repeats and inherited her expanded repeat from her father. Her partner had $(CTG)_{12}$ and $(CTG)_{24}$ repeats. This couple underwent 3 cycles of PGD.

Development and standardisation of TP-PCR protocol at single cell level

The fluorescent triplet-primed PCR (TP-PCR) protocol used in this experiment was adapted from Warner and colleagues (Warner et al 1996), who originally developed the assay using a starting material of 200ng-1μg genomic DNA. A fluorescent forward primer (P2) that flanked the $(CTG)_n$ repeat was used in combination with a pair of reverse primers (P3R and P4CAG) (Warner et al 1996). A schematic diagram depicting the strategy of TP-PCR is shown in Figure 4.1.

Figure 4.1: Scheme of triplet-primed PCR for amplification of expanded *DMPK* repeat



To amplify $(CTG)_{>100}$ repeats, a fluorescent forward-flanking primer P2 to denote the specific sequence of the amplified product was used in conjunction with a pair of primers that have a common non-human 5' tail, P3 and P4. In addition, the P4 primer has $(CAG)_5$ repeats at its 3' end. The concentration of these primers is such that the P4 is used up in early rounds of the reaction thereby stopping a gradual shortening of the product.

A) The specificity of the reaction is dictated by the fluorescent forward- flanking primer P2. Guided by the P2 primer, the repeat-specific P4 primer anneals at multiple sites within the CTG repeat.

B) This produces different lengths of product containing different numbers of CTG repeats.

C) In later rounds, still guided by the P2, the primer P3 preferentially binds to the ends of the products from the previous amplification rounds.

Adaptation of the TP-PCR protocol to permit amplification at the single-cell level, involved testing different cell lysis systems, experimenting with primer extension pre-amplification PCR protocols, adding the P3:P4 paired tail primers after initial cycling had been performed and changing many different components of the PCR reaction and cycling conditions. Specifically, the relative concentrations of the paired primers were varied, different additives such as Betaine, Tricine and dimethylsulphoxide (DMSO) were included in the reaction mixes, different PCR buffers and DNA polymerases (Taq polymerase, Expand Long Template and High Fidelity Taq with proof reading protein) were used, and deaza-7-dGTP replaced or was either added in addition to dGTP.

In order to obtain reproducible results using the TP-PCR approach at the single-cell level 73 PCR reactions were required and a total of 718 samples were tested. This included 356 genomic DNA samples collected from cultured fibroblasts as per standard protocol (Sambrook and Russell 2001) from an CDM1-affected neonate carrying an E3 expansion ($(CTG)_{1100}$) as well as from the blood of 4 DM1 adults carrying E1 and E2 expansions ($(CTG)_{<500}$ and $(CTG)_{500\text{-}1000}$, respectively). The genomic DNA concentration used ranged from 700ng down to 60pg. In addition, 49 buccal cell, 144 fibroblast, 153 lymphocyte and 16 blastomere samples were used in the standardisation step. Initially, for testing the somatic cells, each starting sample consisted of 20 of each cell type and, eventually, this was reduced to the single-cell level. Therefore, apart from the blastomeres all other cell types were divided so that each tube contained 20, 5 or 1 cells from one individual. Once the results from this preliminary standardisation were proven to be reliable, the TP-PCR protocol was used to genotype the *DMPK* repeat in the experimental samples and was also available for clinical diagnosis, through direct detection of the expanded *DMPK* allele, in DM1 PGD.

Genotyping of donated oocytes and embryos

Genotyping of expanded alleles from DM-1 affected females

All embryos diagnosed as DM1-affected after PGD analysis, using the PCR protocol to detect normal-sized *DMPK* repeats designed in Section 2.4, were collected as described previously. Although these embryos had been diagnosed as being DM1-affected, the expanded allele had not been amplified during PGD analysis. Therefore, the degree of repeat expansion, on the embryonic expanded allele, had not been determined. This was because the technique for the diagnosis of unaffected embryos in DM1 PGD was based on the detection of the normal-sized *DMPK* repeat from the unaffected parent.

In addition, all embryos diagnosed as unaffected (those carrying the normal-sized maternal allele) but not suitable for transfer to the patient, all embryos not included in the clinical PGD analysis and all oocytes that were not suitable for insemination, irrespective of their maturity, were collected as above. The blastomeres of each embryo were divided into multiple tubes and amplified separately with 2 - 12 samples collected from each preimplantation embryo. The normal-sized and expanded *DMPK* $(CTG)_n$ repeats of these embryos and oocytes were amplified using the TP-PCR protocol standardised at the single-cell level.

Reactions were carried out in a total volume of 50μl with final concentrations of 5% DMSO, 20mM Tricine [pH4.95], neutralisation and potassium-free PCR buffers (see Section 3.4), 500μM of each dNTP, 1.4 units of DNA polymerase (with Taq and Tgo DNA polymerase) provided in the Expand Long Template kit (Roche Diagnostics, Mannheim, Germany), 0.2μM each of P2 and P3R primers and 0.1μM P4CAG primer (Intergrated DNA Technology, IA, USA). The P2 primer was labelled with CY5

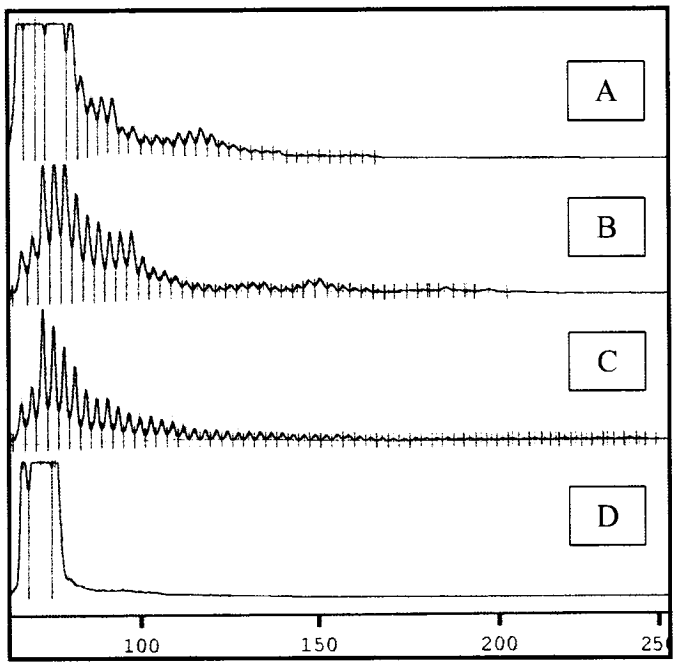
fluorescent dye. The TP-PCR was carried out on PTC-200 thermocycler (as in Chapter 2) using the following program: denaturation at 95°C for 2 minutes (min), followed by 45 cycles of 95°C for 30 seconds (sec), 60°C for 30 sec and 72°C for 60 sec, followed by 72°C for 5 min.

The samples were run on the ALF Automated DNA Sequencer (Section 2.4) including the addition of the base pair marker, using 6μl of the fluorescently-labelled amplified DNA mixed with 5μl of loading dye. This resulted in a characteristic ladder of the amplified product on the fluorescent trace that for normal-sized repeats and small expansions peaked at the largest allele present (Figure 4.2 lines A and B). For larger expansions, the ladder diminished gradually with increasing length of product size (Figure 4.2 line C).

For *DMPK* repeats in the larger normal-size range

In the current study, the results from 220 embryos that inherited a $(CTG)_{19-37}$ repeat for the Group I/Group II genotyped parent were reanalysed to determine if the size of the repeat inherited by the embryo was the same as that seen in the transmitting parent. For any embryo in which the size of the repeat amplified from the blastomere sample appeared different to that amplified from the parent, the embryonic PCR product was re-run using the same protocol as for the original genotyping (Section 2.4) in order to verify the size of the *DMPK* repeat. Each embryo sample was re-run in conjunction with an aliquot of the previously amplified parental cells and a base pair marker, at frequent intervals, to serve as controls.

Figure 4.2: Standardisation of triplet-primed PCR fragment analysis, at the single-cell level, on the *DMPK* $(CTG)_n$ repeat which is expanded in DM1



Amplification of *DMPK* $(CTG)_n$ repeat region from single cells using triplet-primed PCR. The ladder pattern on the fluorescence trace peaks at the largest allele present for normal and small expansions and for larger expansions diminishes gradually with increasing product size

A. Single lymphocyte from individual carrying *DMPK* $(CTG)_{21}$ and $(CTG)_{150}$ repeats.
B. Single lymphocyte from DM1-affected individual carrying *DMPK* $(CTG)_{31}$ and $(CTG)_{500}$ repeats.
C. Single fibroblast from DM1-affected infant carrying *DMPK* $(CTG)_5$ and $(CTG)_{1100}$ repeats.
D. Negative control.

Reliability of sizing of expanded DMPK $(CTG)_n$ repeats

After standardisation of the TP-PCR protocol at the single-cell level, testing of the assay was performed using single blastomeres from IVF embryos donated by DM1-unaffected individuals with known *DMPK* $(CTG)_n$ repeat lengths (control embryos- Table 4.1). Each blastomere gave the same pattern of amplification as all other blastomeres from the same embryo. Further to this, genomic DNA and single lymphocytes which had been collected from DM1-affected individuals as well as single fibroblasts from a CDM1-affected neonate with $(CTG)_{1100}$ repeats were amplified using the above protocol. The genomic DNA and cells from any one individual gave a consistent pattern of amplification, which allowed for further standardisation and also determination of the ladder patterns expected from different sizes of *DMPK* $(CTG)_n$ repeat (Figure 4.2).

From these results, we were able to size the expanded allele in the DM1 oocytes and embryos as having repeat sizes with specific ladder patterns which were categorised into three groups: a) no larger than the mother: *maternal type* b) expansion pattern larger than the mother but smaller than the pattern obtained from a CDM1-affected sample: *intermediate expansion* or c) expansion pattern as observed from a CDM1-affected sample: *in the congenital range*. For maternal-type expansions, the largest product size on "fluorescent trace" was approximately 190 base pairs for Patient A and 170 base pairs for Patient B, for intermediate expansions, the largest product size was between 200-240 base pairs and for expansions in the congenital range, it was ≥250 base pairs.

Table 4.1: Triplet-primed PCR amplification of *DMPK* $(CTG)_n$ repeat in single lymphocytes, cumulus cells, and oocytes and embryos donated from two DM1-affected women

	Level of CTG expansion [a]			
Cell type amplified	Normal $(CTG)_{5-37}$	Maternal type 170-190bp	Intermediate 200-240bp	Congenital ≥250bp
Maternal lymphocyte		147		
Cumulus cell		20		
Germinal vesicle oocyte				1
Metaphase II oocyte	1 [b]	2		1
Embryo 3-4 cell	2 [c]		4	3
Embryo 6-8 cell		2	4	4
Blastocyst		1		2
Control embryos	10 [d]			

(bp: base pairs)

Note: Patient A had 2 donated embryos with a normal-sized repeat, 1 with a maternal expansion, 4 with intermediate expansions and 7 with congenital expansions. Patient B had 2 embryos with maternal expansions, 4 with intermediate expansions and 2 with congenital expansions.

[a] The degree of repeat expansion was classified as:
- "Maternal type": no larger than the mother.
- "Intermediate expansion": larger than the mother but smaller than the pattern obtained from a congenitally DM1-affected sample.
- "Congenital expansion": similar to the pattern seen in a congenitally DM1-affected sample.

[b] "Failed to fertilise" mature oocyte carrying the maternal non-expanded allele.

[c] Diagnosed as normal but unsuitable for transfer to the female.

[d] Donated from genotyped IVF couples who carried $(CTG)_{5-37}$ repeats.

4.5 RESULTS

To analyse the *DMPK* repeat in the study oocytes and blastomeres, 10 PCR reactions were necessary. In addition to the 5 oocyte and 1 polar body samples amplified, a total of 124 samples from the 32 embryos (21 embryos with an expanded *DMPK* repeat and 12 embryos with a normal-sized repeat) were tested.

Instability in the transmission of expanded *DMPK* $(CTG)_n$ repeats

Sixty-two single lymphocytes from patient A, 85 from patient B and cumulus cells from both patients were amplified using the TP-PCR protocol (Table 4.1). All the cells from patient A gave a ladder pattern that related to an expansion in the maternal range, as determined from previously amplified maternal DNA. This was also true for patient B.

Instability in preimplantation embryos

A total of 33 embryos were generated in the 5 PGD cycles. Patient A, with $(CTG)_{444}$ repeat, produced 20 embryos of which 14 were donated to research (11 were PGD genotyped as carrying an expanded repeat; 2 as carrying the maternal $(CTG)_5$ repeat which was confirmed using TP-PCR; and 1 embryo which was not included in the clinical PGD cycle but was subsequently diagnosed as carrying an expanded repeat in the congenital range). Patient B, with $(CTG)_{230}$ repeat, produced 13 embryos of which 8 were donated to research because they had been diagnosed as carrying an expanded repeat. A summary of the $(CTG)_n$ expansion seen in the cohort of embryos donated from both women combined is presented in Table 4.1. All blastomeres from an individual embryo, at any particular stage of development, irrespective of the developmental stage or degree

of expansion, showed the same pattern of amplification indicating that no differential expansion was evident within the cells of a single embryo.

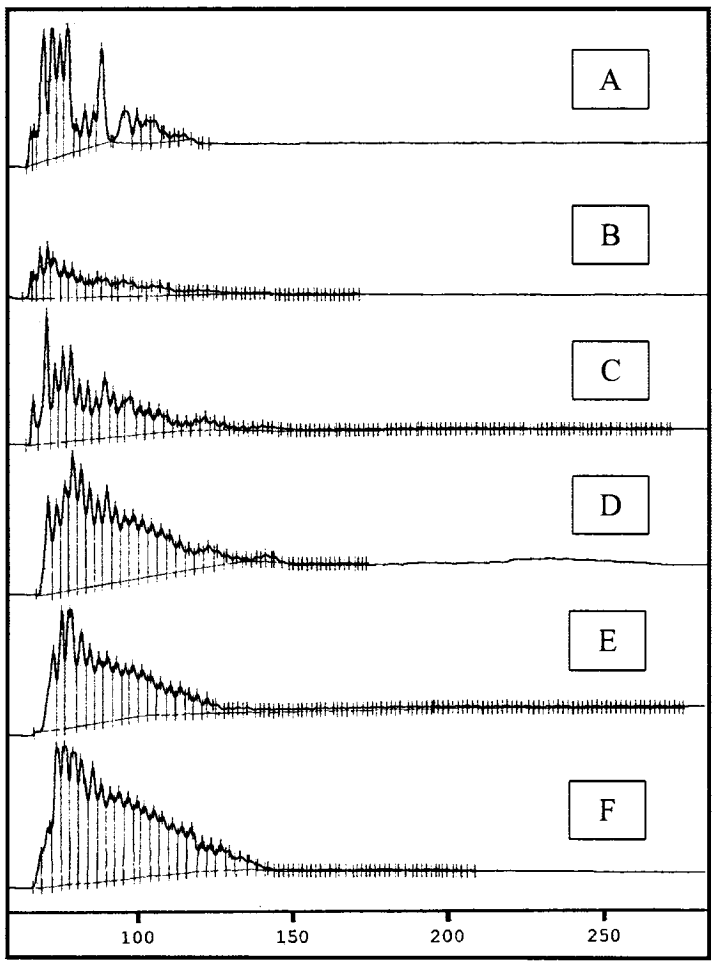
Individually, for patient A, of the 11 embryos which were PGD genotyped as carrying an expanded repeat, 2 three-cell stage embryos had intermediate expansions and the 3 four-cell stage embryos had an expansion in the congenital range. One six- and the seven-cell stage embryo had intermediate expansions whilst another six-cell stage embryo had an expansion in the congenital range. The eight-cell stage embryo had a maternal-type expansion and the 2 blastocysts had expansions in the congenital range. From the 8 embryos donated by patient B, the three- and four-cell stage embryos had intermediate expansions. Of the 5 embryos at the eight-cell stage, 1 had a maternal-type expansion, 2 had intermediate expansions and 2 had expansions in the congenital range (Figure 4.3, lines D,E and F). The blastocyst from this patient had a maternal-type expansion.

For patient A, 7 (58%) of the 12 embryos inheriting the maternal expanded repeat had a ladder pattern in the congenital range and for patient B, 2 (25%) out of 8 embryos inheriting the expanded repeat had an expansion in the congenital range.

Instability in oocytes

An expanded $(CTG)_n$ repeat was found in 4 out of 5 oocytes collected from the two women. This included one gamete at the germinal vesicle stage from patient B that had an expansion in the congenital range (Figure 4.3, line C). Of the 3 mature oocytes with expanded repeats, all from patient A, 2 had maternal-type expansions and 1 had an expansion in the congenital range. The mature oocyte collected from patient B inherited the normal-sized allele. The first polar body from this oocyte was amplified separately and the expanded allele detected, which was found to have undergone an intermediate expansion.

Figure 4.3: Triplet-primed PCR of oocyte and preimplantation embryos, at different developmental stages, from a DM1-affected woman



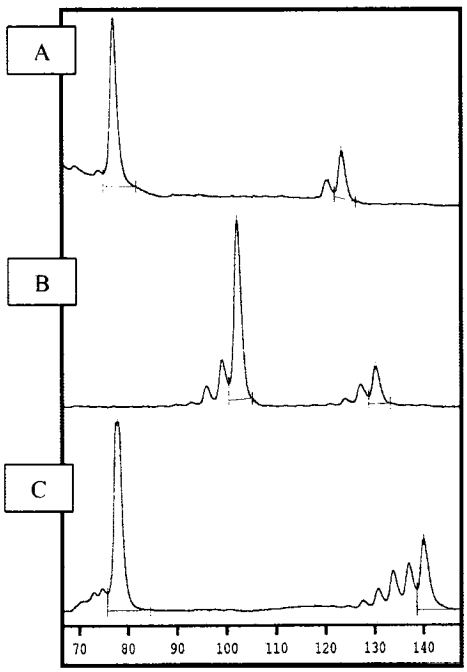
Variable degrees of repeat expansion are evident in the oocytes and preimplantation embryos, at different developmental stages, from a DM1-affected woman

A. Paternal alleles carrying *DMPK* $(CTG)_{12}$ and $(CTG)_{24}$ repeats.
B. Maternal alleles from DM-1 affected women (patient B) carrying *DMPK* $(CTG)_5$ and $(CTG)_{230}$ repeats.
C. Immature oocyte at germinal vesicle stage with an expansion in the congenital range.
D. A cleaved embryo at the eight-cell stage with a maternal-type expansion.
E. A second cleaved embryo at the eight-cell stage with an expansion in the congenital range.
F. A third cleaved embryo at the eight-cell stage with an intermediate expansion.

Instability in the transmission of normal-sized *DMPK* $(CTG)_{19-37}$ repeats

Transmission of the larger normal-sized $(CTG)_{19-37}$ parental repeat was detected in 220 (59%) of 371 preimplantation embryos obtained from the 61 individuals carrying a $(CTG)_{5-18}/(CTG)_{19-37}$ genotype. The size of the $(CTG)_{19-37}$ parental repeat length inherited by the embryo had changed in 7 (7.4%) of 95 paternal transmissions found in the embryos collected from 3 (11.1%) of 27 fathers. Increases of one to four $(CTG)_n$ repeat units were seen in 6 embryos and a contraction of one repeat unit was seen in 1 embryo. The embryos in which the repeat length had increased were at the three-cell, six-cell and blastocyst stages of development whereas the 1 embryo in which the repeat had contracted was at the blastocyst stage. The intergenerational instability seen in the repeat length in five embryos is shown in Figure 4.4 I and II. There was no change in the repeat length in the 125 maternal transmissions from 34 mothers.

Figure 4.4: Intergenerational instability of the larger-normal sized *DMPK* $(CTG)_n$ repeat during paternal transmission in donated IVF embryos



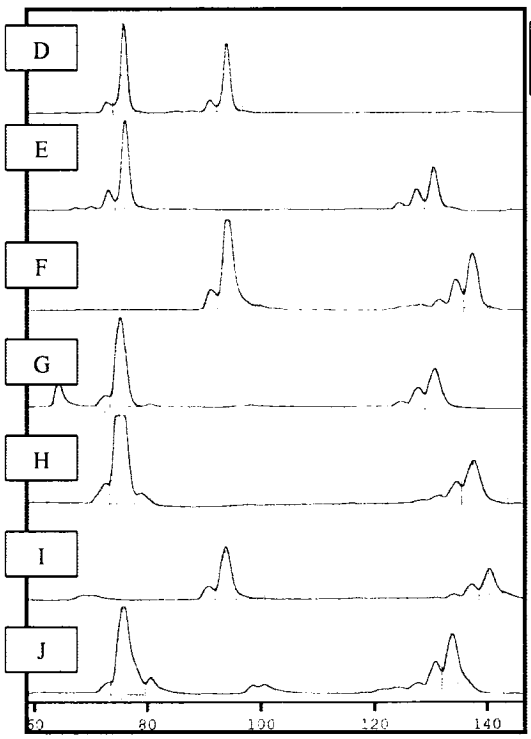
I

Couple who donated 5 embryos and in which an intergenerational paternal expansion was seen in 1 embryo

A. Maternal alleles with *DMPK* $(CTG)_5$ and $(CTG)_{20}$ repeats

B. Paternal alleles with *DMPK* $(CTG)_{13}$ and $(CTG)_{22}$ repeats

C. Embryo with *DMPK* $(CTG)_5$ and $(CTG)_{26}$ repeats, which had expanded by 4 repeat units



II

Couple who donated 9 embryos in which an intergenerational paternal expansion was seen in 4 embryos

D. Maternal alleles with *DMPK* $(CTG)_5$ and $(CTG)_{12}$ repeats

E. Paternal alleles with *DMPK* $(CTG)_5$ and $(CTG)_{23}$ repeats

F. Embryo 1 with $(CTG)_{12}$ and $(CTG)_{25}$ which had expanded by 2 repeat units.

G. Embryo 2 $(CTG)_5$ and $(CTG)_{23}$.

H. Embryo 3 with $(CTG)_5$ and $(CTG)_{25}$ which had expanded by 2 repeat units.

I. Embryo 4 with $(CTG)_{12}$ and $(CTG)_{26}$ which had expanded by 3 repeat units.

J. Embryo 5 with $(CTG)_5$ and $(CTG)_{24}$ which had expanded by 1 repeat unit.

4.6 DISCUSSION

From the limited number of oocytes and preimplantation embryos obtained from 2 DM1-affected women, the pattern of *DMPK* $(CTG)_n$ repeat amplification indicated that the repeat had already expanded in oocytes and embryos, including an immature oocyte, when compared to maternal lymphocytes or cumulus cells. The amplification of maternal lymphocytes and cumulus cells gave ladder patterns shorter than the ones seen in the congenital range. Therefore the enlargement to the congenital range seen in the oocyte most likely occurred during oogenesis, either during premeiotic proliferation of oogonia or during prophase I of meiosis I. These results are in agreement with the recent suggestion that the initial expansion of the *DMPK* $(CTG)_n$ repeat occurs before the completion of the first meiotic division in oogenesis (De Temmerman et al 2004).

A similar timing of maternally transmitted expansion has been suggested in a type I trinucleotide disorder, spino-cerebellar ataxia type 1 (SCA1) (Kaytor et al 1997). In SCA1 mice, $(CAG)_n$ repeat expansion in the *ATXN1* gene has been suggested to occur while the oocytes are arrested in meiosis I, which is similar to the timing of repeat expansion observed in this study, although the mechanism of transmission of SCA1 repeats may be different to DM1. However, contrasting results concerning the timing of repeat expansion have been noted in another type II trinucleotide repeat disorder, Fragile X, where repeat expansion to the full mutation has been shown to be greatest during early post-zygotic cell divisions and, once it has occurred, the repeat is mitotically stable in differentiated tissues (Wohrle et al 1993).

Variability of *DMPK* expansion

Absence of intra-embryonic variability

Variability was demonstrated in the degree of repeat expansion in the embryos from each DM1-affected woman however, no variability in the *DMPK* repeat pattern was detected between blastomeres from any individual preimplantation embryo at any one stage of development. This is in agreement with observations of the earliest somatic mosaicism occurring in affected fetuses between 13 and 16 weeks' gestation (Hecht et al 1993, Jansen et al 1994, Martorell et al 1997, Wohrle et al 1995).

Maternally transmitted inter-embryonic variation

In addition, we compared the expansions seen in the cohort of embryos from each of the DM1-affected mothers and found a variation in the size of the repeat present in each cohort. Patient B's cleavage-stage embryos had expansions from the maternal type to the congenital range and her immature oocyte had a congenital expansion (Figure 4.3) but her blastocyst carried a maternal-type expansion. The variability in the degree of *DMPK* $(CTG)_n$ repeat expansion transmitted to these embryos is similar to the variable DM1 phenotypes observed in the offspring of a DM1-affected mother. This variability can therefore be related back to these early stages of preimplantation development.

Previous studies have shown that a DM1 woman has a 3% to 10% chance of having a neonatally affected first child, but this risk increases to 20% to 58% for future conceptions if she has already had a neonatally-affected child (Bundey 1982, Glanz and Fraser 1984, Grimm and Harper 1983, Harper 1997, Koch et al 1991, Lavedan et al 1993). It has even been suggested that after one congenitally-affected pregnancy, the risk of another is almost 100% (Magee et al 2002). The DM1 used embryos in our study were donated by two women who had previously undergone a pregnancy termination for a

congenitally-affected fetus. Of the 20 affected embryos, 9 (45%) carried a pattern of $(CTG)_n$ repeats consistent with the pattern amplified from a CDM1 neonate's fibroblasts, and even though this represents a small sample, it is within the range estimated for a congenitally-affected conception after a prior congenitally-affected pregnancy. The proportion of embryos with a repeat pattern in the congenital range was higher for the woman with $(CTG)_{444}$ repeats than for the one with $(CTG)_{230}$ repeats. Published findings demonstrate that mothers carrying larger *DMPK* repeats are at increased likelihood of having a CDM1 conceptus (Harley et al 1993, Lavedan et al 1993, Redman et al 1993), which from our study is also shown at the level of the embryo.

Mitotic instability during early development

Although we have shown that expansions in the $(CTG)_n$ repeat length are present in the oocyte and preimplantation embryo, it was not known if these large expansions are the result of one expansion event or whether expansion is a continuous process throughout early development.

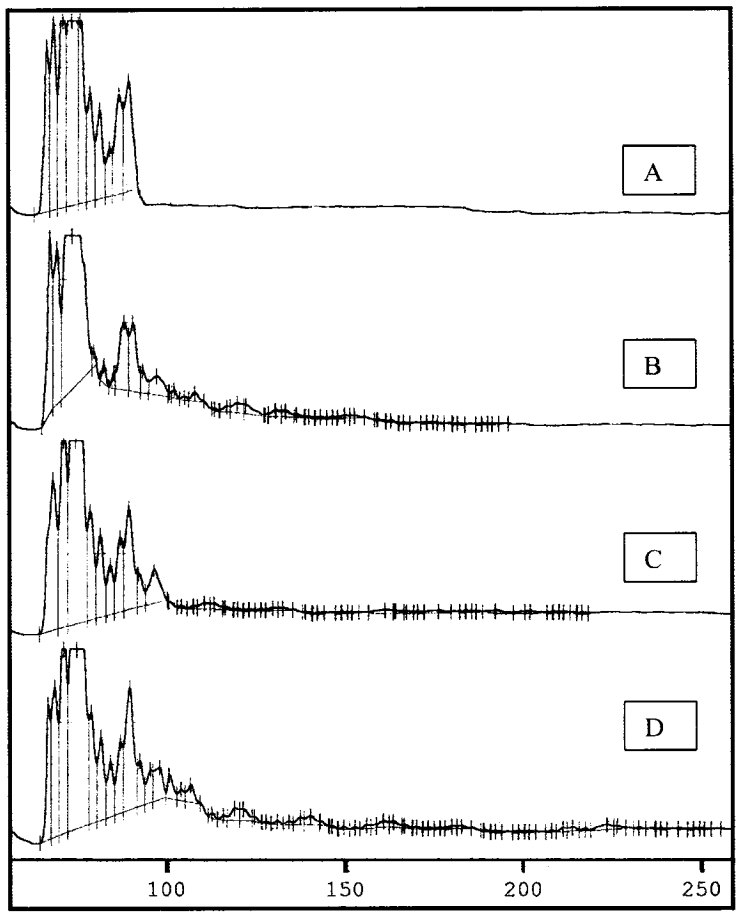
One embryo from patient A was deemed to be of unsuitable quality for inclusion in the clinical treatment cycle. Therefore, one blastomere was collected from this four-cell stage embryo at the time of biopsy. After a further 24 hours of culture, the embryo had undergone a complete round of cell division to the six-cell stage, at which time the blastomeres were collected. The $(CTG)_n$ repeat size in the blastomere collected from the embryo at the four-cell stage and the blastomeres collected from the same embryo at the six-cell stage were tested using the TP-PCR protocol. An intermediate expansion, when compared to the repeat length of the mother, was seen in the blastomere from the four-cell

embryo but the blastomeres from the same embryo at the six-cell stage gave an even larger expansion, in the congenital range (Figure 4.5).

Although it is tempting to speculate that mitotic post-zygotic expansion of the *DMPK* repeat could be possible in the early preimplantation embryo in DM1, we could not draw any conclusion based only on this one embryo. Instability has previously been noted to occur immediately post-fertilisation in two mouse models, arising in the first two embryonic cell divisions for mutations occurring in a tetranucleotide repeat (Gibbs et al 1993) and probably at the one-cell stage for *DMPK* $(CTG)_n$ repeats in a DNA repair-deficient mouse (Savouret et al 2003).

In Fragile-X syndrome, which is also caused by huge expansions of a trinucleotide repeat (Verkerk et al 1991) and has many similarities to DM1 in its mode of transmission, a model has been proposed that assumes repeat expansion occurs equally in oogenesis and spermatogenesis and that post-zygotic expansion to the full mutation is limited to the maternal chromosome (Ashley and Sherman 1995). This model would fit with our findings. In order to determine if $(CTG)_n$ repeat expansion could continue during mitotic cell division in the early DM1 preimplantation embryo, it would be necessary to test a larger sample of suitable embryos. However, because of the limited number of embryos available, further testing was not possible during the course of this study.

Figure 4.5: Instability of expanded *DMPK* $(CTG)_n$ repeat during embryonic cell division



Triplet-primed PCR of a preimplantation embryo from a DM1-affected woman illustrating the continuation of *DMPK* $(CTG)_n$ expansion through mitotic cell division.

Patterns of amplification seen in:

A. Paternal alleles homozygous for *DMPK* $(CTG)_{13}$ repeats.
B. Maternal alleles from DM1 affected mother (patient A) carrying *DMPK* $(CTG)_5$ and $(CTG)_{440}$ repeats
C. Single blastomere from an embryo biopsied at 4-cell stage.
D. A blastomere from the same embryo after it had divided to the 6-cell stage.

Possible mechanisms of repeat expansion

Expansion of the *DMPK* $(CTG)_n$ repeat is known to be responsible for the DM1 phenotype (Brook et al 1992, Buxton et al 1992, Fu et al 1992, Harley et al 1992, Mahadevan et al 1992). However, the mechanism of expansion is not yet well understood, and it is possible that different processes are responsible at separate loci and could occur in a cell type, cell state and species-dependent fashion in both somatic and germline tissue. Repeat expansion has been associated with DNA replication, transcription, recombination and repair (reviewed by Cleary and Pearson 2003, Jakupciak and Wells 2000, Lenzmeier and Freudenreich 2003, Pearson 2003). Structurally, unstable repeats can fold into unusual DNA conformations such as hairpins and slipped-strand structures (Gacy et al 1995, Pearson and Sinden 1996). Molecularly, these unusual DNA structures could be involved in the processes implicated in repeat instability such as replication slippage, the direction of replication fork progression, lagging-strand errors, Okazaki fragment processing, mismatch repair, gap-filling, double-strand break repair or recombination (reviewed by Lenzmeier and Freudenreich 2003).

Various elements possibly related to haplotype background, which flank the repeat, have been proposed to predispose a repeat to expansion. The observation of a familial predisposition in DM1-affected sisters, irrespective of differences in their clinical status and repeat length, for the same DM1 phenotype in their affected children supports the presence of another genetic factor, possibly a *cis*-element, contributing to repeat expansion (Lavedan et al 1993). Such *cis*-elements could include the length of the repeat tract, positioning of nucleosomes within the repeat which may alter the chromatin structure, the presence of closely linked chromosomal elements such as *Alu* repeats or

binding sites for the CCCTC binding factor, and DNA methylation (reviewed by Cleary and Pearson 2003).

DNA methylation in repeat stability

The *DMPK* repeat region lies in a CpG island, although the repeat itself does not contain any CpG methylase recognition sites (Boucher et al 1995). However, hypermethylation of part of the island flanking the repeat is correlated with stabilisation of highly expanded CTG repeats in the somatic tissues of some severely DM1-affected individuals (Steinbach et al 1998) and, in bacteria, *in vivo* CpG methylation of flanking sequences had a mild stabilising effect on expanded $(CTG)_n$ repeats (Nichol and Pearson 2002). This shows that the methylation status of DNA surrounding the *DMPK* repeat can play a role in its stability. Therefore, conversely, demethylation of these regions could induce destabilisation of the repeat and lead to the formation of hairpin and slipped-strand DNA structures. Experimentally, demethylation has been shown to induce repeat instability, mainly in the form of expansion, in the $(CTG)_n$ repeat region of the *DMPK* gene in fibroblast cell lines of DM1 patients (Gorbunova et al 2004).

Demethylation of the genome occurs during two periods of epigenetic reprogramming seen in mammalian development. During gametogenesis, especially oogenesis, genomic patterns of CpG methylation are largely erased in the primordial germ cells (Monk et al 1987) and are then re-established in sex-specific patterns in mature germ cells (Chaillet et al 1991, Kono et al 1996, Walsh et al 1998). An additional wave of demethylation occurs immediately after fertilisation, followed by the re-establishment of the methylation pattern in a tissue-specific manner. The instability of the expanded maternal *DMPK* $(CTG)_n$ repeat in oocytes and early embryos demonstrated in this study coincide with these time points. Although there is no experimental evidence to support it,

it could be that some effect of demethylation on the *DMPK* gene region during oogenesis and immediately post-fertilisation may cause a destabilisation of the $(CTG)_n$ repeat that could result in an increase in the length of the repeat.

DNA mismatch repair system

The observation of somatic *DMPK* instability in non-proliferative tissues in DM1 patients (Ishii et al 1996, Thornton et al 1994) plus the lack of an association between instability and tissue proliferation capacity (Lia et al 1998, Seznec et al 2000) suggests that replication-independent mechanisms could also be involved in *DMPK* repeat expansion. Therefore, one or several DNA repair pathways, linked or not to replication, may be involved in triplet-repeat instability.

In humans, non-polyposis colon cancer is associated with defects in the DNA mismatch repair pathway causing genomewide microsatellite instability in tumours (reviewed by Peltomaki 2001). Transgenic modeling has shown mismatch repair genes to also be important in DM1 repeat instability with different effects of mismatch repair protein disruption seen dependent on the genotype of the offspring (Foiry et al 2006, Savouret et al 2003, van de Broek et al 2002) (Section 1.5.2.4). Theoretically, this could mean that variant alleles of the mismatch repair genes may function as *trans*-acting genetic modifiers, contributing to repeat instability in DM1 families, however, to date, no potential modifiers have been proposed.

Instability in the normal-sized *DMPK* repeat range

Expanded $(CTG)_n$ repeats increase in size in the embryos of DM1-affected women, but instability in larger normal-sized *DMPK* alleles has only been seen at the level of offspring (Dow et al 1997, Martorell et al 2001, Meiner et al 1998, Weber and

Wong 1993). In these studies, only alleles that were $(CTG)_{>24}$ in length showed instability and, in one study, instability was seen in 1.6% of paternal transmissions (Martorell et al 2001). Upon re-analysis of the data generated in Chapter 3, we detected instability during transmission of the parental $(CTG)_{19-37}$ repeat at the level of the preimplantation embryo (Figure 4.4). A change in the number of repeat units was observed in more than 7% of paternal transmissions but not in maternal transmissions. The small increases and one contraction seen in these donated embryos showed that instability of normal-sized *DMPK* alleles can occur during male gametogenesis or early embryogenesis.

Paternal transmission of repeat instability in normal-size range

The instability in paternal repeats in the larger normal-size range was not detected in maternal repeats and is thus consistent with the observation that small-expanded alleles, up to $(CTG)_{100}$, are most unstable when transmitted by males (Brunner et al 1993a, Martorell et al 2001). It is also in agreement with the finding that tandem repeat loci in spermatozoa showed an excess of small mutation events when compared to oocytes (Jeffreys et al 1988). A gradual increase of repeat size within the normal-size range, upon transmission through the male germline, coupled with a preferential transmission of the larger normal-sized alleles would allow maximal dissemination of future disease alleles and thereby help maintain the DM1 mutation in the population. However, it is not known whether the instability in larger normal-sized *DMPK* repeats observed in embryos is due to the same mechanism as that causing the large increases during transmission of already expanded repeats in DM1-affected individuals.

Paternal age effects on instability

It has been noted that with advancing paternal age there is an increased incidence of *de-novo* cases of a number disorders including Apert syndrome, achondroplasia and Crouzon syndrome (reviewed by Crow 2000). Although there is a higher male-to-female mutation rate in the human genome (Penrose 1955), the rate of mutation appears insufficient to explain this rapid rise in the incidence of sporadic cases of certain genetic disorders with advancing paternal age. It has therefore been suggested that there is a subgroup of men who may encounter an increased frequency of spermatozoa mutation at an earlier age or, alternatively, that there is a selection in favour of gametes with mutations occurring in these men (Glaser et al 2003, Goriely et al 2003, Tiemann-Boege et al 2002).

The three males from our study with paternally derived repeat instability were 32, 35 and 36 years old. As the average male age of the study patients was 37.4 years old, it is unlikely that a paternal-age effect would be evident only in these 3 study men and, therefore, the predisposition for *DMPK* repeat instability in these men's spermatozoa could be the effect of an unidentified genetic element. Interestingly, all 9 embryos from the 35-year-old man inherited his $(CTG)_{23}$ repeat in preference to his $(CTG)_5$ repeat and, in 4 of these embryos, the repeat length was longer than detected from the male's spermatozoa (Figure 4.4 II).

This could be due to the *DMPK* repeat length increasing post-zygotically or, alternatively, that repeat instability occurred during gametogenesis but that spermatozoa with longer repeats were not detected. Instability in $(CTG)_{19\text{-}37}$ repeat length has been observed in human male gametogenesis (Zhang et al 1994) and in pre-meiotic spermatogonia in DM1 transgenic mice (Savouret et al 2004). To assess instability at the

spermatozoa level in our male, the original stored sample was re-tested using lower concentrations of starting material than in the original protocol in order to maximise the chance of detecting multiple lengths of products. From over 30 aliquots, the largest repeat detected was the $(CTG)_{23}$ repeat. This does not rule out the presence of spermatozoa carrying longer alleles but suggests that they represent a small proportion of the total spermatozoa present. In Arnheim's study, the frequency of spermatozoa with expansions was detected to be 0.4% to 6.0% (Zhang et al 1994) and, in our sample, the percentage of spermatozoa with larger alleles could be in the same range. If spermatozoa with longer repeat lengths represented such a low proportion of the total spermatozoa in the ejaculate, it would be unexpected for them to fertilise 4 out of 9 oocytes. This could suggest that a selection in favour of spermatozoa carrying expanded repeats had occurred but, as this was an isolated observation, we could not draw any firm conclusions.

Concluding remarks

Through the TP-PCR protocol standardised at the single-cell level, which is also suitable to apply clinically for DM1 PGD to allow direct direction of the expanded *DMPK* allele, we have been able to show that expansion of large *DMPK* $(CTG)_n$ repeats occurs during oocyte development, and a variable degree of expansion is found in the embryos of mothers with different $(CTG)_n$ repeat lengths. Furthermore, although observed only in a single embryo, our results suggest for the first time that in addition to an initial pronounced expansion during oogenesis, repeat expansion could be a continuous process that could occur during mitotic divisions of early cleavage-stage embryos. We have also shown that instability in the paternal transmission of normal-sized $(CTG)_{19\text{-}37}$ repeats in the *DMPK* gene may occur during gametogenesis or early embryogenesis.

Currently, it is not known whether the large expansions observed during oogenesis and possibly embryogenesis, and the instability in normal-sized $(CTG)_n$ repeats occur by the same or distinct mechanisms. Based on the instabilities, both in oogenesis and embryogenesis that coincide with the two periods of epigenetic reprogramming of the mammalian genome, we hypothesise that the expansion mechanism could involve demethylation of the *DMPK* gene region, leading to destabilisation of the $(CTG)_n$ repeat and resulting in an increase in repeat length. There is also a prospect that DNA repair mechanisms may play a role at least in some aspect of *DMPK* repeat instability.

Future studies with further embryonic samples are necessary to determine if $(CTG)_n$ repeat expansion is a continuous process post-fertilisation. However, the definition of more precise timings for $(CTG)_n$ repeat expansion, from the studies carried out on these human oocytes and preimplantation embryos has advanced our understanding of the mode of transmission of *DMPK* repeats.

CHAPTER FIVE

Prospect of preimplantation genetic diagnosis for heritable mitochondrial DNA diseases

A condensed version of the work described in this chapter has been published in the following paper:

Nicola L Dean, Brendan J Battersby, Asangla Ao, Roger G Gosden, Seang Lin Tan, Eric A Shoubridge, Maria J Molnar (2003) Prospect of preimplantation genetic diagnosis for heritable mitochondrial DNA diseases. *Mol Hum Reprod* **9**: 631-638.

A presentation based on this chapter received the Best Basic Science Paper Prize at the 48th Annual Meeting of Canadian Fertility and Andrology Society, Charlevoix, Quebec, Canada, September 25-28, 2002.

5.1 FOREWORD

Defects in mitochondrial function are being increasingly recognised as important causes of disease. The inheritance of mitochondrial DNA (mtDNA) diseases is more complex than even for DM1, as three different types of transmission can be observed: maternal, Mendelian and sporadic. Mitochondrial DNA diseases caused by nuclear gene mutations show a normal Mendelian inheritance pattern and often have a more constant phenotype, whereas, in general, further transmission of sporadic mtDNA mutations does not occur.

One of the most challenging aspects of mtDNA disease is the transmission of maternally inherited pathogenic mtDNA mutations. The inheritance of these mutations has been shown to occur by stochastic segregation, which is generally accepted to result from an mtDNA bottleneck early in development. This can cause a wide variation, of between 0% and 100%, in the level of the mutant mtDNA detected in the offspring of a woman who is heteroplasmic for an mtDNA mutation. Once the percentage of inherited mutant mtDNA exceeds the threshold beyond which energy production is affected, an mtDNA disease phenotype will be expressed. Therefore, for a woman who is heteroplasmic for an mtDNA mutation diagnosing the risk of mtDNA disease in a specific pregnancy is unpredictable.

One reproductive option for a woman carrying a heteroplasmic mtDNA mutation would be to undergo preimplantation genetic diagnosis (PGD). Unlike genotyping for Mendelian disorders, where the presence or absence of the specific mutation is detected from the nuclear DNA, genetic diagnosis for mtDNA diseases aims to detect the proportion of mutant mtDNA in the embryo. The oocytes or embryos carrying the lowest mutant load could then be selected for replacement in the female. Therefore, before PGD

for mtDNA diseases can be offered, it is important to understand the segregation distribution of mutant mtDNA through the female germline by ensuring that the proportion of mutant mtDNA diagnosed in the biopsied cell gives an accurate indication of the mutant load in the remaining oocyte or embryo.

Investigating the segregation of mtDNA in early human development is not practical as human embryos heteroplasmic for pathogenic mtDNA mutations are not readily available. Therefore, the use of an appropriate animal model for such research is of paramount importance, and this study was accomplished by using a mouse heteroplasmic for neutral polymorphic mtDNA sequence variants. The intergenerational transmission of the two mtDNA genotypes in these mice has been shown to occur by a stochastic process determined by random genetic drift similar to that observed in many human mtDNA diseases.

The work presented in this chapter explores mtDNA segregation during meiotic and mitotic division in a heteroplasmic mouse model. Assessing the distribution of the two mtDNA genotypes in the cells of gametes and early embryos at different stages of development would permit determination of whether PGD would be possible for females heteroplasmic for mtDNA mutations. If the proportional levels of the polymorphic mtDNA genotypes were determined to be similar between the polar body and cytoplasm of a mature oocyte and/or between the blastomeres of an embryo, then PGD through polar body and/or blastomere biopsy, respectively, could be a feasible option for heteroplasmic mtDNA mutation carriers.

5.2 ABSTRACT

To determine the feasibility of performing PGD for women heteroplasmic for pathogenic mtDNA mutations, a heteroplasmic mouse model was tested. Mice carrying NZB and BALB mtDNA genotypes with levels of heteroplasmy ranging from 7% - 74% NZB mtDNA were used to study the relative proportions of each mtDNA genotype in the ooplasm and first polar body of mature oocytes and between the blastomeres of early cleavage-stage embryos. Complete results were obtained for 22 mature oocytes and their corresponding polar bodies, 19 two-cell embryos, 18 four-cell embryos and 16 six- to eight-cell embryos with overall levels of heteroplasmy ranging from 1% - 78% NZB mtDNA.

The mean level of heteroplasmy seen in each cohort of embryos was similar to that seen in the mother, but the levels of heteroplasmy varied widely in individual cohorts of gametes and embryos compared with the maternal genotype. However, no variation was seen within each gamete or embryo as demonstrated by the virtually identical distribution of the two mtDNA genotypes between the ooplasm and polar body of a mature oocyte, and also between the blastomeres of each 2-cell, 4-cell and 6- to 8-cell embryo. Therefore, the level of heteroplasmy diagnosed from the polar body of an unfertilised oocyte or from a single blastomere of an embryo is representative of the level in the embryo as a whole. Reliable results were obtained from both polar bodies and blastomeres, but the efficiency of diagnosis was greater with blastomeres. We conclude that PGD is feasible for mtDNA diseases, although, it should be approached with caution, as it is possible that transmission of some pathogenic mutations could behave in a different manner.

5.3 INTRODUCTION

The pattern of transmission for maternally inherited mtDNA mutations, which can lead to a wide range of mtDNA diseases, is different from that seen in genetic disorders caused by mutations of nuclear DNA. Inherited pathogenic nuclear DNA mutations are almost always present in the same number in every cell of the body (one copy for dominant and X-linked conditions and two copies for recessive disorders), and their presence will lead to the manifestation of a phenotype. However, pathogenic mtDNA mutations can be present in any cell or tissue at levels varying from 0% - 100% due to the rapid stochastic segregation of pathogenic mtDNA mutations between generations (Section 1.5.3.2). A phenotype will not be expressed until the mutant level exceeds the threshold beyond which cellular dysfunction will become evident, which for most mtDNA diseases is around 85% mutant load (Boulet et al 1992, Dubeau et al 2000, Hayashi et al 1991, Shoffner et al 1990, Thorburn and Dahl 2001). This threshold can vary according to the particular mutation and the energy needs and characteristics of individual organs and tissues. For example, in Leber Hereditary Optic Neuropathy (LHON), the threshold for the expression of a phenotype can be as low as 60% mutant load (Chinnery et al 2001). Most individuals carrying a pathogenic mtDNA mutation are heteroplasmic for mutant and wild-type mtDNA, and those with mutant levels below the threshold for disease expression will not present with a clinical phenotype. Nevertheless, a heteroplasmic woman who carries an mtDNA mutation but who does not express a phenotype, can transmit dramatically higher levels of mutant mtDNA to her offspring, at or above the threshold that could lead to the manifestation of a clinical disease.

Prenatal diagnosis for detection of mtDNA disease

One way such a woman can reduce the risk of giving birth to an affected child is by undergoing prenatal diagnosis to measure the mutant load in the chorionic villi or amniocytes. Initially there was concern as to whether the level of mutant mtDNA detected in fetal cells at 10 - 18 weeks' gestation could predict the mutant load in other tissues at birth. However, current data from animal and human studies suggest that there is little tissue variation or selection operating on mtDNA mutations *in utero* and that random segregation prevails in early development (Cardaioli et al 2000, Chinnery et al 2000, Dahl et al 2000, Jenuth et al 1997, Matthews et al 1994, White et al 1999). Therefore, the levels found in prenatal samples can be used to predict the mutant load in most tissues at birth. Prenatal diagnosis has been reported occasionally for mtDNA mutations affecting protein-coding genes but is not widely used (Harding et al 1992, Thorburn and Dahl, 2001).

Potential for preimplantation genetic diagnosis in the detection of mtDNA disease

Prenatal diagnosis is only available once a pregnancy is established. If the couple wishes to prevent the birth of an affected child, they will need to decide whether or not to terminate a fetus with a high proportion of mutant mtDNA, or even one with an intermediate mutant load. Alternatively, PGD, used in conjunction with *in-vitro* fertilisation (IVF), is available to diagnose specific genetic disorders by PCR from the polar body of an oocyte or from one or more cells from a preimplantation embryo (Ao and Handyside 1995, Handyside et al 1990, Verlinsky et al 1990). This approach has the advantage of providing a diagnosis before a pregnancy is established and eliminates the dilemma of whether or not to terminate a pregnancy.

Approaches to PGD for mtDNA diseases

Analysis of the first polar body from an unfertilised oocyte may be preferred by some couples who have strong reservations about embryo testing. Unlike Mendelian disorders, where the paternal component can frequently also cause disease, it is generally the maternal mitochondrial contribution that must be determined for mtDNA diseases. One man was identified with 90% mutated mtDNA in his muscle that was of paternal origin (Schwartz and Vissing 2002), however, further studies indicated the occurrence of this event is likely to be infrequent (Johns 2003, Schwartz and Vissing 2004). Therefore, polar-body analysis is theoretically suitable for diagnosing this group of diseases (Briggs et al 2000). The other PGD approaches, namely, blastomere biopsy, which is more widely used for single gene defects, and blastocyst biopsy permit the sampling of mtDNA from a cell or cells within the embryo that has or have the potential to become the fetus or the placenta.

Strategy for PGD in mtDNA diseases

Before considering PGD for mtDNA diseases, it is important to ascertain that the proportional levels of mutant to wild-type mtDNA quantified in the biopsied cell are representative of the levels in the embryo as a whole. Once this has been assured, PGD could then be carried out for inherited human mtDNA disease, and only embryos with undetectable or very low amounts of mutant mtDNA being regarded as suitable for transfer. At the time of this study, it was not known whether mutant and wild-type mtDNA were distributed equally between the ooplasm of an oocyte and its polar body, or among the blastomeres of an early cleavage-stage embryo.

When the results of this work were published, no PGD cycle had been completed for any woman carrying an mtDNA mutation, although one patient with an A3243G

mutation was reported as having undergone treatment, no embryo transfer was performed (ESHRE PGD Consortium, 2002, K de Boer, personal communication).

Model system to assess feasibility mtDNA PGD

Because of the difficulty in obtaining oocytes or embryos from women with mtDNA mutations, use of appropriate animal models is very important in investigating the segregation of mtDNA during early cleavage development. For our experiments, we used a mouse model, which was heteroplasmic for two polymorphic mtDNA sequence variants (NZB and BALB) (Jenuth et al 1996). The mtDNA genotypes in these mice differed at 101 bases, corresponding to about 0.6% of the mouse mitochondrial genome (Loveland et al 1990). This was predicted to result in 15 amino-acid changes, which were nearly all at evolutionary conserved sites and were therefore likely to be neutral polymorphisms. The transmission of these two mtDNA genotypes to offspring in such mice has been shown to be a stochastic process determined by random genetic drift (Jenuth et al 1996).

In the present study, the heteroplasmic animals were used to investigate the distribution of the two genotypes of mtDNA in female gametes and early cleavage-stage embryos. The segregation of the two mtDNA-sequence variants was measured between the ooplasm and first polar body of unfertilised mature oocytes and between blastomeres of early cleavage-stage embryos at different stages of development. The results of these experiments are discussed in context of the feasibility of clinical PGD as a method to prevent inherited mtDNA disorders.

5.4 MATERIALS AND METHODS

Generation of BALB/NZB heteroplasmic mice

Mice heteroplasmic for NZB and BALB mtDNA on a BALB background were generated as previously described (Jenuth et al 1996). Briefly, zona-pellucida-free one-cell embryos were incubated for 30 minutes in 5μg/ml cytochalasin B and 0.1μg/ml actinomyocin D. Cytoplasts were obtained using a 15-20μm glass pipette to aspirate and pinch off an area of cytoplasm and its surrounding plasma membrane. These donor cytoplasts were injected under the zona pellucida of recipient one-cell embryos, which had been treated with 5μg/ml cytochalasin B and 0.1μg/ml colcemid for 30 minutes. To form cytoplasmic hybrid or cybrid embryos, the donor cytoplasts were electrofused to recipient zygotes. After overnight culture, the two-cell embryos were transplanted to the fallopian tubes of pseudopregnant B6 females, resulting in 5 females with levels of donor mtDNA of between 3.1% - 7.1% who were the original founders of the mice generated for this study.

mtDNA genotype analysis of female animals

The NZB and BALB mtDNA genotypes differ in 101 nucleotides, so mtDNA genotyping was performed using a restriction length fragment polymorphism (RLFP) method (Section 1.4.4.2). A *RsaI* site at position 3691 in the ND1 gene present in BALB, but absent in NZB mtDNA was used to genotype individual animals. The mtDNA genotype was determined on a PCR-amplified fragment encompassing the polymorphic site. Tail biopsies were taken from anaesthetised female mice and genomic DNA was extracted according to standard procedure (Sambrook and Russell 2001).

Total genomic DNA was amplified using the following primers: forward MT9 nt 3571–3591 5'-GAG CAT CTT ATC CAC GCT TCC-3'; reverse MT10 nt 4079–4059 5'-CTG CTT CAG TTG ATC GTG GGT-3'. The PCR and cycling conditions were as previously described (Jenuth et al 1996). Briefly, PCR reactions were carried out in a total volume of 50µl with potassium- free PCR buffer (100mM Tris-HCl [pH 8.3]), 2.5mM $MgCl_2$, 200µM of each dNTP, 0.4µM of each of MT9 and MT10 primers and 1.25 units of Taq polymerase (Gibco, Burlington, ON, Canada). The PCR was carried out on a Perkin Elmer 9600 thermocycler (Perkin Elmer, Boston, MA, USA) using the following programme: denaturation at 94°C for 5 minutes (min), followed by 30 cycles at 94°C for 30 seconds (sec), 55°C for 30 sec and 72°C for 30 sec. In the last cycle, 1.5µCi of [α-32P]dCTP was added to the reaction in order to radiolabel the PCR product. A 15µl aliquot of this reaction was digested with 10U of *RsaI* overnight at 37°C and run on a 10% non-denaturing polyacrylamide gel.

Isolation of oocytes and embryos

Ovulated metaphase II oocytes were obtained from mice between 6 weeks and 4 months of age. This work was approved by the McGill University Animal Care Committee. Females with known levels of heteroplasmy were superovulated by intraperitoneal injections of 7.5U of pregnant mares' serum gonadotrophin (PMSG, Sigma, Oakville, ON, Canada) followed by 5U of human chorionic gonadotrophin (hCG, Sigma) 44-48 hours later. Sixteen to eighteen hours later, the oviducts were removed and the oocytes released by tearing the swollen ampulla region of the oviduct into HEPES-

buffered KSOM (H-KSOM) medium. The cumulus cells surrounding the oocytes were removed by brief incubation in 300μg/ml hyaluronidase (Sigma).

To obtain early cleavage-stage embryos, superovulated mice were mated with stud males overnight and checked for the presence of a plug the following morning. Two-, four- and eight-cell embryos were obtained by flushing the oviducts of pregnant females on day -1.5, day -2 and day -2.5, respectively. When more than 4 two-cell embryos were available, some were disaggregated at that stage and the remainder were cultured *in-vitro* for 1 or 2 days to allow further cell division before they were disaggregated. All embryos were cultured in pre-equilibrated fresh KSOM medium under paraffin oil at 37°C in 5% CO_2 in air.

Cell collection

The zona pellucida was removed from oocytes and embryos by a short incubation in acid Tyrode solution (pH 2.5, Sigma) and washed in H-KSOM. The first polar body from each oocyte was carefully aspirated into a fine pulled-glass pipette and transferred to a PCR tube containing 5μl alkaline lysis buffer (LB) (Cui et al 1989). The ooplasm was then pipetted into a separate LB tube using a clean pulled-glass pipette. For the embryos, each zona-free embryo was incubated for 5 minutes in Ca^{2+}- and Mg^{2+}-free media to allow decompaction of blastomeres, and then the cells were disaggregated by gentle aspiration and expulsion from a pulled-glass pipette. Each blastomere was then pipetted into a separate labelled LB tube. Each cell membrane was lysed by heating to 65°C for 15 minutes, after which 5μl neutralisation buffer (Section 2.4) was added (Cui et al 1989).

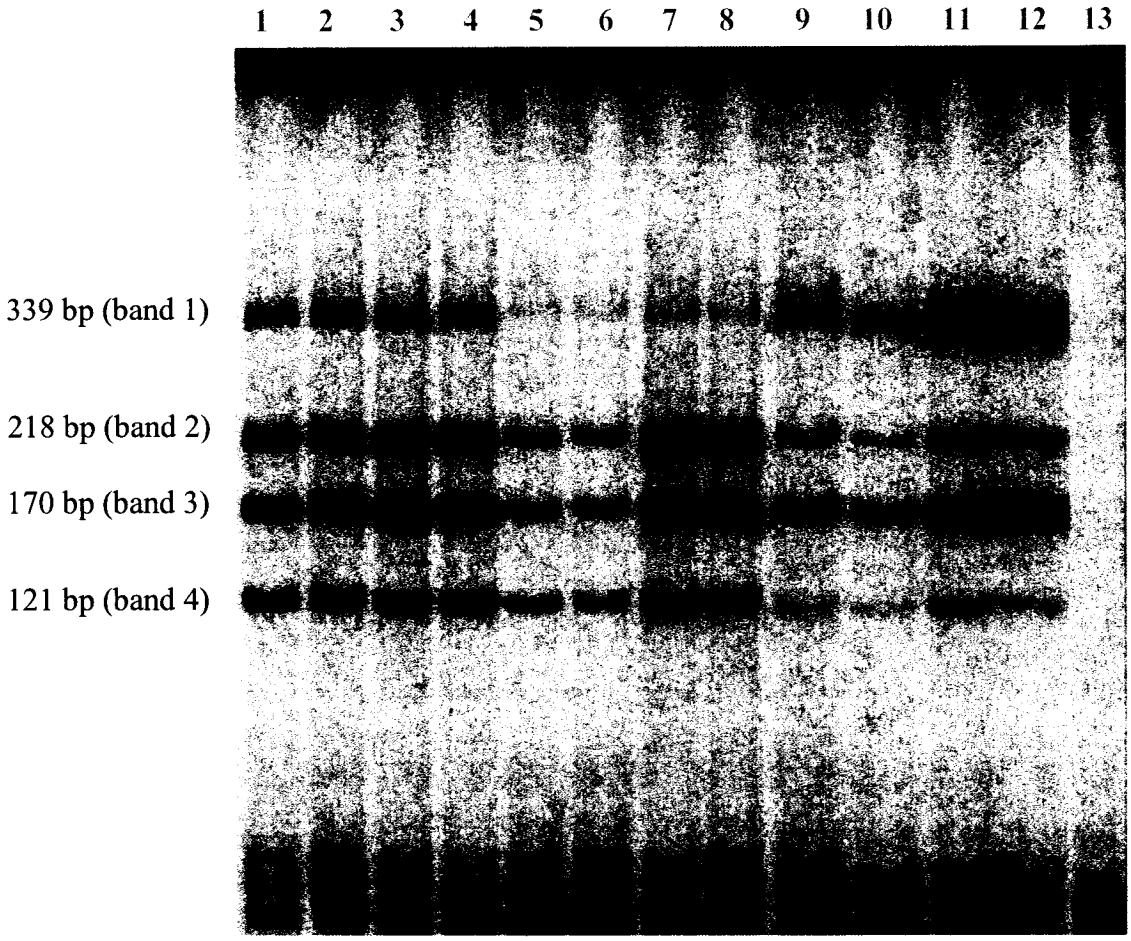
mtDNA genotype PCR of oocytes, polar bodies and blastomeres

The amount of lysed-cell sample necessary for each reaction mix was calculated from pilot data and depended on the size of the initial cell. For oocytes, 1μl was used; for polar bodies, 3μl; and for cleaved blastomeres, 2μl. In each experiment, a negative control consisting of 5μl of ddH_20 was included. Duplicate samples of each lysed cell underwent a PCR assay to amplify the fragment encompassing the polymorphic site, as described for genotyping the female animals except that 35 rather than 30 cycles were used. Overall 131 PCR reactions were necessary to analyse the 58 oocyte and polar body samples, and 448 PCR reactions were necessary to analyse the 211 blastomeres.

Analysis of PCR products

Gels were analysed on a Molecular Dynamics Storm PhosphorImager (GE Healthcare Bio-Sciences Inc., Baie d'Urfé, QC, Canada). The percentage of NZB mtDNA was calculated for each reaction mix by summing the amount of radioactivity in bands 2 and 4 and dividing this by the value in band 1 (Figure 5.1). The product in band 3 is common to both types of mtDNA. The mean %NZB mtDNA was deduced for each cell from the relative amount of NZB mtDNA in each duplicate, and the coefficient of variation (CV) was calculated (Figure 5.1 and Table 5.1). The resulting mean %NZB for each cell was considered to be a reliable value when the CV of duplicate samples was less than 10. When the CV of the duplicates was greater than 10, a triplicate sample of the cell underwent PCR, and if this reduced the CV to below 10, then the results from that cell were considered valid. However, if the CV remained high, the experimental error was considered too great, so the results from the whole oocyte or embryo were considered as unreliable and were excluded from the study.

Figure 5.1: Gel results from 3 oocytes and polar body pairs



Representative gel from the PCR for 3 oocytes and their corresponding polar bodies

Image of gel from radioactive PCR after enzymatic digestion to detect presence of NZB and BALB mtDNA. Band 1 is exclusive to NZB mtDNA. Bands 2 and 4 are the digested products of BALB mtDNA. Band 3 is common to both types of mtDNA.
The results from lanes 1-12 are for duplicate polar bodies and the corresponding duplicate oocyte samples for 3 mature oocytes
Lanes 1 and 2: polar body 1 with 21.5% NZB mtDNA (CV 2.3)
Lanes 3 and 4: oocyte 1 with 22.1% NZB mtDNA (CV 0.4)
Lanes 5 and 6: polar body 2 with 9.2% NZB mtDNA (CV 2.4)
Lanes 7 and 8: oocyte 2 with 4.9% NZB mtDNA (CV 3.6)
Lanes 9 and 10: polar body 3 with 64.1% NZB mtDNA (CV 6.1)
Lanes 11 and 12: oocyte 3 with 64.2% NZB mtDNA (CV1.1)
Lane 13: negative control

(bp: base pairs)

Table 5.1: Example of experimental data to demonstrate the calculation of mean level of heteroplasmy and coefficient of variation

%NZB mother	Cell	Band 1- 339 bp	Band 2- 218 bp	Band 4- 121 bp	%NZB	mean	CV
	Oocyte	8816	14022	6647	29.9	29.1	3.9
67.5		10429	17280	9150	28.3		
	Polar body	4720	7835	4436	27. 8	28.5	3.5
		4100	6348	3604	29.2		
	Oocyte	12199	2506	942	77.9	78.3	0.7
39.7		14245	2658	1195	78.7		
	Polar body	5525	1440	466	74.4	76.3	3. 7
		4608	888	388	78.3		

The raw data of results from 2 oocytes and their corresponding polar bodies from 2 mice with different levels of heteroplasmy.

The figures in the band 1,2 and 4 columns are the intensities of radioactivity measured by the phosphorImager. The %NZB mtDNA was calculated by adding the values in bands 2 and 4 and dividing this by the value in band 1. The mean %NZB mtDNA was deduced from the relative amount of NZB mtDNA in each duplicate and the coefficient of variation (CV) was also calculated.

(bp: base pairs)

5.5 RESULTS

Levels of heteroplasmy in the cytoplasm and polar body of an oocyte

In order to compare the distribution of heteroplasmy between the ooplasm of an oocyte and its corresponding polar body, 29 mature oocytes were successfully collected from 6 mice with maternal levels of heteroplasmy ranging from 17.0% - 67.7%. In addition 8 oocytes were not suitable for the study, of which 5 had a germinal vesicle present while the other 3 were either at the metaphase I stage or the polar bodies had completely disappeared. Overall, 58 oocyte and polar body samples were obtained for analysis. The results were analysed as illustrated in Table 5.1, which shows the raw data from 2 representative oocytes and their corresponding polar bodies obtained from female mice with different levels of heteroplasmy.

This yielded closely duplicated results in 22 oocytes with their corresponding polar bodies while 7 oocytes were excluded because one or both of the cells tested did not yield results, or the results produced did not satisfy the criteria stated in Section 5.4. The average number of oocytes, which gave full results, from each mouse, was 3.7 (range 1 - 7). The cumulative data from all oocyte and polar body pairs is presented in Table 5.2 and Figure 5.2. The coefficient of determination (r^2) for the levels of heteroplasmy in the ooplasm of the oocytes compared to those in the polar bodies was 0.99 (Figure 5.2A), while the value for the level of heteroplasmy in the gamete (the mean value in the ooplasm and polar body) compared to the maternal genotype was 0.32 (Figure 5.2B). Within each gamete, the difference in the levels of heteroplasmy in the ooplasm compared to its respective polar body ranged from 0.1 - 6.1% (median difference 2.5%) (Table 5.2).

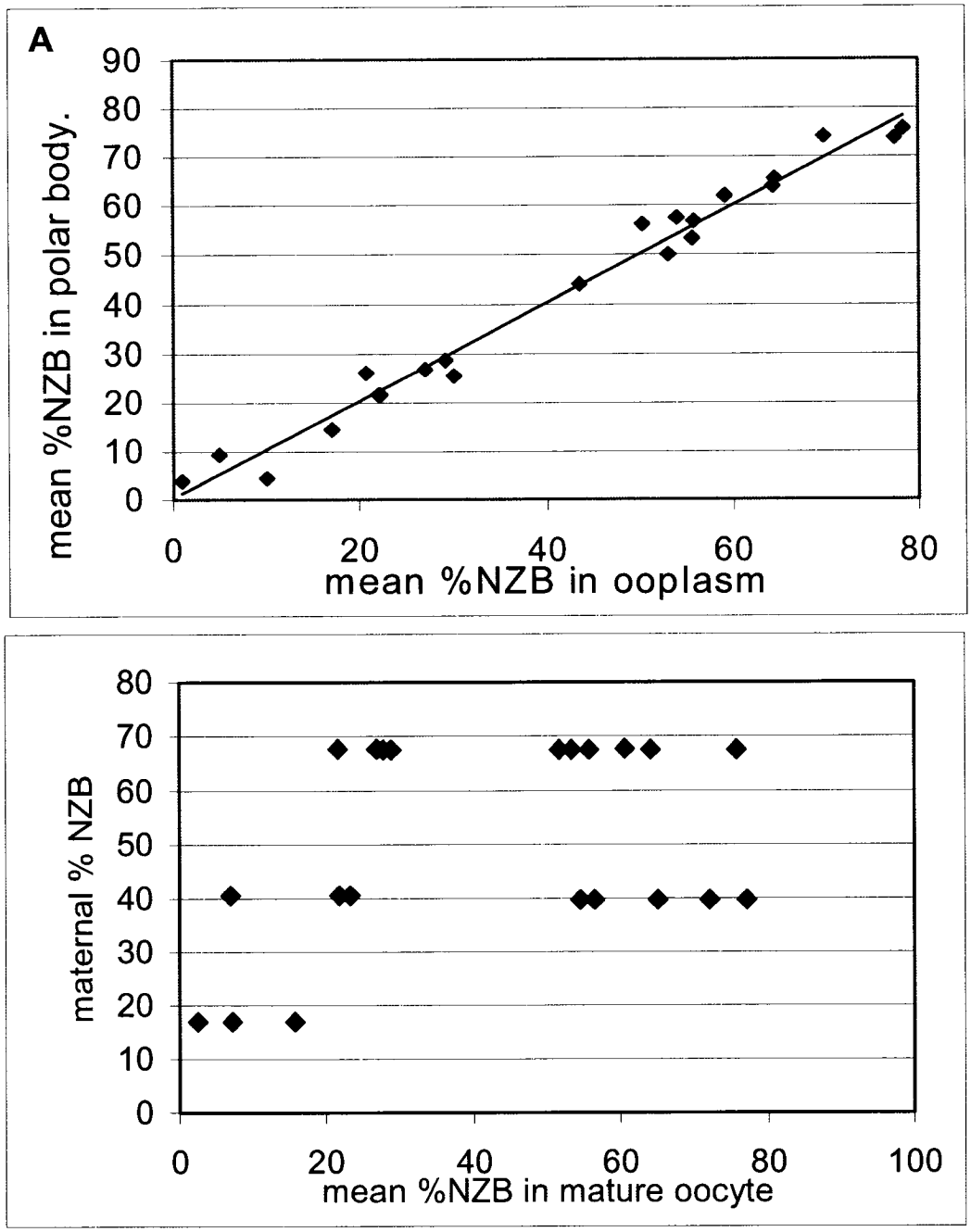
Table 5.2: Results for the levels of heteroplasmy in oocyte and polar body pairs

Maternal % NZB	Oocyte mean % NZB	CV	Polar body mean % NZB	CV
17.0	**1.0**	6.5	**4.0**	4.7
	17.0	0.6	**14.5**	5.5
17.0	**10.0**	1.5	**4.5**	4.9
	55.7	4.4	**53.4**	1.1
	55.9	1.1	**57.0**	3.8
39.7	**59.1**	1.8	**62.1**	3.4
	64.5	0.3	**65.7**	9.4
	69.8	1.3	**74.4**	5.9
	78.3	0.7	**76.3**	3.7
	4.9	3.6	**9.2**	2.4
40.6	**20.6**	0.7	**25.9**	3.9
	22.1	0.4	**21.5**	2.3
	29.1	3.9	**28.5**	3.5
67.5	**30.1**	5.6	**25.4**	8.1
	50.3	4.6	**56.4**	0.3
	53.1	0.4	**50.2**	8.2
	54.0	5.3	**57.5**	2.2
	64.2	1.1	**64.1**	6.1
	77.4	1.2	**74.0**	5.9
	21.9	7.4	**21.4**	6.3
67.7	**27.0**	3.0	**26.7**	1.0
	43.6	8.7	**43.9**	0.2

Levels of heteroplasmy in 22 oocytes and their polar bodies obtained from 6 mice

The figures given are the percentage of NZB present in each of the samples tested. The maternal genotype was deduced from genomic DNA extracted from tail biopsies. Levels of heteroplasmy for oocyte and polar bodies were calculated from the mean of duplicate samples or, if necessary triplicate samples that were amplified for each cell. The coefficient of variation (CV) was calculated for each cell from these duplicate samples.

Figure 5.2: Comparative levels of heteroplasmy between the ooplasm and polar body of an oocyte, and each oocyte and its maternal genotype



Graphical representation of the comparative levels of heteroplasmy between the ooplasm of an oocyte and its corresponding polar body and between each oocyte and the maternal genotype

A) Mean % NZB in each of the 22 oocytes compared to the value in its respective polar body
B) Mean % NZB in each gamete compared to the maternal value

Levels of heteroplasmy in the blastomeres of an embryo

When the levels of heteroplasmy in the blastomeres of cleaved embryos at different developmental stages were investigated, individual cells from 55 embryos were successfully collected from 15 female mice. Levels of heteroplasmy in the mice ranged from 7% - 74% and the levels in the embryos ranged from 3% - 73%.

Closely duplicated results for all cells tested were obtained from 53 embryos at different cleavage stages (Table 5.3). There were 19 two-cell embryos, 18 four-cell embryos and 16 six- to eight-cell embryos. The average number of embryos from each mouse giving a full result was 3.5 with a range of 1 - 13. A cleaved embryo was considered for inclusion in the study only when more than 50% of the blastomeres could be individually collected. The results from two embryos were excluded because one or more cells tested did not give closely duplicated results.

If a small cohort of 2-cell embryos was obtained from one mouse, the embryos were disaggregated and cells collected on the day of retrieval. If a larger cohort of 2-cell embryos was obtained, cells from a proportion of the embryos were collected on the first day and the remainder left in *in-vitro* culture until the following day to allow further division. This acted as an experimental control to ensure that the distribution of heteroplasmy mtDNA in any cohort was independent of embryonic-cleavage stage.

The data presented in Table 5.3 show the range of levels of heteroplasmy detected within all the cells of a particular embryo. This ranged from 0% difference between the cells within each embryo to the largest difference of 6%.

Table 5.3: Results for the levels of heteroplasmy in embryos of different cleavage stages

Maternal % NZB	Developmental stage (number of embryos)	No of blastomeres tested	Range of % NZB in each embryo 1	2	3	4	5
33.2	2 cell (1)	2	23				
40.0	2 cell (3)	6	44-45	55-58	45-50		
	4 cell (1)	4	9-11				
47.0	2 cell (4)	8	21-22	18	72-73	46-47	
	4 cell (1)	4	44-47				
58.0	2 cell (2)	4	43-47	28			
46.0	2 cell (2)	4	17-18	33-37			
64.0	2 cell (3)	6	53	61-62	38-40		
	4 cell (2)	8	59-61	69-71			
	2 cell (4)	8	46	46-47	31-32	34-36	
37.0	4 cell (1)	3	31-32				
	6 cell (1)	6	43-46				
	8 cell (1)	5	42-48				
74.0	3 cell (1)	3	56-57				
08.0	4 cell (1)	4	08-10				
17.0	4 cell (1)	4	14-16				
07.0	4 cell (2)	8	03	02-04			
	8 cell (2)	11	07-09	04-05			
20.0	8 cell (1)	8	16-18				
	4 cell (1)	4	04-05				
33.0	6 cell (1)	6	29-31				
	8 cell (1)	8	14-19				
28.0	4 cell (2)	8	41-42	35-37			
	6 cell (1)	5	20-22				
	3 cell (2)	6	29-33	10-12			
26.0	4 cell (3)	11	26-27	25-28	29-33		
	6 cell (3)	15	19-23	31-35	18-23		
	8 cell (5)	34	31-36	10-12	11-14	30-35	26-28

Levels of heteroplasmy in 53 embryos at the 2-cell, 4-cell and 6-8-cell stages obtained from 15 mice. The figures given are the percentage of NZB present in each of the samples tested. The maternal genotype was deduced from genomic DNA extracted from tail biopsies. The number of embryos tested at each developmental stage and the total number of blastomeres for each mouse are indicated. For each embryo, the lowest and highest levels of %NZB in all the blastomeres are shown.

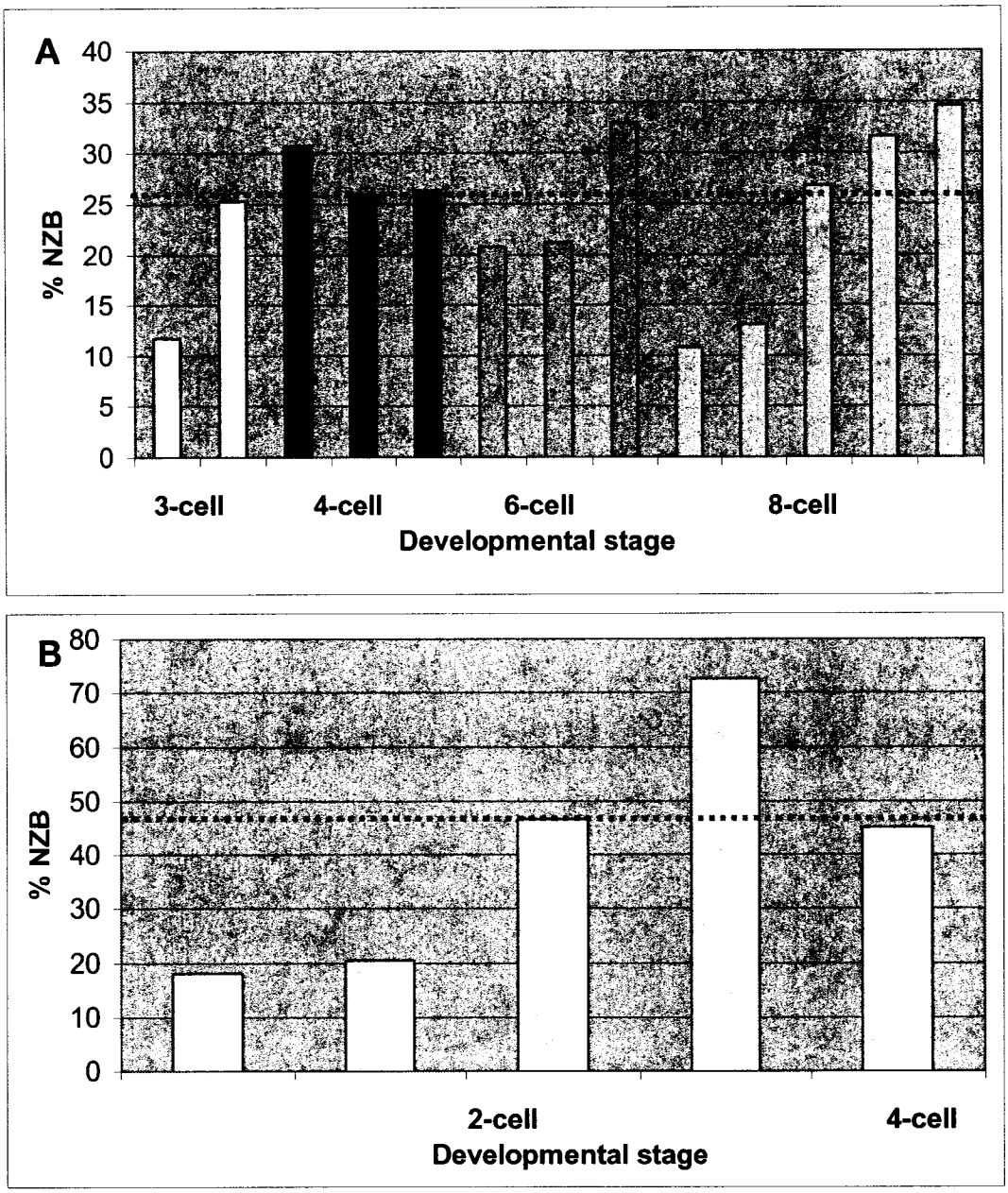
The median difference in all 53 embryos was 2%. Within the cohort of embryos obtained from each mother, a wide variation in the levels of heteroplasmy could be observed between embryos (Figure 5.3A and B). However, the mean level of heteroplasmy seen in any one cohort of embryos was similar to that seen in their mother (median difference = 3.6, $r^2 = 0.94$). This relationship was even stronger (median difference = 2.75, $r^2 = 0.96$) when only those mice (of which there were 8) with 3 or more embryos were included.

Reliability of genotyping

Twenty-four percent (7/29) of the mature oocytes failed to give a result in either one or both of the cells tested. This total comprised 5 polar bodies, 1 ooplasm and one total oocyte (both ooplasm and polar body). The failure rate for the polar bodies alone was 20.7% (6/29). Two of the cleaved embryos failed to give a result. One was a 2-cell embryo in which one cell failed to give a result; the other, a 6-cell embryo in which two cells did not provide any result. Therefore, the failure rate in the case of blastomeres was 1.4% (3/211).

For the oocytes included in the study, it was also necessary to run triplicate samples to verify the results for 13/22 (59.1%) polar bodies and for 2/22 ooplasm samples. Of the 203 blastomere samples included in the study, 5 triplicate samples had to be run for two-cell embryos, 2 for four-cell and 19 for six- to eight-cell embryos, giving a total of 26/203 (12.8%).

Figure 5.3: Variation in levels of heteroplasmy in cohort of embryos from two mothers



Range of heteroplasmy seen in embryos of different developmental stages from 2 mothers. The maternal % NZB is indicated by the blue dotted line.

A) Maternal % NZB was 26% and the level of NZB mtDNA in her 13 embryos ranged from 10-36%
B) Maternal %NZB was 47% and the level of NZB mtDNA in her 5 embryos ranged from 18-73%

5.6 DISCUSSION

In view of the rarity of suitable human clinical material and the limited number of mouse models segregating pathogenic mtDNA mutations (Inoue et al 2000, Sligh et al 2000), a mouse heteroplasmic for two neutral mtDNA polymorphisms (Jenuth et al 1996) was used to in this study to determine the segregation pattern of two genotypes of mtDNA. These mtDNA polymorphisms have been demonstrated to be transmitted to offspring with levels of heteroplasmy largely determined by random genetic drift, similar to what has been observed in many human mtDNA diseases (Chinnery et al 2000, Jenuth et al 1996). Therefore, we used these heteroplasmic mice to determine the segregation pattern of two genotypes of mtDNA in early embryos in order to evaluate the feasibility of PGD for human mtDNA disease. We concluded from the analysis performed using this mouse model that genetic diagnosis from either a polar body or blastomere would give an accurate indication of the level of the two different mtDNA genotypes present in the entire ooplasm or embryo, respectively, from a woman heteroplasmic for a mtDNA mutation.

Preimplantation genetic diagnosis at different stages of cell development

Polar body genetic diagnosis

In our mice, the distribution of the two types of mtDNA was found to segregate evenly between the ooplasm and the first polar body of each mature unfertilised oocyte. The high value of the coefficient of determination verified that the proportion of mtDNA genotypes detected in the polar body and ooplasm of a mature oocyte were almost identical (Figure 5.2A). This demonstrated that the small

number of mitochondria present in the polar body were representative of the entire oocyte. However, the mean level of heteroplasmy in the oocyte (from the ooplasm and polar body combined) was not similar to that of the maternal genotype, most likely as a result of the genetic bottleneck for mtDNA transmission (Jenuth et al 1996) (Figure 5.2B).

Also of note was that mice with different levels of maternal heteroplasmy had different distribution patterns in their oocytes. The maternal genotypes from the cohort of mice studied coincidentally fell into three groups of heteroplasmy (68%, 40% - 41% and 17% NZB mtDNA) (Table 5.2). The 10 oocytes from the two mice with 68% NZB mtDNA showed a range of heteroplasmy distributed on either side of the maternal genotype that was similar to the distribution seen in many human mtDNA mutations (Chinnery et al 2000). The two mice with 40% and 41% heteroplasmy gave six and three oocytes, respectively. Although, both mothers had similar proportions of mtDNA, all the oocytes from one mouse had higher levels of heteroplasmy than the maternal genotype (from 56% - 78%) whereas the all oocytes from the other mother had lower levels (from 5% - 22%). The two mice with the lowest levels of heteroplasmy (17%) had low levels of heteroplasmy in all oocytes tested. These findings are likely due to the small cohort of gametes collected from each mouse rather than any selection of one mtDNA genotype. Data from published human pedigrees supports the notion that transmission of pathogenic mutations is random, as in the mouse model, although a skewed mtDNA segregation has been suggested in one woman whose oocytes all had either extremely high or almost zero levels of mutant mtDNA (Blok et al 1997).

Blastomere genetic diagnosis

Using our mouse model, it was found that the distribution pattern of heteroplasmy within the blastomeres of each cleaved embryo was identical through each cleavage stage examined, which is consistent with our preliminary results (Molnar and Shoubridge 1999). Although the cytoplasmic volumes of blastomeres from embryos at the two-, four-, six- and eight-cell-stage are different, the ability to detect the proportion of the two mtDNA genotypes was not affected. There is reported to be an asymmetrical mitochondrial distribution at the pronuclear stage of human embryos that could lead to varying mitochondrial complements in different blastomeres (Van Blerkom et al 2000). However, unless it is extreme, variance in the total number of mitochondria present in a blastomere should not affect the overall proportion of different genotypes of mtDNA observed in each cell.

Similar to the results observed in oocytes, there was a wide variation in the proportions of mtDNA in cleaved embryos compared to the levels in the mothers. For example a maternal genotype of 47% NZB mtDNA produced embryos with 18% - 73% heteroplasmy (Table 5.3 and Figure 5.3B). Since the publication of our study, our findings have been confirmed in the human model (Steffann et al 2006). The transmission of a mtDNA polymorphism in a set of donated human embryos was generally found to give results similar to those produced by our heteroplasmic mouse model in that the levels of heteroplasmy of the polymorphic mtDNA were similar among the blastomeres of an embryo but varied widely in the cohort of embryos from any one mother (Steffann et al 2006).

Comparison of genetic diagnosis through polar bodies and blastomeres

Accuracy of diagnosis

We have explored the potential of polar body and blastomere analysis in performing PGD for inherited mtDNA diseases in two groups of patients: a) those who are opposed to any manipulation after fertilisation and b) those who are amenable to embryo biopsy, to assess which method permitted greater reliability of diagnosis. Both approaches gave equal accuracy in detecting the percentage of mtDNA genotypes present in the polar body of an oocyte compared to the ooplasm and in one blastomere compared to the remaining cells of the embryo. Although the levels of heteroplasmy among oocytes ranged from 1% - 78%, as expected, the range of difference between each polar body and its respective ooplasm was from 0.1% - 6%. Accordingly, the difference in the detected levels of heteroplasmy among blastomeres of an early cleavage-stage embryo gave a median of 2% and a range of 0% - 6%. This accuracy of diagnosis from the first polar body or blastomere would allow the reliable selection of unaffected embryos and the avoidance of a misdiagnosis. However, cleavage-stage biopsy may allow more accurate determination of the exact proportion of mutant mtDNA load in the resulting fetus if, as suggested from IVF failed to fertilise oocytes and poor quality embryos, human embryos have a significantly reduced level of mtDNA rearrangements than oocytes (Barritt et al 1999).

Efficiency of diagnosis

Although both methods of biopsy permitted accurate results, the genetic analysis of blastomeres showed a higher efficiency of diagnosis. The rate of failure to obtain a result was almost 21% in polar bodies compared to less than 2% in

blastomeres. The results from the polar bodies were also less consistent because it was necessary to run more triplicate samples in order to verify the levels of heteroplasmy in a polar body compared to those in a blastomere (59% to 13%, respectively).

The poorer amplification of polar bodies compared to blastomeres could be due to the difference in cytoplasmic volumes, and therefore the number of mitochondria, in the two cell types. A blastomere from an eight-cell embryo contains approximately 12.5% and a polar body 0.5% of the total ooplasmic volume (Briggs et al 2000). Therefore, a blastomere would be expected to have a higher copy number of mtDNA (approximately 12,500 copies), which could result in more efficient PCR amplification. It is also possible that the mtDNA in the polar body has degenerated compared to the mtDNA in the blastomere of a developing embryo. It has been shown that the first polar body in both mouse and human oocytes undergoes a rapid degeneration process during *in-vitro* incubation (Choi et al 1996, Munne et al 1995).

In humans, a wide variation in the mtDNA copy number can exist between mature oocytes, even among those from the same patient (Barritt et al 2002, Reynier et al 2001, Steuerwald et al 2000b). However, our PGD protocol is based on the proportions of each mtDNA genotype, and detection of a particular genotype is accurate with as few as 10 copies present. Therefore, a low copy number would not affect the sensitivity of our test or interpretation of the result. Efficient diagnosis will result in the availability of more unaffected embryos, allowing a better selection of morphologically good-quality embryos for transfer, thereby increasing the success rate in any given treatment cycle.

Interpretation of results in relation to clinical PGD

Suitability of diagnosed embryos for replacement to the woman

The interpretation of predictive data after PGD for mtDNA diseases could employ methods similar to those used in prenatal diagnosis. For most mtDNA mutations, a mutant load around 30% or above 90% can be interpreted as having low or high probability, respectively, of an affected outcome. Intermediate mutant loads would represent a "grey zone" in which interpretation would be difficult. In genetic prenatal testing, an intermediate mutant load would indicate that the fetus could be phenotypically affected, and this would lead to the dilemma of whether or not to terminate the pregnancy. Based on the available data, it is predicted that embryos diagnosed by PGD as carrying zero or low mutant loads would be regarded as suitable for embryo transfer while embryos diagnosed with intermediate loads would not. This may reduce the number of embryos available for transfer but would not be as serious as the possible termination of a pregnancy at risk of manifestation of an mtDNA disease but that could actually be unaffected. However, it is possible that the presence of a low proportion of mutant mtDNA in the embryo may not indicate the exact mutant load in the resulting individual because significant tissue-specific segregation may occur in some mtDNA mutations (Poulton and Morten 1993, Weber et al 1997). A recent study examining the tissue-specific distribution of the A3243G mutation has determined that the mutational load in post-mitotic skeletal muscle but not in highly mitotic cells, such as leukocytes, may be predicted by determining the mutation load in the embryo (Frederiksen et al 2006). The use of PGD to determine the prospective mutant load of the muscle would provide a more accurate indication

of the risk to the fetus than testing the maternal leukocyte mutant load, which for many mtDNA mutations can decrease over time (Rahman et al 2001).

Suitability of PGD for mtDNA disease

PCR protocols for mtDNA PGD

Technically PGD should be easier for mtDNA mutations than for nuclear gene mutations due to the high copy number of mtDNA in a polar body and particularly an embryonic blastomere. Therefore mtDNA should be less prone to many of the problems encountered in mutation analysis of nuclear gene defects in single cells, such as amplification failure and allele-drop out (ADO).

Patient selection

Patients requesting PGD are generally fertile, nevertheless like infertility patients, they are required to undergo an IVF cycle involving hormonal stimulation, operative procedures and financial costs. Therefore, PGD for mtDNA diseases may be more suitable for women carrying higher proportions of mutant mtDNA, who have a strong likelihood of having a fetus with an intermediate or high mutant mtDNA load. It is thus likely that these women will have a large proportion of oocytes with a substantial mutant load, and they may require multiple cycles of ovarian stimulation to in order to produce a healthy child. Even in such cases, the results from one cycle of PGD would provide valuable information for guiding subsequent reproductive choices. Collecting oocytes from a superovulated cycle and analysing them as a sample of the oocyte population as a whole has been used diagnostically for women with mtDNA deletions (Poulton and Marchington 2002). Although it is not certain that the levels found in these oocytes represent the whole ovarian population, the

amount of mutant mtDNA in superovulated oocytes would give a good indication of the likely content in any child.

Selection of mtDNA diseases for testing

Since the publication of this paper, two cycles of PGD performed for mtDNA disease have been reported. In one paper, PGD was performed for a woman carrying the T8993G mutation seen in neurogenic ataxia retinitis pigmentosa (NARP) (Steffann et al 2006). Of three embryos tested, one was found to contain 100% mutant load whilst the other two carried 0% and were therefore transferred to the woman's uterus leading to the birth of a child in whose cord-blood DNA analysis no mutant mtDNA was detected. The detection of the T8993G mutation may be particularly amenable to PGD, as extreme skewed segregation can occur in the transmission of this mutation (Blok et al 1997, Ferlin et al 1997, Harding et al 1992, Leshinsky-Silver et al 2003, White et al 1999) and, therefore, the results achieved in this PGD may not be applicable to other mtDNA mutations.

The second report on PGD was for sex selection performed in the case of a LHON mutation (Harper et al 2006). No details are presented but, presumably, sexing was performed to avoid the transfer of male embryos, as males have an increased risk of being affected by this disease (Hudson et al 2005). Therefore, once again, the PGD described here is not relevant to PGD for other mtDNA mutations.

Suitability of heteroplasmic mtDNA mice to assess human mtDNA disease

Before offering PGD for mtDNA diseases, we must be confident that the transmission of these neutral polymorphisms in our mouse model is reflective of the transmission seen in human mtDNA disease. Attempts to study the inheritance of mtDNA heteroplasmy in humans have been hampered by a number of problems

including the fact that very few human pedigrees are large enough to permit reliable calculation of the variance between offspring. Two groups have studied either the offspring or primary oocytes of women carrying different mtDNA mutations and have reported that no strong positive or negative selection for pathogenic mtDNAs occurs in the oocyte or in early embryogenesis (Brown et al 2001, Chinnery et al 2000). They concluded that the predominant mechanism behind the transmission of pathogenic heteroplasmic mtDNA point mutations is random genetic drift, as in our mouse model. However, it is possible that some point mutations and mtDNA rearrangements could exhibit different segregation behaviour, with a preferential transmission of mutant genomes (Chinnery et al 2000).

Concluding remarks

In summary, we have shown that heteroplasmic mtDNA is distributed equally between a polar body and ooplasm of a mature oocyte, and also among the blastomeres of an early cleavage-stage embryo in our mouse model. Transmission of heteroplasmic mtDNA in this murine system is similar to that seen in most reported human pedigrees segregating mtDNA mutations. Therefore, we conclude that PGD for inherited human mtDNA disorders is feasible but should be approached with caution, as some pathogenic mutations could behave in a different manner. In the diagnosis of mtDNA diseases, the biopsy and genetic analysis of one or two blastomeres from a cleaved embryo will give results superior to those achieved using the first polar body from an unfertilised oocyte.

CHAPTER SIX

Summary and conclusions

6.1 RECAPITULATION

The ability to sequence human genes has led to the identification of thousands of pathogenic genetic mutations and elucidation of the mode of inheritance for many human diseases. While much of the knowledge on the inheritance of human disease has resulted from the study of mutation transmission from parent to offspring, in order to determine the pattern of inheritance and the mechanism involved in the transmission of many pathogenic genetic mutations, it is of paramount importance to study early embryogenesis. Most of the current information on this subject has been generated through the study of appropriate animal models, due to the unavailability of human material. However, a suitable animal model may not exist for some diseases, or the transmission of the human gene cannot be replicated exactly in an animal model or, alternatively, the genetics behind the mutation have not yet been fully elucidated, even in the animal model. Therefore, there are still many unanswered questions related to the transmission of many genetic mutations in the human.

The use of the *in-vitro* fertilisation technique for assisted conception treatment has provided access to an extremely valuable research material: donated human IVF oocytes and preimplantation embryos. The studies performed for this thesis utilised these gametes and zygotes to advance our understanding of the transmission of disease-causing unstable trinucleotide repeats during early embryonic development. In addition, the segregation of mitochondrial DNA (mtDNA) in the gametes and early cleavage-stage embryos of a heteroplasmic mouse was used as a model to investigate the distribution pattern of mtDNA genotypes in diseases caused by maternally-inherited mitochondrial point mutations.

6.2 DETECTION OF GENETIC MUTATIONS IN HUMAN EMBRYOS

Through the series of experiments described in Chapter 2, we were able to optimise PCR analysis for mutation genotyping at the single-cell level. Analysis of two genes in which two very different types of mutations can occur gave efficient amplification (average 92.4%, range 89.2 - 95.4%), accuracy of detection (average 99.1%, range 98.4-100.0%) and reduced allele drop-out (ADO) (average 5.9%, range 0.8 - 10.0%). The low starting template available for mutation analysis of a single gene defect at the single-cell level can lead to suboptimal PCR genotyping as a result of the amplification of contaminating DNA, increased rates of amplification failure, preferential amplification and the occurrence of ADO. Optimisation of PCR conditions to avoid these problems and errors can take a considerable amount time. This is particularly relevant for the PCR genotyping of single human blastomeres, which was first reported in 1989 (Handyside et al 1989). However, it was a few more years before clinical success was reported for the use of direct mutation analysis for preimplantation diagnosis of a single gene defect (Handyside et al 1992).

Using the strategy developed in Chapter 2, PCR optimisation was achieved through the incorporation of fluorescently-labelled primers and the simultaneous amplification of a linked polymorphic marker. The conclusion from this published study, in which three different detection systems were directly compared for the first time, was that fluorescent fragment analysis gave superior results (Blake et al 2001). However, as demonstrated, with good PCR standardisation, even standard gel electrophoresis with ethidium bromide staining could provide acceptable levels of detection. This is important

for laboratories with a small throughput of testing because the purchase of an automated DNA sequencer would not be cost effective.

This protocol was used as the basis for single-cell genotyping of other genetic mutations, and we were one of the first two groups to explore the inclusion of a linked marker in the genotyping of DNA containing a repeat sequence (Dean et al 2001, Piyamongkol et al 2001). Genotyping DNA containing repetitive sequences can be challenging due to the amplification of stutter peaks which are an artefact of the PCR (Walsh et al 1996) and can be further complicated because DNA sequences with a high CG content are generally refractory to PCR amplification (Fu et al 1991). However, these problems were overcome and this protocol was applied in the first clinical preimplantation genetic diagnosis (PGD) using a multiplex strategy to detect an unstable repeat sequence with a linked polymorphic repeat marker (Dean et al 2001). In the years after the publication of this paper, the majority of PGD laboratories began to routinely incorporate additional linked loci when performing mutation analysis at the single-cell level. This approach has also been recommended in recent PGD guidelines (Thornhill et al 2005, Verlinsky and Kuliev 2003).

6.3 TRANSMISSION OF DYSTROPHIA MYOTONICA TYPE 1 ALLELES

The discovery that the expansion of simple repeat sequences can be responsible for heritable disease (Verkerk et al 1991, La Spada et al 1991) has generated much interest in the study of the genes in which these pathogenic repeats are found. Although all these expanded repeats are transmitted in an essentially Mendelian fashion, their patterns of inheritance do not follow the normal Mendelian rules.

6.3.1 Transmission of normal-sized *DMPK* repeats

It was previously reported that transmission of expanded dystrophia myotonica-protein kinase (*DMPK*) repeats, which are the cause of dystrophia myotonica type 1 (DM1), was observed in DM1 offspring at a level greater than the 50% expected through Mendelian autosomal dominant inheritance (Gennarelli et al 1994, Magee and Hughes 1998, Zatz et al 1997). Transmission ratio distortion (TRD) has also been reported in the inheritance of the larger of two *DMPK* repeats in the normal-size range in families with no DM1 (Carey et al 1994, Chakraborty et al 1996, Shaw et al 1995). Transmission ratio distortion of *DMPK* $(CTG)_n$ repeats was not evident from analysis of sperm and was thus unlikely to occur during male gametogenesis (Leeflang et al 1996). However, the precise timing for TRD had not been further defined and, therefore, an investigation into the developmental timing of TRD of larger normal-sized *DMPK* alleles was performed.

From this study, a statistically significant TRD of larger-normal sized *DMPK* repeats in the early cleavage-stage human embryo was demonstrated for the first time, from both maternal and paternal transmissions (Dean et al 2006a). This suggested that TRD in *DMPK* $(CTG)_n$ repeats of this length most likely occurred around the time of oocyte fertilisation, thereby excluding any mechanism of TRD involving embryonic viability. This TRD was found to be due specifically to the presence of the larger repeat itself and not to an absolute size effect of the repeat length, although the possibility of selection in favour of or against particular combinations of repeat lengths could not be ruled out due to the restricted sample size. Additionally, the preferential transmission of the larger normal-sized allele remained significant when female embryos were considered

alone, which could be the result of sex differences in the mode of repeat transmission or alternatively, could again be due to the limited number of embryos tested.

The occurrence of TRD of larger normal-sized *DMPK* repeats in embryos is consistent with the theory that these alleles act as a future reservoir of expanded *DMPK* alleles in the population to ensure the continued propagation of the DM1 phenotype (Imbert et al 1993). Furthermore, the significant TRD observed in our female embryos adds further support to this hypothesis, as maternally inherited $(CTG)_{19\text{-}37}$ repeats show greater stability during transmission compared to paternally inherited repeats (Dow et al 1997, Meiner et al 1998, Martorell et al 2001), theoretically allowing a wider lateral distribution of the unstable repeat before expansion to a range causing a DM1 phenotype would occur.

Interestingly during this study, instability was detected at the level of the embryo in the paternal transmission of larger normal-sized *DMPK* $(CTG)_n$ repeats (Dean et al 2006b). This agrees with previous findings that paternally inherited pre- and proto-mutation repeats, as well as repeats in the smaller-expanded range, are more unstable (Abbruzzese et al 2002, Brunner et al 1993, Dow et al 1997, Martorell et al 2001, Meiner et al 1998) and that repeat loci in sperm are more liable to expansion than those in oocytes (Jeffreys et al 1988). This instability was found to occur preferentially during transmissions from particular males, which could again be a consequence of the sample size or could indicate a predisposition towards instability in the repeats of some individuals. Our finding of expansion in the $(CTG)_n$ repeat length in almost half of the embryos generated from one particular male adds further interest to the suggestion that a

particular haplotypic background or the presence of other genetic factors may predispose the repeats at the *DMPK* locus to expansion (Imbert et al 1993, Neville et al 1994).

6.3.2 Transmission of expanded *DMPK* repeats

One of the most intriguing uncertainties in DM1 involves the timing of the intergenerational enlargements in repeat length that are seen upon female transmission. The observed increases in *DMPK* repeat length demonstrate some of the largest expansions seen among the unstable repeat diseases. Although these expansions have been suggested to occur before day 10.5 of development (Phillips 1993), no studies had investigated at an earlier developmental time point until researchers started working on PGD for DM1. To investigate the timing of *DMPK* repeat expansion, it was necessary to test those embryos from DM1-affected women that had inherited the expanded allele, making suitable research material extremely limited.

We had access to a number of samples that fulfilled this criterion and were the first to orally report that the large intergenerational increases in *DMPK* repeat length were already present in an oocyte at the germinal vesicle stage of development (Dean et al 2004). This was confirmed a short time later by further publications (Dean et al 2006b, De Temmerman et al 2004). These results demonstrated that pronounced *DMPK* repeat expansion had occurred before the completion of meiosis I of oogenesis.

Variability could also be seen in the degree of repeat expansion in the cohort of embryos from each mother, which could be related to the maternal repeat length. This illustrated that the variation in DM1 phenotypes seen in offspring of a DM1-affected mother could be related back to this initial increase in repeat length during oogenesis.

In addition, we speculated, although only on the findings in one embryo, that the expanded repeat continued to increase in length during early mitotic embryo division (Dean et al 2006b), although this must be verified in additional samples before any firm conclusion can be made. However, the possibility of expansion of DM1-affected alleles in both oogenesis and embryogenesis did permit us to postulate a mechanism of repeat instability involving DNA demethylation of the human genome, but no direct evidence exists to substantiate this hypothesis.

6.4 SEGREGATION OF HETEROPLASMIC MITOCHONDRIAL DNA

The investigations carried out in the majority of this thesis involved the analysis of the transmission of genes encoded by DNA in the nucleus of the cell. In Chapter 5, the research continued to focus on the transmission of genetic mutations, but this study was directed towards the DNA encoded by the mitochondrial genome, and specifically towards mitochondrial mutations that are inherited through the maternal cytoplasm. This project also demonstrates the importance of using an appropriate animal model to further the understanding of the transmission of particular human mutations.

During the last decade, the patterns of inheritance, segregation and distribution of mtDNA heteroplasmy have been studied intensively in both human and mouse, leading to a better understanding of transmission in mtDNA diseases. However, women who are heteroplasmic for mtDNA mutations have had limited reproductive choices because diagnosing the risk for mtDNA disease in specific pregnancies has been unpredictable (Poulton and Marchington 2000, Thorburn and Dahl 2001). In order for prenatal and preimplantation diagnosis to be a viable option for these women, the segregation of

heteroplasmic mtDNA during oogenesis and embryogenesis must be understood. The distribution patterns of two mtDNA genotypes, to the different cells of oocytes and embryos, had not been previously determined.

A mouse model that was heteroplasmic for neutral polymorphic mtDNA sequence variants was used to investigate the segregation of mtDNA to the cells of oocytes and embryos. The murine segregation of this heteroplasmic mtDNA to offspring had previously been shown to be similar to that observed in the transmission of many human mtDNA point mutations (Chinnery et al 2000) and therefore was considered to be a suitable model for this investigation. In this mouse model, we clearly showed that virtually identical segregation patterns of the two mtDNA genotypes occurred between the ooplasm and polar body of a mature oocyte, and also among the blastomeres of individual embryos at different stages of cell division (Dean et al 2003). From these results, it was concluded that PGD using a single blastomere from a cleavage-stage embryo, as blastomeres provided a greater efficiency of amplification than polar bodies, would be feasible to diagnose mtDNA diseases caused by maternally inherited point mutations. It was, however, noted that because some pathogenic mtDNA mutations could segregate in a different fashion, PGD may not be appropriate for detection of some mtDNA mutations.

This work has since been replicated and the results confirmed in human embryos heteroplasmic for two types of mtDNA and a PGD cycle to detect an mtDNA mutation using a method similar to that used in our work has been performed (Steffann et al 2006).

6.5 LIMITATIONS OF THE STUDY

The research in this thesis focused on the transmission of specific genes and pathogenic mutations during early human development. The main limitation, which is also encountered by most other researchers working with donated human embryos, was the limited number of the samples available for testing. Therefore, some of the conclusions were made from the analysis of a relatively small number of oocytes or embryos. To further strengthen some of the conclusions, suitable embryos could be genotyped if they become available.

The use of supernumerary IVF embryos may also have other limitations. The hormone treatment used to stimulate the follicles may promote some to produce mature oocytes that would not have been naturally selected for ovulation. Although it is possible that the findings from these IVF-generated embryos may not exactly represent those seen in naturally ovulated embryos, it is known that these IVF oocytes do have the potential to result in a viable pregnancy and, therefore are a valuable study material.

Furthermore, the embryos analysed in the TRD study were generally of poorer morphological quality than the overall cohort of IVF embryos generated for each couple, as higher-quality embryos were retained for the patient's treatment. However, as discussed in Chapter 3, we were confident in assuming that the findings determined from the embryos tested would be similar to those seen in natural cycles and also to the IVF embryos from each cycle that were not available for research.

6.6 FUTURE DIRECTIONS AND PERSPECTIVES

In addition to providing novel information on different aspects of the transmission of human pathogenic mutations, the data presented in this thesis introduced a number of new questions that warrant further investigation and that could be pursued in future studies.

6.6.1 Transmission of *DMPK* and other trinucleotide repeats

In many trinucleotide repeat disorders, TRD has also been reported for the repeat sizes in which instability has been observed. This could be a coincidental finding, as unstable repeats and trinucleotide repeat disorders have been the subject of many investigations or could be due to a common mechanism underlying both repeat instability and TRD. It would be interesting to determine whether the same or distinct mechanisms are responsible for the instability and TRD seen in both larger normal-sized and expanded *DMPK* repeats and perhaps to widen this search to include other disease-causing trinucleotide repeats. A potential area of study would be to examine whether the population distribution of different repeat diseases varied between those that showed TRD and instability in the normal range and those in which no TRD was seen in this range. Another direction of investigation would be to determine whether methylation could be involved in both the instability and TRD of these alleles.

Smaller, more specific questions related to findings from each of the studies presented in this thesis could also be investigated. For TRD, it would be worthwhile to determine whether a selection either for or against different combinations of repeats within Group I and Group II alleles existed during transmission to embryos, i.e., if a

particular repeat length was driving the TRD. Furthermore, we assumed in our study that TRD did not occur in the female germline because a similar degree of TRD was observed through maternal and paternal transmissions (Dean et al 2006a), and it had previously been shown that TRD did not occur during male gametogenesis (Leeflang et al 1996). However, it would be prudent to test this assumption during different stages of oogenesis.

Continuing repeat instability during mitotic division was observed in a single embryo. To verify our findings, this study could be expanded to test a larger number of embryos from DM1-affected mothers, throughout preimplantation development.

It is interesting to note, of the 7 embryos that inherited a paternally transmitted unstable normal-sized repeat 6 were female. To further define the mode of instability in the transmission of larger normal-sized paternal alleles, a comparison could be made to determine whether there are any differences in the stability of the repeat when transmitted to male versus female embryos.

6.6.2 Segregation of heteroplasmic mtDNA

The segregation patterns of mtDNA genotypes during preimplantation development were investigated in embryos at the cleavage stage of development. This work could be extended to test blastocysts generated from this heteroplasmic mouse model. The levels of heteroplasmy in the trophectoderm in comparison to the inner cell mass would provide valuable information as to the feasibility of chorionic villi sampling for women carrying pathogenic mtDNA mutations.

Finally, on a technical note, simple adaptation of the PCR protocol to avoid the need for radioactive labelling, with the incorporation of fluorescently-labelled primers

and using real-time PCR, would allow this technique to be more widely applicable and may also further increase the efficiency of diagnosis. The feasibility of real-time PCR has been demonstrated for quantifying the mtDNA copy number in blastomeres (Lin et al 2004), thereby opening the possibility of PGD using real-time PCR to diagnose mitochondrial diseases.

6.6.3 Human embryonic stem cells

Currently, studies investigating human embryo development are restricted because of the inaccessibility of human research embryos. However, human embryonic stem (hES) cell lines have recently been derived from fresh or frozen donated IVF embryos (Cowan et al 2004, Reubinoff et al 2000, Strelchenko et al, Thomson et al 1998 2004). Pluripotent hES cell lines have also been created from PGD embryos carrying different well-defined mutations (Mateizel et al 2006, Pickering et al 2005, Verlinsky et al 2005b), including cell lines that carry expanded *DMPK* repeats (Mateizel et al 2006, Verlinsky et al 2005b). These expanded repeat cell lines will be invaluable in studying the mechanisms of repeat instability and could even be differentiated into many different cell types so that the expression pattern and pathophysiology of expanded repeat can be studied in specific types of cells. Overall, the use of hES cell lines carrying different genetic abnormalities is potentially a unique and powerful *in-vitro* tool for modeling human embryogenesis that should hopefully lead to the significant advancement in our understanding of the mechanisms and developmental processes involved in the transmission of DM1 and mtDNA diseases but also for many other human disorders.

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APPENDIX 1: STATEMENT OF ORIGINALITY

According to the *Guidelines for Thesis Preparation* provided by McGill University, "*The elements of the thesis that are considered to constitute original scholarship and an advancement of knowledge in the domains in which the research was conducted must be clearly indicated*". The following is a summary of contributions made to the fields of assisted reproduction and genetics by each of the studies presented in this thesis

Study 1: Detection of mutations based on single cell analysis

This is the first study to compare directly the reliability and accuracy of analysis of fluorescently-labelled products amplified from single cells using 3 different detection systems. These findings can be applied to single cell analysis for basic research and for clinical work. This was also the first study to apply fluorescent multiplex PCR for dystrophia myotonica type 1 (DM1) with a linked polymorphic marker at the level of the single blastomere to clinical PGD.

Study 2: Transmission ratio distortion in the myotonic dystrophy locus in human preimplantation embryos

This was the first study to demonstrate that statistically significant transmission ratio distortion due specifically to the inheritance of larger normal-sized dystrophia myotonica protein kinase (*DMPK*) alleles was evident in the pre-implantation embryo, which remained significant when female embryos were analysed separately. Therefore the developmental time point for the occurrence of this preferential transmission could be placed around the time of fertilisation.

Study 3: Instability in the transmission of the myotonic dystrophy CTG repeat in human oocytes and preimplantation embryos

This was the first presentation showing that the intergenerational enlargements seen upon maternal transmission of expanded *DMPK* repeats occurred during oogenesis, before completion of meiosis I, and that the variability of phenotypic expression seen in the offspring of DM1-affected women can be related back to the level of the embryo. These findings further define the time period for the occurrence of this repeat expansion and advance our understanding of the mode of transmission of expanded CTG repeats.

Study 4: Prospect of preimplantation genetic diagnosis for heritable mitochondrial DNA diseases

This is the first study to determine that the two mitochondrial DNA genotypes present in heteroplasmic mouse zygotes and gametes segregate equally between cells during meiotic and mitotic divisions. This allowed us to suggest that PGD was feasible for women heteroplasmic for some mitochondrial DNA point mutations.

APPENDIX II: SCIENTIFIC COMMUNICATIONS/PUBLICATIONS

Dean NL, Tan SL, Ao A (2001) The development of preimplantation genetic diagnosis for myotonic dystrophy using multiplex fluorescent polymerase chain reaction and its clinical application. *Mol Hum Reprod* **7**(9):895-901.

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Dean NL, Tan SL, Ao A (2006) Instability in the transmission of the myotonic dystrophy CTG repeat in human oocytes and preimplantation embryos. *Fertil Steril* **86**:98-105.

Presented Abstracts

Blake DL, **Dean NL**, Tan SL, Ao A. Single cell PCR: Comparing fluorescent versus conventional PCR in the development of genetic tests used in preimplantation genetic diagnosis. 16th ESHRE Annual Meeting, Bolonga, Italy. June 25-28, 2000.

Dean NL, Battersby BJ, Gosden R, Tan SL, Ao A, Shoubridge EA. Distribution of mitochondrial DNA in heteroplasmic mouse oocytes and their polar bodies. 34th Annual Meeting of the Society for the Study of Reproduction, Ottawa, Ontario, Canada. July 28-August 1, 2001. Oral Abstract 48.

Ao A, Chian RC, Blake DL, Bielenska M, **Dean NL**, Tan SL. Preimplantation genetic diagnosis of embryos produced from in vitro matured (IVM) oocytes. 57th Annual meeting of the American Society of Reproductive Medicine, Orlando, Florida, USA. October 20-25 2001. P-66.

Dean NL, Battersby BJ, Tan SL, Ao A, Shoubridge EA. Distribution of mitochondrial DNA in heteroplasmic mouse oocytes and embryos. 2nd Annual McGill Graduate Biomedical Conference. February 28, 2002. Poster Abstract 25.

Dean NL, Battersby BJ, Tan SL, Ao A, Shoubridge EA. A future prospect of preimplantation genetic diagnosis for heritable mitochondrial DNA diseases using a murine model system. Canadian Fertility and Andrology Society Annual Meeting, Charlevoix, Quebec, Canada, September 25-28, 2002. **The best research oral presentation.**

Dean NL, Naumova AK, Morgan K, Tan SL, Ao A. Transmission ratio distortion in myotonic dystrophy alleles. European Society of Human Reproduction and Endrocrinology Annual Meeting, Madrid, Spain, June 28-July 2003.

Dean NL, Tan SL, Ao A. Transmission of the myotonic dystrophy CTG repeat in early human embryos.18th World Congress on Fertility and Sterility, Montreal, Canada, May 23-28, 2004. Oral abstract 208.1.

Other Presentations

Dean NL. Approaches to Treating Patients with Single Gene Defects for Preimplantation Genetic Diagnosis. Research Dialogue Meeting of Department of Obstetrics and Gynecology, McGill University, Montreal, 7 December 2000.

Dean NL. A Future Prospect for Treatment of Mitochondrial DNA Disease using a Murine Model System. 2nd Research Retreat, Division of Reproductive Biology, Val Morin, Quebec, 22-23 September 2001.

Dean NL. Transmission ratio distortion in myotonic dystrophy alleles. 3rd Research Retreat, Division of Reproductive Biology, Val David, Quebec, 20-22 September 2002.

Dean NL. The expansion of CTG repeats in Myotonic Dystrophy - in the egg or the embryo? 4th Research Retreat, Division of Reproductive Biology, Val David, Quebec, 3-5 October 2003.

Dean NL. Instability in the Transmission of the Myotonic Dystrophy CTG Repeat in Human Oocytes and Preimplantation Embryos. Obstetrics and Gynecology Research Divisional talk, McGill University, Montreal, Quebec. 8 May 2006.

APPENDIX III:

Ethics certificates and copyright waivers

Centre universitaire de santé McGill
McGill University Health Centre

May 10, 2002

Dr. Asangla Ao
Department of Obstetrics and Gynecology
Women's Pavilion F3.16
MUHC – RVH

Dear Dr. Ao:

The research proposal entitled ""Investigation of Segregation Distortion in the Transmission of Myotonic Dystrophy Alleles" REB# SUR 02-014, received Full Board review of the Research Ethics Board, Surgery, at its meeting of February 23, 2002 and was found to be within ethical guidelines for conduct at the McGill University Health Centre.

We are pleased to inform you that final approval was provided on May 10, 2002 for the Clinical Protocol, the revised consent document involving egg retrieval(Version 02/05/03),the revised consent document for investigation of transmission of the myotonic dystrophy gene (Version 02/05/02) and the validated French versions of both consent documents.

We ask you to note that all research involving human subjects requires review at a regular interval and the certification of review will remain in effect until **May 9, 2003**. It is the responsibility of the investigator to submit the appropriate report in a timely manner that does not exceed the expiration date for study approval.

The project has been assigned study REB# SUR 02-014 at the MUHC and should any modification to the research or unanticipated development occur prior to the next scheduled review, please advise the REB promptly and prior to initiating the revision.

In response to your request for a list of the Research Ethics Board members responsible for the review of the above study, for reasons of confidentiality, we do not disclose the names of the members, however the reviewing committee was comprised of:

1 Chair, M.D.
2 physicians
1 ethicist, M.D.
1 lawyer (non-affiliated)
1 scientist
2 non-scientist
1 community representative (non-affiliated)

There are 3 women and 6 men who sit on the Committee.

Journal of Assisted Reproduction and Genetics

"Essenpreis, Alice, Springer DE" <Alice.Essenpreis@springer.com> on 08/01/2006 02:51:33 AM

To: nicola.dean@muhc.mcgill.ca
cc:
Subject: WG: copyright request (Nicola Dean)

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-----Original Message-----
From: Nicola Dean [mailto:nicola.dean@muhc.mcgill.ca]
Sent: Wednesday, July 19, 2006 2:02 PM
To: journals - NY, Springer US; service1, Springer US
Subject: copyright request

sorry to bother you. I sent this previously on 6 july and am now getting a little concerned with the time issue - please could you tell me where to request this permission if this is not the right place Thank you

Dear Sirs

I am submitting my final PhD thesis and need to include some work from a paper that was published by you in the Journal of Assisted Reproduction and Genetics

European Journal of Human Genetics

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Best wishes,

Rebecca

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-----Original Message-----
From: Nicola Dean [mailto:nicola.dean@muhc.mcgill.ca]
Sent: 05 July 2006 21:59
To: Norman, Rebecca
Subject: RE: request for waiver of copyright

Dear Ms Norman

I am submitting my PhD thesis very shortly (sorry for not the longest

notice ever) and wish to ask permission from you to include in my

manuscript a chapter that is based on a paper, including the tables and

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first author, entitled:

Dean NL, Loredo-Osti JC, Fujiwara TM, Morgan K, Tan SL, Naumova AK, Ao A

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Canadian Nuclear Safety Commission | Commission canadienne de sûreté nucléaire

01187-2-08.0

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Montreal Neurological Hospital &
Institute/Institut et Hôpital
neurologiques de Montréal
3801 University Street
Montreal, QC
H3A 2B4
Canada

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II) PERIOD

This licence is valid from: May 1 2003 to April 30 2008.

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This licence authorizes the licensee to possess, transfer, import, export, use and store the nuclear substances and the prescribed equipment listed in section IV) of this licence.

This licence is issued for: consolidated licence: between 11 and 50 locations, inclusive, where radioisotopes are used (849)

IV) NUCLEAR SUBSTANCES AND PRESCRIBED EQUIPMENT

ITEM	NUCLEAR SUBSTANCE	UNSEALED SOURCE MAXIMUM QUANTITY	SEALED SOURCE MAXIMUM QUANTITY	EQUIPMENT MAKE AND MODEL
1	Carbon 14	11 GBq	n/a	n/a
2	Calcium 45	800 MBq	n/a	n/a
3	Chromium 51	2 GBq	n/a	n/a
4	Fluorine 18	2 GBq	n/a	n/a
5	Iron 55	100 MBq	n/a	n/a
6	Gallium 68	400 MBq	n/a	n/a
7	Hydrogen 3	33 GBq	n/a	n/a
8	Iodine 125	4 GBq	n/a	n/a
9	Iodine 131	2 GBq	n/a	n/a
10	Krypton 85	74 GBq	n/a	n/a
11	Phosphorus 32	11 GBq	n/a	n/a
12	Phosphorus 33	2 GBq	n/a	n/a
13	Sulfur 35	22 GBq	n/a	n/a
14	Technetium 99m	11 GBq	n/a	n/a
15	Cesium 137	n/a	1480 kBq	Beckman LS series
16	Radium 226	n/a	370 kBq	Wallac 1200 Series
17	Europium 152	n/a	740 kBq	Wallac 1400 series
18	Germanium 68/ Gallium 68	n/a	1 GBq	n/a
19	Cesium 137	n/a	1 GBq	n/a

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