PREIMPLANTATION DIAGNOSIS

Ke-hui Cui
Department of Obstetrics and Gynaecology,
The University of Adelaide.

A thesis submitted to the University of Adelaide for the degree of Doctor of Philosophy.

March 1993
TABLE OF CONTENTS

Declaration vi
Acknowledgements vii
Publications ix
Abbreviations xi
Thesis summary xii

CHAPTER ONE LITERATURE REVIEW: PREIMPLANTATION EMBRYO AND GAMETE DIAGNOSIS 1

1.1. Introduction 1
1.2 Historical development 3
1.2.1 Animal research 3
1.2.2 Human research 6
1.3 The Choice of Genetic Material and Methods of Sampling 8
1.3.1 Spermatozoa 8
1.3.2 Oocyte (polar body) 10
1.3.3 Embryo 11
1.4 The Choice of Methodology for Diagnosis 17
1.4.1 Metabolic techniques 17
1.4.2 Chromosomal techniques (karyotyping) 21
1.4.3 In situ hybridization 23
1.4.4 PCR amplification 25
### CHAPTER TWO EXperiments to Address the Safety of Embryo Biopsy and to Determine a Marker of Embryo Health Following Biopsy of the Mouse Embryo

<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Introduction</td>
<td>42</td>
</tr>
<tr>
<td>2.2</td>
<td>Materials and Methods</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>Oocyte collection, in vitro fertilization and culture</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>Micromanipulation</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td>Post-biopsy embryo culture, assessment and embryo transfer</td>
<td>46</td>
</tr>
<tr>
<td></td>
<td>Statistical analysis</td>
<td>46</td>
</tr>
<tr>
<td>2.3</td>
<td>Results</td>
<td>46</td>
</tr>
<tr>
<td>2.4</td>
<td>Discussion</td>
<td>53</td>
</tr>
</tbody>
</table>
CHAPTER THREE  SEX SELECTED BIRTHS OF MICE FOLLOWING SINGLE CELL EMBRYO BIOPSY AND Y–LINKED TESTIS SPECIFIC GENE ANALYSIS

3.1 Introduction 56
3.2 Materials and Methods 56
   Selection of gene primers 56
   Blood testing of selected primers 57
   Embryo, embryo biopsy and sexing embryo 58
   General studies after birth 62
3.3 Results 63
   Blood testing of selected primers 63
   Embryo biopsy and sexing embryo 64
   General studies after birth 65
3.4 Discussion 78

CHAPTER FOUR  DETERMINATION OF THE SEX STATUS OF PREIMPLANTATION HUMAN EMBRYOS 84

4.1 Introduction 84
4.2 Materials and Methods 84
   Selection of gene primers 84
   Whole blood DNA testing 85
   Single lymphocyte testing 86
   Human embryo biopsy and embryo sexing 86
4.3 Results 87
   Whole blood testing 87
4.4 Discussion

Specificity of testing–determining gene

Y-repeated sequence

Avoidance of contamination

Embryo biopsy

CHAPTER FIVE
IDENTIFICATION OF HUMAN Y BEARING SPERMATOZOA BY Y–LINKED TESTIS–DETERMINING GENE AMPLIFICATION

5.1 Introduction

5.2 Materials and methods

Testing whole blood DNA

Modified Li–Cui's method and testing single lymphocytes

Sexing motile sperm

Sexing immobilised sperm following photography

5.3 Results

5.4 Discussion

CHAPTER SIX
PREIMPLANTATION DIAGNOSIS OF CYSTIC FIBROSIS:
PCR AMPLIFICATION OF THE ΔF508 MUTATION

6.1 Introduction

6.2 Materials and Methods
Blood testing
Single lymphocyte testing
Human embryo biopsy, blastomere and embryo diagnosis

6.3 Results
Blood testing
Single lymphocyte testing
Diagnosis of human embryos and blastomeres

6.4 Discussion

CHAPTER SEVEN THE FUTURE OF PREIMPLANTATION DIAGNOSIS 123
CHAPTER EIGHT BIBLIOGRAPHY 126
THESIS SUMMARY

The study aimed to develop reliable procedures for determining the genetic status of embryos derived by IVF procedures prior to implantation. This may be a preferred route for prenatal diagnosis as it allows pregnancy to be established using only acceptable embryos, thus avoiding the trauma and risks of artificial termination at later stages of pregnancy.

To achieve this aim, procedures were initially developed using readily available murine embryos which could then be adapted with only minor modification to the human. Early studies of single cell embryo biopsy in the mouse (series 1–8) resulted in a similar rate of blastocyst formation, but a lower rate of hatching of biopsied versus control embryos. This difference was eliminated in subsequent experiences (series 9–13) and safety of the procedure confirmed by showing that embryo transfer of hatching blastocysts resulted in equivalent rates of implantation and fetal development for both biopsied and non-biopsied control groups. These experiments highlight the use of the hatching rate of embryos in culture as a means of providing a simple and reliable guide to evaluating the technical skill of single cell embryo biopsy.

As a basis for the genetic analysis of biopsied cells, procedures based on the polymerase chain reaction (PCR) were developed to amplify fragments of the mouse testis specific gene sequence (pYMT2/B) on the Y chromosome and the ovary specific gene (ZP3) sequence on chromosome 5. Using these oligonucleotides, PCR amplification, allowed
41 male and female mice blood samples to be sexed accurately. The reliability of the procedure was further demonstrated by showing that it was possible to biopsy 164 single cells from 8-cell mouse embryos and successfully amplifying the DNA on every occasion (100%) by PCR. The accuracy of the procedures was further confirmed by establishing that all of the 39 mice born following biopsy and transfer had been sexed correctly. These mice were examined extensively for signs of abnormality. Birthweights, macroscopic, x-ray and histopathological examination together with haematological and biochemical analyses all confirmed essential normality.

As a preliminary to the extension of the procedure to human studies, a PCR protocol was established for primers derived from the motif of human testis-determining gene (SRY) on the Y chromosome and the autosomal gene (ZP3). PCR analysis was performed on blood DNA from 21 men and 20 women and on 20 single lymphocytes using this protocol and on all occasions, the correct sex of origin was confirmed. It was also shown that the PCR procedures developed could be successfully adapted to determine the genetic sex of sperms. Using the modified procedure, 653 of 671 motile human spermatozoa (97.3%) were sexed with 355 (54.4%) determined as Y bearing and 298 (45.6%) as likely X bearing. Interestingly, the length, perimeter and area of the sperm heads and the length of the sperm necks and tails in X bearing human spermatozoa were found to be significantly larger than those of Y bearing spermatozoa.

As a further preliminary test of the reliability and the accuracy of the procedure prior to application to studies with human embryos, PCR
amplification of the targeted SRY and control (ZP3) genes was performed on blood DNA from further 120 men and women, and on 38 single lymphocytes. All results confirmed the correct sex of origin (100%). The sex of 21 single embryo cells biopsied from 4–8 cell human polyspermic embryos was then determined and apart from two degenerate embryo cells (recognised at biopsy) which produced negative results, 19 single embryo cells demonstrated 100% PCR amplification. Eleven of the embryos (57.9%) were determined to be 'male' and 8 (42.1%) 'female'.

Using novel oligonucleotide sequences, the PCR amplification technique was then applied to the diagnosis of the common three point mutation (ΔF508) present in cystic fibrosis, the most common of the non-sex-linked genetic diseases in the Caucasian population. Using a direct PCR amplification method, the results of 22 blood samples and 174 single lymphocytes from homozygous normal, heterozygous and homozygous abnormal subjects for the ΔF508 mutation showed both 100% PCR amplification and the correct diagnosis. Ten human embryos and another 11 selected nucleated blastomeres following single cell embryo biopsy also showed 100% PCR amplification rate and allowed a confident diagnosis.

The study confirms the feasibility of prenatal diagnosis on human preimplantation embryos and describes a protocol allowing precise diagnosis of a single gene defect in a single biopsied cell. The clinical availability of such procedures offers new prospects for couples with sex-linked chromosomal genetic diseases, cystic fibrosis, and, with the ongoing development of suitable techniques, for other clinically important inherent single gene defects.