Treb. Soc. Cat. Biol., Vol. 42 (1992) 163-175

THE PRE-IMPLANTATION MAMMALIAN EMBRYO, ITS USE TO DERIVE CELL LINES AND DIAGNOSE INHERITED GENETIC DEFECTS

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SUMMARY

This manuscript is based on a series of lectures to the students and staff of the Departament de Biologia Fonamental, Universitat Autònoma de Barcelona and forms a review of the progress towards the development of techniques and methods to:

a. Culture cells of the preimplantation mammalian embryo.

b. Use totipotent embryonic stem cells to create animal models for the study of inherited genetic defects and development.

c. Biopsy and diagnosis of genetic diseases using the preimplantation embryo.

Derivation and use of embryonic stem(ES) cell lines from the inner cell mass of the mouse blastocyst

Mouse embryonic stem cell lines provide an in vitro system with which studies may be undertaken on differentiation and development, either by inducing the totipotent cells to differentiate into a variety of different cell types following exposure to retinoic acid, or when injected into murine blastocysts, their contribution to somatic tissues and the germ line. Robertson in 1987 provides details on a practical approach to isolate and culture pluripotential mouse cell lines. Most ES cell lines are poorly characterised in terms of karyotype and stability of totipotent phenotype. One of the major problems is the maintenance

of a normal diploid karyotype as population doubling levels (PDLs) progress. This may explain why only a proportion of ES lines are able to contribute to somatic and germ cells when injected into blastocysts. Recent studies (5) have shown that the E14 cell line (13, 15) is now unable to form chimeras following its injection into blastocysts and concomitant with this has been an increase in the frequency of aneuploidy. The conditions for the derivation of ES cell lines in our own laboratory have recently been described (5) together with full phenotypic characterization in order to optimize their use for genetic manipulation. These studies have defined the phenotype and genotype of mouse ES cell lines MESC. Parameters include 1). an accurate estimate of the replication at each population doubling level (PDL) rather than the vague term of "passage". The term passage reflects the number of times a culture of cells (of unknown number and viability) is trypsinised and subcultured. 2). karyotype and sex, 3). cell size which is measured prior to the time of injection and indicates stage of cell cycle, 4). A biochemical marker e. g. Glucose-6-phosphate-isomerase (GPI), which can verify the genotype of the injected cells. The number of cells injected and their contribution to somatic tissues and the germ line was also noted. These studies showed that the totipotent ES cell phenotype and karyotype are unstable in vitro and that there is an in vitro "window of opportunity" during the culture of ES cells which allows a higher rate of successful integration of injected cells into the germ line (5). The conclusion from these studies was that a full characterisation of the cells is required at selection, transfection and prior to injection into the blastocysts, otherwise the use of ES cell lines will remain inefficient and restricted.

Mouse ES cell lines (MESC) were derived from blastocysts of C57BL/6J, CBA/Ca and 129/J Olac inbred; C57BL/6JxCBA/Ca F_1 hybrids; and MF1 random-bred stocks. Eight of these lines have a normal karyotype, the remainder are aneuploid. The infrequent retention of the normal karyotype in ES cell lines has been previously observed. One of the aneuploid ES cell lines has been shown to contain a single Robertsonian translocation chromosome with a symmetrical, homologous, arm composition Rb(11.11) (7). Although unsuitable for the production of animal models, this spontaneous occurrence and stable retention of the homologous Rb(11.11), as the only metacentric chromosome in an otherwise acrocentric karyotype, provides a potentially useful tool for cytogenetic studies.

Cytogenetic diagnoses of embryonic cells

The most common type of chromosomal abnormality is the presence of an extra chromosome, trisomies. The majority of autosomal trisomies are lethal, and result in failure at implantation or early spontaneous abortion. The three commonest surviving autosomal trisomies in man are those for relatively small chromosomes; trisomy 21 (Down syndrome), 13 (Patau syndrome), and 18 (Edwards syndrome). Even in these cases, most affected embryos abort e.g. only 20% of Down syndrome embryos attain viability. The most common cause of chromosomal failure is nondisjunction, that is, a failure of the relevant chromosome pair to separate at meiosis, usually during the first meiotic division. In 80% of cases of autosomal trisomy, the causative non-

disjunction takes place in the mother and this becomes increasingly common with advancing age. The incidence of these trisomies rises with maternal age. Many of the patients within an IVF programmes are within a vulnerable age area, and thus the incidence of chromosomal abnormalities following fertilisation is increased.

Cytogenetic analysis of biopsied 4, 8 and morula stage mouse preimplanation embryos has demonstrated that air-dried preparations can be G-banded and karyotyped by modifications of routine banding methods. Biopsied material placed in colchicine yielded the greatest mitotic index, however, chromosomal morphology was unsuitable for G-band analysis. 71.4% of fixed 8-cell biopsies were karyotyped (26) although this success rate is not acceptable for the direct application of this technique to human preimplantation diagnosis, it represents a usefuld system to study chromosomal error at the research level and may, with further modifications be improved so that initial studies can be undertaken on "spare" human early cleavage embryos. Cytogenetic analyses may then be performed on biopsies from preimplantation embryos in instances of a parental chromosomal rearrangement, for sex determination in cases where DNA studies are uninformative or in combination with in-situ hybridisation with specific probes for chromosome identification (11). Cytogenetic studies have been undertaken on single blastomeres of a two-cell mouse embryo and on 2 blastomeres from four-cell embryos with 65-70% success. The methods used to achieve this success could be best utilised where additional chromosomes are to be detected (Vidal and Gimenez, personal communication).

If such methods are to be used for human embryos it would be dependant on the production of biopsied material which yields successful karyotypes with a high degree of efficiency. This could be achieved by developing methods which are sensitive enough to detect chromosomal abnormalities in individual or small numbers of cells using mouse and human cell lines with known chromosomal defects (Trisomy 21, Robertsonian translocation) for in situ hybridisation, cytogenetic and other analyses. A variety of cells and tissues could be used for example: *a*) A mouse embryonic cell line has been derived which has a known Robertsonian translocation; this cell line will be a useful tool in studying the behaviour and detection of cells with a translocation in vitro.; *b*) Human cell lines which have various trisomies; *c*) "Spare" human embryos from IVF facilities which have been shown to have major abnormalities (triploid); and *d*) Material derived from amniocentesis and chorionic villus sampling (CVS) from patients undergoing prenatal diagnosis.

There is the possibility of using techniques for the DNA amplification of particular cell chromosomes in a similar manner to studies recently undertaken on the Y chromosome repeat in a single blastomere which detected the sex of the mouse embryo (11). Biopsied human embryos have established succesful pregnancies and babies have been born (13). This technique allows those embryos with the possibility of sex linked defects e.g. Lesch-Nyhan to be identified and not returned to the patient. Cytogenetic analysis of sperm from a human male heterozygous for a 13:14 Robertsonian translocation has used sperm and the in vitro penetration of hamster eggs (16).

Creating mouse models for inherited genetic diseases

Animals with modified genomes can be created from genetically manipulated embryonic stem (ES) cells or by the direct injection of DNA into the pronuclei of fertilized eggs. The ES cell approach allows the creation of transgenic animals with predetermined genetic modifications. The ES cells have the distinct advantatge in that they can be selected for specific genetic modification before producing transgenic animals. The genetic modifications may be achieved by manipulation of endogenous genes by targeting and disruption by homologous recombination, or mutagenic agents

Animal models of human disease may be used for molecular, biological and physiological characterisation of the defect where the developmental consequences can be studied. Also, an evaluation of therapeutic procedures and the possibility of gene therapy could be undertaken to correct or modify the defect in mouse models.

Lesch-Nyhan mouse model

The structural gene for hypoxanthine guanine phosphoribosyl transferase (HPRT) is X-linked in mice and humans and HPRT variants occur at a relatively high frequency in cultured cells of male origin. The disease is characterized by mental retardation and self mutilation and death before puberty in human males. Chimeric mice deficient for the HPRT enzyme which is encoded by a gene on the X-chromosome were derived using the stem cell line strategy (15). The mutant has a large gene deletion involving the first two exons of the coding sequence, which prevents transcription at the mutant locus. The chimeras have been used to: *a.* establish a breeding colony on MF1 and 129/J backgrounds and *b.* undertake studies on their breeding performance and examine the fate of normal and HPRT deficient cell lineages (1).

Tay-Sachs mouse model

In humans, the onset of the infantile form of Tay Sachs disease occurs within the first year of life leading to death in early childhood after progressive motor and mental deterioration. In adults the disease onset usually occurs in the second or third decade with motor neuron, pyramidal tract, and cerebellar deterioration.

The properties of A and B forms of the enzyme provided the biochemical basis for the first simple screening tests for Tay-Sachs carriers and prenatal diagnosis by amniocentesis and chorionic villus biopsy. The main questions of interest about Tay-Sachs disease concerns the mechanism(s) of dysfunction which are presently unknown and maybe attributable to: various neurological symptoms or simply to storage of GM2 gangliosides within lysosomes and the concomitant mechanical effects of this engorgement. With a mouse models it should be possible to analyze the relationship between GM2 accumulation in various parts of the brain with the observed dysfunction.

To obtain models for the infantile and adult forms of Tay Sachs disease we are targeting the Hexosaminidase A gene by homologous recombination and injection of the transfected and selected ES cell into mouse blastocysts.

Culture of preimplantation human embryos

The majority of early cleavage human embryos block and/or degenerate when cultured

from the one-cell stage. Due to the lack of human embryos available for basic research very little is known of the factors affecting the viability of the cultured human embryo; whereas, a variety of conditions are known which can influence or overcome the in vitro 2-cell block observed in the mouse (21). The murine in-vitro 2-cell block coincides with the time when the embryonic genome is first expressed. Human gene expression first occurs between the four -and eight-cell stages of preimplantation development (4), which is when an in-vitro block also occurs.

Only 17% of cultured human embryos reach the blastocyst stage in Earle's medium. We have shown that a dramatic improvement in the culture of the early human pre-implantation embryo can be achieved using commercially available Ham's F-12 medium (Figure 1). This data shows that there is a variability in the in-vitro development of the embryos even when similar staged embryos were used at the initiation of the culture period in Ham's F-12 (22, 23). The greatest variability observed is at the 8-12 stage which is where the greatest percentage of human embryos "blok"in-vitro. The viability of the cultured blastocysts was assessed by allowing them to outgrow on different substrates in vitro. Table 1 shows the attachment and outgrowth of cultured zona-intact and zona-free or thinned (acid tyrode's treated) human blastocysts on different substrates.

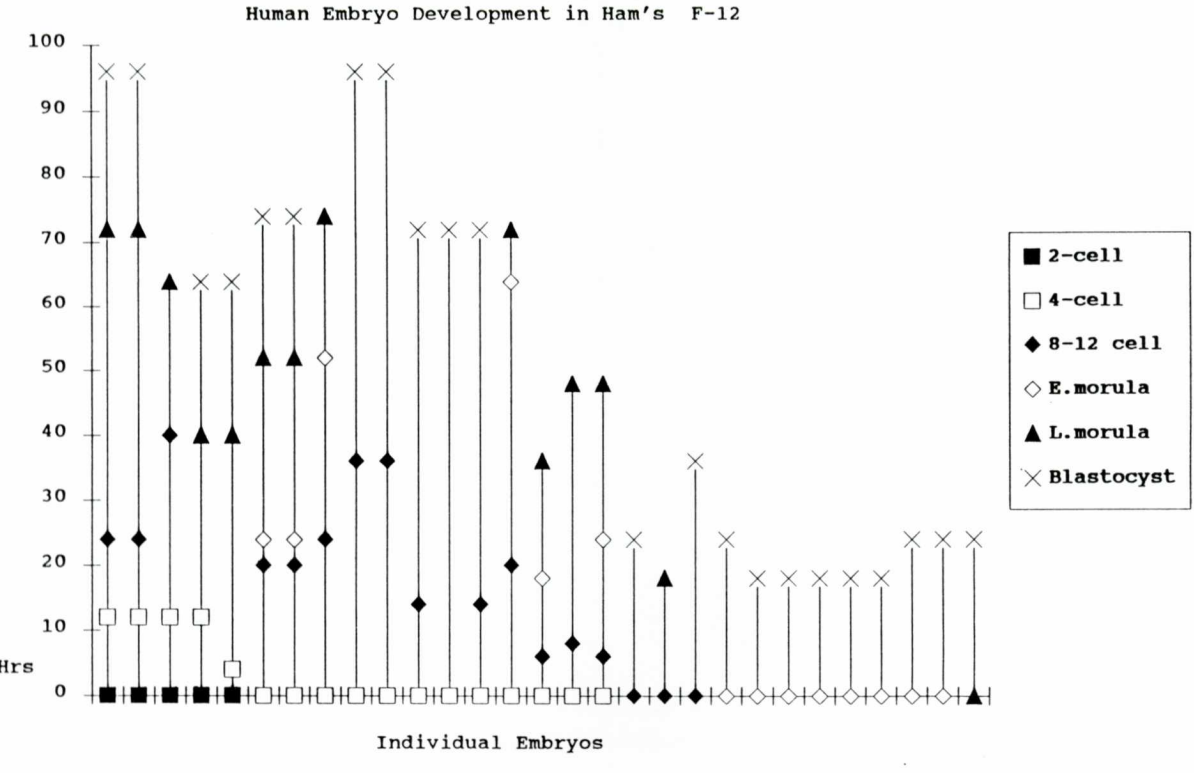

FIGURE 1. These data demonstrate that a variability in rate of cleavageand development occurs in Ham's F-12 media despite using similat staged embryos at the initiation of the culture period. The gratest variability occurs at the 8- to 12- cell stage of development. Thirty-six individual embryos obtained from different donors were used for these experiments. (Reproduced from Muggleton-Harris, et al. Hum. Reprod. 5, 2, 217-220, 1990.)

TABLE I

Attachment and outgrowth of zonae intact and AT treated Grade 1 and 2 human blastocysts on different substrates

	Cultured on feeder cells (A), fibronectin (B) or gelatin (C) substrates in F-12/BRL media	Attached outgrowth degenerated (t)[b]	Attached outgrowth disaggregated (t)[b]
Acid tyrodes treated embryos (AT)	A	—	+(48)
1			
2	A	+(48)	—
3	A	+(48)	—
4	B	+(48)	—
5	B	+(48)	—
6	B	–	+(72)
7	B	+(72)	—
8	B	—	—
9	B	—	—
10	B	—	+(24)
11	C	—	+(72)
12	C	+(48)	—
13	C	+(24)	—
14	C	—	—
15	C	—	—
16	C	—	—
17	B[a]	+(48)	—
18	B[a]	+(48)	—
19	B[a]	—	+(90)
20	B[a]	+(72)	—
21	B[a]	+(48)	—
Zonae intact embryos			
1	C	—	+(48)
2	C	+(48)	—
3	C	—	—
4	C	—	—
5	C	—	—
6	C	—	—
7	C	—	—
8	C	—	—

The table shows the attachment and outgrowth of AT-treated and zona-intact blastocysts on feeder cell, fibronectin and gelatin substrates. The majority of the blastocysts either began to attach and form an outgrowth, then degenerated before disaggregation could occur, or attached and formed outgrowths which could be disaggregated. The time of degeneration or disaggregation is also shown. Some blastocysts from both groups did not form outgrowths and are represented by (-) in both columns.
[a]Cultured in F-12 media plus 170 nanolitres/l of progesterona on Fibronectin. [b]t, time of disaggregation of outgrowth or degeneration of outgrowth in hours. Embryos from both AT-treated and zonae intac degenerated and did not form outgrowths.
(Reproduced from Muggleton-Harris and Findlay, Hum. Reprod. 6, 1, 85-92, 1991.)

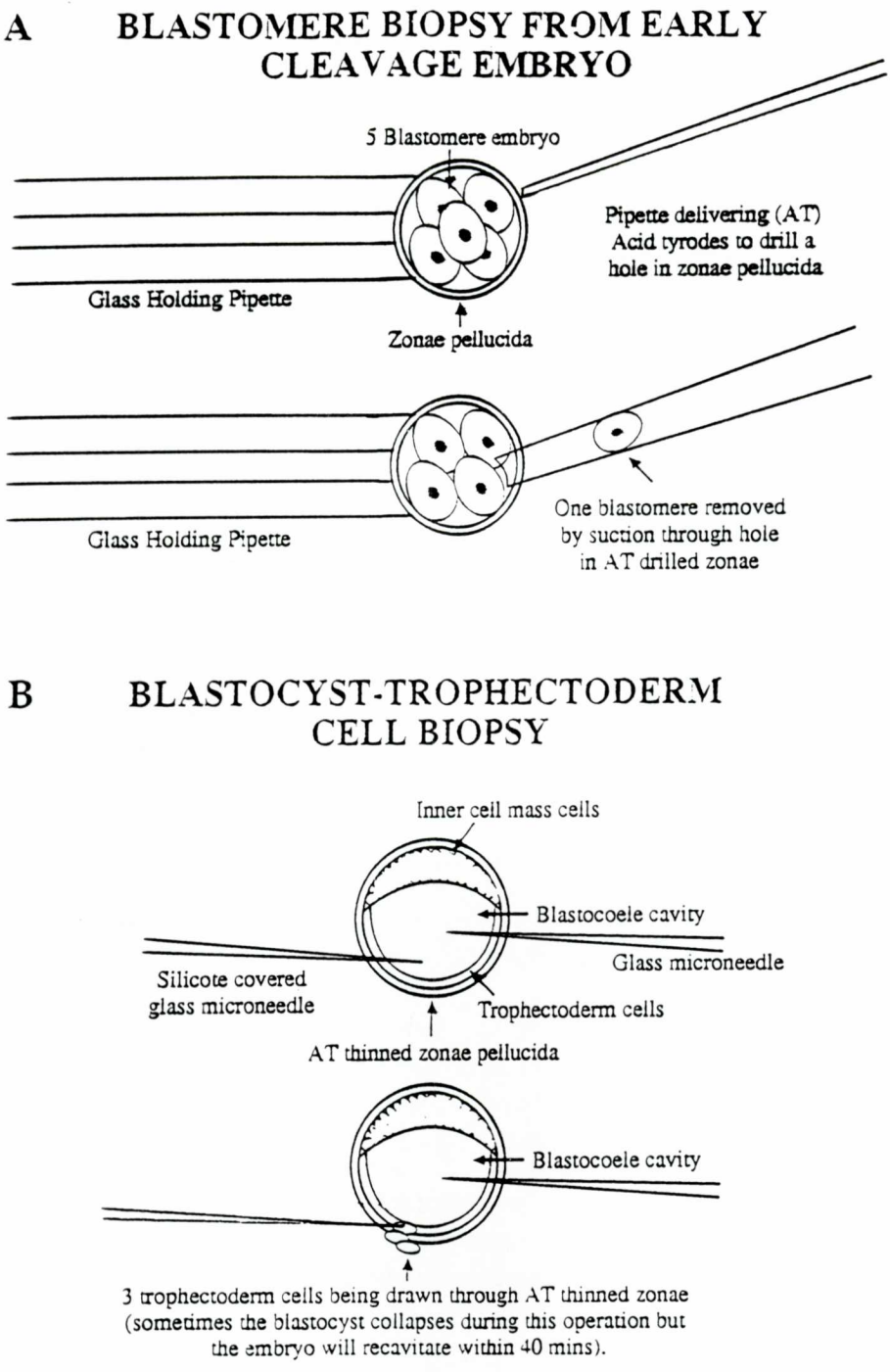

FIGURE 2. (Reproduced from Muggleton-Harris and Findlay, Hum. Reprod. 6: 85-92, 1991.)

Biopsy of the pre-implantation mammalian embryo

There are two approaches to obtaining embryonic cells for preimplantation diagnosis, either during early cleavage or at the blastocyst stage (Figure 2). Each has advantages and problems and we have examined ways to improve the viability of biopsied embryos using both approaches.

Cleavage stage biopsi

There are two major advantages of cleavage stage biopsy. The totipotency of blastomeres up to the 8-cell stage is well established and, depending upon stage, one blastomere can be removed without impairment of subsequent development. Also, the transfer of early cleavage stage biopsied human embryos to the uterus has resulted in established pregnancies and live offspring (13). The major limitation is the small amount of biopsy material which allows only a single analysis to be undertaken. It is always desirable to be able to perform replicate diagnoses, (in case of ambivalent results). Another drawback is the removal of blastomers that would have contributed to the fetus which may have ethical ramifications.

Blastocyst stage biopsy

The advantages of blastocytst stage biopsy are: trophectoderm cells can be removed easily with simple glass microneedles rather than having three different sized glass micropipettes attached to syringes (this is an important consideration if these techniques are to be used in IVF facilities). Also the trophectoderm cells do not contribute to the embryo proper and sufficient number of cells can be initially removed allowing for more than one diagnoses. Many genetic diseases show extensive heterogeneity, and additional testing can be undertaken to defect for other genetic diseases. This strategy may be applied to the screening of human embryos obtained by IVF or uterine lavage (6, 3) to identify those embryos appropriate for transfer. We have shown that the trophectoderm biopsy procedure readily allows sufficient cells to be removed which could be used for duplicate analyses in both mouse and human embryos (22, 23, 24). Recent results has shown that 5 pregnancies were established from 21 transfers of early cleavage stage embryos whereas 9 pregnancies were established when 27 blastocysts were transferred.

In vitro studies on biopsied cels

The growth and proliferation of extraembryonic cells from the preimplantation mouse blastocyst have been examined in vitro. Micromanipulation procedures were used to isolate various subsets of trophectoderm cells. A mitotically active population of polar trophectoderm cells has been identified which proliferate rapidly in suitable in-vitro conditions; these cells were only observed when the majority of the mural trophectoderm was removed from the ICM/polar trophectoderm cell populations (22, 23). Unfortunately we have not been successful in establishing cultures from extraembryonic mouse trophectoderm cells or as shown in Table II, human abembryonic trophectoderm cells.

Biopsied mouse and human cells have been cultured using a variety of substrates and culture conditions to stimulate replication of blastomeres (22, 23, 24). Using "spare" human embryos which have been donated for research

TABLE II

Fate of zonae thinned blastocysts following biopsy of three to five trophectoderm cell

	Cultured on feeders (A), fibronectin (B), gelatin (C) in F-12/BRL	Attached outgrowth degenerated	Attached/ outgrowth disaggregated	Fate of biopsied cells 24h post-biopsy
Biopsied embryos				
1	A	+	—	not recovered
2	A	+	—	not recovered
3	A	+	—	not recovered
4	A	+	—	not recovered
5	B	+	—	not recovered
6	B	+	—	not recovered
7	C	+	—	not recovered
8	C	+	—	ells attached. Replication
9	C	+	—	cells attached. Replication
10	C	—	+	cells attached. Replication
11	C	—	+	cells attached but degenerated
12	C	—	+	cells attached but degenerated
13	C	—	+	cells attached but degenerated
14	C	—	+	cells attached but degenerated
15	C	+	—	cells recovered and placed in microcaps
16	C	+	—	cells recovered and placed in microcaps
17	C	+	—	cells recovered and placed in microcaps
18	C	+	—	cells recovered and placed in microcaps
19	C	+	—	cells attached but degenerated
20	C	+	—	cells attached but degenerated
21	C	+	—	cells attached but degenerated
Non-biopsed				
1	A	+	—	
2	C	+	—	
3	C	+	—	
4	C	+	—	

The table shows the fate of biopsied, zona-thinned blastocysts and non-biopsied, zona-thinned controls on feeder cell, fibronectin and gelatin substrates. The majority of the blastocysts either began to attach and form an outgrowth and then degenerated before disaggregation could occur, or attached and formed outgrowths which could be disaggregated. The fate of the biopsied trophectoderm cells is also shown.
(Reproduced from Muggleton-Harris and Findlay, Hum. Reprod. 6: 1, 85-92, 1991.)

from an IVF facility, we have shown (Table III) that a single human blastomere can replicate within 18-24 hours in vitro (23, 24). This would provide the means of ensuring that duplicate cells were available when a single human blastomere is removed for diagnosis. Since it has been shown that a human blastomere can be removed at the 4-8 cell stage without adverse affects (13, 14), this is now a realistic approach to diagnosis.

TABLE III

Representative sample of replicating individual blastomeres from grade 3 embryos cultured on a gelatin substrate in F-12/BRL media

Embryo	No. of blastomeres in embryo	Finite number of replications of each blastomere *(t)*
1	4	2(48)
2	4	1(24)
3	6	4(18)
4	8	1(20)
5	4	1(18)
6	4	3(72)
7	4	3(18)
8	6	4(24)
9	8	4(18)
10	4	2(48)

The table shows a representative sample of replicating individual blastomeres on a gelatin substrate in F-12/BRL. The number of regular sized blastomeres within the embryo is shown and the number of replications observed from each individual biopsied blastomere. The time for the first replication to be observed is also shown.
(*t*), time of first replication in hours.
(Reproduced from Muggleton-Harris and Findlay, Hum. Reprod. 6: 1, 85-92, 1991.)

Analysis of inherited genetic defects in the pre-implantation embryo

Detection of an enzyme deficiency in the HPRT deficient mouse

Hypoxanthine phosphoribosyl transferase (HPRT) deficient male embryos derived from heterozygous (carrier) female mice have been diagnosed by a biochemical microassay of the X-cromosome coded HPRT activity in a single blastomere and in 5-10 trophectoderm cells sampled from the blastocyst (17, 18, 19, 20). The biopsy of trophectoderm cells at the blastocyst stage distinguished carrier female embryos from affected males, and normal males and females as having intermediate HPRT activity. Confirmation of the diagnosis was made on fetuses developing from the biopsied embryos after transfer into pseudopregnant recipient mice. All HPRT negative and carrier female embryos were correctly diagnosed from the enzyme analysis of the biopsied cells.

Detection of a gene deletion in the shiverer mouse mutant

The mouse mutant shiverer (shi/shi) is characterized by tremors, hypomyelination of the central nervous system and a shortened life-span was selected for use. The gene encoding myelin basic protein (MBP) a major component of the central nervous system myelin, has been largely deleted in shiverer mice. The normal MBP gene has seven exons which have been sequenced while the MBP gene has only exon 1 and 2, the 5 prime breakpoint of the MBP gene has been sequenced. The aim was to identify the mutant and normal MBP genes in biopsied trophectoderm cells obtained from the preimplantation blastocyst using the biopsy procedures developed previously (Figure 3).

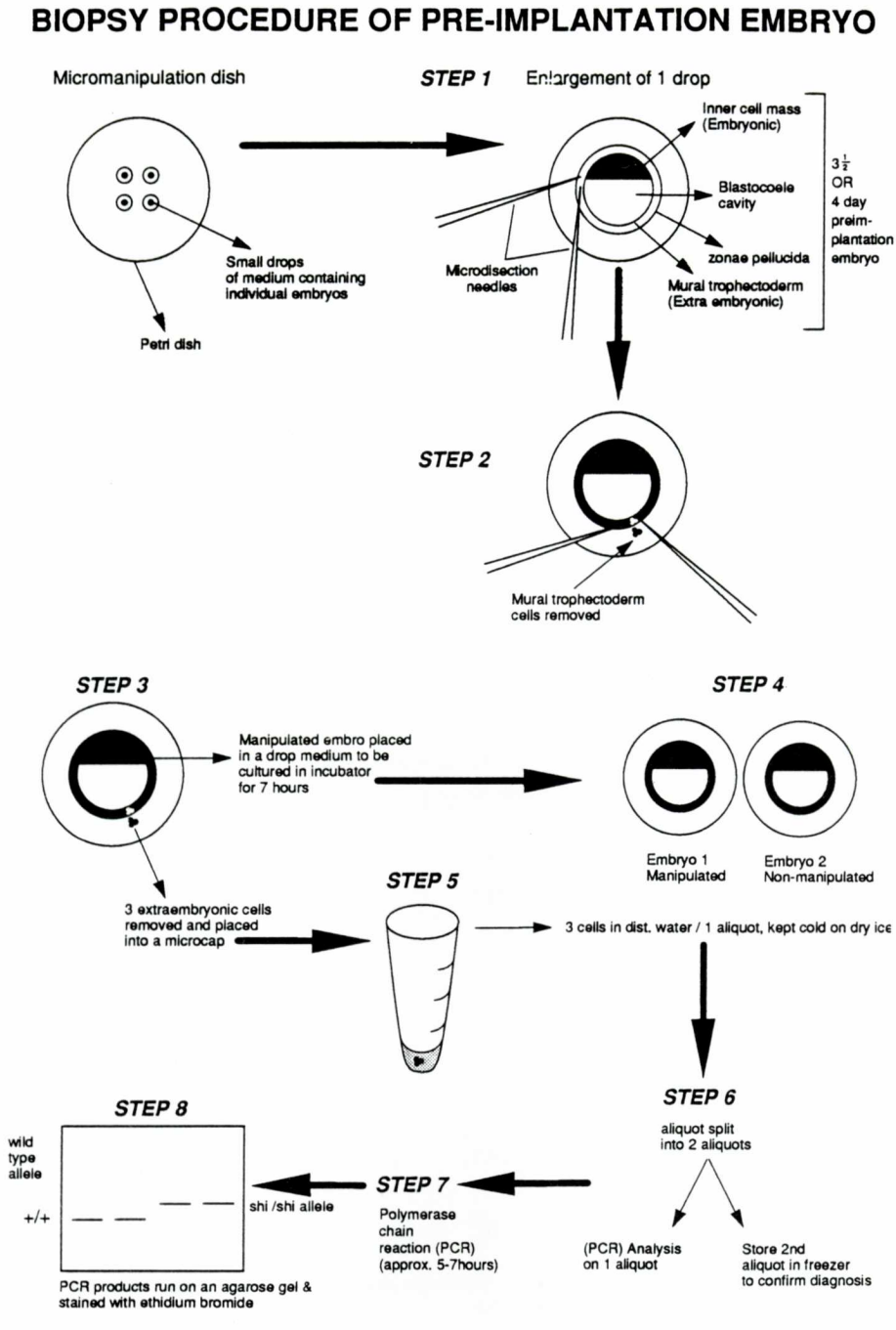

FIGURE 3. A flow diagram demonstrating the PCR assay of trophectoderm biopsy samples. (Reproduced from Readhead and Muggleton-Harris, Hum. Reprod. 6: 93-100, 1991.)

The results demonstrated that preimplantation diagnosis using polymerase chain reaction (PCR) could be completed in less than seven hours. The rapid preimplantation detection of mutant (shiverer) and normal alleles of the mouse myelin basic protein gene allowed selection of the non-affected blastocysts for transfer into recipient mice, the implantation of the biopsed embryos to establish pregnancies and subsequent birth of live young (8, 9, 25). In these studies, the identity of either amplification product was proved conclusively by direct sequence analysis of amplified products. The possibility for germ line therapy of this disorder has already been demonstrated by producing transgenic mice for the wild type MBP gene (25).

Detection of single-base-pair mutations in a Hb^s transgenic mouse

In humans, the most common form of sickle cell anaemia results from a single base pair mutation in codon 6 of the human β-globin gene causing a single amino-acid substitution. Sickling of cells in small vessels causes painful crisis and other life threatening complications. We are developing a clinical test involving PCR amplification of the relevant part of the Hb^s gene to detect in the human embryo. To devise appropiate analysis procedures we obtained a transgenic mouse carrying the human Hb^s gene and manifesting the sickle cell trait (10). Oligonucleotides have been constructed to amplify a fragment of DNA from the Hb^s gene at a high level of sensitivity. Our eventual aim is to be able to distinguish between normal, carrier and defective in transgenic mice and to apply these techniques to human embryos from "at risk" patients.

Acknowledgments

I wish to thank the members of staff of the Departament de Biologia Fonamental, Universitat Autònoma de Barcelona, for their interest and hospitality during my visit.

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