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# Mechanisms of cell allocation in mouse and human preimplantation development

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# Abstract

Cell allocation to the inner cell mass (ICM) and trophectoderm (TE) of mouse and human blastocysts has been examined by differential labelling with polynucleotide specific fluorochromes. In human blastocysts, total cell number increased from ≈58 on day 5 to 126 on day 7. The proportion of ICM cells was high, ranging from one third on days 5 and 7 to one half on day 6. Cell death was evident in both lineages. In the mouse, allocation occurs by the differentiative and conservative divisions of totipotent 8-cell blastomeres with the number of cells allocated to the TE thought to be controlled by the surface area of the embryo. Manipulation of the surface area:volume ratio by aggregating and bisecting 8-cell embryos, and analyzing the resulting blastocysts demonstrates that allocation was unaffected by changes in this ratio.

Pyruvate uptake, measured using a non-invasive ultramicrofluorescence assay, increased from ≈30 to 40 pmoles/embryo/h between days 2.5 and 4.5, before declining, whereas glucose uptake was initially low (<10 pmoles/embryo/h) before increasing between days 4 and 5. Embryos which arrested failed to show this increase in glucose uptake and also exhibited lower levels of pyruvate uptake throughout preimplantation development indicating that nutrient uptake reflects embryo viability. Manipulation of the surface area:volume ratio as above, and measurement of nutrient uptake over the subsequent 48h demonstrates that nutrient uptake is controlled at the membrane level.

Preimplantation diagnosis of genetic disease in the human involves removing one or two cells from an 8-cell embryo, analyzing the cells for the suspected defect and transferring unaffected embryos. The effect of removing cells on subsequent nutrient uptake, development to the blastocyst stage and allocation to the ICM and TE was examined. The proportion of embryos developing to the blastocyst stage was unaffected by biopsy. Both the nutrient uptake and the blastocyst cell number on days 5 and 6 in biopsied embryos were reduced but only in proportion to the reduction in cellular mass. Importantly, the ICM, which gives rise to the fetus, was not disproportionately reduced.

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# Abbreviations

| | |
|---|---|
| ATP | Adenosine triphosphate |
| BSA | Bovine Serum Albumin |
| CC/hMG | Superovulation regimen involving clomiphene citrate and human menopausal gonadotrophin |
| DNA | Deoxyribonucleic acid |
| FITC | Fluorescein Isothiocyanate, a fluorochrome. |
| G6PDH | Glucose-6-phosphate dehydrogenase |
| hCG | Human chorionic gonadotrophin |
| HEPES | (N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]): Buffer used for the making of handling medium. |
| HK | Hexokinase |
| HLT6 | Modified T6 embryo culture medium |
| hMG | Human Menopausal Gonadotrophin |
| ICM | Inner Cell Mass |
| IVF | In Vitro Fertilization |
| LHRH | Luteinizing Hormone Releasing Hormone |
| LHRH agonist/hMG | Superovulation regimen involving luteinizing hormone releasing hormone (LHRH) agonist and human menopausal gonadotrophin |
| M2 | Medium M2 |
| M16 | Medium M16 |
| NAD | Nicotinamide adenine dinucleotide |
| NADH | Nicotinamide adenine dinucleotide: reduced form |
| NADP | Nicotinamide adenine dinucleotide phosphate |
| NADPH | Nicotinamide adenine dinucleotide phosphate: reduced form |
| NC ratio | Nucleo-cytoplasmic ratio |
| PHA | Phytohemagglutinin |
| PMS | Pregnant mares' serum gonadotrophin |
| PVP | Polyvinylpyrrolidone |
| RNA | Ribonucleic acid |
| T6 | Human embryo Culture Medium |
| TCA Cycle | Tricarboxylic Acid Cycle. Cycle whereby there is a stepwise oxidation of organic intermediates derived from pyruvate, in which electrons are transferred to coenzymes. Subsequent reoxidation of reduced coenzymes is accompanied by the generation of ATP. |

| | |
|---|---|
| TE | Trophectoderm |
| UV | Ultraviolet |
| ZP | Zona Pellucida |

# §1 INTRODUCTION

## §1.1 Introduction

Until recently, relatively little was known about human preimplantation development, mainly due to the inaccessibility of the human embryo after normal conception. In a classic study, some descriptive anatomical and morphological information was obtained from a limited series of embryos both at preimplantation and early postimplantation stages, which were fortuitously obtained over many years from uteri removed at hysterectomy (Hertig *et al*, 1954). However, it was not until the advent of *in vitro* fertilization (IVF) over a decade ago, whereby techniques were developed to fertilize oocytes *in vitro* and culture the resulting embryos to early preimplantation stages before transfer back to the oocyte donor, that the human preimplantation embryo became accessible for study. Subsequently, methods have been refined and developed further, however, the success rate of IVF and embryo transfer remain disappointingly low (the overall figures for 1989 show that 21% of patients who have embryos transferred become pregnant; ILA, 1990).

There are many possible reasons for the low success rate of human IVF. Chromosome analysis of surplus embryos after transfer have shown that a high proportion (up to 40%) of these embryos are chromosomally abnormal (Plachot *et al*, 1987). As the incidence of congenital abnormality after IVF (1.4%) is not higher than that after natural conception (1.8%) (ILA, 1990), these embryos probably contribute to some of the embryonic loss. In addition, further culture of surplus embryos shows that *in vitro* many embryos arrest, and only a low proportion (between 17% (Bolton *et al*, 1989) and 57% (Dawson *et al*, 1988)) are capable of developing to the blastocyst stage. This has led to the suggestion that the culture media, developed from those used for other species such as mouse, are suboptimal for the culture of human embryos. Extensive work on the metabolism of preimplantation mouse embryos has shown that they require three energy substrates, pyruvate, glucose and lactate, and the composition of mouse culture media have been designed accordingly. However, it is not known whether human embryos have the same nutrient requirements, and this area requires further study. Such work may, ultimately, provide information which can both elucidate developmental mechanisms and lead to the improvement of the *in vitro* culture of human embryos.

Apart from a series of ultrastructural studies of human blastocysts (Lopata *et al*, 1982; Mohr and Trounson, 1982), there is relatively little information about cleavage rates in late preimplantation development, and no information about the total numbers of cells in human blastocysts or about the first differentiative divisions which give rise to the trophectoderm (TE) and inner cell mass (ICM). In the mouse preimplantation embryo, the division of polarized cells at the 8-cell stage generates phenotypically distinct outer and inner cells which differentiate into the outer epithelial layer of TE, and the ICM at the blastocyst stage. This first differentiative division, allocating cells to the TE and ICM, is of fundamental importance for later development. At the 32-cell stage, during blastocyst formation, the TE is responsible for blastocyst fluid accumulation and becomes specialized for implantation. Following blastocyst expansion, hatching from the zona and implantation, the TE gives rise to components of the placenta and extraembryonic membranes whilst the ICM gives rise to the fetus. The human blastocyst is morphologically similar to the mouse blastocyst. There is some ultrastructural evidence which shows that blastomeres of human 8-cell embryos undergo a similar process of surface polarization as described for mouse embryos at this stage, and that inner cells have different plasma membrane characteristics than outer cells (Lopata *et al*, 1983; Tesarik, 1989). Beyond this, nothing is known about the stage at which blastocyst formation occurs or how many cells are allocated to the TE and ICM, or indeed whether the embryos are undergoing active cell division in culture.

In the mouse, the cellular mechanisms of polarization at the 8- to 16-cell stages, and the subsequent divisions of these cells to generate phenotypically distinct inner (ICM) and outer (TE) precursor cells are well understood (reviewed by Johnson and Maro, 1986). However, whilst much is known about the mechanisms of allocation, very little is understood about the control of allocation. Experiments involving either perturbations of the proportion of the two lineages with regard to one another, or manipulations of preimplantation embryonic size, provide important insights into this question, and, as a result, two contrasting theories for the control of allocation have been proposed. Firstly, the small amount of existing embryo aggregation data suggests that the differentiative divisions of polarized cells are not autonomous and that they are controlled by the position and packing of polarized cells within the embryo (Buehr and McLaren, 1974), with the number of TE cells being proportional to the available surface area of the embryo. However, the observation

that *isolated* 8-cell stage cells polarize and can divide to form both ICM- and TE-like cells, suggests that allocation occurs irrespective of cell position within the embryo (Johnson and Ziomek, 1981a), leading to the possibility that polarization controls allocation.

As well as providing a useful model to examine the control of allocation in the preimplantation mouse embryo, changing embryonic volume by aggregating cleavage stage mouse embryos also provides a model for studying the control of nutrient uptake. The uptake of nutrients could either be regulated at the membrane level (where uptake rates will be determined by the existence or efficiency of transport mechanisms), or at the intracellular level (where they will be determined by the rate of utilization of substrates (Gardner and Leese, 1988; Biggers *et al*, 1989)). Embryos aggregated at the 8-cell stage form single morulae with altered surface area:volume ratios, allowing examination of whether uptake is related to the surface area, suggesting control at the membrane level, or the volume, for example, by intracellular utilization.

Finally, the effects of changing the size of preimplantation mouse embryos has much relevance to the clinical application of preimplantation diagnosis. The ability to remove a few cells from preimplantation embryos made accessible by IVF, assay the cells for a suspected genetic defect and replace only unaffected embryos would obviate the traumatic decision of whether or not to terminate a pregnancy after current prenatal diagnosis techniques at between 8 and 20 weeks of gestation (Penketh and McLaren, 1987). However, before this technique can be used clinically, it is vital to ascertain how the reduction in cellular mass resulting from the removal of cells affects the subsequent development of biopsied embryos as far as is practicable. It is also important to investigate how biopsy affects allocation to the ICM, as this is the lineage which is thought to give rise to the fetus.

## §1.2 Comparative mammalian preimplantation and early development

Much of our knowledge of mammalian embryology has been derived from studies on the mouse. The reasons for this are numerous. Mice are small, relatively inexpensive, easy to maintain and breed and have a short gestation period of around 21d, allowing short experimentation times. The development and identification of many genetic variants over the years have meant that the genetic makeup is the most well understood apart from humans, and the use of inbred strains decreases experimental variation and can provide genetic markers to identify the origin and fate

of cells. Mice have short oestrus cycles and, under laboratory conditions, are not seasonal breeders. It is possible to superovulate mice, mate them with a high success rate, and obtain relatively large numbers of oocytes and embryos. The culture of preimplantation embryos of certain inbred strains from the 1-cell to the blastocyst stage in defined culture medium with high success (>80%) is now routine. Studies of the preimplantation development of other mammalian species such as rat, hamster and rabbit are hampered by the fact that either their development is retarded *in vitro* (rabbit: Fischer, 1987), or the embryos block completely at a specific stage, such as the 4-cell stage in the rat (Mayer and Fritz, 1974) or the 2-cell stage in the hamster, although this block has recently been overcome by altering the composition of the culture medium (McKiernan *et al*, 1991). The only other species where it is possible to culture from the 1-cell to the blastocyst is the human (Steptoe *et al*, 1971; Edwards *et al*, 1981; Mohr and Trounson 1984). Similarly, transfer of mouse preimplantation embryos to pseudopregnant recipients results in high rates of implantation making it possible to examine the effects of experimental manipulations at preimplantation stages on postimplantation development. Finally, the mouse preimplantation embryo is remarkably resilient to a variety of experimental manipulations.

However, if hypotheses or theories of development are based on studies of one species it is desirable to test their general applicability by comparing other species. Such studies may reveal diverse mechanisms for similar developmental events. Among mammals the variety of placentation types alone (Wimsatt, 1975), necessitates the study of representative species. For example, human blastocysts implant at the embryonic pole whereas in the mouse the cells at the abembryonic pole are the first to penetrate the endometrium. Also, human and mouse placentas, although similar at later stages, have different developmental sequences. In the human, until the advent of IVF for the treatment of infertility, it was virtually impossible to obtain any information on preimplantation human development. However, IVF has made the human preimplantation embryo accessible. Ethical considerations prevent the application of many experimental techniques to human embryos, and numbers of surplus embryos available after transfer for study are low, necessitating the development of sensitive techniques which provide maximal information from minimal amounts of material. However, it is hoped that such studies, supported by the wealth of knowledge on mouse embryo development, will

increase understanding of why so few human embryos develop to the blastocyst stage *in vitro* and why so few implant after transfer.

### §1.2.1 Mouse preimplantation development

In the ovary, oocytes are arrested in the dictyate stage of the first meiotic division until stimulation of the surrounding granulosa cells by luteinizing hormone brings about completion of their maturation (Edwards and Gates, 1959). Meiosis resumes and one set of homologous chromosomes is expelled in the first polar body. Shortly afterwards, ovulation occurs and oocytes are released from the ovary, arrested at the second metaphase stage of meiosis. Meiosis is only completed two to three hours after fertilization, with one set of sister chromatids being expelled as the second polar body and the remaining haploid set being retained by the oocyte which form the female pronucleus, lying close to the second polar body.

The freshly ovulated oocyte is enveloped by an acellular layer known as the zona pellucida (ZP) and further by a dense cluster of follicular cells, the cumulus oophorus. The ZP, which is approximately 7 μm thick, is made up of three glycoproteins, ZP1, ZP2 and ZP3. It is permeable to large macromolecules such as immunoglobulins and enzymes, and is synthesized by the oocyte during follicular growth within the ovary (reviewed by Wassarman, 1990). Fertilization takes place in the proximal region of the oviduct known as the ampulla. As the spermatozoa are transported to the site of fertilization they undergo capacitation, ie. they undergo a maturation process in the female reproductive tract, which enables them to fertilize. Principal events in capacitation are believed to include a biochemical alteration in the sperm plasma membrane (Yanagimachi, 1989) and alterations in sperm motility (reviewed by Fraser and Ahuja, 1988). Capacitated sperm bind to sperm receptors (ZP3) in the ZP. ZP3 causes bound sperm to undergo the acrosome reaction whereby the acrosome (a lysosome-like organelle overlying the sperm head) undergoes signal-transduced exocytosis releasing hyaluronidase and acrosin (a proteinase) (reviewed by Fraser and Ahuja, 1988). Acrosome-reacted sperm penetrate the ZP, aided by the released acrosin, reach the oocyte plasma membrane and fuse with it. Fusion of the sperm and oocyte results in the activation of the oocyte, and development ensues. The engulfment of the fertilizing sperm head by the oocyte leads to the formation of the male pronucleus. In mammals, the male and female pronuclei do not fuse but, shortly before the first cleavage division, the nuclear membranes break down and the

chromosomes from each pronucleus come together on a single mitotic spindle, whereupon diploidy is restored.

Fertilization by a single sperm induces the oocyte to undergo the cortical reaction, whereby lysosome-like cortical granules underlying the oocyte membrane, exocytose, releasing various enzymes into the perivitelline space. These contents enter the ZP and induce the zona reaction, whereby the ZP hardens, as detected by decreased solubility to proteolytic enzymes and acidic solutions, and loses its ability to bind sperm by inactivation of ZP3 (the sperm receptor). This constitutes the 'slow' block to polyspermy (reviewed by Wassarman, 1990). The 'fast' block occurs within seconds of sperm fusion at the level of the oocyte membrane. Whilst the plasma membrane block is not electrically mediated as in other non-mammalian species (reviewed by Jaffe and Gould, 1985), there does appear to be changes in the organization of the membrane at fertilization (Wolf *et al*, 1981). One possible mechanism which may contribute to the 'fast' block may be a post-fertilization reduction in sperm binding to the plasma membrane (reviewed by Jaffe and Gould, 1985).

The rate of polyspermy in the mouse *in vivo* ranges from ≈2 to 5% (Fraser and Maudlin, 1979; Santalo *et al*, 1986) and *in vitro* is between 2 and ≈ 14% (Fraser and Maudlin, 1979; Santalo *et al*, 1986; Carroll *et al*, 1989). Examination of the chromosome complement of mouse oocytes fertilized *in vitro* indicates that the incidence of polyspermy increases with increasing concentration of sperm (Fraser and Maudlin, 1978), a phenomenon also observed after human IVF (§1.2.2; Wolf *et al*, 1984; Englert *et al*, 1986).

After fertilization, the cumulus oophorus slowly disperses and the free-floating fertilized zygotes begin their passage down the oviduct to the uterus, where implantation occurs. The zona pellucida is thought to have several functions, firstly, as part of the 'slow' block to polyspermy, discussed above; secondly, to maintain the normal cleavage pattern, by restraining the fairly loosely associated blastomeres in a spherical space; thirdly, to prevent fusion of embryos to each other; and finally, to protect the embryo during the passage down the oviduct, which is undergoing muscular contractions, moving the embryos vigorously backward and forward during the slow forward progress down the oviduct. This protective facility has been confirmed by the observation that zona-free mouse embryos transferred to the oviducts of pseudopregnant recipients between the 1- and 4-cell stage fail to implant

and cannot be recovered intact from the oviduct (Modlinski, 1970; Bronson and McLaren, 1970).

The one-cell embryo undergoes a series of cleavage divisions, at each of which the cells or blastomeres approximately halve in size. Up until the 8-cell stage the blastomeres are spherical, phenotypically identical and totipotent (Kelly, 1977). The divisions, after the 2-cell stage, can be asynchronous by up to 2h (Pedersen, 1986), but a relatively large proportion of each cleavage stage is spent at the 4-, 8- or 16-cell stages: for example, the situation where some blastomeres in an embryo are at the 4-cell stage whilst some are at the 16-cell stage will never occur during normal development. At the 8-cell stage the embryo undergoes the process of compaction, forming a morula, whereby the spherical cells flatten on each other, maximizing cell-cell contacts and minimizing intercellular spaces (Lewis and Wright, 1935). Focal tight junctions form at the apical margins of adjacent cells, and by the 16- to 32-cell stage these have become zonular, forming a tight permeability seal between the external environment and the inside of the embryo (Ducibella *et al*, 1975). In addition, gap junctions form between cells (Lo and Gilula, 1979). The establishment of such a permeability barrier allows the development of a blastocoel cavity by internal fluid accumulation. Initially, small fluid-filled cavities develop intercellularly between blastomeres, a process which requires activity of the membrane associated $Na^+K^+$-ATPase localized on the basal surfaces of the blastomeres (Wiley, 1984). These small cavities eventually coalesce to form a single, large blastocoel cavity. The cell number at which blastulation occurs varies between species: in the mouse it occurs at the 32-cell stage (Smith and McLaren, 1977), in the rabbit when the embryo has 128 cells, in the pig at the 16-cell stage and in the sheep at the 64-cell stage (Papaioannou and Ebert, 1986). The blastocyst is characterized by an outer epithelial layer of trophectoderm surrounding the blastocoel cavity, with an eccentrically placed, internal clump of cells called the inner cell mass, from which the fetus is derived (Gardner and Papaioannou, 1975). The blastocyst hatches from the zona pellucida and implants in the uterus. In rodents and primates the preimplantation period lasts between 4 and 7 days and implantation occurs at the blastocyst stage. However, in ungulates, the blastocyst continues to grow before implantation as an elongated chorionic vesicle with an allantois, with the preimplantation phase lasting up to 60 days in the horse (Wimsatt, 1975)

### §1.2.2 Human preimplantation development

The use of *in vitro* fertilization (IVF) and embryo transfer for the therapeutic treatment of infertility provides a unique opportunity to study the early development of the human embryo in culture. As a result, it has been possible to extensively describe the early cleavage divisions *in vitro*, before transfer (Sundstrom *et al*, 1981; Edwards *et al*, 1981; Trounson *et al*, 1982; Mohr and Trounson, 1984) (see §1.3.2). In addition, there have been improvements in superovulation protocols. The resulting increase in the number of oocytes collected and fertilized, coupled with increasing success at embryo transfer, has decreased the number of transferred embryos required to establish a pregnancy. The increase in the number of surplus embryos after transfer has facilitated analysis of later development *in vitro*.

The human preimplantation embryo developing in culture appears to undergo a similar sequence of morphological changes as in the mouse. Human fertilization appears to be morphologically very similar to that occurring in other mammalian species: sperm fusion to the oocyte plasma membrane, cortical granule exocytosis, sperm chromatin decondensation and second polar body formation have all been described in electron micrographs of human oocytes fertilized *in vitro* (Soupart and Strong, 1975; Lopata *et al*, 1980; Sathananthan and Trounson, 1982; Sathananthan *et al*, 1982a). Cleavage and blastocyst formation proceeds as in the mouse although compaction occurs later, at the 16-cell stage. However, there are differences such as an increased level of abnormal fertilization and more variable morphology during cleavage, compaction and blastocyst formation.

The ability to observe pronucleate stages after IVF has shown that in up to 12% of cases (Angell *et al*, 1986) fertilized oocytes have three or more pronuclei. The percentage of tripronucleate oocytes generally observed is variable, ranging from 1 - 7% (Mohr and Trounson, 1984; Englert *et al*, 1986; Van Blerkom *et al*, 1987; Plachot *et al*, 1987). This could either be due to diandry, where the embryo has been fertilized either by more than one sperm or a single diploid sperm, or to digyny, where the oocyte has failed to extrude its second polar body. In the mouse it is possible to identify the origin of the pronucleus by its size and shape; the female pronucleus is close to the second polar body and is smaller, whereas the male pronucleus is larger, and sometimes can be seen to be associated with a sperm tail. In contrast, in the human, the pronuclei lie in the centre of the zygote, and it is virtually impossible to identify which one is male or female on the basis of size (Wiker *et al*,

1990). Plachot *et al* (1989) have attempted to identify whether multipronucleate oocytes are diandric or digynic by carrying out a histological study to check for the presence of one or two polar bodies. 14% had a single polar body, suggesting that these tripronucleate oocytes had resulted from digyny after non-extrusion of the second polar body. Thus, the remaining majority (86%) result from polyspermy. This is not surprising in that whilst *in vivo* only a few hundred sperm reach the vicinity of the egg, up to 500,000 sperm are inseminated with each oocyte at IVF (reviewed by Van Blerkom, 1989). Indeed, there is a positive correlation between multipronucleate oocytes and both high fertilization rates generally in a cohort of oocytes and inseminations with high numbers of sperm (Wolf *et al*, 1984; Englert *et al*, 1986). In addition, ruptured zonae have been associated with multipronucleate oocytes, possibly because part of the block to polyspermy at the zona level has been physically interrupted (Soupart and Strong, 1974; Lowe *et al*, 1988). Finally, there are increased levels of multipronucleate oocytes found after early insemination. Ultrastructural observations of multipronucleate zygotes inseminated immediately after oocyte retrieval showed delayed cortical granule release, and evidence that the cortical granules were still migrating to the surface at the time of oocyte retrieval (Sathananthan and Trounson, 1982). Plachot *et al* (1987) found strong evidence that one embryo had indeed been fertilized with two Y-bearing sperm in that the chromosome complement was 69, XYY.

Polyspermic embryos cleave normally and are indistinguishable from normal embryos (Van Blerkom *et al*, 1984). In some cases, diploidy is restored in tripronucleate embryos, others are triploid or have highly abnormal chromosome complements (Trounson, 1982; Rudak *et al*, 1984; Angell *et al*, 1986; Plachot *et al*, 1987). Triploidy generally results in spontaneous abortion during the first trimester, or, more rarely, can lead to possible hydatiform mole formation or anomalous fetal development, thus multipronucleate embryos are avoided for transfer.

A second abnormality of development is that of parthenogenesis, whereby nuclear activation is not induced by sperm penetration but by another factor. In the mouse, oocytes can be parthenogenetically activated by a variety of physical (thermal, electrical, osmotic) and chemical (alcohol or enzyme treatment) stimuli. These embryos can cleave to varying extents depending on the activating stimulus and can even, upon transfer, reach early somite stages (reviewed by Whittingham, 1980). In the human, approximately 1% of oocytes examined histologically show the formation

of a single female pronucleus (Plachot *et al*, 1985). Chromosome analysis of such embryos which have reached the 2- to 4-cell stage show that the majority (69%) are indeed haploid with a chromosome complement of 23, X, confirming that the second polar body was extruded without fertilization. Interestingly, however, 13% were diploid, possibly as a result of non-extrusion of the second polar body, or possibly they were fertilized but the male pronucleus was not visible initially (Plachot *et al*, 1985). Oocytes showing evidence of parthenogenetic activation in the form of a single pronucleus can continue to cleave, although only a low proportion (11%) of them reach the blastocyst stage (Hardy *et al*, 1989a; §2.2).

In contrast with the mouse, human preimplantation embryos have a highly variable morphology. Many embryos exhibit blastomeres of uneven size, some of which appear to be fragmenting. At compaction in mouse embryos, the cells flatten onto each other to such an extent that the embryo almost appears as though it has only one cell. In contrast, compaction in human embryos is variable. Some embryos will form very compacted morulae where it is virtually impossible to discern cell outlines, whilst the remainder will only exhibit slight flattening of cells, and cell outlines will still be visible. In addition, many blastomeres in both *in vivo* and *in vitro* fertilized embryos have been observed to have two or more nuclei (Hertig, 1954; Sathananthan *et al*, 1982b; Lopata *et al*, 1983; Trounson and Sathananthan, 1984; Tesarik *et al*, 1987; Braude *et al*, 1990).

Finally, whereas the rates of blastocyst formation in the mouse can reach 75% in culture after IVF (Edgar *et al*, 1987), the percentage of surplus human embryos developing to the blastocyst stage *in vitro* is variable and ranges from 17% (Bolton *et al*, 1989) to 57% (Dawson *et al*, 1988). The variable morphology and lower rate of blastocyst formation in the human could be due to many reasons including chromosome abnormalities and suboptimal culture conditions (§4.1).

### §1.3 Timing and regulation of preimplantation development

During early embryonic development, cell differentiation must be regulated both temporally and spatially. As well as the correct differentiative events occurring, it is of importance that they occur in the correct order and at the correct time. In addition to absolute time measured in hours elapsed since a finite event such as fertilization, there are also postulated to be biological clocks which rely on cyclic events such as cell division, nuclear division, chromosome or DNA replication, or on intracellular relationships such as the nucleo-cytoplasmic ratio (Smith and McLaren, 1977).

Events in the cell cycle are usually analysed in relation to the nuclear events of DNA synthesis (S-phase) and mitosis (M-phase). These events are separated in most cells by a post-mitotic gap ($G_1$) and a second gap ($G_2$) between DNA synthesis and mitosis.

In addition to the specific cell cycle events described above, more subtle cyclic changes are being found. Whether they are themselves biological clocks, or dependent on other timing systems is not known. Extensive studies on the cell cycle of yeasts (*Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*) have allowed the isolation of cell division cycle (cdc) mutants which have allowed the identification of a large number of gene products necessary for cell cycle progression. Homologues of some of these genes are also being found in other species, such as human (reviewed by McConnell and Lee, 1989). It has also been demonstrated that the *Xenopus* homologue of one of these genes, $cdc2^+$, is in fact a component of maturation-promoting factor (MPF). MPF induces the breakdown of the nuclear membrane and condensation of chromosomes in the maturing oocyte (Ford, 1985). MPF activity peaks immediately preceding mitosis and declines thereafter. Evidence for the existence of a similar cytoplasmic factor in mouse embryos is provided by the observation that the developmental 'block' of embryos from certain outbred strains of mice at the 2-cell stage can be alleviated by injecting cytoplasm from embryos of non-blocking strains. By injecting cytoplasm from donor 2-cell embryos at different stages of the cell cycle, it was found that this cytoplasmic component, which released the 2-cell block in recipient 2-cell embryos, peaked in activity just before mitosis, in an analogous fashion to MPF (Pratt and Muggleton-Harris, 1988).

A further example is the occurrence of surface contraction waves in *Xenopus* embryos which recur in each cell cycle, even in the absence of a nucleus. If these are inhibited, repeated DNA synthesis and mitosis do not occur, suggesting the presence of an autonomous cytoplasmic oscillator which directs the orderly repetition of DNA synthesis and mitosis (reviewed by Ford, 1985). Observations of nucleated and anucleated halves of fertilized mouse oocytes show that there are similar cyclic surface contractions suggesting that such a cytoplasmic clock exists in mammalian embryos (Waksmundzka *et al*, 1984).

As early cleavage embryos do not grow, but undergo a series of divisions reducing the volume of the cell with each cleavage (Abramczuk and Sawicki, 1974; Lehtonen, 1980), there is a continuing change in the ratio of the volume of the

nucleus, which remains the same size, to the volume of the cell, which is steadily decreasing. Therefore, the nucleo-cytoplasmic (NC) ratio gradually increases with every DNA replication step, reaching the somatic value when the embryo has approximately 120 cells (Surani, cited in Smith and Johnson, 1986). This ratio could act as a trigger for events during preimplantation development. The effect of the NC ratio is thought to be based on the relative dosage of factors, stimulatory or inhibitory, produced by either the nucleus or the cytoplasm. In *Xenopus* embryos it was shown that the achievement of a critical NC ratio or DNA/cytoplasmic ratio was required for the event of midblastula transition and the activation of the embryonic genome (Newport and Kirschner, 1982ab). Evidence for a similar triggering mechanism in the mouse is provided by the observation that whilst haploid embryos (with a half normal-sized diploid nucleus, and therefore an decreased NC ratio) rarely develop beyond the 2-cell stage, reducing the amount of cytoplasm to a near normal NC ratio greatly improves development (reviewed by Howlett *et al*, 1987). However, in the mouse embryo, alterations to the NC ratio have no apparent effect on the timing of stage specific gene expression, or on protein synthesis patterns during the first four cell cycles (Petzoldt and Muggleton-Harris, 1987), supporting the idea that stage specific gene activity during early cleavage might proceed in parallel to DNA replication cycles and is independent of the NC ratio.

### *§1.3.1 Timing of mouse preimplantation development*

The first cell cycle is unusually long, with times ranging from 14h (Molls *et al*, 1983) to 20h (Bolton *et al*, 1984, Howlett and Bolton, 1985). There is a more extended $G_1$ phase than succeeding cycles and no $G_2$ phase. During this cycle there is minimal embryonic transcription, and development is controlled exclusively by the maternal genome utilizing RNA transcripts and stored proteins synthesized during oogenesis (reviewed by Johnson *et al*, 1984). The majority of molecular changes observed during the one-cell stage occur irrespective of fertilization or activation suggesting that the oocyte is undergoing events triggered by the terminal events of oogenesis, such as germinal vesicle breakdown or ovulation. Fertilization induces a sequence of changes that are superimposed on this programme, and the fertilization programme assumes the dominant role, as it is the time of fertilization or activation and not ovulation which determines the overall length of the first cell cycle (Howlett and Bolton, 1985).

The second cell cycle is slightly longer, ranging from 18 - 22h (Molls *et al*, 1983). The second cleavage division, from 2- to 4-cell, is asynchronous by about 2h (Pedersen,1986). The transition from maternal to embryonic control takes place during the 2-cell stage. This occurs 8-9h after first cleavage, during $G_2$, and results in a major transition in polypeptide synthesis which is necessary for second cleavage (Flach *et al*, 1982; Bolton *et al*, 1984). Maternal transcripts decline during this period. The switch-on of the embryonic genome has an absolute requirement for the first round of DNA replication, ie S-phase of the first cell cycle (Howlett, 1986), although it is independent of the second S-phase (Bolton *et al*, 1984).

The third and subsequent cell cycle lengths, up to the expanded blastocyst stage (64 cells +), are shorter and in the region of 10-12h (reviewed by Pedersen, 1986). At the 16-cell stage and after blastocyst formation, with the differentiation of the trophectoderm (TE) and inner cell mass (ICM), the cell cycle lengths become dependent on the cell type, with outer TE cells initially dividing faster than ICM cells (MacQueen and Johnson, 1983; Handyside and Hunter, 1986).

Compaction begins at the 8-cell stage. Exposure of embryos to inhibitors of protein synthesis as early as the late 2-cell / early 4-cell stage does not prevent cell flattening or surface polarization, and gap junction formation still occurs in embryos inhibited from the mid 4-cell stage. Thus it appears that most embryos possess sufficient of the proteins required to achieve compaction well in advance of the normal time of compaction (Levy *et al*, 1986). Interestingly, incubation of embryos at the early 4-cell stage in anisomycin or puromycin (both inhibitors of protein synthesis) prematurely compacts the majority of embryos, indicating that some of the proteins synthesized during the 4-cell stage may be involved in suppressing compaction (Levy *et al*, 1986).

Blastocyst formation occurs with the completion of the fifth cleavage division. Nascent blastocysts have 30-34 cells (Smith and McLaren, 1977; Chisholm *et al*, 1985). Experiments involving aggregating 2- or 8-cell stages or bisecting 2-cell stages show that manipulated embryos cavitate at the same time as control embryos, even when they have twice or half the number of cells. This shows that the process of blastocoel formation is not dependent on cell number. Embryos incubated in Cytochalasin B, which prevents cell division by inhibiting microfilaments, for one cell division, also cavitate at the same time as controls, showing that the number of cell divisions is not a factor controlling the timing of cavitation either. It was

concluded that the time of formation of the blastocoel is controlled by either the nucleo-cytoplasmic ratio or the number of nuclear or chromosomal divisions or DNA replications since fertilization (Smith and McLaren, 1977). The latter possibility was eliminated by experiments whereby S-phase of the fourth cleavage division was inhibited with aphidicolin (a specific and reversible inhibitor of DNA polymerase α, therefore DNA synthesis). Cavitation was not delayed, indicating that the time of blastocyst formation was not controlled by the number of DNA replicative cycles completed since fertilization (Dean and Rossant, 1984). Evidence that the nucleo-cytoplasmic ratio is indeed crucial for the timing of blastocyst formation was found in the cow. When donor nuclei are fused to enucleated oocytes with only half the normal amount of cytoplasm, the nucleo-cytoplasmic ratio has effectively been doubled. Manipulated oocytes develop to the blastocyst stage approximately one cell cycle earlier than control oocytes and have half the number of blastomeres (First and Barnes, 1989).

### *§1.3.2 Timing of human preimplantation development*

In the human, information on the timing of preimplantation developmental events is sparse, due to a variety of reasons. There is an obvious reluctance to disturb embryos which are to be transferred more than absolutely necessary, thus observations on their development are made only once or twice a day, so that precise timings of cleavage divisions are not available. In addition, many embryos arrest during cleavage, so accurate lengths of cell cycles are difficult to obtain. The timings in Table 1.1 are based on the earliest times at which particular stages have been observed.

**Table 1.1:** *Approximate timing of human preimplantation development in vitro*

| Stage | Day post insemination | Time post insemination (h)* |
|---|---|---|
| 2-cell | 2 - 3 | 22-43 |
| 4-cell | 2 - 3 | 32-65 |
| 8-cell | 2 - 3 | 45-73 |
| 16-cell | 3 - 4 | 68-85 |
| Cavitating morula | 4 | 90 |
| Blastocyst | 5 | 110 |

* Trounson *et al*, 1982; Laufer *et al*, 1984; Mohr and Trounson, 1984.

Several pieces of evidence suggest that in the human the embryonic genome is activated between the 4- and 8-cell stages of preimplantation development. At this stage ribosomal particles mature into a functional morphological form (Tesarik *et al*, 1988), uptake and incorporation of uridine into ribosomes increases (Tesarik *et al*, 1986) and development is blocked for the first time by transcriptional inhibitors (Braude *et al*, 1988; Tesarik, 1989). Accompanying these events are changes in the types of proteins that are synthesized (Braude *et al*, 1988).

Compaction, in contrast with the mouse, appears to occur mainly later at the 16-cell stage (Dawson, personal communication; personal observation).

Blastocysts are seen from day 5 onwards (§2.3; §4.2; §6.3). This appears to be comparable to the few reliably timed observations *in vivo*. For example, Buster *et al* (1985) recovered 19 embryos by uterine lavage 5 days after artificial insemination, 5 of which (26%) had developed to the blastocyst stage. This is comparable with our observations in which 18/75 (24%) reached the blastocyst stage by day 5 *in vitro* (§4.2; §6.3).

It appears, therefore, that whilst the first three cell cycles *in vitro* are similar in the mouse and human, cell cycle lengths in human embryos lengthen after the 8-cell stage, with blastocysts appearing a day later than in the mouse (Fig. 1.1).

### §1.4 Allocation of cells to trophectoderm and inner cell mass lineages

The formation of the blastocyst represents the first differentiative step of mammalian embryogenesis, in which a morphologically identical cluster of cells develops, over the fourth and fifth cleavage divisions, into an asymmetrical structure consisting of two phenotypically distinct cell types. The mouse blastocyst consists of an outer layer of trophectoderm (TE) cells specialized for implantation and an inner cell mass (ICM) from which the fetus is derived (Gardner and Papaioannou, 1975). The TE transports metabolites between the maternal tissues and the ICM, initiates chemical and physical communication with the mother and provides a proliferative source of cells for the placental TE (reviewed by Johnson, 1989).

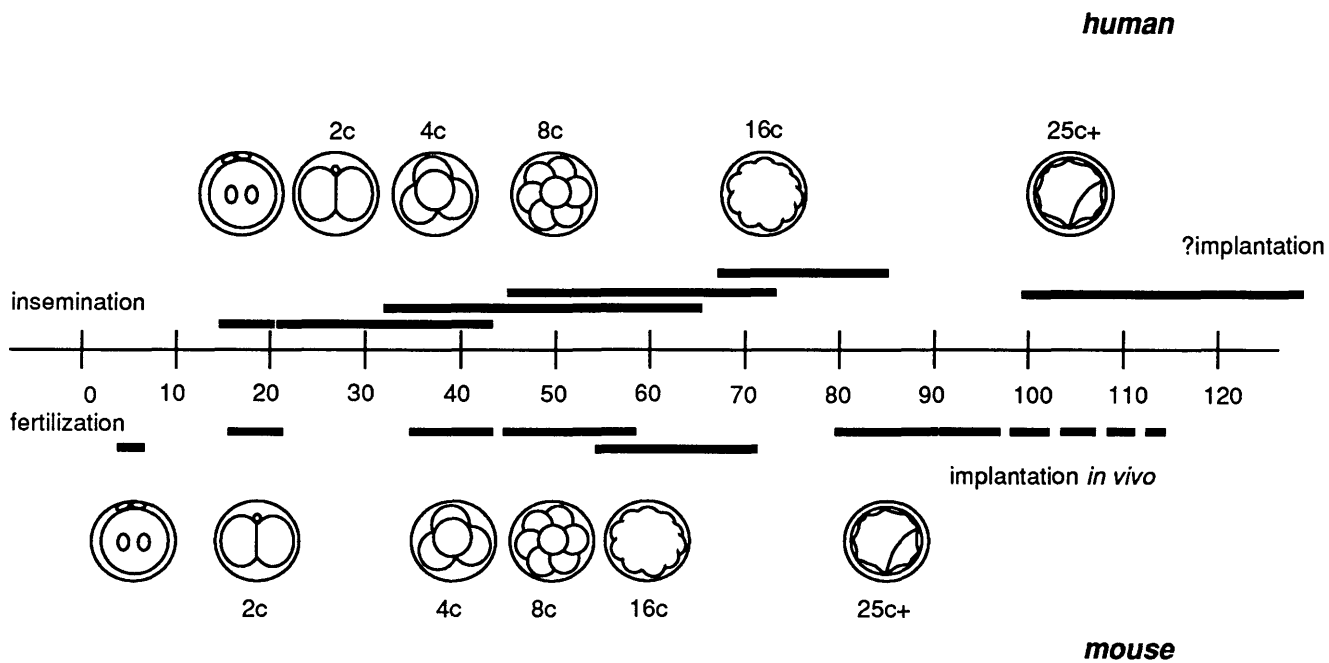



**Figure 1.1:** *Comparative timing of preimplantation development of human and mouse embryos in vitro. For human development, the bars represent the range of times at which these stages have been observed, and are not accurate because, firstly, human embryos are only scored once a day, and secondly, because some embryos will arrest at any preimplantation stage. For mouse development, the bars represent the range of times at which cleavage to a particular stage occurs. Timing of human development from Trounson et al, 1982; Laufer et al, 1984; Mohr and Trounson, 1984; Hardy et al, 1989a. Timing of mouse development from Bolton et al, 1984; Howlett and Bolton, 1985; Molls et al, 1983; Smith and Johnson, 1986*

### §1.4.1 Oocyte cytoplasmic gradients

Allocation to the TE and the ICM was originally thought to be determined in the fertilized oocyte. On the basis of histochemical studies on the distribution of acid and alkaline phosphatases, Dalcq (1957) postulated that the fertilized oocyte is bilaterally symmetrical and that one half, the "dorsal" zone, gives rise to the ICM and the other "ventral" zone, the trophoblast. It was proposed that as cleavage proceeds, the regionalised cytoplasm is partitioned between blastomeres, such that some blastomeres give rise to ICM and others the TE. Tarkowski (1959) examined whether such a proposal was valid by destroying, at random, one blastomere in embryos at the 2-cell stage. If regionalised cytoplasm was partitioned during cleavage, the resulting embryos should, at the blastocyst stage, be predominantly either ICM or TE. However, this was not the case, the majority of blastocysts which developed from "half" embryos contained both ICM *and* TE, suggesting that there was no partitioning of cytoplasm at the 2-cell stage. Furthermore, later experiments found no variation in the distribution of acid or alkaline phosphatases in the oocyte cytoplasm (Solter *et al*, 1973). In addition, Kelly (1977) isolated 4-cell and 8-cell blastomeres and found that such single cells, upon reaggregation with genetically distinct blastomeres to restore cell number, were totipotent and capable of contributing to both the TE and the ICM, effectively discounting any possibility of early segregation of blastomeres much before the 16-cell stage.

### §1.4.2 Inside:outside hypothesis

A more enduring hypothesis was proposed by Tarkowski and Wroblewska (1967) which postulated that the restriction in developmental potential occurred later in preimplantation development. They proposed that allocation to the ICM and TE depended on the number of enclosed cells at the morula stage with enclosed inner cells subject to a microenvironment which caused them to form ICM whilst exposed outer cells formed TE (Tarkowski and Wroblewska, 1967). This "inside:outside" or epigenetic hypothesis provided a convincing explanation for the observation that whereas blastomeres isolated from 2-cell embryos gave rise to smaller but morphologically normal blastocysts, blastomeres isolated from later stages gave rise, at the light microscope level, to an increasing proportion of TE vesicles with no visible ICM. Cavitation was not significantly delayed in blastocysts derived from isolated blastomeres, indicating that it was dependent on time, rather than cell

number. It was suggested that blastomeres isolated from later stages would have to pass through fewer intervening cells divisions before the time dependent event of cavitation, thus there would be a smaller number of cells at cavitation, fewer totally enclosed cells, resulting in embryos in which the ICM was reduced or totally eliminated.

Support for the inside:outside hypothesis was provided by Hillman *et al* (1972). The progeny of 4-cell blastomeres were followed either in the short term with [$^3$H] thymidine labelling or through to the postimplantation period by combining cells which synthesized different electrophoretic variants of glucose phosphate isomerase. It was found that when labelled 4-cell blastomeres were placed on the outside of other 4-cell embryos they (a) tended to form the outside layer of the blastocyst and (b) tended to develop into the yolk sac and trophoblast on the 10th day of pregnancy, suggesting that outside cells gave rise to the TE of the blastocyst and extraembryonic membranes of the fetus.

Barlow *et al* (1972) serially sectioned paraffin-embedded embryos and found that, on average, 16-cell embryos had one cell enclosed. Similarly analysed early blastocysts at around the 32-cell stage had approximately 6 cells inside. The inner cell would have to undergo very rapid division to produce this number of inside cells between the 16- and the 32-cell stage, therefore Barlow *et al* proposed that there was a first allocation to the inner position during the fourth cleavage division (8- to 16-cell stage) and that there was a subsequent internalization event during the fifth cleavage division. Also, labelling of the embryos with [$^3$H]thymidine showed that the inner cells had a higher labelling index, suggesting that the inner cells were indeed dividing faster than the outer cells (this, however, was later disputed by MacQueen and Johnson (1983), who isolated and identified inner and outer cells on the basis of size, inner cells being smaller, and observed that smaller cells had longer cell cycle lengths than larger cells). These estimates for the numbers of inner cells at the 16- to 32-cell stage are low compared with later studies. Handyside (1981) fluorescently labelled the outer surface of 16-cell stage morulae before disaggregating them and scoring the number of labelled, outer and unlabelled, inner cells. The number of inner cells ranged from 4.6 to 6.6 (range 1-8), depending on the method of blastomere separation and labelling used. Johnson and Ziomek (1981a) labelled polar cells with FITC-concanavalin A and found that in the majority of 16-cell embryos, the number of inside cells ranged from 6 to 8 (on average 7). Fleming (1987), using similar surface

labelling techniques in combination with marking the cytoplasm of outer cells with fluorescently labelled latex microparticles (which are endocytosed exclusively into the outer cells) found that the number of unlabelled, inside cells at the 16-cell stage ranged from 2 to 7 (on average 5.2). At the 32-cell stage Copp (1978) estimated approximately 13 inside cells on the basis of serial sectioning; Handyside (1978) observed 13-14 cells in air-dried immunosurgically isolated inner cell masses from early blastocysts and 12 in differentially labelled early blastocysts (Handyside and Hunter, 1986). Thus it has been consistently shown that at the 16-cell stage there are between 5 and 8 inner cells, and at the 32-cell stage there are between 12 and 14. One explanation for why Barlow's estimates are so low is that paraffin sections can shrink, exposing inner cells to the outside. Such exposed inner cells would be scored as outer cells reducing the number of observed inner cells.

Interestingly, Barlow's estimates do closely fit the inside:outside hypothesis on the basis of the packing of spheres. The closest way in which spheres can pack together is for four spheres to form a tetrahedron and larger arrays to be built up on the basis of this packing pattern (Sloane, 1984). By building plasticine models of "embryos" made up of equally sized spheres, it is possible to show that there are no internal spheres at the 8-cell stage (Daunter, 1986). The lowest number of spheres possible to enclose one sphere is thirteen (twelve on the outside, one inside). Structures built of nineteen spheres have two enclosed spheres; 23 have 3 enclosed; 28 have 4 enclosed and 29 (ie. approximately the 32-cell stage) have 5 enclosed spheres. This estimate of 5 enclosed cells at the 32-cell stage on the basis of sphere packing is lower than the observed number of inner cells of between 12 and 14 (Copp, 1978; Handyside, 1978; Handyside and Hunter, 1986). This discrepancy led investigators to re-examine the inside:outside hypothesis.

### *§1.4.3 Polarization*

Wilson *et al*, (1972) first proposed that there was a regionalization within the cells themselves which predisposed descendents towards an ICM or TE fate. They found that oil injected into the outside cortical region of 2- or 8-cell embryos led to the appearance of oil droplets in the TE, thus the outer part of the cell becomes predisposed to a TE fate and early cells pass this information on to their descendants. These results were confirmed by Graham and Deussen (1978), whereby oil droplets were injected either deep into the cytoplasm or into the cortical region of 8-cell blastomeres. Marker drops in the peripheral half of the cell usually contributed to the

TE (16/17 cases) whilst drops in the internal half of the cell segregated to the ICM (11/12 cases).

The 8-cell stage sees a change in the physical organization of the embryo. The blastomeres, which up to this stage have been morphologically identical cells, flatten upon one another to maximize intercellular contact, a process known as compaction. In addition, cells at the 8-cell stage develop a polar phenotype both cytoplasmically and at the membrane level (reviewed by Johnson and Maro, 1986). This polarization manifests itself in many ways: the cytoplasm exhibits a redistribution of organelles; cells are subject to an asymmetry of cell contacts; the apical membrane shows a markedly nonadhesive microvillous pole whilst the adhesive basolateral domain is relatively smooth, these two membrane zones being kept separate by zonular tight junctions. The axis of polarity develops radially in the intact embryo and the polarity appears to be induced by asymmetrical contacts with other cells, as shown by studying the axis of polarity which develops in couplets of 8-cell blastomeres, such that the microvillous apical pole develops at the surface most distant from all contact points (Ziomek and Johnson, 1980). The axis of polarity, once it has been laid down in the early 8-cell blastomere, is stable (Johnson and Ziomek, 1981b) and isolation of a cell or alteration of its position relative to other cells does not appear to disturb the axis and the cell realigns itself appropriately. This ability to realign is presumably as a result of the differential adhesivity of the apical and basolateral surfaces of the polarized cells (Kimber *et al.*, 1982; Johnson, 1985).

When the polarized 8-cell blastomeres cleave, two types of cleavage occur. If the cleavage plane is oriented parallel to the axis of polarity, two similar daughter cells result, both of them polar. This type of cell division is termed a conservative division, where the polarity of the 8-cell blastomere has been conserved in the two daughter progeny. If however, the 8-cell blastomere divides with the cleavage plane perpendicular to the axis of polarity, one daughter cells receives the outer polar portion of the cell, whilst the other daughter cell receives only the basolateral domain and becomes apolar and symmetrical (Fig. 1.2). This type of cleavage division is known as a differentiative division, because one polar and one apolar cell has resulted. Approximately 50% of 8-cell blastomeres undergo differentiative divisions, whilst the rest undergo conservative divisions, thus the resulting 16-cell embryo should consist of approximately 25% inner cells: 75% outer cells (Johnson and Maro, 1986). These observations were confirmed by Balakier and Pedersen (1982) where

single 8-cell blastomeres were labelled by iontophoretic injection of horseradish peroxidase. Cell division of the labelled cells gave rise to labelled cells in either just the TE or both the TE and ICM (in approximately 50% of the cases).

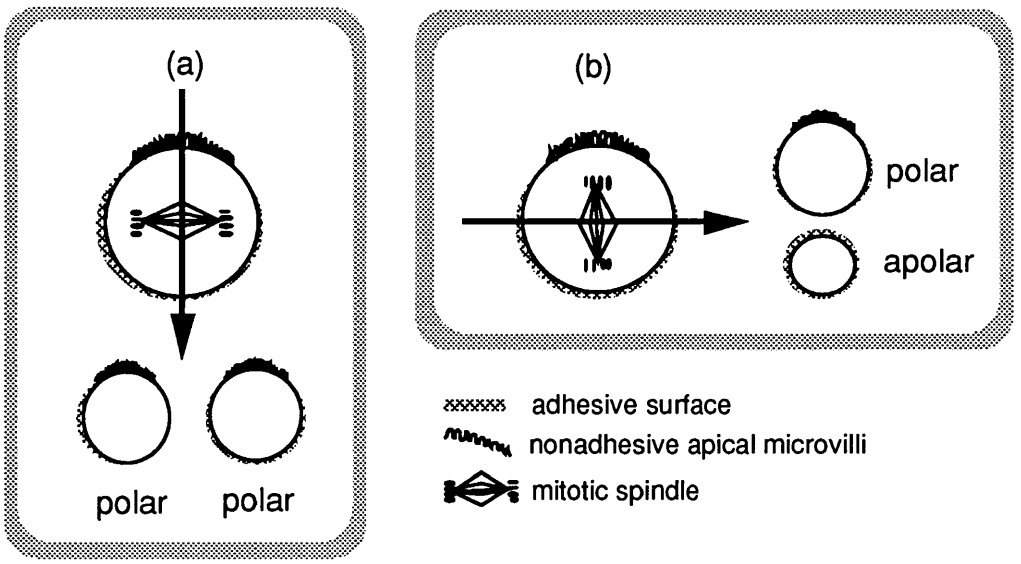



**Figure 1.2:** *Diagram to show the two different types of cleavage division which an 8-cell blastomere can undergo: (a) conservative, where the plane of the division is parallel to the axis of polarity, producing two polar progeny and (b) differentiative, where the plane of division is perpendicular to the axis of polarity, producing an apolar and a polar cell.*

### §1.4.4 Maintenance of TE and ICM lineages

The 16-cell embryo consists of two phenotypically distinct cell types: polar and apolar cells which differ in their adhesiveness and junctional properties. The differential adhesiveness of different domains of the 8-cell parent blastomere, with the microvillous apical pole being relatively nonadhesive and the basolateral domains being adhesive, is passed on to the 16-cell stage progeny, so that apolar daughter cells are uniformly adhesive whilst polar cells have adhesive basolateral surfaces and nonadhesive apical poles (Ziomek and Johnson, 1981; Kimber *et al.*, 1982). In the 16-cell embryo, the blastomeres try to maximize cell contacts between the adhesive surfaces, with the result that the uniformly adhesive apolar cells become internalized, whilst the polar cells remain at the surface of the embryo with their apical microvillous surface exposed. Polar and apolar cells maintain their relative positions within the embryo by their different adhesive properties (Burgoyne and Ducibella, 1977; Ziomek and Johnson, 1981, 82; Kimber *et al,* 1982; Surani and Handyside,

1983; Surani and Barton, 1984). Thus the number of inside cells in the 16-cell embryo will be determined by the ratio of differentiative to conservative divisions by polarized cells at the fourth cleavage division and the movements within the embryo of cells of different phenotypes to positions governed by their surface adhesiveness.

Surani and Handyside (1983) showed that when isolated, fluorescently labelled, polar and apolar 16-cell blastomeres are aggregated with unlabelled, partially decompacted 8-cell morulae, the majority of these phenotypically distinct cells relocate to their original position. They concluded that this confirmed the view that allocation partly depends on adhesiveness. However, the polar and apolar cells at the 16-cell stage are not totally committed to ICM or TE lineages, and if the ratio of polar to apolar cells is seriously perturbed, the embryo corrects for it. It is possible to identify polar outer cells and apolar inner cells on the basis of size. In 96% of 2/16 natural couplets (progeny of single 8-cell blastomeres), the larger cell is polar and the smaller is apolar (Johnson and Ziomek, 1981a). Ziomek *et al.* (1982) separated pure populations of outer and inner cells, on the basis of size, from 16-cell embryos and reaggregated them into 16-cell "embryos" of either inner or outer cells only. A pure population of apolar 16-cells forms a compact aggregate in which the outer cells are exposed to asymmetric contacts. These then proceed to polarize, like early 8-cell blastomeres, and so generate an outer layer of polar cells - reconstituting an embryo of both cell types. Conversely, an aggregate consisting exclusively of polar cells tends to generate a large number of apolar cells during the next mitotic division. These aggregates thus formed blastocysts which developed normally after transfer to pseudopregnant recipient mice. Another example of embryonic regulation of gross disturbances in the balance of early TE and ICM cells is the regeneration of TE-like cells by ICMs isolated later from early blastocysts and the regeneration of blastocyst-like structures (Handyside, 1978). However, ICMs isolated from expanded blastocysts were not able to reform blastocysts, suggesting that during blastulation TE and ICM cells finally become irreversibly committed to their fates (see below). Thus a dynamic process of cell distribution occurs to continually adjust the number of blastomeres in the two lineages.

Fleming (1987) developed a lineage marker for outer cells at the 16-cell stage and their progeny. Morulae incubated in medium containing fluorescently labelled latex microparticles took up the label by endocytosis into the outer cells. The label remained exclusively in the outer cells and was passed on to the progeny. Following

the fate of these outer cells established that the TE was derived exclusively from the outer polar blastomeres at the 16-cell stage. The ICM was mostly derived from the unlabelled inner population of cells, with evidence that about two cells were allocated in addition from the outer cells during the fifth cleavage division. Similarly, Balakier and Pedersen, (1982) found that injections of horseradish peroxidase into outer cells at later stages (16-cell/late morula) contributed fewer cells to the ICM. Dyce *et al* (1987) using the same technique of labelling outer cells, examined the 32- to 64-cell stage and found that during the sixth cleavage division there was very little exchange of cells between ICM and TE. Thus at the 16-cell stage the qualitative differences between the prospective TE and ICM lineages are established, at the 16- to 32-cell stage the embryo fine-tunes the allocation and after the 32-cell stage, during undisturbed embryonic development, the lineages are established and discrete, with no exchange of cells between them.

### *§1.4.5 Cell division order and allocation*

The allocation of cells to the ICM lineage appears to be influenced by the division order of cells at early cleavage stages. The second and subsequent cleavage divisions in the mouse are asynchronous, and one 2-cell blastomere divides before the other. It was found that there was a tendency for the progeny of the earlier dividing cell to contribute more to the inner cell mass. This was demonstrated by a series of elegant experiments. Barlow *et al.* (1972) serially sectioned nineteen embryos which had between 11 and 15 cells and contained at least one inside cell. Cells which had divided since the 8-cell stage could be clearly identified because of their smaller volume (cell volume is approximately halved during each of the first five cleavage divisions of mouse development). In these sections the inside cell was always a small cell and they concluded that the inside cell originated from the first 8-cell blastomere to divide. Kelly *et al.* (1978) labelled the progeny of the earlier dividing 2-cell blastomere with [$^3$H] thymidine and reaggregated these cells at the 8-cell stage with unlabelled host blastomeres. They found an increased contribution to the ICM of labelled cells. Surani and Barton (1984) confirmed this result by labelling late dividing 2-cell blastomeres with FITC and reaggregating them with unlabelled early dividers. In this case they found an increased contribution of unlabelled cells to the ICM. In these above experiments the differences were small, in the order of 2-3 cells. A greater effect was shown by Spindle (1982). Entire 8-cell embryos were labelled with [$^3$H] thymidine and aggregated with unlabelled 4-cells with the result that the

ICM at the expanded blastocyst stage consisted almost entirely of 8-cell descendants. In an attempt to counter any adverse effects of labelling cells with radiolabelled compounds, injected oil droplets were used as a marker (Graham and Deussen, 1978; Graham and Lehtonen, 1979). It was found that the earlier dividing progeny of the 2-cell stage moved inside and had more cell contacts with other cells. Garbutt *et al* (1987) examined early dividing cells later in cleavage, at the 8-cell stage. Using Fleming's (1987) technique of labelling outer cells with fluorescently labelled microparticles, the contribution of early dividing 4-cells to the inner cells was examined and it was found that these first formed 8-cell blastomeres contributed more to the ICM ie. they underwent more differentiative divisions than 8-cell blastomeres formed later. This was associated with earlier flattening of these cells. In conclusion, these experiments show that early in cleavage,cells divide asynchronously and that there is not a random distribution of cells to the ICM or TE: the progeny of earlier dividing cells tend to contribute to the ICM.

### *§1.4.6 Allocation in the human blastocyst*

The human blastocyst is morphologically similar to the mouse blastocyst (Edwards *et al*, 1981; Lopata *et al*, 1982; Mohr and Trounson, 1984; Tesarik, 1988). However, there is very little information on allocation to the ICM and TE. Hertig *et al* (1954) recovered two blastocysts, estimated to be 5 days post fertilization, from uteri removed at hysterectomy. Cell counts were made by counting nuclei in serial sections and were 58 and 107 cells of which 5 and 8, respectively, were considered ICM cells on the basis of their position. There is some ultrastructural evidence which shows that blastomeres of human 8-cell embryos undergo a similar process of surface polarization as described for mouse embryos at this stage, and that inner cells have different plasma membrane characteristics than outer cells (Tesarik, 1989). Beyond these sparse pieces of information, nothing is known about at what cleavage stage blastocyst formation occurs, how many cells are allocated to the TE or ICM, or about whether inner and outer cells are formed by differentiative and conservative divisions of polarized cleavage stage blastomeres.

### *§1.4.7 Overview of allocation*

Both the epigenetic and the polarization hypotheses focus on the importance of position as providing the information for differentiation. With the inside:outside hypothesis the positional information is acquired at the morula stage after the ICM

progenitors have been internalized and presumes that this positional information takes the form of microenvironmental signals. This theory obviously requires that there are sufficient cells in the morula such that some cells are enclosed. In contrast, the polarization hypothesis acts at the 8-cell stage, when all the cells are exposed. Here, the positional information takes the form of asymmetric cell contacts between blastomeres which positions a radial axis of polarization; inner cells derived from the differentiative cleavage of some of these polarized 8-cell blastomeres then assume inner positions. The inside:outside hypothesis is based on the position of whole cells relative to each other, the polarization hypothesis relies on differences within cells, from the apical surface to the basolateral surface.

In summary, during early cleavage stages, each cell is morphologically equivalent and totipotent. Following compaction at the 8-cell stage, cells polarize radially from the centre of the embryo, this polarization being determined by asymmetric cell contacts. The 8-cell blastomeres divide, either differentiatively to produce inner and outer progeny, or conservatively to produce outer progeny only. Thus at the 16-cell stage two phenotypically distinct cell types are present: small, apolar, uniformly adhesive inner cells and larger, polar, relatively non-adhesive outer epithelial cells. During the fifth cleavage division, from the 16- to the 32-cell stage, most outer and all inner cells undergo conservative divisions and "breed true", whilst one or two outer cells undergo differentiative divisions and contribute a few cells to the inside. In the normal, undisturbed embryo, there is little further exchange between the two lineages after the 32-cell stage. However, if the 16-cell stage embryo is grossly perturbed, for example all the polar or apolar cells removed, the embryo is capable of regulating and producing the necessary cell types before blastulation and implantation. Even ICMs isolated from early blastocysts are capable of regenerating into blastocysts, though this ability is lost with increasing age. It is thus possibly here, at the late blastocyst, that the two lineages are finally committed: the TE as an implanting and transporting epithelium, the ICM as a group of cells capable of forming all three germ layers in the fetus.

Whilst the generation of the two cell types which constitute inner and outer cells in the mouse is well understood, there is little information on the control of these divisions, and therefore the regulation of how many cells are allocated to the ICM and TE. The inside:outside hypothesis (§1.4.2) predicts that the packing of cells within the embryo determines the number of cells allocated to these two lineages, with the

number of cells in the TE being proportional to the surface area (Buehr and McLaren, 1974). However, the observation that isolated polarized 8-cell blastomeres can divide to produce daughter progeny of both inner and outer phenotype suggests that the packing of cells may not be such a dominant influence (Johnson and Ziomek, 1981a). Finally, in the human, there is virtually no information on allocation to the ICM and TE.

## §1.5 Metabolism of pyruvate and glucose by preimplantation embryos

### §1.5.1 Substrate requirements during preimplantation development *in vitro*

The first success at growing preimplantation mouse embryos *in vitro* in a defined culture medium was achieved by Whitten in 1956 when mouse embryos were grown from the 8-cell to the blastocyst stage in a Krebs-Ringer bicarbonate solution supplemented with glucose and bovine serum albumin (BSA). However, such a medium containing glucose as sole energy substrate did not support development before the 8-cell stage, an observation later confirmed by Brinster (1965a). The addition of lactate allowed a proportion of 2-cell embryos to develop to the blastocyst stage (Whitten, 1957), then Biggers *et al* (1967) found that pyruvate and oxaloacetate alone would support the first cleavage division. Late 2-cell stage embryos can only use lactate, pyruvate, phosphoenolpyruvate (PEP), and oxaloacetate (Brinster, 1965b). Successful development of mouse embryos from the 1-cell stage through to the blastocyst stage was finally achieved in medium containing 0.3mM pyruvate, 5.56mM glucose (used because of the similarity with plasma concentrations),and 21.5mM lactate (Whitten and Biggers, 1968), concentrations still widely used in embryo culture media.

In summary, the one-cell mouse embryo has an obligatory requirement for pyruvate (Cross and Brinster, 1973), a requirement which extends to the developing oocyte (Brinster and Harstad, 1977). The second and subsequent cleavage divisions can also be supported by lactate. It is not until the 8-cell stage that glucose can support development as sole energy substrate.

### §1.5.2 Methods of analysing metabolism of preimplantation embryos

Most studies of preimplantation metabolism have been carried out in the mouse, mainly because of the relative ease of collection of large numbers of embryos in this species and also because of the effective *in vitro* culture conditions which have been developed on the basis of the early investigations into the metabolic requirements of

these early embryos. Due to their small size and the relative insensitivity of conventional assay techniques, large numbers of embryos were required initially to carry out biochemical experiments. Such studies involved radiolabelled substrates with high specific activities. The fate of glucose in the embryo, for example, was studied by incubation in [U-$^{14}$C]glucose followed by extraction into the following components: glycogen (acid-soluble), proteins and nucleic acids (acid-insoluble) and lipids (extracted from the acid-insoluble fraction) (Pike and Wales, 1982). Radioactive $CO_2$ released can also be absorbed and measured with hyamine (Brinster, 1967a) or sodium hydroxide (Wales, 1986). However, this approach has some disadvantages. Within a population of embryos (even from the same female), there will be a degree of developmental asynchrony due to variations in the time of ovulation, fertilization, and the maturity of oocytes, resulting in some embryos being at more advanced stages than others. It is thus difficult to accurately assign a feature of embryonic metabolism to a particular stage. In addition, radioisotopes of high specific activity can damage development as demonstrated by Wiebold and Anderson (1985). Preimplantation mouse embryos were incubated with a tritiated amino acid which resulted in decreased development *in utero* after embryo transfer. This suggests that the embryo is being compromised by the exposure to labelled substrates of high specific activity, which makes it probable that under these suboptimal conditions the embryo metabolism is being disturbed,

During the last decade, sensitive microtechniques have been developed to analyse the metabolism of single or small groups of preimplantation embryos. Mroz and Lechene (1980) developed micropipettes capable of accurately delivering volumes in the nano and picolitre range, to assay femtomole amounts of urea. Pipettes delivered droplets of sample and reagent onto siliconized slides under mineral oil. Urea assays were carried out in siliconized capillary tubes and the reaction product quantified using a fluorescence microscope. This technique was modified by Leese *et al* (1984) to measure the nucleotide content of single mouse oocytes and embryos. Reactions are carried out in nanolitre-sized droplets on siliconized microscope slides under a layer of mineral oil, rather than in capillary tubes. The assays are based on a miniaturised version of the conventional methods of enzymatic analysis in which NADH or NADPH are generated or consumed in coupled reactions. These reduced pyridine nucleotides have absorption maxima at 340 nm and when excited with light in the UV range, emit fluorescence at 459 nm and above. By attaching a

photomultiplier to the fluorescence microscope to amplify the fluorescence output and a photometer to give a digital readout, the amount of fluorescence can be quantified and compared to standard curves. This technique has been extended to measure the uptake of glucose and pyruvate by single or small numbers of mouse embryos (Leese and Barton, 1984; Gardner and Leese, 1986). The advantages of this technique over conventional biochemical assays are that it can be applied to single embryos and is non-invasive, allowing repeated measurements to be made on the same embryo over several days. The ability to assess the metabolism of single embryos is particularly useful in domestic species and man in which one or only a few oocytes are produced in each cycle.

### *§1.5.3 Substrate utilization by preimplantation embryos*

Glucose has two main metabolic functions in preimplantation development: firstly as a source of energy and, secondly, as a precursor for the synthesis of macromolecules. The utilization of glucose can be studied by incubating embryos in radiolabelled glucose and measuring its incorporation into various metabolic products (see §1.5.2). Although glucose as sole substrate is unable to support development until the late 4-cell/early 8-cell stage, it can be metabolised to a limited extent at the zygote and early 2-cell stages. The production of energy from [U-$^{14}$C]glucose can be assessed either by measuring the production of organic acids such as lactate, an end product of glycolysis, or by measuring the formation of $^{14}CO_2$ via the tricarboxylic acid (TCA) cycle. $CO_2$ production from labelled glucose is low during the early cleavage stages but increases markedly at the 8-cell and morula stages (Brinster, 1967a). Wales (1986) incubated single mouse embryos in 500 nl microdrops of medium containing 5.56mM [U-$^{14}$C]glucose of high specific activity and measured both $^{14}$C-lactate production and $^{14}CO_2$ production. An increase in $CO_2$ production was found throughout early development, in agreement with the work of Brinster (1967a). However, quantitatively the levels were only 20 - 30% of those reported by Brinster (1967a). Lactate formation takes place throughout preimplantation development, with a marked rise at the 8-cell to morula stage which coincides with the switch to the preference for glucose (Brinster, 1967a; Wales, 1969; Wales, 1986). It appears that the increased production of $CO_2$ and lactate from glucose during preimplantation development parallels the embryo's increasing ability to utilize glucose as sole substrate. Thompson (1967) reported that fluoride ions, which inhibit the formation of phosphoenolpyruvate from 2-phosphoglycerate, arrest embryo development at the

8-cell stage. This observation is consistent with the idea that glycolysis becomes particularly important at this stage.

Similarly, the accumulation of radiolabelled glucose-derived carbon into potential energy stores such as glycogen and triglyceride or its incorporation into structural and informational molecules such as proteins, nucleic acids and phospholipids has been assessed. Brinster (1969) studied the appearance of carbon from glucose or pyruvate in glycogen throughout preimplantation development in the mouse. Glucose-derived carbon was accumulated by the embryo to a much greater extent than pyruvate-derived carbon throughout the preimplantation period, with a sharp increase in carbon incorporation at the 8-cell/morula stage.

The utilization of glucose by preimplantation embryos at the 8-cell to morula stage was followed in more detail using pulse-chase techniques, whereby embryos were incubated in [$^{14}$C]glucose for 1h followed by a 24h chase incubation in unlabelled glucose (Pike and Wales, 1982). Fractionation and assay of the embryos at intervals throughout the chase period allowed the radiolabel to be followed through a number of the embryos' metabolic pathways. Initially the glucose carbon enters the non-glycogen pool either in the form of glucose or as glucose-6-phosphate. The labelling in this pool decreases as the glucose is metabolized, either to glycogen or, via glycolytic and TCA cycle intermediates, to lipids, nucleic acids and proteins. However, despite optimum conditions, a large proportion of the glucose label was lost to the surrounding medium during the chase, and not used to synthesize glycogen.

When both pyruvate and lactate are present in the medium, the zygote and 2-cell stages preferentially metabolize pyruvate to $CO_2$. Throughout development, pyruvate is more effective in maintaining the activity of the TCA cycle. Both lactate and pyruvate may be utilized by the preimplantation embryo. When provided as sole substrates, incorporation of carbon from lactate is less than that from pyruvate. However, if both substrates are supplied together, overall incorporation is increased, with more carbon incorporated from lactate than from pyruvate (Quinn and Wales, 1973).

### *§1.5.4 Substrate uptake by preimplantation embryos*

The uptake of pyruvate and glucose from the culture medium by single mouse embryos was measured non-invasively throughout preimplantation mouse development. Pyruvate uptake exceeded that of glucose until the 8-cell to morula stage, whereupon glucose consumption increased dramatically and pyruvate uptake

fell (Leese and Barton, 1984; Gardner and Leese, 1986). It is interesting to note that despite the inability of embryos to survive before the 8-cell stage in the presence of glucose alone, the uptake of this sugar was detected at low levels prior to this stage.

### §1.5.5 Transport of substrates by preimplantation embryos

Glucose as sole energy substrate is unable to support preimplantation mouse development before the 8-cell stage (§1.5.1), its utilization increases after this stage (§1.5.3) as does its uptake (§1.5.4). It is possible that the embryo is unable to transport glucose from the extracellular environment across the plasma membrane at early stages. The mechanisms by which nutrients can enter cells and their characteristics are summarized in Table 1.2.

**Table 1.2:** *Summary of membrane transport mechanisms for small molecules*

| **Passive Diffusion** | **Carrier mediated transport** | |
|---|---|---|
| • Lipid-soluble solute diffuses across membrane. | • Solute's transport across membrane is facilitated by a carrier protein. | |
| • Non-specific | • Specific | |
| • Non-saturable: dependent on the concentration gradient of solute across the membrane | • Saturable: uptake dependent on a discrete number of carriers in the membrane. | |
| • Rate proportional to concentration gradient | • Initial rate of transport is higher by carrier mediated transport than by simple diffusion. | |
| | **Facilitated diffusion** | **Active Transport** |
| • Energy independent | • Energy independent | • Energy dependent |
| • Transport from higher to lower concentrations | • Transport from higher to lower concentrations | • Transport from lower to higher concentrations. |
| | • Can be specifically inhibited, eg. glucose transport in erythrocytes can be inhibited by phloretin. | • Can be specifically inhibited eg. by phlorizin |
| | | **Primary** / **Secondary** |
| | | Energy provided directly by hydrolysis of ATP eg. $Na^+$-$K^+$ ATPase / Active transport creates ion gradient to which transport.of other molecules is coupled |

There is often more than one process involved in the transport of a substance into a cell. Simple diffusion of substrates into the cell, from a high to a low concentration, can be complemented by carrier transport. In addition, the same substrate can be transported into different cell types by different mechanisms, depending both on the environment and the function and needs of the cell type. In erythrocytes, the rate of uptake of D-glucose is a typical example of facilitated diffusion. After glucose is transported into the erythrocyte, it is rapidly phosphorylated, forming glucose 6-phosphate, which is unable to leave the cell. Glucose concentration in the cell is lowered, increasing the concentration gradient for this sugar across the plasma membrane and therefore allowing facilitated diffusion to continue. In contrast, in the small intestine, where the role of the intestinal epithelium is to transport substrates from the intestinal lumen to the bloodstream, glucose can be transported against its concentration gradient by a specific glucose-$Na^+$ symport protein, ie. by a secondary active transport mechanism. The transmembrane movement of glucose is coupled to the transport of one $Na^+$ ion. A low $Na^+$ concentration is maintained in intestine cells by a $Na^+K^+$-ATPase located in the basal lateral membranes. At the apical luminal surface of the cell, $Na^+$ transport into the cell is driven both by concentration gradient (the $Na^+$ concentration is lower inside the cell) and by the negative inside membrane potential. This combined electrochemical gradient can pump glucose into the cell against a concentration gradient. It is thought that both $Na^+$ and glucose bind to the same carrier protein, induce a conformational change which generates a transmembrane pore allowing both glucose and $Na^+$ to be transported simultaneously into the cytosol. Glucose leaves the cell by facilitated diffusion at the baso/lateral membrane.

Until recently, the mechanism of glucose transport into preimplantation embryos has been poorly understood. The first evidence that glucose might be transported by a carrier mediated mechanism was provided by Wales and Brinster (1968). The same amount of labelled glucose was taken up by 2-cell and 8-cell embryos after a 30 minute incubation in [U-$^{14}$C]glucose. However, with longer incubations there was an increase in 8-cell but not 2-cell uptake, which led to the hypothesis that glucose entry was limiting up to the 8-cell stage and responsible for the failure of glucose to support early cleavage stage development.

By incubating embryos in increasing substrate concentrations, Wales and Whittingham (1974) demonstrated that pyruvate and lactate can enter the 1- and 2-cell

embryo by simple diffusion. However, this cannot be the only mechanism for pyruvate entry, since more pyruvate than lactate accumulated in the embryo when the two nutrients were present at equal concentrations in the medium (Wales and Whittingham, 1967). Leese and Barton (1984) investigated transport mechanisms in unfertilized mouse oocytes by incubating them in increasing concentrations of pyruvate (up to 5mM). Pyruvate uptake increased with increasing medium concentration but not linearly as would be expected for simple diffusion, nor did it saturate as would be expected if facilitated diffusion were the sole uptake mechanism; rather it was a combination of the two. With α-cyano-4-hydroxycinnamate, an inhibitor of facilitated pyruvate transport in erythrocytes and mitochondria, there was a decrease in pyruvate uptake to a rate directly proportional to pyruvate concentration in the medium, indicating that pyruvate was transported into mouse oocytes by a combination of simple and facilitated diffusion. Similar studies were undertaken by Gardner and Leese (1988) with fertilized mouse embryos throughout preimplantation development. Measurements of glucose uptake by mouse blastocysts at increasing concentrations of exogenous glucose, with and without phloretin (an inhibitor of glucose facilitated diffusion), showed a similar pattern of uptake, again suggesting a combination of facilitated and simple diffusion was operating. From the 2-cell stage onwards, the majority of glucose uptake is via a facilitated mechanism, with a sharp increase between the 8-cell and the blastocyst stage. Simple diffusion in the presence of phloretin was negligible at early stages but also increased sharply at the blastocyst stage, presumably due to the increased utilization of glucose occurring at this stage. Increased utilization would decrease the concentration of glucose in the embryo, maintaining a concentration gradient relative to the medium and increasing passive diffusion. Thus, whilst simple diffusion is negligible up to the 8-cell stage, the embryo can take up glucose via a facilitated mechanism, suggesting that the embryos' low utilization of glucose early on is not due to the absence of transport mechanisms. Incubating embryos with either the active transport inhibitor phlorizin or in the absence of $Na^+$ ions (which inhibits $Na^+$-coupled glucose transport) had no effect on glucose uptake, showing that the entry of this sugar into the blastocyst was not an active process. In addition, the inhibition studies with α-cyano-4-hydroxycinnamate were extended to preimplantation mouse embryos, confirming that there was also a carrier mediated mechanism operating for pyruvate uptake. Similarly, the bulk of pyruvate uptake from the 1-cell stage onwards is via a facilitated transport

mechanism, which increases steadily at the blastocyst stage. This indicates that the switch to a glucose-based metabolism after compaction is not due to the loss of a pyruvate transport system. In addition, if the embryo is deprived of glucose after compaction, the characteristic decline in pyruvate uptake does not occur, showing that the embryo can adapt to its environment in culture by altering its substrate preference.

In summary, it appears that in addition to simple diffusion into embryonic cells, particularly at the blastocyst stage, carrier mediated (facilitated) transport mechanisms are operating for both pyruvate and glucose which increase at the blastocyst stage. Active transport does not appear to play a role.

### *§1.5.6 Human preimplantation embryo metabolism*

In contrast with the detailed information available on the changing metabolic requirements of mammalian embryos, particularly those of the mouse, little is known about the metabolism of human preimplantation embryos. Using a non-invasive technique (§1.5.2), Leese *et al* (1986) found that pyruvate consumption by single human oocytes and embryos was high in the mature oocyte, fell after fertilization, then increased to a peak just prior to the morula stage before declining again. This pattern is similar to that identified in mouse oocytes and embryos (Leese and Barton, 1984; Gardner and Leese, 1986), but the overall consumption of pyruvate by human embryos is higher at all stages than in mouse embryos, possibly due to the greater size of the human embryo. However, no information was obtained on glucose uptake. Wales *et al* (1987) found that glucose turnover, as measured by $CO_2$ production, lactate accumulation and carbon incorporation from labelled [U-$^{14}$C]glucose was initially low, before increasing dramatically between days 2 and 3. Thus it appears that, as in the mouse, there may be an initial blockade to glucose utilization in early human embryos.

### *§1.5.7 Overview of embryonic metabolism*

Preimplantation mouse embryos can be successfully cultured from the 1-cell to the blastocyst stage in a simple salt solution supplemented with the energy substrates glucose, pyruvate and lactate (Whitten and Biggers, 1968; Whittingham, 1971). During early cleavage, pyruvate is the major energy substrate (Brinster, 1965a). At the 8-cell/morula stage there is a switch in substrate preference in favour of glucose, which becomes the predominant energy source at the blastocyst stage (Gardner and

Leese, 1986). Entry of pyruvate and glucose is via both simple diffusion and a carrier-mediated (facilitated) mechanism (Gardner and Leese, 1988).

The inability of preimplantation embryos to take up and utilize glucose during early stages could result from one of several possibilities. Firstly, by measuring levels of some of the metabolites of the glycolytic pathway (glucose-6-phosphate, fructose-1,6-bisphosphate, citrate and malate) in individual embryos during starvation and refeeding with glucose or glucose+pyruvate, Barbehenn *et al* (1974) obtained evidence which suggested that glucose utilization during early preimplantation stages was blocked at the level of the glycolytic enzyme 6-phosphofructokinase. Secondly, although glucose can be transported into the embryo during early stages by simple and facilitated diffusion, both these transport systems increase markedly at the 8-cell to morula stage. This does not provide unequivocal evidence that a lack of transport mechanisms inhibits glucose uptake early on, but it is certainly feasible that glucose uptake is enhanced after the morula stage by an increased transportation of glucose.

The reasons for this switch from a pyruvate-based to a glucose-based metabolism remain obscure, but it is notable that several important events occur at this stage of development. Firstly, the embryonic genome is activated (Flach *et al*, 1982; West and Green, 1983; Bolton *et al*, 1984) at the late 2-cell/early 4-cell stage, from which there follows a surge of transcriptional activity and protein synthesis. Glucose is a precursor of many macromolecular cell constituents, such as ribose moieties for nucleic acid biosynthesis, glycerol phosphate for the formation of phospholipids; complex sugars in mucoproteins and mucopolysaccharides; the surge in synthesis may, therefore, demand an increased requirement for glucose. Secondly, the process of compaction and blastocoel cavity formation impose a heavy demand for ATP, therefore increasing the embryo's metabolic requirements. Finally, the switch in substrate requirement coincides with the time at which the embryo moves from the oviduct to the uterus. Brinster (1967b) has suggested that this change of environment could modify the embryo's metabolic requirements.

In the mouse preimplantation embryo much is known about the pattern of substrate uptake and utilization. However, it is not known how nutrient uptake is controlled, whether at the membrane level, by the existence or efficiency of transport mechanisms; or at the intracellular level, by the utilization of substrates (Biggers *et al*, 1989). In the human embryo, there is very little information on substrate uptake or utilization, and, if the *in vitro* environment of embryos after IVF is to be improved,

with a resulting improvement in transfer success rate, this is an area which needs further study.

### §1.6 Size regulation

In mammals, given adequate diet and health, the progression to final size, ie. growth, is sufficiently characteristic of a species or strain to enable the prediction of what the length, height, width and weight of an individual should be at a given age (Snow *et al*, 1981).

It is possible to manipulate the mammalian preimplantation embryo and either decrease or increase the cell number, both techniques being valuable in elucidating mechanisms of size regulation, twinning, allocation to the inner cell mass (ICM) and trophectoderm (TE) in the blastocyst and subsequent cell potential and cell lineage analysis. Reduction in cell number can be brought about by the destruction of blastomeres within the zona pellucida (Tarkowski, 1959; Hoppe and Whitten, 1972; Rands, 1985/6b; Papaioannou *et al*, 1989), separation of blastomeres (Rossant, 1976; Fiser and McPherson, 1976; Tsunoda and McLaren, 1983), bisection of compacted embryos (Moustafa and Hahn, 1978; Nagashima *et al*, 1984; Nakagawa *et al*, 1985), or bisection of blastocysts (Gardner, 1974; Ozil, 1983). Increase in cell number can be brought about by embryo aggregation at cleavage stages (Tarkowski, 1961; Mintz, 1962; Markert and Petters, 1978) or injection of cells into blastocysts (Gardner, 1978). Methods have been adapted and applied to rats (Matsumoto *et al*, 1989), rabbits (Moore *et al*, 1968; Yang and Foote, 1987) and domestic species (reviewed by Papaioannou and Ebert, 1986; Fehilly and Willadsen, 1987). Most importantly, transfer of such manipulated embryos produces viable offspring of normal size, which are born at the expected time, showing that embryos are capable of regulating their size during gestation.

In the mouse, Buehr and McLaren (1974) examined the timing of size regulation in 'double' embryos made from two aggregated 8-cell embryos, or 'single' control embryos, by estimating the volume of the ICM or egg cylinder in histological sections. They concluded that size regulation was completed before proamniotic cavity formation, which was earlier than suggested by Lewis and Rossant (1982) in similar experiments. Lewis and Rossant (1982) found that 'double' embryos remained twice the size of 'single' embryos until at least 5d 16h post coitum (p.c.), and that size regulation occurred rapidly over the next 24h period, so that by 6d 16h p.c. there was no difference in cell number between double and control embryos. In

the strain of mice used, proamniotic cavity formation occurred around 5d 6h p.c. Size regulation was found to occur in all tissues of the embryos over roughly the same period. As well as examining the precise timing of size regulation, Lewis and Rossant (1982) attempted to elucidate the mechanisms which control size regulation by labelling embryos with [$^3$H]thymidine to assess the proportion of actively dividing cells within the embryo, and treating embryos with colcemid to analyse mitotic activity. Several different mechanisms were postulated which could account for size regulation. Firstly, it was suggested that double embryos might experience enhanced cell death, although no obvious increased level of necrosis was observed in double embryos. Also, labelling with [$^3$H] thymidine showed that >90% of the cells were synthesizing DNA. This evidence also discounted the second possibility, that there was a large non-dividing population of cells. The final possibility was that double embryos might show an increase in cell cycle length compared to that of controls. Indeed, double embryos incubated in colcemid, an inhibitor of microtubule assembly which blocks cells in mitosis, showed a much lower mitotic index in all tissues, indicating a longer cell cycle, in comparison to single embryos. It was concluded that the proliferative burst seen around 6d 8h p.c. in 'single' embryos did not occur in 'double' embryos, so that by 6d 16h p.c. they were equivalent in size to 'single' embryos. However, it was not possible to ascertain whether the control of the proliferative activity in 'double' embryos is exerted by the embryo itself or by the uterine environment.

The observation that whilst larger embryos down-regulate in size between approximately 5.5 and 6.5d p.c. (Lewis and Rossant, 1982), embryos reduced in size during cleavage appear to up-regulate at a later time, between 9 and 10.5d p.c. (Tarkowski, 1959; Rands, 1986b) may reflect the changing conditions of maternal-fetal relationships. Between implantation and fertilization, whilst the embryo is spending 3 days passing down the oviduct and a further 2 days "free-living" in the uterus, it is thought to derive its nutrients from oviduct and uterine fluid, depending to a much lesser extent on its own endogenous energy stores. Between implantation and placentation the embryo is dependent on nutrients supplied from the tissues of the uterine wall. In the mouse, implantation involves the invasion of trophoblast through the endometrium into the uterine decidual tissue followed by permeation of the trophoblast by a system of cavities known as the trophoblastic lacunae. Further trophoblastic invasion between 6 and 6.5 days p.c. erodes the endothelium of the

uterine capilliaries and maternal blood floods into the lacunae, allowing maternal nutrients in the blood to become available to the fetal tissues over the large surface area of the reticulated trophoblast. The downregulation in the size of double embryos (Lewis and Rossant, 1982) between 6 and 6.5 days therefore coincides with the dependence of embryonic nutrition on the diffusion of substrates across the decidual tissue, prior to placentation. Thus, as embryos increase in size, their surface area to volume ratio decreases and the availability of nutrients may become limited, affording a possible explanation for downregulation (Snow *et al*, 1981; Rands,1986a). Conversely, upregulation of half embryos coincides with the development of a fully functioning chorio-allantoic placenta at between 9.5 and 10.5 days. It may be that for the embryo to "catch up" and effectively double its size, its nutritional requirements are above average and cannot be satisfied until a fully functioning placenta is formed (Snow *et al*, 1981; Tarkowski, 1959).

### §1.7 Preimplantation biopsy

Currently, in the human, prenatal diagnosis of inherited disease involves recovering cells of fetal origin by chorion villus sampling or amniocentesis between 8 and 20 weeks of gestation, prior to cytogenetic, biochemical or molecular analysis. If the fetus is diagnosed as affected, therapeutic termination can be offered. However, the accessibility of preimplantation embryos after *in vitro* fertilization (IVF) raises the possibility of preimplantation diagnosis, in which embryos are biopsied to remove a cell or cells for genetic analysis and unaffected embryos identified and transferred to the uterus to establish a pregnancy. In this way, many first and second trimester abortions could be avoided.

Edwards (1965) first suggested that sex-linked recessive disorders could be diagnosed in the preimplantation embryo by sexing blastocysts and discarding those of affected sex. Soon after, rabbit embryos were successfully sexed by examining sex chromatin in trophoblast cells removed by micromanipulation from blastocysts (Gardner and Edwards, 1968). In the mouse, cleavage stage embryos have been sexed by dislodging one blastomere from a zona-free 8-cell embryo, by gentle pipetting, and assaying the single blastomere for the presence of an X-linked enzyme, hypoxanthine phosphoribosyl transferase (HPRT) using a biochemical microassay (Monk, 1987). As both X-chromosomes are active at the 8-cell/morula stage (Epstein *et al*, 1978), female embryos can be identified on the basis of having a double dose of the X-linked product, HPRT, and on this basis it was possible to accurately predict

the sex of fetuses after transfer of the biopsied embryos (Monk and Handyside, 1988). Using the same technique it has also been possible to diagnose specifically HPRT-deficient embryos (a deficiency which, in the human, causes Lesch-Nyhan Syndrome) (Monk *et al*, 1987).

Theoretically, embryos can be biopsied at any stage between the 2-cell and the expanded blastocyst stages, ie. between days 2 and 7 for embryos fertilized and cultured *in vitro*. The advantages and disadvantages of biopsy at cleavage stages or at the blastocyst stage will be discussed below.

### §1.7.1 Cleavage stage biopsy

At cleavage stages, each cell of the mammalian embryo remains totipotent and can contribute to all extraembryonic and embryonic lineages. There is evidence that the preimplantation embryo is able to survive having cells removed, reducing the cellular mass, both in the mouse and the human. Other mammalian embryos also have a remarkable capacity to compensate for reduced cellular mass at preimplantation stages (§1.6). For example, single early cleavage stage blastomeres transferred to recipient females have produced live offspring in a variety of laboratory and domestic species (reviewed by Papaioannou and Ebert, 1986). However, as cleavage proceeds, the proportion of cellular mass in each blastomere is progressively reduced (Lehtonen, 1980), and there is a limit to how much the cellular mass can be reduced without affecting implantation and postimplantation development. In the mouse, single blastomeres isolated at the 2-cell stage (1/2) give rise to live offspring (Tarkowski, 1959; Hoppe and Whitten, 1972; Papaioannou *et al*, 1989). Single blastomeres isolated from 4-cell stage (1/4) embryos implanted but only in one case was a retarded egg cylinder formed and blastomeres from 8-cell stage embryos (1/8) only rarely implanted (Rossant, 1976). This clearly indicates that there appears to be a lower limit to the cellular mass of the embryo compatible with implantation and development. Therefore, if cells are to be removed during cleavage stages, the reduction in cellular mass should be minimized, thus it is preferable to biopsy a cell at the 8-cell stage, when a lower proportion ie. 1/8 of the embryo, will be removed, rather than at the 2-cell stage, when half will be removed.

Any effect of biopsy on preimplantation embryos on implantation or postimplantation development can only be assessed after transfer. In the mouse, biopsy at the 4- or 8-cell stages and removal of 1/4, 1/8 or 2/8 has no effect on blastocyst formation, implantation or postimplantation development after transfer to

pseudopregnant recipients (Monk and Handyside, 1988; Wilton *et al*, 1989). In the human, there is anecdotal evidence that embryos with reduced cellular mass are capable of implantation and normal development. Also, the first pregnancy to be reported for a cryopreserved embryo resulted from the transfer of an embryo in which only five out of the eight blastomeres survived thawing (Trounson and Mohr, 1983). Although the patient suffered a late miscarriage, examination of the fetus indicated that there were no gross abnormalities.

There are several techniques by which one or two cells can be removed from cleavage stage embryos. The simplest is to remove the zona pellucida totally and to dislodge a cell by gentle pipetting with a flame polished pipette of diameter just smaller than the embryo (Monk *et al*, 1987; Monk and Handyside, 1988). This technique has the disadvantage that the embryo would then be transferred without the protection of the zona. Whilst transferring embryos to pseudopregnant recipients after allowing them to develop to the blastocyst stage is compatible with postimplantation development in the mouse, there is nothing known about the ability of zona-free embryos to survive after transfer in the human. A second technique, which while technically more difficult, retains most of the zona pellucida, involves immobilizing the embryo by suction using a micromanipulator controlled flame-polished holding pipette. A hole can be made in the zona pellucida, either mechanically using a sharpened, bevelled aspiration pipette, which is pushed through the zona pellucida (Wilton and Trounson, 1989), or by "drilling", whereby a square-ended pipette is placed against the zona pellucida and acidic medium is expelled locally, dissolving a hole (Gordon and Talansky, 1986; Handyside *et al*, 1989). In both cases an aspiration pipette can be inserted through the hole and one or two blastomeres aspirated.

This technique has the disadvantage that cellular mass is reduced and one or two totipotent cells which are likely to contribute to both embryonic and extra-embryonic lineages are removed. However, transfer of human embryos at cleavage stages appears to be optimal for pregnancy success after transfer.

### *§1.7.2 Blastocyst stage biopsy*

An alternative to cleavage stage biopsy is to remove only trophectoderm (TE) cells from blastocyst stage embryos. It has been observed that TE cells will herniate spontaneously out of the zona pellucida during the process of hatching (Monk *et al*, 1988; Summers *et al*, 1988). This phenomenon can be utilized by allowing TE cells

to extrude through an artificially created hole or slit in the zona pellucida, produced as described in §1.7.1, and excising the herniated cells using glass needles. This technique has several advantages: firstly, more cells can be biopsied than at cleavage stages leading to a more reliable diagnosis and secondly, only TE cells, which in rodents form exclusively extraembryonic tissues, are removed, leaving an intact ICM, from which the fetus is derived.

TE cells have been biopsied from rabbit (Gardner and Edwards, 1968), mouse (Monk *et al*, 1988), marmoset (Summers *et al*, 1988) and human embryos (Dokras *et al*, 1990). In the case of mouse, rabbit and marmoset, pregnancies were established after transfer of the biopsied blastocysts. However, in the human, this approach is currently confounded by the disappointing pregnancy success rate after transfer of blastocysts during routine IVF (Dawson *et al*, 1988; Bolton *et al*, 1991). In addition, only a low percentage of embryos reach the blastocyst stage *in vitro* (Bolton *et al*, 1989; Dawson *et al*, 1988), thus there will only be a limited number of blastocysts to biopsy and the number of unaffected embryos available for transfer is likely to be reduced.

Because of the limited success of transferring blastocysts and obtaining pregnancies, the main interest has been focussed on cleavage stage biopsy. However, before attempting transfer of biopsied embryos, any resulting damage needs to be carefully assessed, as far as possible, *in vitro*. One obvious parameter which can be examined is the ability of biopsied embryos to develop to the blastocyst stage. Further, as cells which have the potential to give rise to both the embryonic and extra-embryonic lineages are removed by this procedure, the effect on the ICM, from which the fetus is derived, needs to be carefully assessed, to check that allocation to this lineage is not compromised by the procedure, which would lead to biochemical pregnancies.

# §2 Allocation to trophectoderm and inner cell mass in human blastocysts

## §2.1 Introduction

In the mouse blastocyst, there are two distinct cell types: an outer epithelial layer of trophectoderm (TE) responsible for blastocoel fluid accumulation and specialized for implantation, and an inner cell mass (ICM). Following implantation, the TE only gives rise to components of the placenta and extraembryonic membranes whereas the ICM forms all three germ layers of the fetus as well as complementary contributions to the extraembryonic membranes (Gardner and Papaioannou, 1975). Allocation of cells to these two primary lineages occurs at the preceding morula stage mainly during the fourth cleavage division (Handyside, 1981), although an additional contribution from the outer cells to the ICM has been demonstrated during the fifth cleavage division (Pedersen, 1986; Fleming, 1987; §1.4).

The numbers of TE and ICM cells in mouse blastocysts developing *in vivo* have been studied by serial sectioning (Copp, 1978) and more recently by differential labelling of the nuclei with polynucleotide-specific fluorochromes (Handyside and Hunter, 1984, 1986). Over a period of 36h following blastocyst formation, the number of TE cells increases exponentially before reaching a plateau. This coincides with the initiation of endoreduplication and giant cell formation in the mural TE surrounding the blastocoel cavity during implantation (Barlow *et al.* 1972). At the same time, cell number increase in the ICM slows down and there is a wave of embryonic cell death, or apoptosis (El-Shershaby and Hinchliffe, 1974; Copp, 1978; Handyside and Hunter, 1986). Hence, the proportion of ICM cells steadily declines as the blastocyst expands, hatches and begins to implant.

With the human embryo developing *in vitro*, compaction occurs late on day 3 following *in vitro* fertilization (IVF), between the 8- and 16-cell stages, blastulation on day 5 or 6 (§4.2; Hardy *et al*, 1989b), and implantation *in vivo* between days 7 and 10. The time taken to complete the first three cleavage cycles is similar to the mouse, on average about 60h (§1.3; Edwards *et al*, 1981; Sundstrom *et al*, 1981; Trounson *et al*, 1982). At later stages there is very little information about cell numbers or the allocation of cells to the TE and ICM at the blastocyst stage. By differentially labelling the nuclei of the TE and the ICMs of human blastocysts developing *in vitro*, it has been possible to assess cell numbers in newly expanded

blastocysts on day 5, the increase in cell numbers on days 6 and 7, and the allocation of cells to the two lineages. In addition, polyspermic and parthenogenetic blastocysts and the nuclei of a series of embryos arrested at cleavage and morula stages have been examined. The results suggest that the numbers of cells allocated to the TE and ICM of the human blastocyst are similar to the mouse. However, despite evidence of increasing cell death between days 5 and 7 in both the TE and ICM, the proportion of ICM cells remains higher.

## §2.2 Method

Both normally and abnormally fertilized surplus human embryos were allowed to develop either in 1ml of culture medium (§8.1.2) or in 20µl of the same medium under paraffin oil. All embryos which developed to the blastocyst stage were selected for labelling on days 5 to 7. The TE and ICM nuclei of blastocysts were differentially labelled with polynucleotide specific fluorochromes (§8.4.3) using a modification of the method described for mouse embryos (Handyside and Hunter, 1984; §8.4.2). The modification involved treating human blastocysts with trinitrobenzenesulphonic acid (TNBS) to label cell surface proteins with covalently bound trinitrophenol (TNP) groups (§8.4.3) (Fig. 2.1). This allowed substitution of a rabbit antiserum against dinitrophenol (DNP) (which cross-reacts with TNP-labelled proteins, for the anti-species antiserum used with mouse blastocysts for immunosurgery (Surani and Handyside, 1983; §8.4.2). Preliminary experiments with fluorescein conjugated goat anti-rabbit antiserum demonstrated uniform labelling of the outer cells of human embryos at cleavage and blastocyst stages.

Labelled blastocysts were examined as described in §8.5.2 and the numbers of TE and ICM cells counted. In addition, the numbers of dead TE and ICM cells and the number of those in metaphase were ascertained. Estimates of cell numbers in blastocysts are based on the assumption that they are equivalent to the number of nuclei counted. Multinucleate cells are commonly observed in normally fertilized early cleavage stage embryos. However, they are considered to result from defective migration of chromosomes at mitotic anaphase (Tesarik *et al*, 1987) and are therefore unlikely to make a major contribution to more advanced embryos.

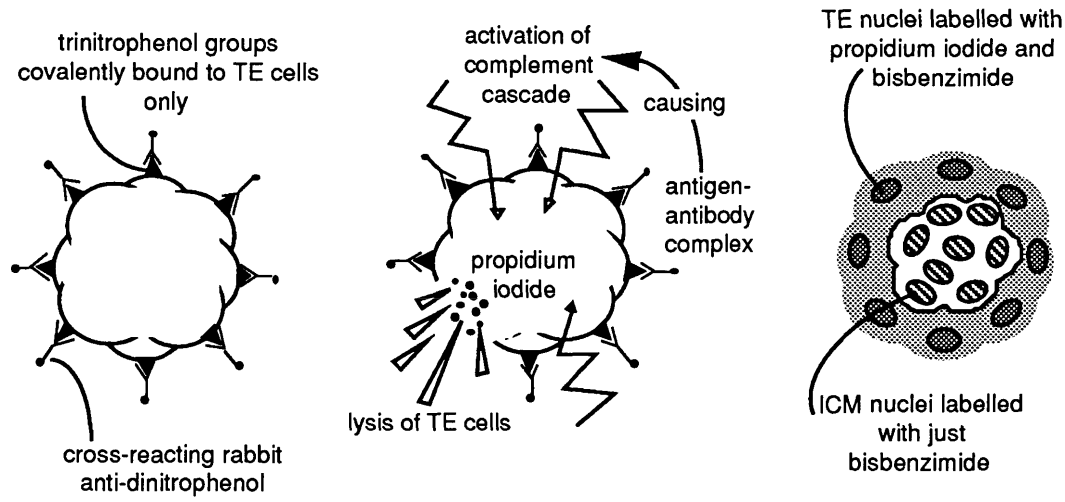



**Figure 2.1:** *Diagram showing differential labelling of trophectoderm (TE) and inner cell mass (ICM) nuclei of human blastocysts. The surface of the TE cells is labelled with covalently bound trinitrophenol (TNP) groups. Cross-reacting anti-dinitrophenol is bound to the TNP groups. Upon exposure of the embryo to complement, the antigen-antibody complexes activate the complement cascade, selectively lysing the TE cells, allowing entry of the fluorochrome propidium iodide, so that the TE nuclei are labelled. The whole embryo is then fixed in alcohol and bisbenzimide, resulting in the ICM nuclei being labelled with bisbenzimide only and the TE nuclei being labelled with both bisbenzimide and propidium iodide.*

## §2.3 Development of human preimplantation embryos *in vitro*

The development of a total of 181 surplus embryos of various classifications from 42 patients (4.9 ± 0.5 surplus embryos per patient, range 1-17), was observed from day 2 to day 5, 6 or 7 *in vitro* (Table 2.1). 63 out of 149 (42%) normally fertilized embryos developed to the blastocyst stage by day 5 or 6, and 6 of these hatched (Fig. 2.2A). Some of these blastocysts (15/63), however, were morphologically abnormal and were characterized by one or more of the following features: unincorporated blastomeres, multiple cavities (possibly intracellular vacuoles), no discrete ICM visible or low numbers of mural TE cells. The remainder of the normally fertilized embryos arrested at cleavage and morula stages. Of 21 polyspermic embryos, 6 reached the blastocyst stage by day 6. Three of these were morphologically abnormal. Two out of a total of 11 parthenogenetic embryos developed to the blastocyst stage. Both were morphologically abnormal.

**Table 2.1:** *Development of human embryos in vitro*

| No. of pronuclei | Total no.of embryos (% of total) | No. observed to day 5, 6 or 7 *in vitro*[1] | No.arrested | Total no. to blastocyst on day 5 and 6[2] |
|---|---|---|---|---|
| 2 | 279 (89%) | 149 | 86 (58%) | 63 (42%) |
| >2 | 22 (7%) | 21 | 15 (71%) | 6 (29%) |
| <2 [3] | 12 (4%) | 11 | 9 (82%) | 2 (18%) |
| Total | 313 | 181 | 110 (61%) | 71 (39%) |

Unless otherwise stated numbers in parentheses are % of number of embryos observed to day 5,6 or 7 *in vitro* .

[1] 109 2-pronucleate embryos were transferred on day 2 or 3 and 23 embryos were allocated to other studies.

[2] 3 embryos were observed as blastocysts on day 7, 2 of which were not scored on days 5 or 6.

[3] Embryos derived from oocytes with one or no pronuclei but which subsequently cleaved.

### §2.4 Cell allocation to the trophectoderm and inner cell mass

Differential labelling of 50 out of 71 blastocysts was attempted (Fig. 2.2B). Four were lost during processing. In 17 cases, careful examination in whole mount suggested that some of the TE nuclei had not been differentially labelled. The majority of these (10 out of 17) were either morphologically abnormal blastocysts, polyspermic or parthenogenetic blastocysts. Among the remaining 7 normally fertilized blastocysts, remnants of the zona pellucida after acid Tyrodes treatment appeared to have prevented the immunosurgical lysis of part of the TE. This phenomenon has been observed in the pig (Papaioannou and Ebert, 1988). These blastocysts were, however, adequately labelled after fixation for determination of total cell numbers (see below). The numbers of TE and ICM cells in the remaining 30 normally fertilized blastocysts on days 5-7 are presented in Table 2.2. Newly expanded blastocysts on day 5, with a total of 58.3 ± 8.1 cells, had 37.9 ± 6.0 and 20.4 ± 4.0 TE and ICM cells, respectively. Amongst expanded blastocysts on day 6 (some of which had reached this stage on day 5) the number of TE cells was virtually unchanged, 40.3 ± 5.0; however, the number of ICM cells, 41 9 ± 5.0, was double. By day 7, TE cell number was double (80.6 ± 15.2), but the number of ICM cells was almost the same (45.6 ± 10.2) as on day 6. All of these normally fertilized blastocysts had ICMs although the range, from 5 to 113 cells, was considerable. The proportion of ICM cells varied from about a third on day 5 and 7 to a half on day 6. Cell death, characterized by

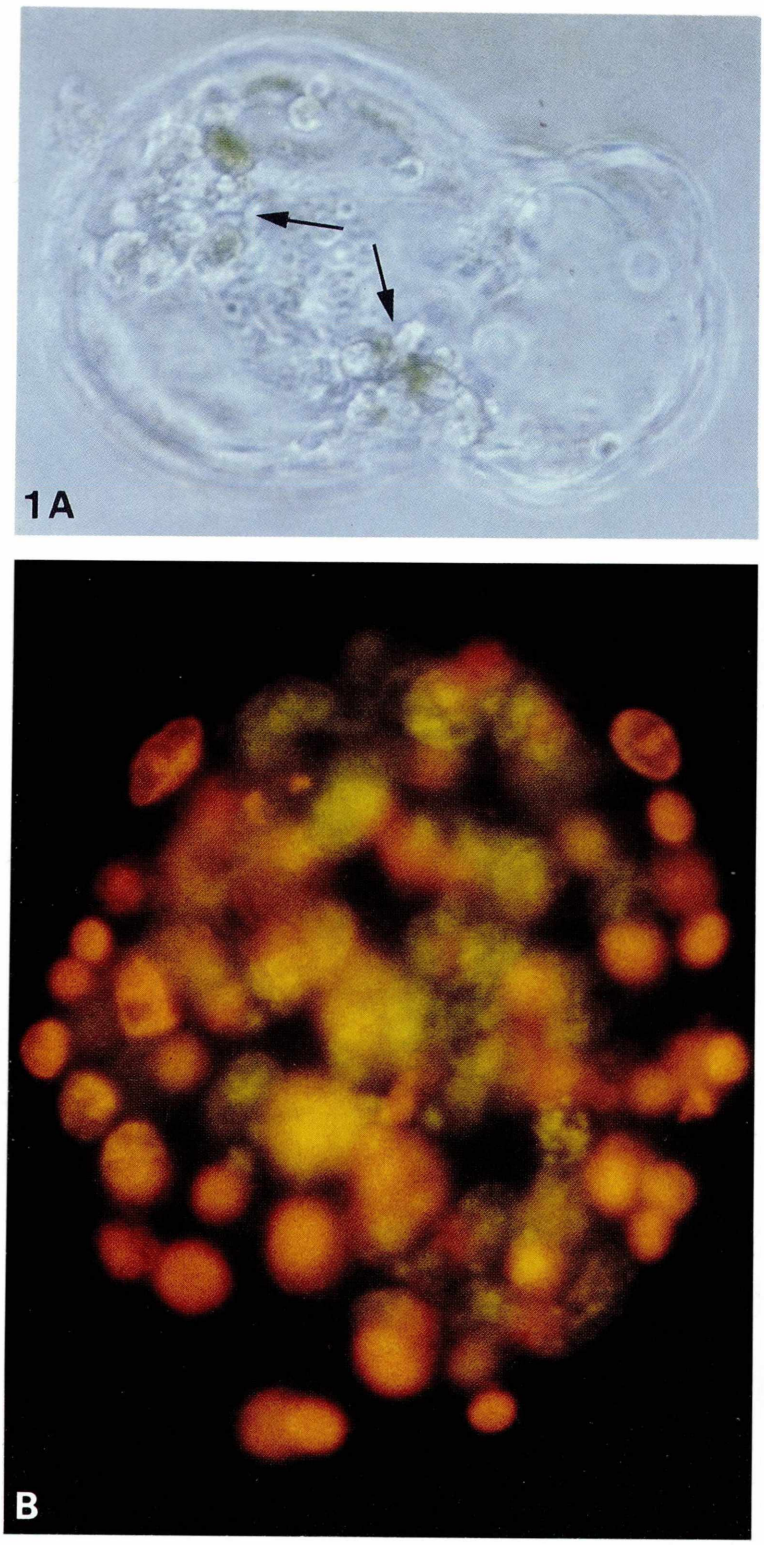



**Fig 2.2:** *Phase contrast and fluorescence micrographs of human blastocysts and arresting embryos. (A) Normally fertilized human blastocyst hatching from the zona pellucida on day 6. Note the apparently double ICM (arrows). This blastocyst was differentially labelled and found to have 32 and 46 TE and ICM nuclei, respectively. Phase contrast (×330). (B) Whole mount of a differentially labelled normally fertilized human blastocyst on day 6. The green ICM nuclei labelled with bisbenzimide and orange TE nuclei are easily distinguished. There were 64 TE and 41 ICM nuclei. Some fragmented nuclei characteristic of dead cells are visible in both the TE and ICM. Fluorescence (×860).*

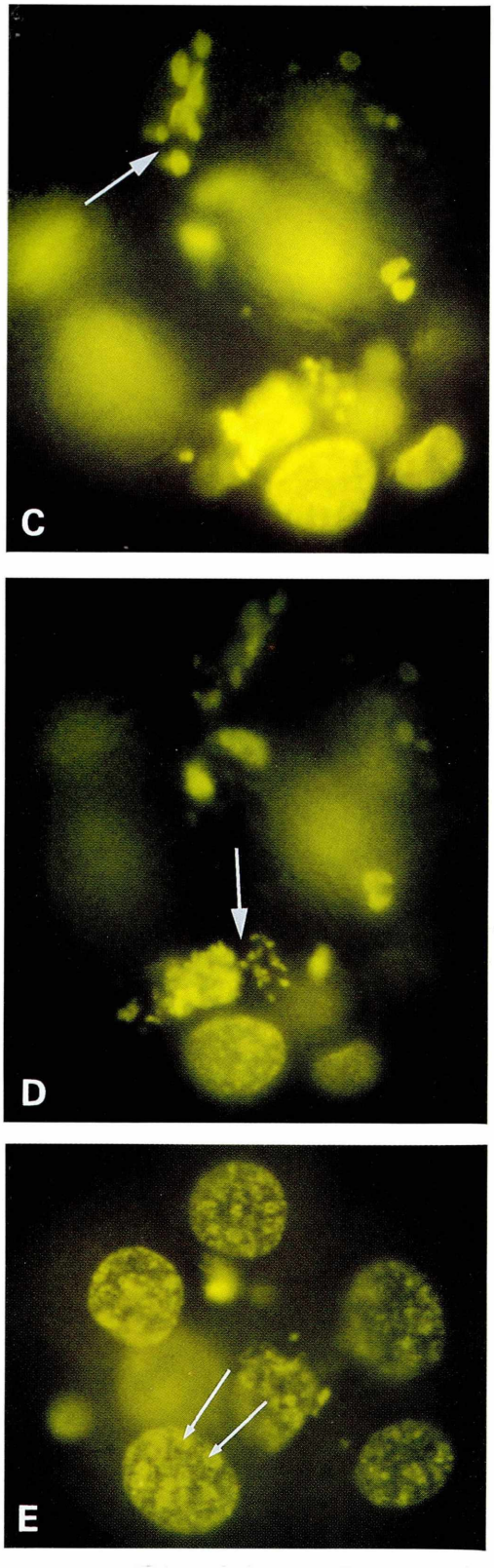



**Fig 2.2 (continued):** *(C) Whole mount of an arrested 8-cell human embryo labelled with bisbenzimide on day 6. Labelled nuclear fragments characteristic of degenerating cells are arrowed. Fluorescence (×720). (D) As C. An abnormal metaphase containing an excess of 100 chromosomes (arrow). Fluorescence (×720). (E) An arrested human 8-cell embryo labelled as in (C). Note the vesicles of labelled chromatin (arrows) within the nuclear membrane giving the characteristic 'flocculent' appearance frequently found in arrested embryos. Fluorescence (×720).*

fragmenting nuclei (Fig. 2.2C), was widespread and increased between days 5 and 7; 28 out of the 30 blastocysts examined between days 5 and 7 had evidence of cell death in both the ICM and the TE. The mitotic index of both the TE and the ICM was relatively high on day 5 but was reduced by day 7. Differential labelling of two morphologically abnormal blastocysts on day 6 revealed 15 and 9, and 51 and 35 cells in the TE and ICM, respectively.

**Table 2.2:** *Numbers of TE and ICM cells in normally fertilized expanded blastocysts grown in vitro to days 5, 6 and 7.*

| | Trophectoderm | | | Inner cell mass | | | Total |
|---|---|---|---|---|---|---|---|
| Day | cell number | mitotic index | dead cell index | cell number | mitotic index | dead cell index | cell number |
| Day 5 n=9 | **37.9$^{a}$** ±6.0 | 3.0$^{a}$ ±1.8 | 6.8$^{ab}$ ±2.3 | **20.4$^{c}$** ±4.0 | 3.8$^{a}$ ±1.8 | 8.0$^{ab}$ ±4.2 | **58.3** ±8.1 |
| Day 6 n=11 | **40.3$^{ab}$** ±5.0 | 0.8$^{aa}$ ±0.4 | 6.0$^{ac}$ ±2.3 | **41.9$^{cd}$** ±5.0 | 1.1$^{aa}$ ±0.5 | 9.2$^{ad}$ ±2.8 | **82.2** ±7.8 |
| Day 7 n=10 | **80.6$^{b}$** ±15.2 | 0.4$^{a}$ ±0.3 | 16.3$^{bc}$ ±2.7 | **45.6$^{d}$** ±10.2 | 0.3$^{a}$ ±0.2 | 27.8$^{bd}$ ±5.3 | **126.2** ±21.0 |

values are means ± standard errors
[a] not significant
[bd] $P<0.05$
[c] $P<0.01$

## §2.5 Total cell numbers in blastocysts developing in vitro

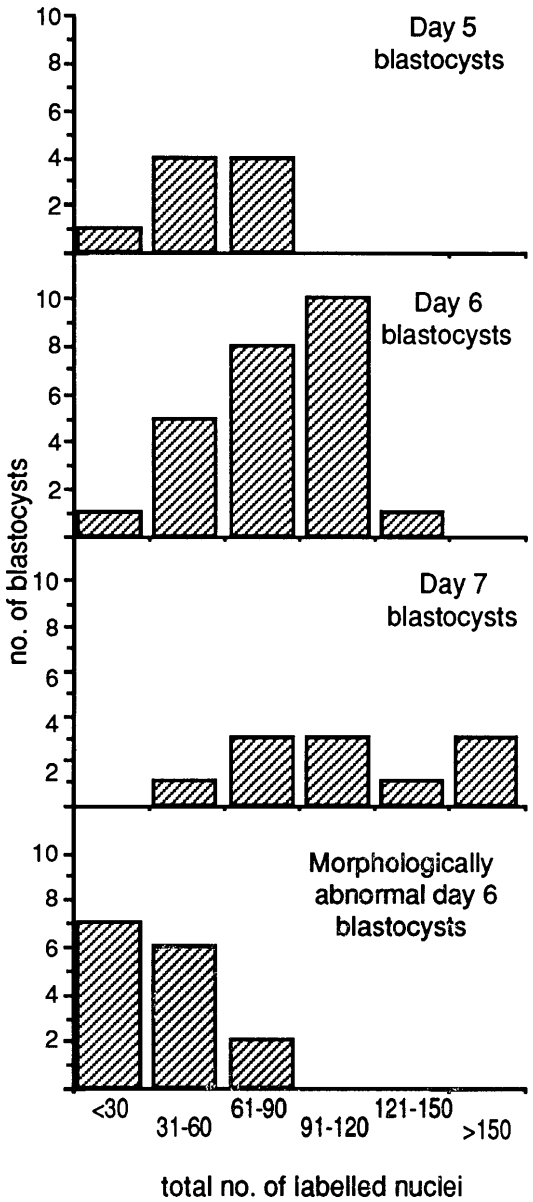



**Fig. 2.3:** *Distribution of total cell number in normally fertilized human blastocysts grown in vitro.*

The total cell numbers, mitotic and dead cell indices for 45 normally fertilized blastocysts, 16 with abnormal morphology and 6 abnormally fertilized blastocysts on day 5, 6 and 7 are summarized in Table 2.3. This series of embryos included all 50 blastocysts which were differentially labelled (see above) together with 17 others that were simply fixed and labelled with a single fluorochrome. Total cell numbers in this larger series of normally fertilized blastocysts on days 5 to 7 were almost identical to those ascertained after differential labelling (Table 2.2). The range of total cell numbers in normally fertilized blastocysts increased progressively from day 5 to day 7 (Fig. 2.3). Newly expanded blastocysts on day 5 ranged from 24 to 90 cells, on day 6 from 27 to 136 cells and on day 7 from 60 to one hatched blastocyst with 283 cells.

Normally fertilized blastocysts on day 6, which were morphologically abnormal, had only half the cells of their morphologically normal counterparts (Fig. 2.3, Table 2.3). One morphologically abnormal blastocyst on day 5 had only 18 cells. Polyspermic blastocysts on day 6 had a similar number of cells to morphologically abnormal blastocysts and large numbers of dead cells. Two parthenogenetic blastocysts had a total of only 6 and 13 cells.

**Table 2.3:** *Total numbers of nuclei in expanded and abnormal human blastocysts grown in vitro to days 5, 6 & 7.*

| Age and morphology (No. of pronuclei) | no. of embryos | total no.of nuclei (range) | mitotic index | dead cell index |
|---|---|---|---|---|
| Day 5 Newly expanded blastocysts (2) | 9 | 58.3[a] ±8.1 (24-90) | 3.5 ±1.6 | 7.0 ±3.1 |
| Day 6 Expanded blastocysts (2) | 25 | 84.4[abc] ±5.7 (27-136) | 1.5 ±0.4 | 9.1[de] ±1.6 |
| Day 7 Expanded blastocysts (2) | 11 | 125.5[b] ±19.0 (60-283) | 0.4 ±0.2 | 17.6[d] ±2.8 |
| Day 5 Morphologically abnormal blastocyst (2) | 1 | 18 | 5.6 | 0 |
| Day 6 Morphologically abnormal blastocysts (2) | 15 | 38.3[c] ±6.2 (3-86) | 2.1 ±0.7 | 27.0[e] ±5.6 |
| Day 6 Polyspermic blastocysts (>2) | 4 | 41.5 ±22.9 (0-103) | 2 ±1.4 | 38.5 ±30.8 |
| Day 6 Parthenogenetic blastocysts (<2) | 2 | 6 & 13 | 0 | 33±8.0 |

values are means ± standard errors
[ab] $P<0.05$
[c] $P<0.001$
[de] $P<0.01$

### §2.6 Nuclear morphology in arrested embryos

46 arrested embryos were labelled to examine nuclear morphology on days 6 and 7. These included eleven 2- to 7-cell-stage embryos, ten 8-cells, four uncompacted morulae with greater than 8 cells, seven morulae, eight cavitating or vacuolated late morulae and six totally fragmented embryos. Ten of the arrested embryos had more nuclei than would be expected on the basis of their morphology. Four arrested 4-cell embryos had between 7 and 35 nuclei including one with 23 normal nuclei and 12 dying nuclei in one cell and none in the other three cells. One 8-cell embryo had 16 normal nuclei with a few additional fragments of nuclear material. Two morulae contained large numbers of nuclei; one with 90 including 35 degenerate nuclei, the other 30 including 7 degenerate nuclei. Interestingly, three embryos that were scored as being totally fragmented had 20, 24 and 45 normal nuclei. Also, seven arrested

embryos had no nuclei apart from a few bright specks of fluorochrome-labelled material. One arrested embryo had a highly polyploid metaphase (Fig. 2.2D). Finally, six other arrested embryos had "flocculent" nuclei, with labelled chromatin clumped on the nuclear membrane (Fig. 2.2E).

## §2.7 Conclusion

The early cleavage divisions in the human, before transfer on day 2, have been extensively described (Sundstrom *et al.* 1981; Edwards *et al.* 1981; Trounson *et al.* 1982; Mohr and Trounson, 1984). However, apart from a series of ultrastructural studies of human blastocysts (Lopata *et al.* 1982; Mohr and Trounson, 1982) there is relatively little information about cleavage rates in late preimplantation development, mainly due to the paucity of surplus embryos after transfer or cryopreservation.

The timing of development *in vitro* from day 2 onwards appears to be comparable to the few reliably timed observations *in vivo*. For example, Buster *et al* (1985) recovered 19 embryos by uterine lavage 5 days after artificial insemination, 5 of which (26%) had developed to the blastocyst stage. This is similar to the proportion reported in §4.2, in which 10/43 (23%) reached the blastocyst stage by day 5 *in vitro* (Hardy *et al.* 1989b). Notably, amongst the embryos examined here, those reaching the blastocyst stage by day 5 were already more advanced on days 3 and 4 than those embryos not reaching the blastocyst stage until day 6. The total cell numbers in these day 5 blastocysts from individual patients were remarkably similar and not significantly different, whereas there was significant between patient variation ($P<0.001$). This suggests that among embryos from individual patients, the timing of fertilization and cleavage may be similar. However, on days 6 and 7, there was significant variation both within and between groups of embryos from different patients. This increase in variability may be caused by the increased cell death at these stages (Tables 2.2 and 2.3).

The minimum number of cells we observed in normally fertilized, expanded blastocysts on days 5 and 6 was 24 and 27 cells, respectively (Fig. 2.3). It is likely, therefore, that blastulation is initiated between the fourth and fifth cleavage divisions. This is similar to the mouse, where blastulation coincides with completion of the fifth cleavage division (Smith and McLaren, 1977; Handyside and Hunter, 1986); but later than the pig, where it occurs mainly after the fourth cleavage division (Papaioannou and Ebert, 1988).

Total cell numbers in normally fertilized blastocysts increased from day 5

onwards and significantly between days 6 and 7 ($P<0.05$) (Table 2.3). Thus cell division continues *in vitro* at least up to day 7, although the mitotic index steadily declined to a low level. The hatched blastocyst with 283 cells on day 7 is particularly encouraging for these *in vitro* culture conditions. On average, the first three cleavage divisions take about 60h (Edwards *et al.* 1981; Sundstrom *et al.* 1981; Trounson *et al.* 1982). Between days 3 and 7, the increase in total cell numbers indicates that, on average, a further four cleavage divisions have been completed in the same number of days. Thus, the overall cleavage rate of human embryos *in vitro* at morula and blastocyst stages appears to be no faster than the previous cleavage divisions, with one division every 24h. However, this must be an underestimate because of the extensive cell death occurring at these stages. In the mouse, cleavage rates over this period are initially higher, with about one division every 12h at the morula stage. At the blastocyst stage, the rate decreases because of a combination of giant cell formation in the mural TE and slower division and cell death in the ICM (Handyside and Hunter, 1986). Cleavage rates in sheep embryos, however, are equally slow (Handyside *et al.* 1987).

Previous data on the numbers of cells in human blastocysts are sparse; however, these results are comparable. Hertig *et al.* (1954) recovered two blastocysts, estimated to be 5 days post-fertilization, from uteri after hysterectomy. Cell counts were made by counting nuclei in serial sections and were 58 and 107 cells of which 5 and 8, respectively, were considered ICM cells on the basis of their position. Croxatto *et al.* (1972) obtained one blastocyst from uterine flushing which upon serial sectioning was found to have 186 cells but was thought to be more than 5 days old. Finally, Steptoe *et al.* (1971) counted the nuclei of two blastocysts grown *in vitro* for 7 days after IVF. One had 112 nuclei, 16 of which were in mitosis, the other 110 nuclei.

Not surprisingly, morphologically abnormal blastocysts and those derived from abnormally fertilized embryos had lower total cell numbers (Table 2.3). However, one blastocyst derived from a polyspermic embryo had 103 cells on day 6 which is well within the range for normally fertilized blastocysts (Fig. 2.3) suggesting that it had undergone normal cleavage. Van Blerkom *et al.* (1987) reported that some zygotes, which at the light microscope level appeared to have multiple pronuclei, have upon closer examination at the electron microscope level the normal two pronuclei closely associated with a membrane-bound cytoplasmic vacuole. These pseudo-multipronuclear embryos developed normally and have given rise to

pregnancies. Therefore, it is possible that this blastocyst was in fact derived from a pseudo-multipronucleate embryo.

The allocation of cells to the TE and ICM of the blastocyst is of fundamental importance for later development. In the mouse, allocation to the two lineages occurs during the fourth (Handyside, 1981) and fifth cleavage divisions (Pedersen, 1986; Fleming, 1987) at the morula stage. Following implantation, the TE only gives rise to the placenta and extraembryonic membranes whereas the ICM forms all three germ layers of the fetus as well as complementary contributions to the extraembryonic membranes (Gardner and Papaioannou, 1975). With human blastocysts, apart from sporadic estimates of the number of these cells in sectioned embryos (see above) and descriptions of the ultrastructural features of the two cell types, cell allocation has not previously been examined in detail.

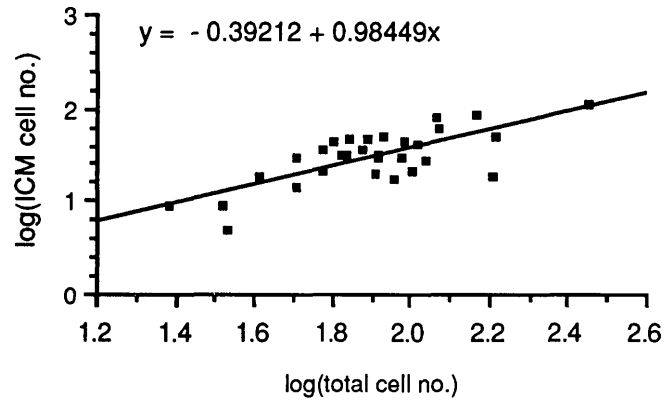



**Fig. 2.4:** *Logarithm (base 10) of total cell numbers of 30 day 5, 6 and 7 blastocysts plotted against the logarithm of their ICM cell numbers. The straight line is a least squared fit to the observed data.*

The timing and numbers of cells initially allocated to the inner and outer positions at the morula stage may be difficult to ascertain in the human embryo. Focal intercellular contacts and junctions in 8- to 16-cell human morulae have been observed (Lopata *et al.* 1983; Tesarik, 1989; Hardy, unpublished observations). However, compaction, in terms of the close apposition of cells and loss of spherical shape, is less complete than in the mouse embryo. Specific antibody labelling of the outer morula cells may, therefore, not be a reliable approach (Handyside, 1981). Instead, an attempt has been made to estimate cell allocation at the 16- and 32-cell stages by extrapolating from TE and ICM numbers in day 5-7 blastocysts (Fig. 2.4).

On this basis, and ignoring cell death, at the 16-cell stage there would be approximately 6 cells allocated to the ICM, and 10 to the TE and, at the 32-cell stage, there would be approximately 12 and 20 cells allocated to the ICM and TE, respectively. These numbers are similar to the numbers found in the mouse (Handyside, 1981; Handyside and Hunter, 1986).

Between days 5 and 7, the numbers of TE and ICM cells in normally fertilized human blastocysts both doubled. This contrasts with mouse blastocysts in which, over a similar period, the numbers of TE cells quadruple, before reaching a plateau coinciding with the initiation of giant cell formation, whilst the number of ICM cells only doubles (Handyside and Hunter, 1986). Also, the maximum number of TE and ICM cells in mouse blastocysts was 147 and 49, respectively. With human blastocysts, the maximum number of TE cells was similar, 170, whereas the maximum number of ICM was higher, 113. The overall proportion of ICM is high: 34% on day 5, 51% on day 6 and 37% on day 7. This contrasts with both the pig, where the proportion of ICM declines from ≈25% at the expanded blastocyst stage to ≈17% at the hatched blastocyst stage (Papaioannou and Ebert, 1988), and the mouse, where the proportion declines from ≈40% in nascent blastocysts to ≈17% before implantation (Handyside and Hunter, 1986). However, the high proportion of ICM in the human is similar to that in the sheep, where the ICM constitutes ≈44% of the expanded blastocyst (Handyside *et al.* 1987).

The widespread cell death, which we have observed as fragmented labelled nuclei in human blastocysts at all stages (Fig. 2.2C), was equally prominent in both the TE and ICM and increased markedly with culture of blastocysts to day 7 (Table 2.2). Cell death is a widespread feature of embryogenesis in both vertebrates and invertebrates (Glucksmann, 1951; Saunders, 1966) and generally occurs by apoptosis, characterized by cellular shrinkage with condensation of nuclear chromatin and cleavage of chromatin into oligonucleosome chains (Kerr *et al.* 1972; Wyllie, 1980). It has been observed in a wide variety of mammalian embryos at the blastocyst stage. Ultrastructural studies show cell death in the ICM of the cow and the human (Mohr and Trounson, 1982), the mouse (El-Shershaby and Hinchliffe, 1974) and in both the TE and the ICM of the Rhesus monkey (Enders and Schlafke, 1981). Differential labelling confirmed that cell death occurs in the ICM of the mouse (Handyside and Hunter, 1986) but, in contrast, using a similar technique, none was observed in the pig blastocyst (Papaioannou and Ebert, 1988). In the cow, Rhesus monkey and mouse, the blastocysts had been flushed directly from the uterus, suggesting that cell death is

a normal phenomenon occurring *in vivo*. The significance of these dead cells is not fully understood but it has been suggested that, in the mouse, it may involve the elimination of ICM cells retaining the potential to form TE (Handyside and Hunter, 1986). With human embryos, some of the degenerating nuclei may be derived from the multinucleate blastomeres observed at cleavage stages (Lopata *et al.* 1983; Tesarik *et al.* 1987).

Embryos arrested for several days at cleavage or morula stages had extensive cell death but also other nuclear and chromosomal abnormalities (Fig. 2.2C-E). The "flocculent" nuclei observed in six arrested embryos on day 6 had the appearance of DNA clumped in vesicles on the nuclear membrane. It is of interest that in ultrastructural studies of cell lines undergoing induced apoptosis, margination of nuclear heterochromatin in dense clusters on the nuclear membrane has been seen (Bardon *et al.* 1987). Thus it is possible that these "flocculent" nuclei are a feature of an intermediate stage of apoptosis, before fragmentation of the nuclei, or simply the early signs of degeneration after embryonic arrest.

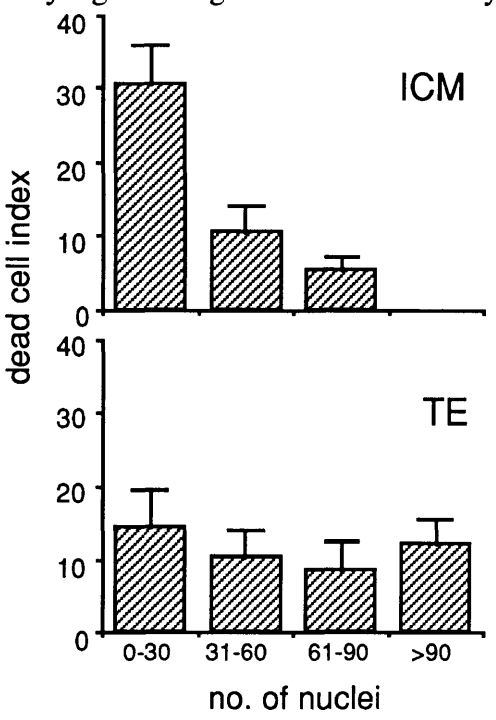



**Fig. 2.5:** *Comparison of the mean dead cell index ± standard error with the number of nuclei in the ICM and the TE of days 6 and 7 normally fertilized human blastocysts.*

Over a third, 63/149 (42%), of normally fertilized embryos examined developed to the blastocyst stage on day 5 or 6 *in vitro* (Table 2.1). Assuming that a similar proportion of embryos would have developed to the blastocyst stage *in vivo* and that all of these blastocysts had a high probability of implanting and establishing a pregnancy, the expected pregnancy rate, including multiple pregnancies, after transfer of three embryos would be about 70% (ie. if the probability of an embryo arresting is 2/3, the probability of all three embryos arresting is $(2/3)^3 = 0.296$, assuming that they are independent of each other. Thus 29.6% of the women would not become pregnant). In fact, of the 41 patients for whom information is available, only 17 (41%) became pregnant, as determined by raised hCG levels. However, 15 out of these 63 blastocysts were morphologically abnormal and had significantly fewer cells and high levels of cell

death (Table 2.3). In addition, amongst the other blastocysts low numbers of ICM cells on day 6 and 7 were associated with a high proportion of dead cells (Fig. 2.5). This raises the possibility that blastocysts with smaller numbers of ICM cells may be less viable, and responsible for biochemical pregnancies where there is an initial increase in the levels of hCG but no fetal development. Whether these blastocysts with low cell numbers result from suboptimal conditions *in vitro* or inherent defects will require further investigation.

# §3 Control of allocation to the trophectoderm and inner cell mass in the mouse blastocyst

## §3.1 Introduction

The two phenotypically distinct cell types present in the mouse blastocyst, the inner cell mass (ICM) which gives rise to the fetus, and the trophectoderm (TE), which gives rise to the extraembryonic membranes and the placenta (Gardner and Papaioannou, 1975), are thought to result from differentiative divisions of polarized 8-cell blastomeres (Johnson and Ziomek, 1981a). Polarized cells at this stage can divide to form either one polarized epithelial-like cell and one smaller apolar cell (this type of division being termed a differentiative division), or two polarized cells (a conservative division) (§1.4.3, Fig. 1.2). The uniformly adhesive apolar cells tend to remain on the inside of the embryo forming ICM, whilst the polar cells, with their non-adhesive microvillous apical pole, remain on the surface as TE precursors. However, the control of the number of conservative and differentiative divisions at the 8- to 16-cell transition and consequently the number of cells allocated to the ICM and TE is not well understood.

There are three possible mechanisms which could control allocation to the ICM and TE. The first is based on simple packing and the geometry of the embryo at the compact morula stage, with the number of cells allocated to the TE being controlled by the available surface area. In this case, the number of TE cells achieves an optimal packing density and is proportional to the surface area. It is possible to test this "inside:outside" hypothesis (§1.4.2) by changing the surface area:volume ratio of preimplantation embryos. Embryos aggregated or bisected at the 8-cell stage will compact to form single spherical morulae of proportionally greater or lesser volume respectively, with correspondingly altered surface area: volume ratios. As the volume and surface area of a sphere are proportional to the cube and the square of the radius, respectively, embryos of twice the volume, for example, have less than twice the surface area (Appendix 9.1). Thus, it would be expected that morulae created by aggregating two embryos ('double' embryos) would have twice the total cell number but less than twice the number of TE cells resulting in a corresponding increase in the number of ICM cells. Buehr and McLaren (1974) provided evidence for this model by showing that the ICMs of double embryos did indeed have more than twice the number of cells in control

single embryos and proposed that there were lower numbers of outside cells proportional to the surface area. As a result the remainder of the cells would be enclosed, producing a greater than double number of ICM cells.

An alternative to simple packing as a mechanism for allocation has been suggested by observations of Johnson *et al* (1984) that surface area could influence the division of polarized cells. Polar cells on the surface of the embryo which are at less than optimal packing density (ie. flattened), may be more likely to undergo conservative divisions to produce more polar cells, and in so doing attain optimal packing density. Conversely, polar cells at greater than optimal packing density could be more rounded and more likely to undergo differentiative divisions, producing both polar and apolar (inside) cells (Fig. 3.1). Double embryos with a smaller surface area:volume ratio would therefore more quickly attain optimal packing density at the surface, more polar cells would undergo differentiative divisions and a larger than normal ICM would result. This could occur at either initial allocation (the 8- to 16-cell transition), or later at the 16- to 32-cell transition. In either case, however, the expectation would be a balanced shift in the number of cells in the TE and ICM compartments.

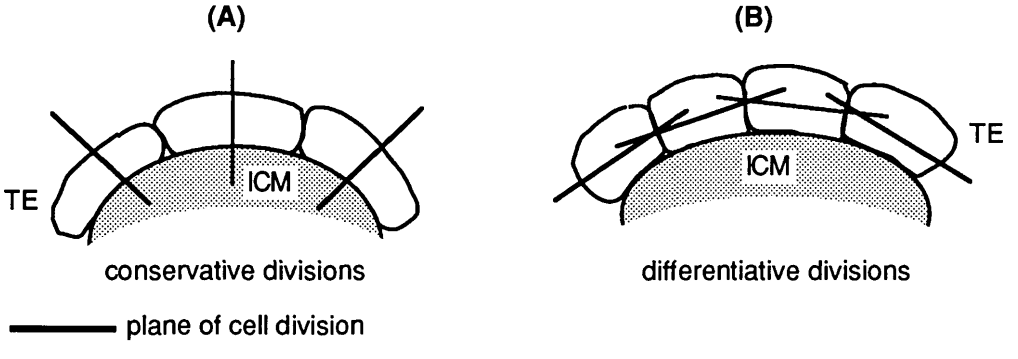



**Figure 3.1:** *Schematic representation of (A) flattened TE cells at less than optimal packing density dividing conservatively and (B) closely packed, rounded TE cells at greater than or equal to optimal packing density dividing differentiatively.*

A final possibility for the control of allocation is that the cells are pre-programmed at polarization to undergo either differentiative or conservative divisions and that, irrespective of packing density and environment, a certain proportion of ICM and TE cells is produced. Some evidence for this has been provided by the observation that 8-cell stage blastomeres *in isolation* polarize to form both ICM- and TE-like inner and outer cells, suggesting that allocation occurs

irrespective of cell position within the embryo (Johnson and Ziomek, 1981a). In this case, the number of TE and ICM cells in double or half embryos would simply be twice or half, respectively, those in control embryos.

Recently, Rands (1985) re-examined allocation to the TE and ICM in mouse embryos where the surface area: volume ratio had been altered by both reducing and increasing cell number. Half embryos were produced by lysing one cell of a 2-cell embryo within the intact zona pellucida using a micromanipulator-controlled glass needle. Half and control embryos were then separately transferred, within an hour, to the oviducts of recipient mice on the first day of pseudopregnancy, and morulae and blastocysts recovered 48 and 72h later, respectively. Quadruple embryos were produced by aggregating four zona-free 8-cell embryos, which were then cultured for 24 and 48h to the morula and blastocyst stages, respectively. The numbers of cells in morulae were examined by disaggregating embryos after incubation in pronase and calcium-free medium and counting cells. The numbers of cells in blastocysts were counted by reconstructing serial sections of paraffin-embedded embryos. In this case, cells were assigned to the TE or ICM on the basis of nuclear morphology and cell boundaries.

At the morula stage, half embryos had approximately half and quadruple embryos approximately four times the total number of cells of control morulae. At the expanded blastocyst stage half embryos still had approximately half the control number of cells, but quadruple embryos had only 3.3 times the number. The proportions of ICM and TE cells in these manipulated embryos differed significantly from those in the controls. In half embryos, the proportion of ICM cells was reduced, whilst the proportion of TE cells was increased. In quadruple embryos the converse was true, with a larger ICM and disproportionately fewer TE cells. Indeed, when compared with the number of cells expected on the basis of the altered surface area it was found that the observed number of TE cells in half and quadruple blastocysts did not differ significantly from those predicted by the inside:outside hypothesis.

Although a selective analysis of the number of TE cells in Rands' (1985) study appears to support a surface area mediated mechanism, a more detailed analysis indicates that the effects on allocation were not straight forward. For example, the increase or reduction in the number of TE cells in half and aggregated embryos respectively was not balanced by a decrease or increase in the number of

cells in the ICM. If the number of TE and ICM cells in quadruple embryos is divided by four, it can be seen that on a "per-single-embryo" basis, approximately 21 cells have been lost from the TE whilst only approximately 10 cells have been gained by the ICM. A major problem with this study was the necessarily low numbers of embryos analysed at the blastocyst stage by serial reconstruction. Combined with the absence of any attempt to synchronise the developmental stage of embryos before manipulation and analysis mostly at a relatively late blastocyst stage when cell death occurs in the ICM along with differential rates of division between the TE and ICM, this seriously complicates any interpretation. Thus, although the observed ratio of TE cells did not differ significantly from that expected by the inside:outside hypothesis, it cannot be excluded that this was fortuitous since under these conditions the proportions of TE and ICM cells and the ratio with unmanipulated controls is likely to vary continuously.

Here, the dependence of allocation to the TE and ICM on the position and packing of cells within the embryo has been investigated *in situ* in synchronised mouse embryos carefully selected at completion of their third division at the early 8-cell stage, bisected or aggregated in various combinations and cultured to the 32-cell (late morula/early blastocyst) stage (§8.3). The numbers of TE and ICM nuclei were then examined at this stage by differential labelling with polynucleotide specific fluorochromes (Handyside and Hunter, 1984). There are several advantages of this technique over serial sectioning: firstly, it is rapid, allowing relatively large numbers of embryos to be analysed; secondly, visualization of the nuclei by fluorescence microscopy allows accurate counting of the number of nuclei without having to serially reconstruct sections; finally, the TE nuclei are labelled before fixation so that there is a decreased probability of artifacts caused by fixation and embedding. Counting nuclei at the 32-cell stage allows examination of initial allocation, avoiding the possibility of cell death and different rates of cell division within the two lineages over subsequent cleavage divisions. In addition, at the equivalent of the 32-cell stage, all the cells within the embryo should have completed the fifth cleavage division (assuming there are no effects on the rate of division), and the proportion of ICM and TE cells is a true proportion, rather than a distorted proportion that may be expected in the transition between cleavage stages resulting, for example, from different rates of cell division between inner and outer cells (MacQueen and Johnson, 1983). However, to examine how the proportions of TE and ICM cells

change in aggregated embryos in relation to unmanipulated controls during the expansion of the blastocyst and for comparison with Rands' (1985) study, a smaller series were differentially labelled at the expanded blastocyst stage after a further 24h in culture.

## §3.2 Allocation to trophectoderm and inner cell mass

### §3.2.1 Method

Two-cell embryos were flushed from oviducts on day 2 (§8.3) and cultured overnight in M16 + BSA (§8.1.1) under oil. By the next morning the embryos consisted of a mixed population of between 4-cell and morula stages. In an attempt to synchronize embryos undergoing the third cleavage division to within a period of 2h, 8-cell and more advanced embryos were initially eliminated from the pool of embryos. 2h later uncompacted 8-cell embryos were selected for manipulation (≈66 - 67h post hCG). Zona-free embryos (§8.3.1) were either bisected by disaggregation into two half-embryos consisting of four 8-cell blastomeres each (§8.3.2), or were aggregated as closely as possible into "double", "triple" or "quadruple" embryos (§8.3.3) consisting of sixteen, twenty-four or thirty-two cells each. Bisected, aggregated and zona-free unmanipulated control embryos were processed as far as possible in the same way and cultured in drops of M16 + BSA (§8.1.1) under oil overnight. In the majority of cases, half embryos were cultured and processed so that twin halves of the same embryo could be identified and maintained throughout the labelling procedure. In culture, the embryos rapidly compacted to form single, spherical morulae and the following morning, at the 32-cell stage, when 50% of the embryos had cavitated (26.00 ± 0.2h post aggregation, 93.00 ± 0.9h post hCG), the TE and ICM nuclei of both cavitating blastocysts and late morulae were differentially labelled (§8.4.2). Individual nuclei of both cell populations including those in mitosis, were counted using fluorescence microscopy (§8.5.2).

### §3.2.2 Control embryos

Overall, the total number of cells in 127 unmanipulated embryos from sixteen experiments was 31.21 ± 0.33. This is very close to the number expected at the time of cavitation confirming that blastocoel formation normally occurs on

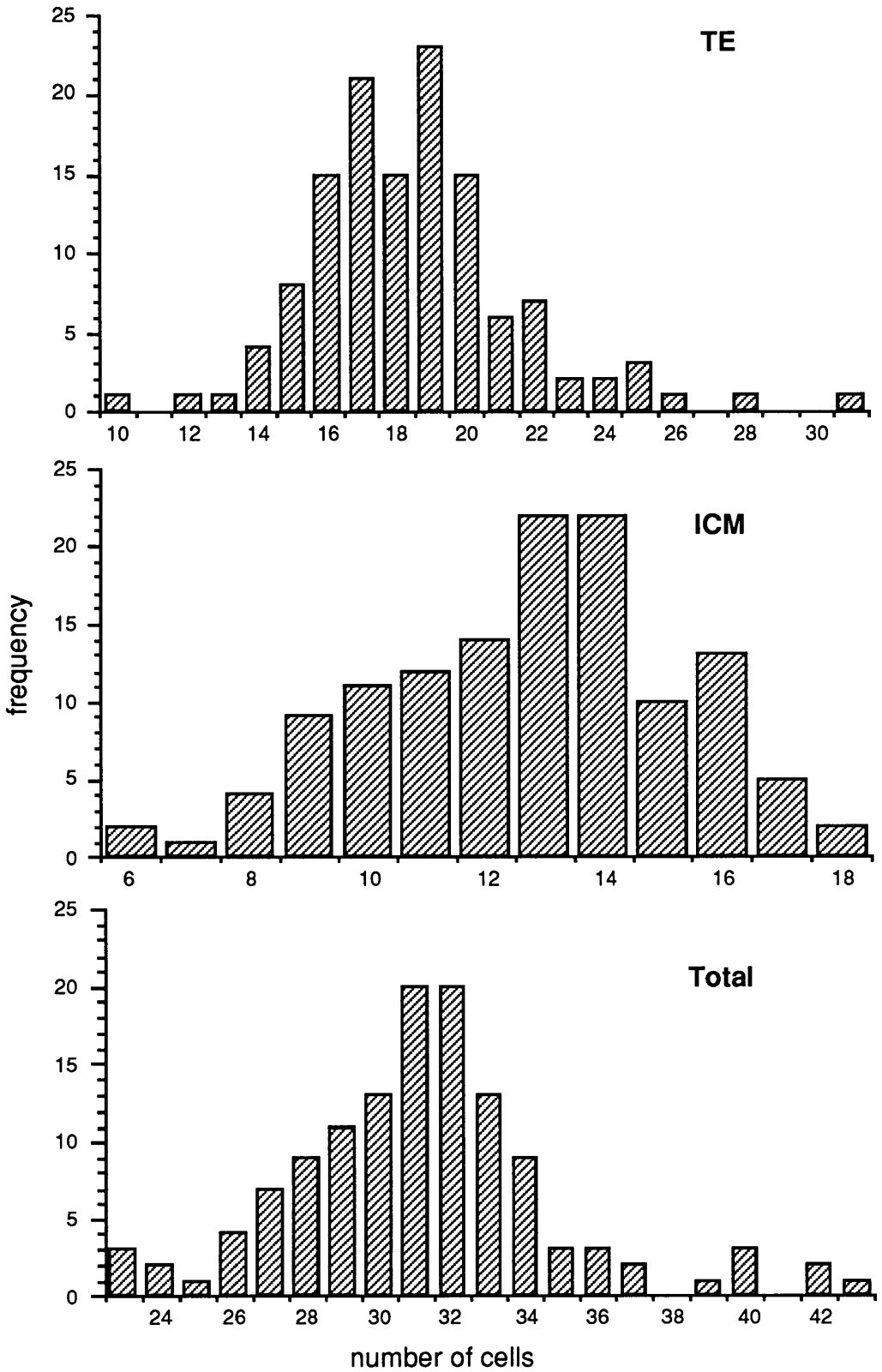



**Figure 3.2:** *Distribution of cell numbers in the TE and ICM of 127 unmanipulated control embryos of which 50% had cavitated. These embryos were synchronised to within 2h of each other at the 8-cell stage.*

completion of the fifth cleavage division at the 32-cell stage (Handyside, 1978; Smith and McLaren, 1977; Handyside and Hunter, 1986). Fig. 3.2 shows the distribution of TE, ICM and total cell numbers in these control embryos. The number of TE cells, 18.5 ± 0.27, and ICM cells, 12.72 ± 0.25, is very close to Handyside and Hunter's (1986) findings of a total cell number of 31.1 ± 3.6 (S.D.) with 18.8 ± 2.9 TE cells and 12.3 ± 2.6 ICM cells.

The average percentage of cells in mitosis (mitotic index (§8.5.2)) in the ICM (8.7 ± 1.0) was significantly higher than that in the TE (2.89 ± 0.38) ($P \leq 0.001$). Handyside and Hunter (1986) also observed high mitotic indeces in *in vivo* blastocysts, however, they were of the order of 4% in both the TE and ICM at 84h post coitum, when the majority of embryos were at the early blastocyst stage.

### §3.2.3 Bisected embryos

Total, TE and ICM cell numbers in a total of 21 intact control, 22 twin half embryos and 11 random half embryos were examined at the time of cavitation, at the equivalent of the 32-cell stage. There was no apparent difference in the timing of cavitation between the half and control embryos. The cell numbers in both twin and random halves are presented in Table 3.1 and for twin half embryos only in Table 3.2.

**Table 3.1:** *Numbers of TE and ICM cells in single control and half embryos at the 32-cell stage.*

| | n | TE cell no. (range) | ICM cell no. (range) | Total cell no. (range) |
|---|---|---|---|---|
| Singles | 21 | 17.5 ± 0.8* (10 - 28) | 11.9 ± 0.5 (6 - 17) | 29.4 ± 0.8 (23 - 37) |
| Halves | 33 | 10.2 ± 0.3 (7 - 15) | 5.4 ± 0.3 (2 - 8) | 15.6 ± 0.3 (12 - 22) |
| Halves × 2 | 33 | 20.4 ± 0.6* | 10.9 ± 0.5 | 31.2 ± 0.65 |
| Ratio of halves:singles | | 0.58 | 0.45 | 0.53 |

Values are expressed as means ± standard errors
Values compared with control using the Mann-Whitney U test; those which differ significantly from control values bear an asterisk: *$P < 0.001$

Multiplying the number of TE cells in half embryos by two and comparing them with the number of TE cells in single control embryos shows that there are

significantly more TE cells in half embryos ($P < 0.001$) (Table 3.1). A similar comparison between ICM cells in half and control embryos shows that the number of ICM cells is slightly reduced in half embryos, but not significantly so (Table 3.1). Also, there is a small, but not significant, increase in the total cell number in half embryos. Because only the increase in TE cell number in half embryos was significant, it is not possible to decide whether this effect is compensated for by reduction in the numbers of ICM cells or whether this increase simply accounts for the increase in total cell number. However, if the differences in the numbers of cells in the TE and ICM are calculated on the basis of the numbers of cells shifted between the two lineages per individual embryo (at the equivalent of the 32-cell stage), it appears that the increase in the numbers of TE cells (≈3) is not balanced by the reduction in numbers of ICM cells (one) (see conclusions §3.3; Table 3.9).

**Table 3.2:** *Comparison of the sum of ICM or TE numbers in twin half embryos with single control embryos*

| | n | TE cell no. (range) | ICM cell no. (range) | Total cell no. (range) |
|---|---|---|---|---|
| Singles | 16 | 17.9 ± 0.9* (13 - 28) | 12.0 ± 0.7 (6 - 17) | 29.9 ± 0.8 (23 - 37) |
| Sum of twin halves | 11‡ | 20.1 ± 0.7* (18 - 25) | 11.5 ± 0.7 (7 - 15) | 31.5 ± 1.15 (26 - 40) |

Values are expressed as means ± standard errors. Values compared with control using the Mann-Whitney U test; those which differ significantly from control values bear an asterisk: *P<0.01
‡22 half embryos

In the majority of cases, half embryos had been cultured and processed so that it was possible to identify the twin halves of the same embryo. If the numbers of TE and ICM cells in each twin half of the original embryo are added together, and compared with those in control embryos (Table 3.2), a similar result is obtained. The number of ICM cells, combining twin halves, is not significantly different from the ICM cell number in intact controls. In contrast, the number of TE cells, combining twin halves, is significantly larger ($P < 0.01$) than that in control embryos. This increase in the number of TE cells in half embryos is clearly shown in Fig. 3.3.

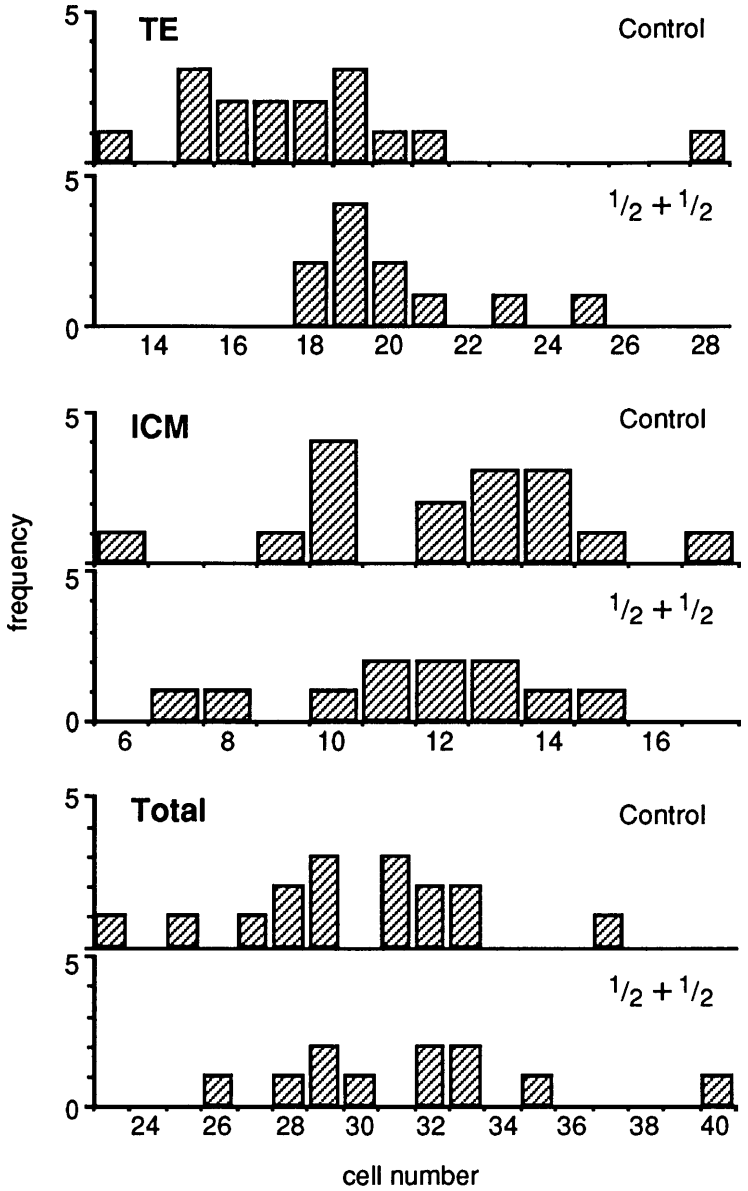



**Figure 3.3:** *Distribution of total, TE and ICM cell numbers in 16 control and both halves of 11 twin half embryos at the equivalent of the 32-cell stage.*

Interestingly, in both twin halves of an embryo, the ICM cell numbers were similar to each other, as were TE cell numbers, and they were rarely different by more than two cells (Fig. 3.4). In only 2/11 cases was there a reciprocal imbalance of more than two cells between the TE and ICM in two halves having similar total cell numbers (arrowed A, Fig. 3.4), and in one case there was an imbalance of TE only between the two halves (arrowed B, Fig. 3.4).

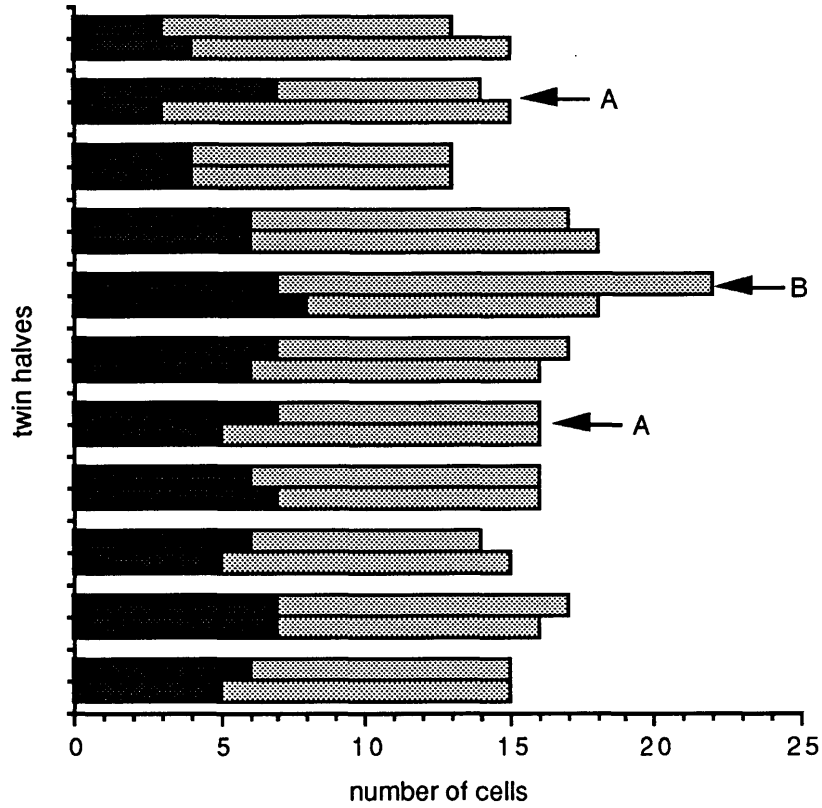



**Figure 3.4:** *Demonstration of similarity of numbers of ICM (■) and TE (▩) cells in twin half embryos*

The average percentage of cells in mitosis (the average mitotic index (§8.5.2)) in the TE of half embryos (3.2 ± 1.1) was similar to that in control embryos (3.4 ± 0.8). The mitotic index in the ICM of half embryos (8.0 ± 2.4) was lower than that in control embryos (10.8 ± 3.4), but not significantly so. Whilst there is no evidence from these values of increased mitotic activity in the TE, this technique is probably not sufficiently sensitive to detect small differences in the rates of mitosis due to the small numbers of cells being observed. An alternative would be to incubate half and control embryos for 12h in colchicine (an inhibitor of microtubule assembly and therefore spindle formation which blocks cells in mitosis), and count the number of mitoses which have accumulated. It would then be possible to detect whether more cells in the TE of half embryos had entered mitosis in the preceding 12h compared with control embryos.

### *§3.2.4 Aggregated embryos at the 32-cell stage*

The allocation of cells to the TE and ICM of control and double aggregates at the time of cavitation is shown in Table 3.3. The total cell number in doubles is just in excess of twice that of controls, however, there are less than twice the number of TE

cells (not significant) and significantly more than twice the number of ICM cells ($P<0.01$).

**Table 3.3:** *Numbers of TE and ICM cells in control and double embryos at the 32-cell stage.*

| | n | TE | ICM | Total |
|---|---|---|---|---|
| control | 45 | $18.69^{a}$ ±0.45 | $12.69^{b}$ ±0.33 | $31.38^{c}$ ±0.53 |
| doubles | 40 | 35.30 ±0.81 | 28.03 ±0.84 | 63.33 ±1.45 |
| doubles/2 | 40 | $17.65^{a}$ ±0.41 | $14.01^{b}$ ±0.42 | $31.66^{c}$ ±0.72 |
| ratio doubles: singles | | 1.89 | 2.21 | 2.02 |

values are means ± standard errors
Pairs of values bearing the same superscript compared by Mann-Whitney U test.: $^{ac}$not significant, $^{b}P<0.01$

Triple and quadruple embryos show a similar pattern of allocation, with about three and four times the total cell number, respectively, a decreased number of cells in the TE and a significantly increased number in the ICM (Tables 3.4 and 3.5).

**Table 3.4:** *Numbers of TE and ICM cells in control and triple embryos at the 32-cell stage.*

| | n | TE | ICM | Total |
|---|---|---|---|---|
| control | 21 | $18.62^{a}$ ±0.74 | $13.86^{b}$ ±0.43 | $32.48^{c}$ ±0.76 |
| triples | 24 | 53.71 ±1.00 | 45.42 0.84 | 99.13 ±1.56 |
| triples/3 | 24 | $17.90^{a}$ ±0.33 | $15.14^{b}$ ±0.28 | $33.04^{c}$ ±0.52 |
| ratio triples: singles | | 2.88 | 3.28 | 3.05 |

values are means ± standard errors.
Pairs of values bearing the same superscript compared by the Mann-Whitney U test: $^{ac}$not significant, $^{b}P<0.05$

**Table 3.5:** *Numbers of TE and ICM cells in control and quadruple embryos at the 32-cell stage.*

| | n | TE | ICM | Total |
|---|---|---|---|---|
| control | 40 | $18.73^a$ ±0.40 | $12.58^b$ ±0.49 | $31.30^c$ ±0.60 |
| quads | 34 | 65.35 ±1.12 | 61.24 ±1.18 | 126.59 ±1.93 |
| quads/4 | 34 | $16.34^a$ ±0.28 | $15.31^b$ ±0.30 | $31.65^c$ ±0.48 |
| ratio quads: singles | | 3.49 | 4.87 | 4.04 |

values are means ± standard errors
Pairs of values bearing the same superscript compared by the Mann-Whitney U test: $^c$not significant, $^a P<0.00005$, $^b P<0.0001$

If the differences in the numbers of cells in the TE and ICM are calculated on the basis of the numbers of cells shifted between the two lineages per individual embryo (at the equivalent of the 32-cell stage), the decrease in the number of TE cells by approximately one cell in double and triple embryos is balanced by an increase of approximately one cell in the ICM. In quadruple embryos the differences are larger, the TE is reduced and the ICM increased by between two and three cells (see conclusions §3.3; Table 3.9).

**Table 3.6:** *Average percentage of cells in mitosis (mitotic index) in the TE and ICM of control and aggregated embryos at the 32-cell stage.*

| | | Mitotic index | | |
|---|---|---|---|---|
| | n | TE | ICM | Total |
| control | 106 | 2.79 ±0.43 | 8.28 ±1.01 | 4.88 ±0.49 |
| doubles | 40 | 1.53 ±0.34 | 6.74 ±0.97 | 3.74 ±0.42 |
| triples | 24 | 3.22* ±0.57 | 7.61 ±1.22 | 5.22 ±0.58 |
| quads | 34 | 3.64** ±0.51 | 6.14 ±0.87 | 4.77 ±0.55 |

values are means ± standard errors.
Values compared with control using the Mann-Whitney U test; those which differ significantly from control values bear an asterisk: *P<0.05, **P<0.01.

In single control, double, triple and quadruple embryos the ICM has a higher mitotic index than the TE, which is not affected significantly by size. This is in

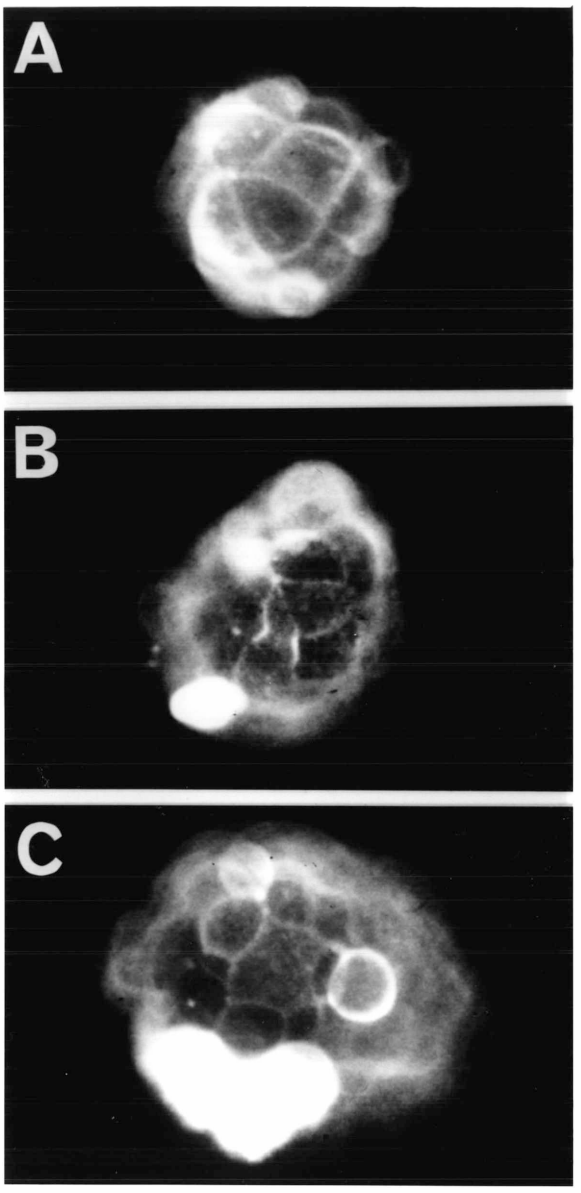
contrast to the mitotic index in the TE, which appears to increase with increasing embryonic size (Table 3.6).

### §3.2.5 Density of outer cells

A second method of visualising whether or not the number of TE cells is influenced by the surface area of the embryo involved labelling the surface of the embryo with FITC conjugated goat anti-rabbit IgG after incubating zona-free control and aggregated embryos in rabbit anti-mouse antiserum, at the equivalent of the 32-cell stage before the expansion of the blastocoel (§8.4.1). The entire exposed surface of the embryo is labelled but the cleavage furrows are clearly visible allowing traces of the outline of individual TE cells from photographs from which estimates of the exposed apical surface area in control and aggregated embryos could be calculated (§8.5.1). These estimates are based on the assumption that the surface of the embryo is flat, and do not allow for the spherical nature of the embryo, or the curvature of the surface of the individual cells themselves.

Fig. 3.5 shows examples of such labelling patterns in single, double and quadruple embryos. It can clearly be seen that the TE cells in quadruple embryos are more tightly packed than in single embryos. Indeed, calculations of the areas of the exposed apical surfaces of the TE cells show that in aggregated embryos, the apical surfaces are significantly smaller (Fig.3.6). This shows that the packing density of TE cells is not uniform and confirms the observation that in larger embryos the number of TE cells is not linearly proportional to the surface area (Fig. 3.7).

**Figure 3.5:** *Fluorescence micrograph of single (A), double (B) and quadruple (C) aggregated mouse embryos at the equivalent of the 32-cell stage. The embryo surface has been labelled with FITC conjugated goat anti-rabbit IgG after incubation in rabbit anti-mouse antiserum. The cleavage furrows are clearly visible (350×)*


A
B
C

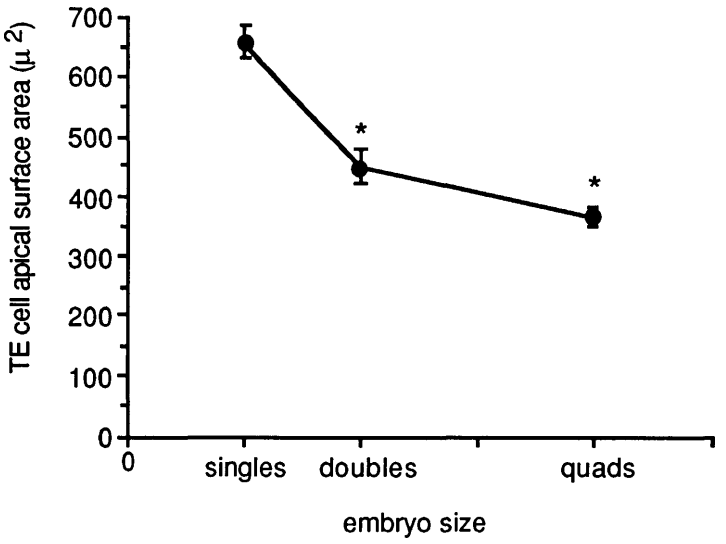



**Figure 3.6:** *Mean apical surface area ($\mu^2$) of individual TE cells in single, double and quadruple embryos at the equivalent of the 32-cell stage. Values compared with single controls using the Mann Whitney U test. * $P < 0.0001$. Doubles and quads differ significantly from each other ($P < 0.01$).*

### §3.2.6 Aggregated embryos at the expanded blastocyst stage

A second series of experiments was carried out to investigate how the numbers of cells in the TE and ICM changed over 24h *in vitro* following the 32-cell stage. Embryos were aggregated as previously (§3.2.1); some control, double and quadruple embryos were differentially labelled at the time of cavitation and the rest labelled 24h later. Table 3.7 confirms that even in this smaller group of embryos, at the 32-cell stage, there is a decreased proportion of TE cells and an increased number of ICM cells in larger embryos.

**Table 3.7:** *Numbers of TE and ICM cells in control, double and quadruple embryos at the 32-cell stage.*

| | n | TE | ICM | Total |
|---|---|---|---|---|
| control | 25 | 18.76 ±0.53 | 13.24 ±0.64 | 32.00 ±0.91 |
| doubles | 12 | 34.58 ±1.08 | 30.33 ±1.12 | 64.92 ±1.65 |
| doubles/2 | 12 | 17.29 ±0.54 | 15.17* ±0.56 | 32.46 ±0.82 |
| quads | 6 | 75.00 ±1.71 | 66.67 ±3.64 | 141.67 ±2.75 |
| quads/4 | 6 | 18.75 ±0.43 | 16.67** ±0.91 | 35.42* ±0.69 |
| ratio double:singles | | 1.84 | 2.29 | 2.03 |
| ratio quads:singles | | 4.00 | 5.04 | 4.43 |

values are means ± standard errors
values compared with control using the Mann-Whitney U test; those which differ significantly from control values bear an asterisk: *P<0.05, **P<0.01

**Table 3.8:** *Numbers of TE and ICM cells in control, double and quadruple embryos 24h after the 32h stage, at the expanded blastocyst stage.*

| | n | TE | ICM | Total |
|---|---|---|---|---|
| control | 21 | 61.81 ±2.86 | 13.95 ±0.83 | 75.76 ±3.14 |
| doubles | 17 | 128.29 ±5.87 | 40.59 ±1.79 | 168.88 ±7.15 |
| doubles/2 | 17 | 64.15 ±2.93 | 20.29*** ±0.9 | 84.44* ±3.58 |
| quads | 9 | 260.00 ±6.99 | 103.56 ±4.05 | 363.56 ±0.84 |
| quads/4 | 9 | 65.00 ±1.75 | 25.89*** ±1.01 | 90.89** ±2.13 |
| ratio double:singles | | 2.08 | 2.91 | 2.23 |
| ratio quads:singles | | 4.21 | 7.42 | 4.8 |

values are means ± standard errors
values compared with control using the Mann-Whitney U test; those which differ significantly from control values bear an asterisk: *P<0.05, **P<0.01, ***P<0.001

Table 3.8 shows that in control embryos in the next 24h, there is a threefold increase in the number of TE cells, but no change in the number of ICM cells. Handyside and Hunter (1986) noted that the mean ICM cell number remained fairly

constant in blastocysts *in vivo* over the equivalent period, whilst the number of TE cells increased. Double and quadruple embryos exhibit a similar threefold increase in TE cell number but the number of ICM cells also increases, so that, in quadruple embryos, on a per-single-embryo basis there are nearly twice as many cells in the ICM as in single control embryos.

## §3.3 Conclusions

### §3.3.1 32-cell stage analysis

The inside:outside hypothesis (§1.4.1) proposes that cells on the outside of an embryo form TE whilst enclosed, inner cells form ICM. The number of cells allocated to the TE is thus dependent on simple packing and is proportional to the surface area of the embryo. Bisection and aggregation of embryos at preimplantation stages alters the surface area:volume ratio of resulting morulae and provides a useful model for examining the effect of surface area on the allocation of cells to the TE. In order to satisfy the inside:outside hypothesis several predictions must be fulfilled. The first is that the change in volume of the manipulated embryo will be conserved for at least the next three cleavage divisions, ie. that a double embryo created by aggregating two 8-cell embryos will still have double the volume at the stage at which it is examined, and, consequently, double the number of cells. The second is that the number of cells in the TE is proportional to the surface area (Appendix 1). Thus, for instance, in a double embryo there will be fewer than twice (1.59 times) the number of TE cells. It follows that, in order to maintain the double volume and double the number of cells, there must be an *equivalent* increase in the number of ICM cells.

The results presented here do indicate a statistically significant increase in the number of TE cells in halves and conversely a significant increase in the number of ICM cells in aggregates. However, these changes are much smaller than predicted on the basis of the alteration in surface area. If the numbers of TE and ICM cells (18.5 and 12.72, respectively) in the complete series of control embryos examined (§3.2.2) are used to calculate how closely the number of TE cells in aggregated embryos relates to the surface area of aggregated embryos, the observed data are closer to the values predicted on the basis of volume (—●—) than to those predicted on the basis of surface area (—○—) (Fig. 3.7). Similarly, if the TE is proportional to the surface area, the remaining cells would be enclosed and form ICM cells, and

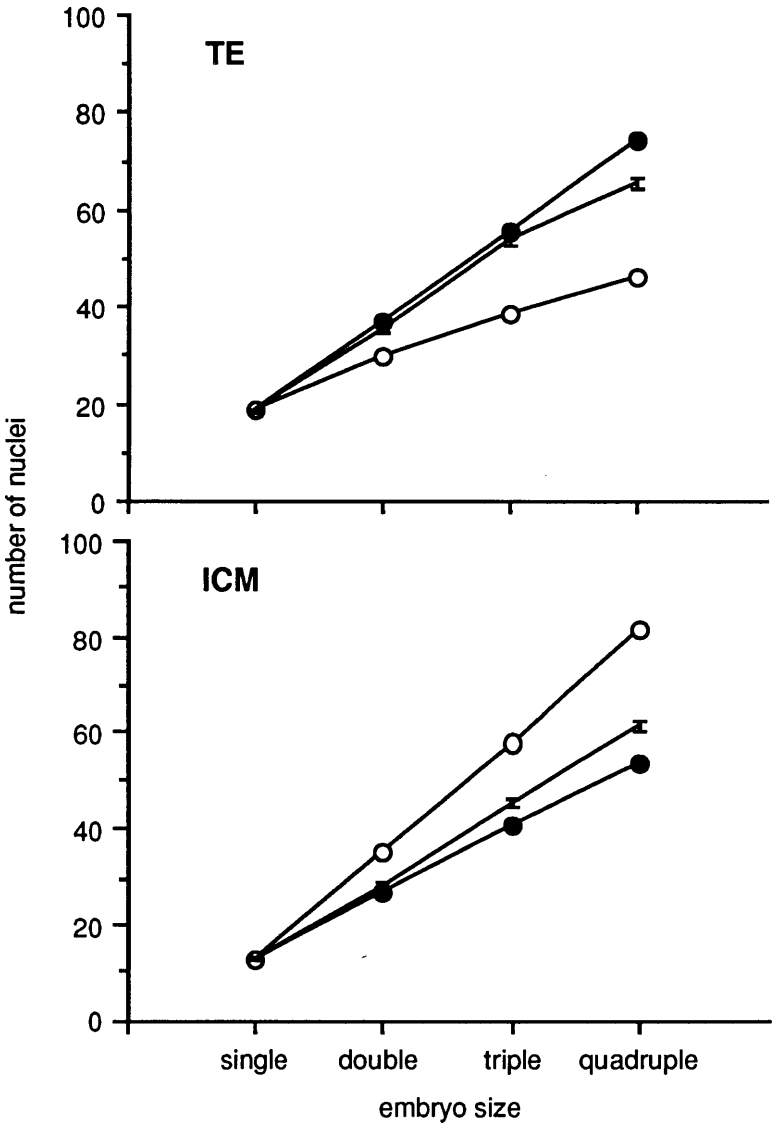



**Figure 3.7:** *Number of cells in the TE and ICM of control and aggregated embryos at the 32-cell stage. (——) observed data; (—●—) predicted on the basis of volume‡; (—○—) predicted on the basis of surface area**

**Calculated by multiplying the control TE cell number of 18.5 by $2^{2/3}$, $3^{2/3}$ and $4^{2/3}$ for double, triple and quadruple embryos respectively. ‡Calculated by multiplying the control TE cell number of 18.5 by 2, 3 and 4 for double, triple and quadruple ebmryos respectively.*

the number of ICM cells would be elevated, as predicted by the surface area related line (—○—) in the lower graph. Again, the observed data do not fit this predicted line. Thus it appears that the allocation of cells to both lineages is not simply determined by the surface area of the embryo.

This is substantiated by surface labelling of intact embryos at the equivalent of the 32-cell stage, allowing direct visualization of the apical surfaces of the TE cells (Fig. 3.5). In larger embryos the TE cells are clearly more closely packed, and the apical surface area of individual cells is significantly smaller (Fig. 3.6).

If the number of cells in both lineages at the time of cavitation in double, triple and quadruple embryos is divided by 2, 3 and 4 respectively (Tables 3.3, 3.4 and 3.5), it is possible to compare directly, first, the numbers of cells in the TE and ICM, and second, how changes in embryo volume have affected these numbers on a "per-single-embryo" basis (Table 3.9).

**Table 3.9:** *Changes in cell number in ICM and TE lineages per equivalent single embryo at the 32-cell stage*

| | Predicted* | Observed | | |
|---|---|---|---|---|
| | TE | TE | ICM | Total |
| Halves[1] | ↑ 4.82 | ↑ 2.90 | ↓ 1.00 | ↑ 1.80 |
| Doubles[2] | ↓ 3.82 | ↓ 1.04 | ↑ 1.32 | ↑ 0.28 |
| Triples[3] | ↓ 5.67 | ↓ 0.72 | ↑ 1.28 | ↑ 0.56 |
| Quads[4] | ↓ 6.94 | ↓ 2.39 | ↑ 2.73 | ↑ 0.35 |

* Predicted if the TE were proportional to the surface area of the embryo.
[1] Table 3.1
[2] Table 3.3
[3] Table 3.4
[4] Table 3.5

Again, it is clear from these observations that the changes in number of TE cells are not proportional to the surface area of the embryo, suggesting that the simple packing as originally proposed by the inside:outside hypothesis is not the mechanism by which allocation to the TE and ICM is controlled, as previously suggested (Buehr and McLaren, 1974, Rands, 1985).

An alternative possibility is that altered cell-cell interactions in bisected and aggregated embryos change the proportion of conservative and differentiative divisions during the fourth and fifth cleavage divisions at the 8- to 16- and 16- to 32-cell stages. At the 8- to 16-cell transition approximately half the polarized 8-cell blastomeres (3 or 4 cells) undergo conservative divisions to form two polar daughter cells, whilst the other half (4 or 5 cells) undergo differentiative divisions, to form one polar and one apolar inner cell (Johnson and Maro, 1986) (§1.4.3). This results, at the 16-cell stage, in approximately 75% (11 or 12 cells) polar outer cells, and 25% (4 or 5 cells) inner cells, which is close to the number of these cells observed at this stage (Handyside, 1981; Johnson and Maro, 1986; Fleming, 1987). By labelling outer cells at the 16-cell stage with fluorescently labelled latex

microparticles (FL), which are taken exclusively into the outer cells by endocytosis, and labelling the surface of outer cells again at the 32-cell stage with rhodamine conjugated concanavalin A (Rh-Con A), it has been possible to follow the fate of inner and outer cells at the 16- to 32-cell transition (Fleming, 1987). All of the outer cells at the 32-cell stage were both FL and Rh-Con A labelled, showing that they had been exclusively derived from outer cells at the 16-cell stage. The majority of inner cells at the same stage were totally unlabelled, indicating that they resulted from the division of unlabelled inner cells at the 16-cell stage. However, a variable but low number of inner cells are labelled with FL only, showing that a minority of outer cells at the 16-cell stage had divided differentiatively to give rise to inner and outer cells. Thus it appears that at the 16- to 32-cell transition there is a second allocation of, on average, two cells to the ICM, resulting in the 32-cell stage embryo having a predicted modal number of 20 outer cells and 12 inner cells.

If a single polarized 8-cell blastomere underwent a differentiative division rather than a conservative division as a consequence of aggregation, there would be one extra inner cell at the 16-cell stage, and one less outer cell. Thus after the next cleavage division, at the 32-cell stage, there would be two more ICM cells and two less TE cells than normal. The magnitude of the shift between the two lineages only reaches this level in the case of quadruple embryos (Table 3.9). Alternatively, if the 8- to 16-cell allocation is unaffected by aggregation but at the 16- to 32-cell transition a single outer cell underwent a differentiative instead of a conservative division, there would only be one extra ICM and one less TE cell at the 32-cell stage. In double and triple embryos, the reduction in TE cells and gain in ICM cells is in the region of one cell, suggesting that allocation results in limited re-allocation at this stage. However, the numbers of outer and inner cells at the equivalent of the 16-cell stage would need to be analysed to distinguish between infrequent effects at the fourth cleavage division versus an effect on the second ICM input during the fifth division.

In half embryos, the increase in the number of TE cells at the equivalent 32-cell stage, of almost three cells (Table 3.9) may be accounted for by one or occasionally two 8-cell blastomeres undergoing conservative instead of differentiative divisions, producing, by the 32-cell stage, two or more extra outer cells. Another explanation could be that at the 16- to 32-cell transition the second allocation of two cells to the ICM does not occur (Fleming, 1987). In either case,

however, the ICM itself does not decrease, as would be expected, by two cells, but only by one cell. This suggests that in half embryos, in addition to a relatively infrequent switch in the allocation of a cell from the ICM to the TE, there is also an effect on the rate of division of TE cells. The rate of division of anchorage-dependent adult cells is proportional to spreading (Folkman and Moscona, 1978). It is, therefore, possible that the TE cells in half embryos, which may be less crowded and more spread than in control embryos due to the greater surface area:volume ratio, may divide more rapidly.

### *§3.3.2 Expanded blastocyst analysis*

The proportion of ICM cells in single embryos at the 32-cell stage (41%) is similar to that expected from previous observations (40%: Handyside and Hunter, 1986). There is an increase in the proportion of ICM cells in double and quadruple embryos but it is not as great as would be expected on the basis of the inside:outside hypothesis (54% in doubles and 64% in quadruple embryos).

**Table 3.10:** *Proportion of TE and ICM in aggregated embryos at the equivalent of the 32-cell stage expressed as a percentage of the total cell number.*

| | 32-cell stage | | Expanded blastocyst stage | |
|---|---|---|---|---|
| | TE (%) | ICM (%) | TE (%) | ICM (%) |
| Single | 58.6 | 41.4 | 81.6 | 18.4 |
| Doubles | 53.3 | 46.7 | 76.0 | 24.0 |
| Quads | 52.9 | 47.1 | 71.5 | 28.5 |

After 24h the proportion of ICM cells in single embryos has decreased to 18.4%. This again is comparable to the proportion found previously in expanded blastocysts at an equivalent stage *in vivo* (102h post coitum: Handyside and Hunter, 1986). Over this 24h culture period there has been no apparent increase in the ICM cell number (Tables 3.7 and 3.8). This is thought to result from a combination of slower division (MacQueen and Johnson, 1983) and cell death predominantly in the ICM (Handyside and Hunter, 1986). Similarly, the proportion of ICM cells in double and quadruple embryos at the expanded blastocyst stage has decreased markedly from about 47% to 24 and about 29%, respectively, indicating that in these embryos similar mechanisms are operating. However, there is no evidence for a

disproportionate reduction indicative of size regulation prior to implantation (§1.6). It appears that in double and quadruple embryos the reverse is true and the proportion of ICM cells remains higher. On a per-single-embryo basis, quadruple embryos have approximately 26 ICM cells compared with approximately 14 in single control embryos, ie. nearly twice as many (Table 3.8). It may be that in single embryos the absence of an increase in ICM cell number during blastocyst expansion may be influenced either by the TE or by the blastocoel fluid. In the larger ICMs of quadruple embryos, with approximately 100 ICM cells, many cells may not be in intimate contact with either the TE or the blastocoel fluid and, therefore, not subject to any regulative process.

Finally, these changes in the proportions of the two lineages between the completion of allocation at the equivalent of the 32-cell stage and the expanded blastocyst stage provide a possible explanation for the discrepancy with the results of Rands (1985). Since the reduction in proportion of TE, and increase in ICM cell numbers, are accentuated in quadruple embryos in comparison with single control embryos (Table 3.10), it is clear that analysis of effects on allocation *per se* cannot be analysed at the expanded blastocyst stage.

### *§3.3.3 Implications for preimplantation biopsy in human preimplantation embryos*

These observations have important implications for the preimplantation diagnosis of genetic defects in human embryos (§1.7). If one or two cells are biopsied from an 8-cell embryo, the inside:outside hypothesis would predict that in addition to a reduction in the total cell number, there would be a disproportionate decrease in the number of ICM cells. As the ICM forms the fetus, such an effect would be undesirable. However, here it has been shown that allocation to the TE and ICM is not simply related to surface area and there is no significant decrease in the proportion of cells allocated to the ICM in half embryos.

# §4 Uptake of glucose and pyruvate by human preimplantation embryos

## §4.1 Introduction

The success of fertilizing oocytes *in vitro* in both the mouse (85%; Carroll *et al*, 1989) and the human (56%; Appendix 9.2) is high. However, unlike the mouse, where rates of blastocyst formation can reach 75% in culture after *in vitro* fertilization (IVF) (Edgar *et al*, 1987), the proportion of surplus human embryos developing to the blastocyst stage *in vitro* ranges from 17% (Bolton *et al*, 1989), 25% (Fehilly *et al*, 1985), 39% (Braude *et al*, 1990), to 57% (Dawson *et al*, 1988). This variable and reduced rate of blastocyst formation in the human could be due to several reasons including chromosome abnormalities and suboptimal culture conditions. The incidence of chromosome abnormalities in normally fertilized human embryos after IVF is high, ranging from 12.5% for embryos of good quality to 37% for those which are fragmenting (Plachot *et al*, 1987). This high incidence of chromosomal defects could account for the failure of many embryos to develop to the blastocyst stage. In addition, relatively little is known about the specific culture requirements of human embryos. As a result, most of the culture media used for human IVF have been modified from those developed for mouse embryos. Uneven cleavage and fragmentation, frequently observed with human embryos *in vitro*, may be indicative of suboptimal media conditions. In comparison, the metabolism of early mouse embryos, which are more easily obtainable in large numbers, is relatively well understood. During early cleavage, pyruvate, rather than glucose, is the major energy substrate for mouse embryos (Brinster, 1965b; §1.5). There is then a switch in substrate preference at the 8-cell/morula stage in favour of glucose, which becomes the predominant energy source at the blastocyst stage (Gardner and Leese, 1986).

It is only recently with the development of increasingly sensitive assay techniques that examination of the substrate requirements of single human preimplantation embryos has been possible. Using a non-invasive technique, Leese *et al* (1986) found that pyruvate consumption by single human oocytes and embryos was high in the mature oocyte, fell after fertilization then increased to a peak just prior to the morula stage before declining again. Wales *et al.* (1987) found that glucose turnover as measured by $^{14}CO_2$ production, lactate accumulation and carbon incorporation from labelled [U-$^{14}$C]glucose was initially low, before increasing dramatically between days

2 and 3 (day 0 = day of insemination). Thus it appears that, as in the mouse, there may be an initial blockade to glucose utilization and that pyruvate is the major energy substrate in early human embryos.

Here, the uptakes of both pyruvate and glucose by single human oocytes and embryos have been measured simultaneously for the first time from days 1 or 2 respectively to day 6 post insemination. Abnormally fertilized embryos and those which arrested have also been examined. These studies were carried out during the transition from a superovulation regimen involving clomiphine citrate (CC)/human menopausal gonadotrophin (hMG) to one where patients were initially suppressed with a luteinising hormone releasing hormone (LHRH) agonist prior to being given hMG. Individual embryos were incubated for successive 24h periods, between days 2 and 6, in 4µl droplets of a modified T6 medium (HLT6) containing 5mM lactate, 0.47mM pyruvate and 1mM glucose (§8.6). On day 6 embryos were categorized as "normal" or "arrested", on the basis of whether they reached the blastocyst stage by day 6 or stopped cleaving, respectively. Depletion of pyruvate and glucose from the medium by the embryos was measured using an ultra-micro fluorescence assay (Leese and Barton, 1984; Gardner and Leese, 1986) (§8.6.1).

## §4.2 Development of unfertilized oocytes and preimplantation embryos in vitro

The morphology of nine unfertilized oocytes and a total of 64 normally and abnormally fertilized embryos was scored each day. Nine patients treated with the CC/hMG regimen donated 13 normally fertilized embryos (1.4 surplus embryos per patient). Twelve patients treated with the LHRH agonist/hMG regimen donated 30 normally fertilized embryos (2.5 surplus embryos per patient).

The majority of fertilized embryos which arrested (9 out of 17) stopped cleaving between days 3 and 4 at the 4- to 8-cell stage. (One fertilized embryo which developed normally to the morula stage by day 5, but could not be examined on subsequent days, was included in the analysis of those that progressed to the blastocyst stage). The polyspermic embryos which arrested did not progress beyond the morula stage. Four of the seven parthenogenetic embryos arrested at the 4-cell stage and the other three reached the 8-cell or morula stages.

**Table 4.1:** *Development of normally fertilized and abnormally fertilized surplus human embryos in 4µl microdrops in vitro*

| Superovulation regimen | No. of pronuclei | No. of embryos | No. to blastocyst by day 5 | No. to blastocyst by day 6 | Total no. to blastocyst |
|---|---|---|---|---|---|
| CC/hMG | 2 | 13 | 3 (23%) | 7 (54%) | 10 (77%) |
| Buserelin/hMG | 2 | 30 | 7 (23%) | 8 (27%) | 15 (50%) |
| Total | 2 | 43 | 10 (23%) | 15 (35%) | 25 (58%) |
| Either | >2 | 14 | 0 | 1 (7%) | 1 (7%) |
| Either | <2 | 7 | 0 | 0 | 0 |

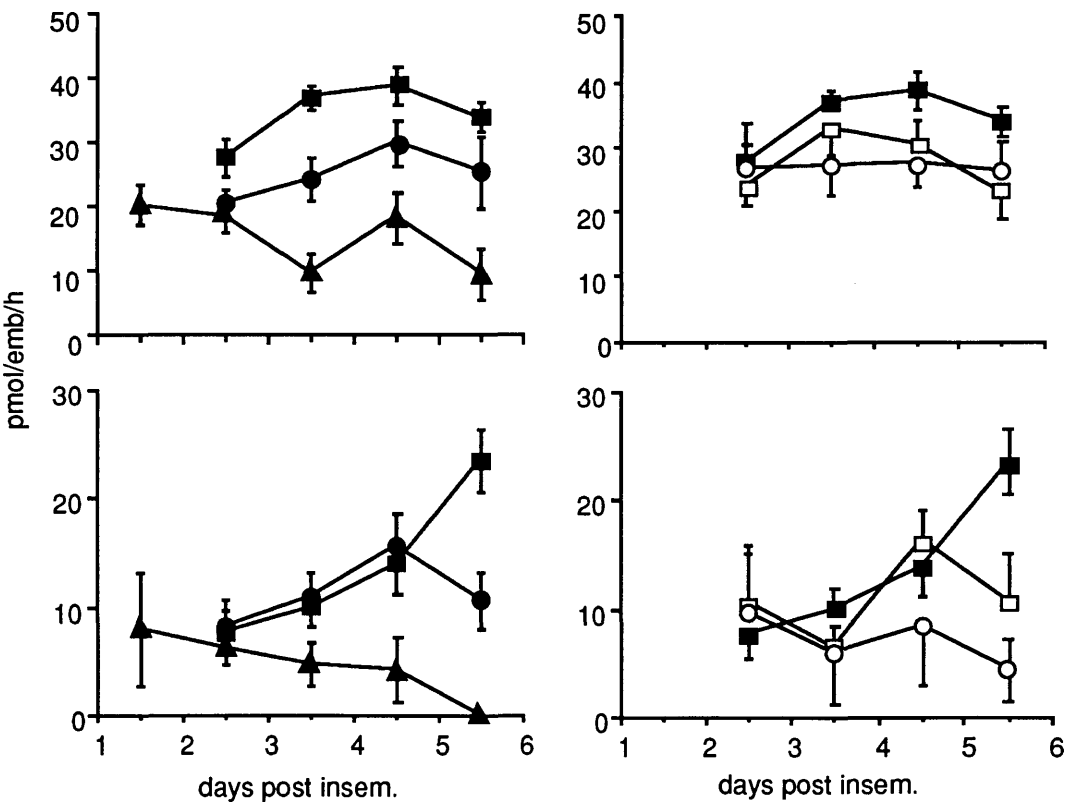



**Figure 4.1:** *Pyruvate (upper panel) and glucose (lower panel) uptake by normally fertilized embryos which developed to the blastocyst stage by day 6 (■), embryos which arrested during cleavage (●), unfertilized oocytes (▲), polyspermic embryos(□) and parthenogenetic embryos(○).*

## §4.3 Pyruvate uptake

The pyruvate uptake of normally fertilized embryos which developed to the blastocyst stage by day 6, increased from ≈28 to a maximum of 40 pmole/embryo/h between days 2.5 and 4.5 and then started to decline (Fig. 4.1). There was no significant difference in uptake by these fertilized embryos from patients treated with the CC/hMG or LHRH agonist/hMG regimens, so the data were pooled. Fertilized embryos which arrested before the blastocyst stage had a lower pyruvate uptake throughout the period examined, with a peak consumption on day 4.5 of only 30 pmole/embryo/h. Ageing unfertilized oocytes had the lowest uptakes which declined from 20 to 10 pmole/embryo/h. Uptake by polyspermic embryos showed a similar profile to that of normally fertilized embryos but was consistently lower between days 2 and 6. Pyruvate consumption by polyspermic embryos began to decline from 33 pmole/embryo/h on day 3.5. The uptake by parthenogenetic embryos did not change from its value of ≈28 pmole/embryo/h on day 2.5.

## §4.4 Glucose uptake

The glucose uptake of normally fertilized embryos which developed to the blastocyst stage by day 6, increased from ≈8 to 14 pmole/embryo/h between days 2.5 and 4.5 and then increased further to 24 pmole/embryo/h on day 5.5 at the blastocyst stage (Fig. 4.1). Again, there was no significant difference in uptake between the embryos from patients treated with the CC/hMG or LHRH agonist/hMG regimens, except on day 4.5 when embryos from the latter patients had a higher uptake. However, because of the overall similarity in the uptake profiles the data were pooled. The glucose uptake of fertilized embryos which arrested and of polyspermic embryos was similar to that of fertilized embryos developing to the blastocyst stage except on day 5.5, when they failed to demonstrate the final upsurge in consumption. Uptake by unfertilized oocytes was low and decreased steadily from initial levels of <10 pmole/embryo/h. Uptake by unfertilized embryos which cleaved (parthenogenetic embryos) was higher than that of ageing unfertilized oocytes but also declined throughout the preimplantation period.

## §4.5 Conclusions

This study confirms that nutrient uptake by single human oocytes and preimplantation embryos can readily be measured non-invasively. Furthermore, a large proportion of

the fertilized embryos cleaved and appeared to develop normally when cultured in the microdrops needed to facilitate measurements of nutrient uptakes.

These results support the observations of Gardner and Leese (1986) who demonstrated that pyruvate is taken up preferentially during early cleavage stages in the mouse, but with a switch to glucose uptake during the transition between morula and blastocyst stages on days 3 to 4 post-fertilization (§1.5.4). Although the pattern of nutrient consumption by fertilized human embryos which developed to the blastocyst stage reported here is qualitatively similar to the mouse, there are a number of important differences. For example, there is significant uptake of glucose throughout the period examined including the early cleavage stages (Fig. 4.1) whilst the fall in pyruvate uptake at the blastocyst stage is less dramatic, and the rise in glucose uptake is not as marked.

It is notable that the levels of uptake of pyruvate by human embryos are 15 - 20 times higher than those of preimplantation mouse embryos (≈2pmole/embryo/h) (Gardner and Leese, 1986), when the difference in volume between human and mouse embryos is only 4- to 5-fold. In contrast, the final value for glucose uptake on day 5.5, 24 pmole/embryo/h, corresponds closely with those given by mouse blastocysts on the basis of this difference in volume (≈4 pmole/embryo/h; Gardner and Leese, 1986).

Finally, it is interesting that the pyruvate uptakes of embryos which arrested is in the region of 20% lower than of embryos which developed to the blastocyst stage. This suggests that the pyruvate uptake of preimplantation human embryos reflects their viability, and is a useful method for assessing the effects of manipulations, such as the removal of one or two cells during preimplantation biopsy, on the health of the embryo.

# §5 Metabolism of aggregated preimplantation mouse embryos

## §5.1 Introduction

Preimplantation mouse embryos can be successfully cultured from the 1-cell to the blastocyst stage in a simple salt solution supplemented with the energy substrates glucose, pyruvate, lactate and a protein source such as bovine serum albumin (BSA) (Whitten and Biggers, 1968; Whittingham, 1971; §1.5.1). During early cleavage pyruvate is the major energy substrate for mouse embryos (Brinster, 1965a; §1.5). There is then a switch in substrate preference at the 8-cell/morula stage in favour of glucose, which becomes the predominant energy source at the blastocyst stage (Gardner and Leese, 1986). Nutrient uptake could be regulated at two levels: either (1) at the membrane level where uptake rates will be determined by the existence or efficiency of transport mechanisms or (2) at the intracellular level where uptake rates will be controlled by rates of utilization of substrates (Gardner and Leese, 1988; Biggers *et al*, 1989). Regulation by a combination of (1) and (2) is also possible. Studies using inhibitors of facilitated transport showed that transport mechanisms for pyruvate and glucose do exist and that in the preimplantation mouse embryo uptake of these substrates is both by passive diffusion and by carrier-mediated (facilitated) transport (§1.5.5) (Gardner and Leese 1988). It appears that the glucose carrier-mediated mechanism operates from the 2-cell stage, increasing markedly between the 8-cell and blastocyst stages and that the pyruvate carrier is present throughout preimplantation development.

Preimplantation embryonic size can be changed either by aggregating two or more 8-cell stage embryos, or by disaggregating cleavage stage embryos (§1.6). Numerous studies in a variety of species have shown that such size-manipulated preimplantation embryos are capable of regulating their size after implantation and giving rise to normal sized young (reviewed by Papaioaonnou and Ebert, 1986). Two embryos aggregated at the early 8-cell stage compact to form a single larger morula, which has twice the volume of a single embryo. These develop into morphologically normal but larger blastocysts. As the volume and surface area of a sphere are proportional to the cube and square of the radius respectively, embryos of twice the volume have less than twice the surface area (Appendix 9.1). Such aggregated embryos therefore provide a useful model with which to study the uptake of nutrients at preimplantation stages, and to investigate whether uptake is regulated at the

intracellular level, or at the membrane level. If substrate uptake is regulated at the intracellular level it would be expected that the nutrient requirements would be proportional to the volume of the embryo, whereas, if substrate uptake is regulated at the membrane level, it would be expected to be proportional to the surface area.

## §5.2 Nutrient Uptake

### §5.2.1 Method

Two-cell embryos were flushed from oviducts on day 2 (§8.3) and cultured overnight in M16 + BSA (§8.1.1) under oil. In an attempt to synchronize embryos to within a period of 2h, 8-cell and more advanced embryos were initially eliminated from the pool of embryos. 2h later, uncompacted 8-cell embryos were selected for manipulation (≈66-67h post hCG). Zona-free embryos (§8.3.1) were aggregated into "double", "triple" or "quadruple" embryos (§8.3.3), consisting of sixteen, twenty-four or thirty-two cells each. Groups of single and aggregated preimplantation embryos were incubated for successive 24h periods from the morula (day 3) to the expanded blastocyst (day 5) stage in 3µl droplets of a modified T6 medium (HLT6; §8.6; Hardy *et al*, 1989b) containing 5mM lactate, 0.47 mM pyruvate and 1mM glucose. Depletion of these substrates from the medium by the embryos was measured using an ultramicrofluorescence assay (§8.6.1; Leese and Barton, 1984; Gardner and Leese, 1986).

### §5.2.2 Volume and surface area of single and aggregated embryos

Double, triple and quadruple embryos were allowed to compact before measuring nutrient uptake. Before interpretation of the data on nutrient uptake, it was important to verify the assumption that the aggregated embryos had formed spherical morulae with the surface areas predicted on the basis of their increased volume. Measurements were made before cavitation, since varying blastocoel cavity sizes could have distorted the true embryonic volume. The mean volumes and surface areas of single and aggregated embryos are shown in Table 5.1 along with the values expected from theoretical calculation. It can be seen that the observed values are very close to those predicted.

**Table 5.1:** *Observed and expected volumes and surface areas of single and aggregated embryos*

| | | Single | Double | Triple | Quadruple |
|---|---|---|---|---|---|
| | n | 48 | 28 | 22 | 30 |
| volume | observed ($\mu^3$) | $1.76 \times 10^5$ $\pm 5.12 \times 10^3$ | $3.52 \times 10^5$ $\pm 1.13 \times 10^4$ | $5.36 \times 10^5$ $\pm 1.56 \times 10^4$ | $7.02 \times 10^5$ $\pm 1.9 \times 10^4$ |
| | expected ($\mu^3$)* | $1.76 \times 10^5$ | $3.52 \times 10^5$ | $5.28 \times 10^5$ | $7.04 \times 10^5$ |
| surface area | observed ($\mu^2$) | $1.51 \times 10^4$ $\pm 2.95 \times 10^2$ | $2.40 \times 10^4$ $\pm 5.09 \times 10^2$ | $3.18 \times 10^4$ $\pm 6.28 \times 10^2$ | $3.80 \times 10^4$ $\pm 6.89 \times 10^2$ |
| | expected ($\mu^2$)† | $1.51 \times 10^4$ | $2.40 \times 10^4$ | $3.14 \times 10^4$ | $3.81 \times 10^4$ |

values are means ± standard errors
* Calculated by multiplying the observed volume of single embryos by 2, 3 and 4 for double, triple and quadruple embryos respectively.
†Calculated by multiplying the observed surface area of single embryos by 1.59, 2.08 and 2.52 for double, triple and quadruple embryos respectively.

### §5.2.3 Substrate uptakes of single control embryos

Pyruvate uptake by single control embryos decreased from 3.44 ± 0.34 pmol/embryo/h on day 3.5 to 2.35 ± 0.53 pmol/embryo/h on day 4.5 (at the expanded blastocyst stage). In contrast, glucose uptake increased from 3.17 ±0.64 pmol/embryo/h on day 3.5 to 4.23 ±0.45 pmol/embryo/h on day 4.5. The decrease in pyruvate uptake and increase in glucose uptake is in agreement with the observations of Gardner and Leese (1986) on mouse embryos cultured from the 2-cell stage and confirms that mouse embryos undergo a switch in their nutrient requirements from pyruvate to glucose. However, pyruvate uptakes were higher than those observed by Gardner and Leese (1986) (<0.25 pmol/embryo/h at the morula and blastocyst stage), although glucose uptakes were similar at the blastocyst stage. Gardner and Leese

**Table 5.2:** *Concentrations of glucose, pyruvate and lactate in the embryo culture media M16 and HLT6 and in mouse oviduct fluid.*

| Substrate | M16* (mM) | HLT6† (mM) | Oviduct Fluid§ (mM) |
|---|---|---|---|
| Glucose | 5.56 | 1.00 | 3.40 |
| Pyruvate | 0.33 | 0.47 | 0.37 |
| Lactate | 23.28 | 5.00 | 4.79 |

* Whittingham, 1971; † Hardy *et al*, 1989b; § Gardner and Leese, 1990. (in the presence of cumulus cells).

(1986) carried out their incubations in medium M16 (Whittingham, 1971; §8.1.1) containing 23.3 mM lactate, whereas in this study, incubations were in HLT6 medium containing only 5mM lactate, a concentration closer to that found in mouse oviductal fluid (4.79mM lactate; Gardner and Leese, 1990) (Table 5.2). Lactate ($CH_3CHOHCOO^-$) and pyruvate ($CH_3COCOO^-$) are structurally similar; their interconversion is catalyzed by lactic dehydrogenase and both are utilized by preimplantation embryos during early cleavage (although lactate alone cannot support the first cleavage division (Biggers *et al.*, 1967)). The higher pyruvate uptakes described above, in the presence of the low lactate concentrations in HLT6, may be explained by studies carried out by Wales and Whittingham (1973), where two to three times as much carbon was accumulated from radiolabelled pyruvate (0.5mM) in the presence of 5mM lactate as in the presence of 25mM lactate. Thus high levels of lactate in the culture medium such as those used in the studies of Gardner and Leese (1986) appear to depress pyruvate uptake, which is increased in the lower concentrations of lactate used in this study.

### *§5.2.4 Substrate uptake of aggregated embryos*

Larger embryos took up significantly more pyruvate and glucose ($P<0.05$, Kruskal Wallis test for more than two groups) It is possible to predict what the uptakes of double, triple and quadruple embryos would be if their uptake was related to volume by multiplying the uptake of single control embryos by 2, 3 and 4 respectively (—▲—; Fig. 5.1 and 2). Similarly, if related to surface area; double, triple and quadruple embryos would increase by 1.59, 2.08 and 2.5 times respectively the single embryo uptake (—■—; Fig. 5.1 and 2).

If the observed uptakes of aggregated embryos are plotted with these predicted uptakes, it can be seen that between days 3 and 4, during cavitation, the uptake of glucose and pyruvate by aggregated embryos appears to be related to the surface area of the embryos rather than their volume (Figs. 5.1 and 2). Interestingly, between days 4 and 5, during blastocyst expansion, both pyruvate and glucose uptake by double embryos is closer to the uptake expected if it is related to volume. However, uptakes of triple and quadruple embryos appear to be surface area related.

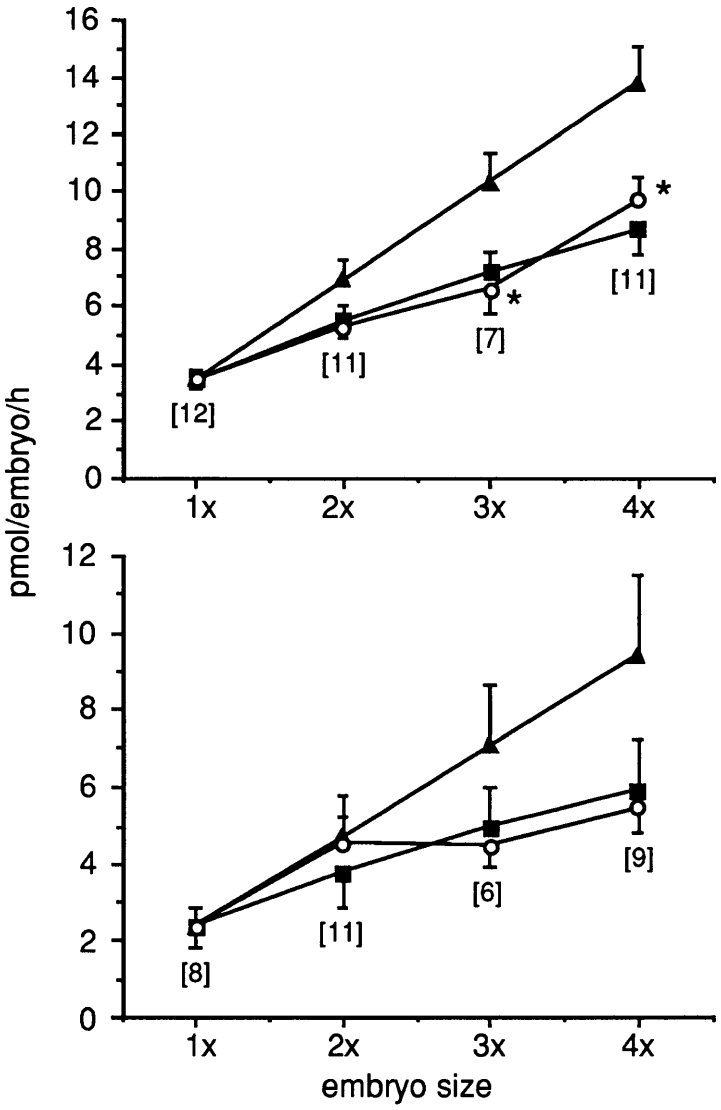



**Figure 5.1:** *Pyruvate uptake by single and aggregated embryos between days 3 and 4 (upper panel) and days 4 and 5 (lower panel) post hCG. (—○—) observed data; (—▲—) expected uptakes if volume dependent; (—■—) expected uptakes if surface area dependent. Numbers in parentheses are the number of observations. Values labelled with * are significantly different (P<0.05) from the expected volume dependent values.*

## §5.3 Conclusions.

These results show that overall the uptake of glucose and pyruvate, which are vital for preimplantation development, is closely related to the surface area rather than the volume of the embryo. Glucose functions as a source of energy (as seen by the production of lactate and $CO_2$), a precursor of the energy store glycogen, and as a source of macromolecular constituents. Pyruvate readily enters the TCA cycle and is oxidized at all stages of development (§1.5.3).

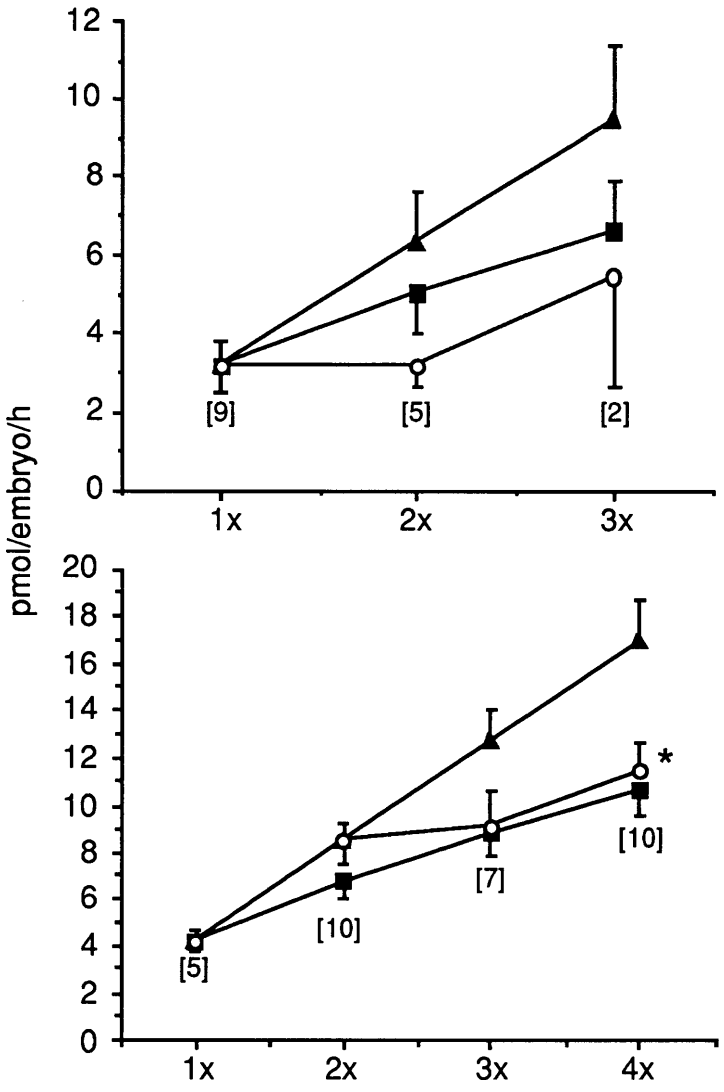



**Figure 5.2:** *Glucose uptake by single and aggregated embryos between days 3 and 4 (upper panel) and days 4 and 5 (lower panel) post hCG. (—○—) observed data; (—▲—) expected uptakes if volume dependent; (—■—) expected uptakes if surface area dependent. Numbers in parentheses are the number of observations. Values labelled with * are significantly different (P<0.05) from the expected volume dependent values.*

The major metabolic pathways fed by glucose and pyruvate are in place at all stages of preimplantation development and all avenues of substrate utilization are open to the embryo. Aggregating preimplantation embryos as described in §8.3.3 is not detrimental to their viability and they have the potential to give rise to normal sized young. It therefore seems unlikely that artificially large embryos created by aggregation would have different nutrient requirements from single embryos. If this is the case, it might be predicted that uptake values would be linearly related to size, and that the values would increase in proportion to the volume of the embryo. Indeed, after cavitation, it appears that the nutrient uptake of double embryos is

double that of single embryos, so that a change in volume of this magnitude can be compensated for by the embryo. However, if the volume is increased by a factor of greater than two, the uptakes are clearly related to the surface area, thus the control of substrate uptake would appear to lie mainly at the membrane level.

An alternative hypothesis is that the crowded state of the cells in the trophectoderm (TE) is controlling nutrient uptake. The number of cells in the TE is not proportional to the surface area of the embryo ie. a quadruple embryo of four times the single control embryo volume has approximately four times the number of TE cells, which are crowded into a surface area of only 2.5 times that of the single control embryo. When TE cells become more crowded, a smaller area of the individual cell surface is exposed (§3.2.5). From this it can be inferred that TE cells are unlikely to be stretched over the surface of the embryo but are nearly spherical. Folkman and Moscona (1978) noted that cells which were nearly spherical were inhibited from proliferating and hypothesized that a possible factor could be a decrease in the rate of nutrient transport. Thus, crowded, nearly spherical, TE cells could be inhibited from transporting nutrients across the plasma membrane.

Finally, it has been suggested that nutrient availability is one possible mechanism by which larger embryos regulate their size (§1.6; Snow *et al*, 1981). Although regulation in embryonic size has not been demonstrated before implantation (Buehr and McLaren, 1974; Lewis and Rossant, 1982), it is possible that embryos are already nutritionally compromised before implantation, and the effects of this do not manifest themselves until after implantation.

# §6 Cell allocation and nutrient uptake in biopsied human embryos

## §6.1 Introduction

The prenatal diagnosis of chromosomal abnormalities or inherited diseases currently involves recovering cells of fetal origin by chorion villus sampling or amniocentesis between 8 and 20 weeks of gestation, prior to karyotyping, biochemical or genetic analysis. If the fetus is diagnosed as affected at these stages, the couple is faced with the decision of whether or not to terminate the pregnancy. The early diagnosis of genetic defects in embryos within days of fertilization and before implantation, ie. preimplantation diagnosis, would allow the selection and transfer of unaffected embryos and the establishment of a normal pregnancy (Penketh and McLaren, 1987).

Before attempting diagnosis and transfer of biopsied embryos, any resulting damage needs to be carefully assessed *in vitro*. Here, the effects of biopsy and removal of one or two cells at the 8-cell stage (1/8 and 2/8) from normally fertilized human embryos three days after *in vitro* fertilization (IVF) on viability and development *in vitro* have been examined. The viability of biopsied embryos was assessed by daily non-invasive measurement of the uptake of two energy substrates, glucose and pyruvate (§1.5.2; §4; Leese and Barton, 1984; Leese *et al*, 1986; Hardy *et al*, 1989b). In addition, the cleavage rate and allocation of cells to the trophectoderm (TE) and inner cell mass (ICM) of blastocysts was studied by counting nuclei after differential labelling (§2; Hardy *et al*, 1989a)

## §6.2 Preimplantation biopsy

Normally fertilized embryos of good morphology, i.e. those with fairly even blastomeres and little fragmentation, which had reached the 6-10 cell stage by day 3 were studied. Embryos were manipulated singly to minimize the length of time spent at room temperature. After removal of adherent cumulus cells with a finely drawn Pasteur pipette of internal diameter just larger than the zona pellucida, embryos were transferred to a drop (≈10µl) of M2+BSA (§8.1.1) in a petri dish under paraffin oil. The embryo was immobilized by gentle suction on a flame polished holding pipette (O.D. 100µm) controlled by a Leitz micromanipulator. A second micromanipulator with a double holder system controlled a drilling pipette (diameter 10-20µm) containing acid Tyrodes (§8.3.1) and a biopsy pipette (diameter 30-50µm) containing M2+BSA. A hole was

made through the zona pellucida of the embryo by bringing the drilling pipette into contact with the zona and gently expelling the acidified medium. The drilling pipette was withdrawn and the biopsy pipette pushed through the hole in the zona and one or two blastomeres were removed. Biopsied embryos and unmanipulated control embryos of similar morphology were cultured to the expanded blastocyst stage to measure nutrient uptake (§8.6), before being differentially labelled to ascertain the numbers of inner cell mass (ICM) and trophectoderm (TE) cells (§8.4.3).

Eighty one normally fertilized surplus embryos which had reached the 8-cell stage (a few embryos were at intermediate 6- to 10-cell stages) on day 3 were selected for study. Forty five of these embryos, from randomly selected patients, were biopsied and one or two cells at the 8-cell stage (1/8 or 2/8) removed. In three cases, 3/8 cells were removed. Four other embryos were partially destroyed during biopsy and were eliminated from the study. The remaining 32 unmanipulated embryos were used as controls.

## §6.3 Development of biopsied embryos

Nineteen out of 32 (59%) unmanipulated embryos developed to the blastocyst stage by day 5 or 6; the remainder arrested at earlier stages (Table 6.1). The proportion of 7/8 and 6/8 biopsied embryos which developed to the blastocyst stage was similar and exceeded that of the unmanipulated embryos; overall 33 out of 45 (73%) reached this stage. Nineteen of these 33 blastocysts from biopsied embryos (58%) hatched out of the zona pellucida.

**Table 6.1:** *Development of biopsied 8-cell human embryos in vitro*

| | Total number of embryos | Number arrested | Number to blastocyst | | |
|---|---|---|---|---|---|
| | | | By day 5 | By day 6 | Total |
| Control | 32 | 13 (41%) | 8 (25%) | 11 (35%) | 19 (59%) |
| 7/8/ biopsied | 28 | 6 (21%) | 11 (39%) | 11 (39%) | 22 (79%) |
| 6/8 biopsied | 14 | 4 (29%) | 3 (21%) | 7 (50%) | 10 (71%) |
| 5/8 biopsied | 3 | 1 | 1 | 1 | 2 (67%) |

Numbers in parentheses are the percentage of the total number of embryos

## §6.4 Metabolism of biopsied embryos

The uptake of pyruvate and glucose (Fig. 6.1) by unmanipulated embryos which developed to the blastocyst stage by day 5 or 6 followed a similar pattern to that reported previously (§4; Hardy *et al*, 1989b).

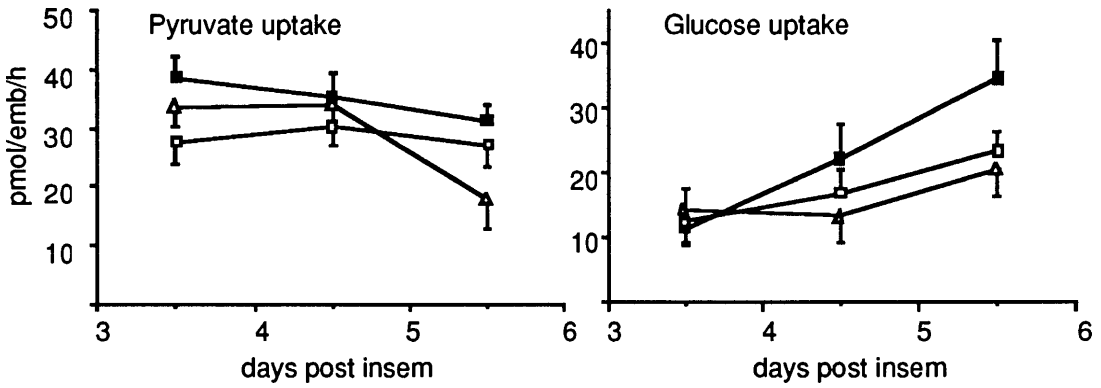



**Fig. 6.1:** *Pyruvate and glucose uptake by nineteen unmanipulated (-■-), twenty 7/8 (-□-) and ten 6/8 (-△-) biopsied embryos which had developed to the blastocyst stage by day 5 or 6.*

Pyruvate uptake declined from 38.8 to 31.3 pmol/embryo/h between days 3.5 and 5.5, and over the same period, glucose uptake increased steadily from about 11 to 35 pmol/embryo/h. The uptakes of both substrates by 7/8 or 6/8 biopsied embryos which developed to the blastocyst stage were generally lower (Fig. 6.1) but followed a similar pattern to the unmanipulated embryos and were within the range expected on the basis of the reduced cellular mass. Biopsied and unmanipulated embryos which arrested before the blastocyst stage had lower pyruvate uptake throughout this period and no increase in glucose uptake associated with blastocyst formation (data not shown).

## §6.5 Cell numbers in blastocysts

The total numbers of cells in biopsied and unmanipulated control embryos which reached the blastocyst stage on day 5 or 6, analysed by counting labelled nuclei, is summarized in Table 6.2. With the majority of blastocysts (44/46), total cell numbers were determined after differential labelling of nuclei to examine cell allocation (§2.4.3); the other two blastocysts were simply fixed and labelled with a single fluorochrome.

**Table 6.2:** *Total numbers of cells in newly expanded blastocysts after biopsy at the 8-cell stage.*

| | Newly expanded blastocysts on day 5 | | Newly expanded blastocysts on day 6 | |
|---|---|---|---|---|
| | No. of embryos | Total no. of nuclei (range) | No. of embryos | Total no. of nuclei (range) |
| Control | 6 | $60.5^{a,b} \pm 5.1$ (41-78) | 10 | $78.5^{c,d} \pm 8.8$ (30-116) |
| 7/8 biopsied | 9 | $60.7^{a} \pm 6.3$ (34-97) | 9 | $64.2^{c} \pm 7.7$ (35-102) |
| 6/8 biopsied | 3 | $49.7^{b} \pm 2.7$ (47-55) | 7 | $55.4^{d} \pm 7.4$ (37-82) |
| 5/8 biopsied | 2 | 24 | 1 | 114 |

Six blastocysts were lost during labelling. Values are means ± standard errors.
Differences between pairs of values with the same superscript were tested for significance.
$^{a,b,c}$not significant; $^{d}P < 0.05$.

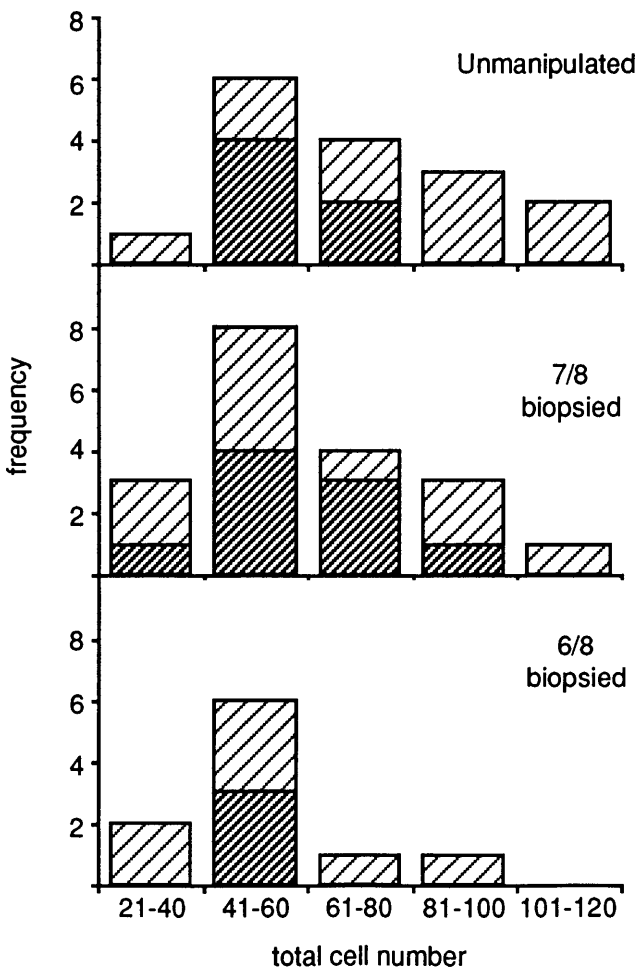



**Fig. 6.2:** *Distribution of total cell number in newly expanded blastocysts on day 5 (▨) and 6 (▨)*

The total numbers of cells in unmanipulated embryos at the blastocyst stage increased from 60.5±5.1 to 78.5±8.8 between days 5 and 6 and were similar to the numbers reported previously (§2.5; Hardy *et al* 1989a). In general, total cell numbers in 7/8 and 6/8 biopsied embryos at the blastocyst stage were fractionally reduced, but this was only significant in 6/8 biopsied embryos on day 6 ($P<0.05$). The range and distribution of cell numbers in blastocysts on days 5 and 6 from unmanipulated and 7/8 biopsied embryos was similar but was lower in 6/8 blastocysts (Figure 6.2).

## §6.6 Cell allocation in biopsied embryos

The numbers of TE and ICM cells, analysed by differential labelling of the nuclei, in biopsied and unmanipulated embryos are summarized in Table 6.3.

**Table 6.3:** *Numbers of TE and ICM cells in blastocysts after biopsy at the 8-cell stage*

| | Newly expanded blastocysts on day 5 | | | Newly expanded blastocysts on day 6 | | |
|---|---|---|---|---|---|---|
| | No. of embryos | TE cell no. (range) | ICM cell no. (range) | No. of embryos | TE cell no. (range) | ICM cell no. (range) |
| Control | 6 | 38.0 ± 4.4 (24 - 55) | 22.5 ± 4.1 (11 - 36) | 8 | 42.5 ± 8.0 (14 - 81) | 34.8 ± 7.9 (13 - 78) |
| 7/8 biopsied | 9 | 36.0 ± 4.1 (22 - 51) | 24.7 ± 3.5 (13 - 50) | 9 | 34.3 ± 6.5 (5 - 55) | 29.9 ± 4.9 (3 - 51) |
| 6/8 biopsied | 3 | 31.7 ± 1.9 (28 - 34) | 18.0 ± 2.6 (13 - 22) | 7 | 31.9 ± 3.6 (24 - 47) | 23.6 ± 4.9 (9 - 40) |
| 5/8 biopsied | 1 | 20 | 4 | 1 | 70 | 44 |

Values are means ± standard errors

As with total cell numbers, the numbers of these cells in blastocysts from unmanipulated embryos on days 5 and 6, 38.0 ± 4.4 and 42.5 ± 8.0 TE cells and 22.5 ± 4.1 and 34.8 ± 7.9 ICM cells respectively, were similar to those reported previously (§2.4; Hardy *et al*, 1989a). In blastocysts from biopsied embryos, the numbers of both TE and ICM cells, as with total cell numbers, were fractionally lower although in no case were the differences significant. The ratio of ICM:TE cells increased in unmanipulated embryos between days 5 and 6 and was not affected in either 7/8 or 6/8 biopsied embryos. Most strikingly, comparison of the numbers in blastocysts from 7/8 and 6/8 biopsied with unmanipulated embryos demonstrated that the numbers of both

**Table 6.4:** *Cell death and mitosis in blastocysts after biopsy at the 8-cell stage*

| | Newly expanded blastocysts on day 5 | | | | Newly expanded blastocysts on day 6 | | | |
|---|---|---|---|---|---|---|---|---|
| | TE | | ICM | | TE | | ICM | |
| | mitotic index | dead cell index | mitotic index | dead cell index | mitotic index | dead cell index | mitotic index | dead cell index |
| Control | 5.6±2.1 (n = 6) | 10.7±2.9 | 0.5±0.5 | 17.9±6.9 | 2.6±1.1 (n = 8) | 14.5±5.9 | 1.2±0.9 | 14.1±3.3 |
| 7/8 biopsied | 1.3±0.6 (n = 9) | 12.3±4.1 | 0.5±0.5 | 8.0±3.2 | 2.1±0.7 (n = 9) | 13.9±5.8 | 1.3±1.1 | 19.3±8.8 |
| 6/8 biopsied | 1.0±1.0 (n=3) | 7.9±1.2 | 1.8±1.8 | 13.2±6.6 | 1.8±1.2 (n = 7) | 16.2±5.3 | 0.5±0.5 | 18.9±7.6 |

Values are means ± standard errors.

of these cell types were only reduced in proportion to the reduction of cellular mass at biopsy in each case (Fig. 6.3). The numbers of dead cells and the mitotic index of both the TE and the ICM were not significantly altered by biopsy (Table 6.4) and although very variable were not dissimilar from our previous report (§2.4; Hardy *et al*, 1989a).

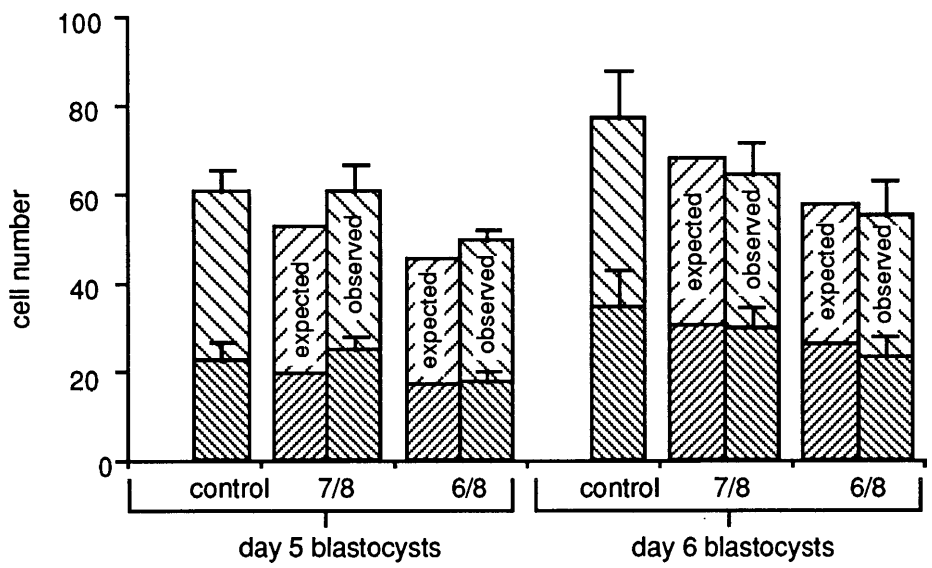



**Fig. 6.3:** *Comparison of observed and expected total ( ) and inner cell mass ( ) cell numbers in newly expanded blastocysts on day 5 and 6 derived from unmanipulated and 7/8 and 6/8 biopsied embryos. Expected values are calculated as proportions (7/8 and 6/8 of the control values. Error bars are standard errors.*

## §6.7 Conclusions

For the diagnosis of genetic defects in human preimplantation embryos, one or more cells needs to be biopsied from each embryo for analysis. Theoretically, embryos could be biopsied at any stage between the 2-cell and the expanded blastocyst stages, ie. between days 2 and 7 for embryos fertilized and cultured *in vitro*. However, biopsy at either early cleavage or blastocyst stages may be harmful to the subsequent development of the biopsied embryos. Removal of even a single cell from early cleavage stages substantially reduces the cellular mass of the embryo which may affect blastocyst formation and allocation to the trophectoderm (TE) and inner cell mass (ICM) (Nijs and Van Steirteghem, 1987). On the other hand, excision of TE cells from blastocysts is likely to damage the remaining TE which may affect implantation. At whichever stage embryos are biopsied, therefore, any resulting damage must be carefully assessed *in vitro* before attempting diagnosis and transfer of biopsied embryos. These observations here on normally fertilized embryos biopsied at the 8-cell stage demonstrate that neither the trauma of micromanipulation nor reduction in cellular mass by 1/8 or 2/8 have serious adverse effects on viability, metabolism, blastocyst development *in vitro* or allocation of cells to the TE or ICM.

The viability of biopsied embryos was assessed by non-invasive measurement of metabolic activity as reflected in the uptake of two energy substrates, pyruvate and glucose, from the culture medium (Leese and Barton, 1984; Leese *et al*, 1986). Between days 2 and 6, the uptake of pyruvate is significantly lower and the increase in glucose uptake at the blastocyst stage abolished in human embryos which arrest during cleavage (§4; Hardy *et al*, 1989b). The uptake of both energy substrates by biopsied embryos developing to the blastocyst stage was generally lower than their unmanipulated counterparts developing to the same stage. However, uptakes were within the range expected on the basis of reduced cellular mass and there was no indication of an additional effect on viability either immediately following biopsy or later in culture.

Biopsy at the 8-cell stage had no effect on the proportion developing to the blastocyst stage. Indeed the development of 7/8 and 6/8 biopsied embryos exceeded that of unmanipulated embryos (79, 71 and 59%, respectively). Most importantly, the proportion of morphologically abnormal blastocysts was not increased and only accounted for 10 - 20% of blastocysts in each group. The total numbers of cells at the blastocyst stage were fractionally lower in both 7/8 and 6/8 biopsied embryos, but only

in proportion to the reduction in cellular mass (Fig. 6.3). Mammalian embryos, in general, have a remarkable capacity to regulate for reduction of cellular mass at cleavage stages. Normal offspring of the same weight and size as control litter mates have been obtained after transfer of single cells from cleavage stages in a number of laboratory and domestic species (§1.6; reviewed by Papaioannou and Ebert, 1986). The mechanism of regulation is poorly understood. However, as observed here with biopsied human embryos, there is no evidence that the rate of cleavage of preimplantation embryos is altered to compensate for reduced cellular mass. In the mouse, reduced cell numbers are maintained throughout preimplantation development (§3.2.3) and compensation occurs in the ICM lineage soon after implantation (§1.6; Lewis and Rossant, 1982).

The reduction in cell numbers at the blastocyst stage in both 7/8 and 6/8 biopsied embryos was equally distributed between the TE and ICM so that the ratio of ICM:TE cells was not altered (Fig. 6.3). This is important because the fetus is derived from the ICM after implantation. In contrast, biopsy of mouse embryos and removal of 1/4 at the 4-cell stage caused a disproportionate reduction in the numbers of ICM cells and the ICM:TE ratio of 3/4 biopsied embryos was significantly lower (Somers *et al*, 1990). In the mouse, the allocation of cells to the TE and ICM involves the polarization of cells during compaction at the 8-cell stage which generates phenotypically distinct outer and inner cells in the subsequent division (§1.4.3; Handyside, 1980). This process is influenced by a series of cell-cell interactions from the 4-cell stage onwards (reviewed by Johnson and Maro, 1986). It is possible, therefore, that the lower ICM:TE ratio in the biopsied mouse embryos resulted from biopsy at the 4-cell stage which could effect the extent of these interactions and the allocation of cells in the blastocyst especially after complete removal of the zona and treatment with calcium- and magnesium-free medium.

Any effect of biopsy of preimplantation embryos on implantation or postimplantation development can only be assessed after transfer. In the mouse, biopsy at the 4- or 8-cell stages and removal of 1/4, 1/8 or 2/8 has no effect on blastocyst formation, implantation or postimplantation development after transfer to pseudopregnant recipients (Monk and Handyside, 1988; Wilton *et al*, 1989). In the human, there is evidence that embryos with reduced cellular mass are capable of implantation and normal development. The first pregnancy to be reported for a cryopreserved embryo resulted from the transfer of an embryo in which only five out of

the eight blastomeres survived thawing (Trounson and Mohr, 1983). Although the patient suffered a late miscarriage, examination of the fetus indicated that there were no gross abnormalities.

For accurate diagnosis, it may be necessary to biopsy more than one cell from each embryo. DNA amplification of unique sequences, for example, is not successful in every case (reviewed by Handyside, 1991). The finding that biopsy and removal of 2/8 cells did not affect development or the ratio of ICM:TE may provide a solution. Thus, preimplantation diagnosis both indirectly for X-linked disease by amplification of a Y-specific repeat (Handyside *et al*, 1989) and directly in single gene disorders, for example by amplifying sequences associated with cystic fibrosis or Duchenne muscular dystrophy (Coutelle *et al*, 1989) may now be possible. Normally fertilized embryos biopsied early on the third day after IVF could be returned to culture while the DNA from the 1/8 or 2/8 biopsies is amplified and analysed. Amplification can be completed in 3-5h which should allow unaffected biopsied embryos to be identified and transferred later the same day when embryo transfers are known to be successful.

# §7 Conclusions

## §7.1 Allocation to the inner cell mass and trophectoderm

Human blastocysts are morphologically very similar to mouse blastocysts. More detailed examination of total cell numbers and the numbers of trophectoderm (TE) and inner cell mass (ICM) cells, by differential labelling with polynucleotide specific fluorochromes, indicates that there are other similarities. The cell number at which cavitation occurs varies considerably between species, ranging from 16 cells in the pig to 128 cells in the rabbit (Papaioannou and Ebert, 1986). However, in the human and the mouse, the minimum total cell numbers in blastocysts of 25 and 24 cells respectively, suggests that cavitation occurs during the same (fifth) cleavage division. Further, examination of the total cell numbers in expanded human blastocysts reveals an agreement with those published for the mouse. In the human, cell numbers range from 58 in newly expanded blastocysts on day 5 to 126 on day 7. In the mouse, the total cell number in *in vivo* expanded blastocysts ranges from 55 (at 87h post coitum, when the majority of blastocysts have expanded) to approximately 120, before implantation (Handyside and Hunter, 1986). However, the length of time taken to reach the blastocyst stage is longer in the human than in the mouse. This appears to be due to the longer cell cycle times after the 8-cell stage (Fig. 1.1). Up to the 8-cell stage mouse and human embryos have comparable cell cycle lengths. After the 8-cell stage, mouse embryos cleave approximately every 12h whilst human embryos appear to cleave approximately every 24h, meaning that human embryos cavitate in the region of one day later than mouse embryos.

In addition to the total cell numbers in mouse and human blastocysts being comparable, it seems that the initial number of cells allocated to the ICM in the human corresponds to the number in the mouse. Both newly expanded mouse and human blastocysts have approximately twenty ICM cells. Although the number of ICM cells has not been directly measured in the human, at the 16- and 32-cell stages retrospective extrapolation indicates that there are six and twelve ICM cells, respectively. This is in agreement with the number observed in mouse embryos at the 16- and 32-cell stages (§1.4.2; Handyside, 1981; Johnson and Ziomek, 1981a; Fleming, 1987; Copp, 1978; Handyside, 1978; Handyside and Hunter, 1996). However, in the mouse, the proportion of ICM cells declines from approximately 40% in nascent blastocysts to approximately 17% before implantation. In contrast, in

the human, the proportion of ICM remains high, between one third and one half. In the mouse, cell death is seen predominantly in the ICM while in the human it is evident in both lineages, increasing on day 7.

In the mouse, the generation of phenotypically distinct inner (ICM) and outer (TE) precursor cells by differentiative divisions of polarized 8- and 16-cell blastomeres is now well understood. However, little is known about what controls the proportion of cells allocated to the ICM and TE. The inside:outside hypothesis (§1.4.1) proposes that cells on the outside of an embryo form TE whilst enclosed, inner cells form ICM. The number of cells allocated to the TE would thus be proportional to the surface area of the embryo. However, bisection and aggregation of embryos at the 8-cell stage which alters the surface area:volume ratio of resulting morulae, shows that whilst there is a disproportionate increase and decrease in the number of TE cells in half and aggregated embryos respectively, the number of TE cells is *not* proportional to the surface area. The relatively small changes in the numbers of TE and ICM cells in bisected and aggregated embryos could be due to alterations in the cell-cell interactions resulting in a change in the proportion of polarized cells undergoing differentiative divisions.

Furthermore, in expanded blastocysts derived from aggregated embryos, the proportion of ICM does not decrease as dramatically as it does in single control embryos. This may be due to the ICM in aggregated embryos being too large to be influenced by any regulative process initiated by either the TE or the blastocoel cavity. Further work on the development of ICMs in the presence and absence of TE cells needs to be carried out to elucidate the properties of such a putative regulatory mechanism.

### §7.2 Substrate uptake

Qualitatively, the nutrient uptake pattern exhibited by human embryos which reach the blastocyst stage is similar to that of the mouse (Leese and Barton, 1984; Gardner and Leese, 1986), in particular, the pronounced increase in the uptake of glucose on day 5. However, in the mouse, concomitant with the increase in glucose is a decline in pyruvate uptake. This is not observed in human embryos. Overall, total nutrient uptake is greater for human than mouse embryos, even if the difference in size of the two types is taken into account. The human embryo is approximately three times the volume of a mouse embryo. However, average pyruvate uptake by single human embryos is about 35 pmol/embryo/h compared with 1-3 pmole/embryo/h in the mouse

(Leese and Barton, 1984; Gardner and Leese, 1986,88), a greater than 10-fold difference.

The uptake of pyruvate by embryos which reach the blastocyst stage is consistently about 20% higher than for those which arrest. In addition, they were the only embryos to exhibit a pronounced increase in glucose uptake at the blastocyst stage. This suggests that nutrient uptake reflects the viability of the embryo and allows an assessment of the effects of manipulations, such as the removal of one or two cells during preimplantation biopsy, on the health of the embryo (see below).

The uptake of nutrients by the preimplantation embryo could either be regulated at the membrane level (where uptake rates will be determined by the existence or efficiency of transport mechanisms), or at the intracellular level, where they will be determined by the rate of utilization of substrates). Manipulation of the surface area:volume ratio of embryos by aggregating 8-cell stage mouse embryos, allowing them to compact and subsequently assessing nutrient uptake suggests that it is regulated at the membrane level.

While the human embryo is three times the volume of the mouse embryo, the surface area, over which substrate uptake occurs, is only approximately twice (Appendix 9.1) that of the mouse. The observation that pyruvate uptake is ten-fold higher in the human has interesting implications for facilitated transport mechanisms. Further work needs to be carried out to investigate the presence and efficiency of facilitated transport mechanisms in the human embryo, using similar techniques to those described by Gardner and Leese (1988).

### §7.3 Preimplantation biopsy

The advent of in vitro fertilization (IVF) has made the human preimplantation embryo accessible for diagnosis of genetic disease, allowing the replacement of only unaffected embryos. This would obviate the traumatic decision of whether or not to terminate a pregnancy after current prenatal diagnosis techniques such as chorionic villus sampling or amniocentesis at between 8 and 20 weeks of gestation. Cleavage stage biopsy involves the removal of one or a few cells from the embryo, assay of the cells for a suspected genetic defect and replacement of the unaffected embryos. Here, the effects of removal of one or two cells from embryos at the 8-cell stage on viability, metabolism, blastocyst development in vitro, and allocation of cells to the TE and ICM were examined. The 8-cell stage was chosen for several reasons. Removal of a cell at an earlier stage, such as the 2-cell stage, would reduce the mass

of the embryo too drastically, in this case by half, which might affect blastocyst formation and allocation of cells to the ICM. Removal of TE cells from blastocysts is an attractive possibility. Firstly, more cells would be obtained, which can only increase the accuracy of a diagnosis. Secondly, only TE cells would be removed, leaving the ICM, which gives rise to the fetus, unaffected. However, removal of too large a number of TE cells could compromise implantation, and, most importantly, pregnancy success after transfer of intact blastocysts is low.

Biopsy at the 8-cell stage had no effect on the proportion developing to the blastocyst stage. As has been shown earlier, the nutrient uptake of embryos appears to reflect their viability, with embryos which arrest showing lower pyruvate uptakes and failing to show the upsurge in glucose uptake on day 4 onwards which is characteristic of blastocyst formation. Biopsied embryos show reduced pyruvate and glucose uptakes, in comparison with the unmanipulated control embryos. However, uptakes were within the range expected on the basis of reduced cellular mass. The total numbers of cells at the blastocyst stage were lower in biopsied embryos, but again, only in proportion to the reduction in cellular mass. In conclusion, removal of one or two cells from 8-cell stage embryos does not appear to have serious adverse effects on the metabolism, blastocyst development *in vitro* or allocation of cells to the TE and ICM.

Recently, pregnancies have been achieved after replacement of biopsied embryos (Handyside et al, 1990). However, the analysis of only one or possibly two cells is carried out at the limits of sensitivity of the analytical techniques available. The possibility of biopsying cells from 8-cell stage embryos, and allowing these cells to divide in culture over a further 24-48h, before replacement of unaffected embryos on day 4 or 5, needs further investigation.

# §8 Materials and Methods

## §8.1 Embryo culture media

### §8.1.1 Mouse embryo handling and culture media

M16, a bicarbonate buffered salt solution containing energy substrates and antibiotics, is used for long-term culture of mouse embryos for up to five days. A pH of between 7.2 and 7.4, optimal for successful development of mouse zygotes to the blastocyst stage, is maintained in an atmosphere of 5% $CO_2$-in-air under this buffering system. However, exposure of this medium to air causes the pH to become alkaline (approx pH 8.0). To avoid such possibly detrimental pH changes, a medium of similar composition, M2, where the bicarbonate buffer has been replaced with HEPES, is used for the short-term handling and manipulation of embryos outside the controlled atmosphere of the incubator. Both M16 and M2 are supplemented with 4 mg $ml^{-1}$ bovine serum albumin (BSA; Crystallized Albumin, ICN Flow, 81-001) to make embryo handling easier and to prevent embryos sticking to each other and to glassware.

Both M2 and M16 are made from diluting concentrated stock solutions (Table 8.1) in water of high purity, either double glass distilled water or pharmaceutical "Water for Injections" B.P.(Boots, U.K.) (Table 8.2)

**Table 8.1**: *Composition of concentrated stocks for media used for handling (M2) and culturing (M16) mouse embryos. (M2: Whittingham, 1971; M16: Quinn et al, 1982)*

| Stock (concentration) | Component | Amount | Molecular weight | Final media mM | Supplier & catalogue number |
|---|---|---|---|---|---|
| ***A*** | | *g 100ml$^{-1}$* | | | |
| (10×) | NaCl | 5.534 | 58.44 | 94.66 | BDH 10241 |
| | KCl | 0.356 | 74.55 | 4.78 | BDH 10198 |
| | $KH_2PO_4$ | 0.162 | 136.09 | 1.19 | BDH 10203 |
| | $MgSO_4.7H_2O$ | 0.293 | 246.47 | 1.19 | BDH 10151 |
| | Na Lactate | 4.349 of 60% syrup | 112.1 | 23.28 | Sigma L-1375 |
| | Glucose | 1.000 | 180.16 | 5.56 | BDH 10117 |
| | Penicillin | 0.060 | 1625 U ml$^{-1}$ | 97.5 U ml$^{-1}$ | Sigma PEN-K |
| | Streptomycin | 0.050 | 750 U ml$^{-1}$ | 37.5 U ml$^{-1}$ | Sigma S-6501 |
| ***B*** | | *g 100ml$^{-1}$* | | | |
| (10×) | $NaHCO_3$ | 2.101 | 84.01 | 25.00* 4.00‡ | BDH 10247 |
| | Phenol Red | 0.010 | | | BDH 20091 |
| ***C*** | | *g 10ml$^{-1}$* | | | |
| (100×) | Na Pyruvate | 0.036 | 110.0 | 0.33 | Sigma P2256 |
| ***D*** | | *g 10ml$^{-1}$* | | | |
| (100×) | $CaCl_2.2H_2O$ | 0.252 | 147.02 | 1.71 | BDH 10070 |
| ***E*** | | *g 100ml$^{-1}$* | | | |
| (10×) | HEPES§ | 5.958 | 238.3 | 20.85‡ | Calbiochem 391338 |
| | Phenol Red | 0.010 | | | BDH 20091 |

* M16, ‡ M2, § pH adjusted to 7.6 with 1N NaOH before making up to 100ml. Stocks prepared with Boots "Water for Injections" B.P. throughout; filter-sterilized by passing through a 0.22μ Millex-GS disposable Millipore filter (SL GS 025 BS) using a 20ml Monoject disposable syringe; stored in 6ml and 14ml Falcon (Becton Dickinson, U.K.) tubes at 4°C. Stocks A, D & E are kept for 3 months, B & C for 2 weeks.

**Table 8.2**: *Preparation of M2 and M16 from concentrated stocks*

| | M2 (100ml) | M16 (100ml) |
|---|---|---|
| Stock | ml | ml |
| A | 10 | 10 |
| B | 1.6 | 10 |
| C | 1.0 | 1.0 |
| D | 1.0 | 1.0 |
| E | 8.4 | 0 |
| $H_2O$ | 78.0 | 78.0 |

Osmolality 288-294 (M16); 283-289 (M2)
Media are filter-sterilized by passing through a 0.22μ Millex-GS disposable Millipore filter (SL GS 025 BS) using a 20ml Monoject disposable syringe and stored in 6ml and 14ml Falcon (Becton Dickinson, U.K.) tubes at 4°C.

### §8.1.2 Human embryo handling and culture media

Initially, all human embryos were cultured in a bicarbonate-buffered modified T6 medium (Hiller *et al*, 1984; Table 8.3) and handled in a HEPES buffered T6 medium of similar composition. During the course of this work, however, the media were changed, to facilitate ease of making up large volumes of media, to those based on Earle's Balanced Salt Solution (Gibco, Table 8.4) supplemented with sodium bicarbonate, antibiotics, sodium pyruvate, and, in the case of embryo handling medium, HEPES.

Both T6 and Earle's culture media are made by diluting concentrated stocks with pharmaceutical "Water for Injections" B.P. (Boots, U.K.) and stored without pyruvate. The pyruvate stock, made weekly, is added 24h before use. In addition, at this time, the culture medium is supplemented with maternal serum (10% v/v) which has been heat-inactivated for 30min at 56°C and filter sterilized using a 0.22μ filter.

All pipettes and storage vessels used to prepare medium are Falcon disposable plastic (Becton Dickinson). All new batches of media and items which come into contact with the embryos, such as plasticware, syringes for filtering medium and collecting serum, filters, water, transfer catheters etc. are tested for toxicity by culturing 2-cell mouse embryos (§8.1.1) to the expanded blastocyst stage.

**Table 8.3**: *Composition of concentrated stocks for T6 medium used for human IVF and embryo culture (Hillier et al, 1984, modified from T6 (Quinn et al, 1982), adapted from Tyrode's solution (Whittingham, 1971))*

| Stock (conc$^{n.}$) | Component | Amount | Molecular weight | Final media mM | Supplier & catalogue number |
|---|---|---|---|---|---|
| ***A*** | | *g 100ml$^{-1}$* | | | |
| (10×) | NaCl | 5.719 | 58.44 | 97.86 | BDH 10241 |
| | KCl | 0.106 | 74.55 | 1.42 | BDH 10198 |
| | $MgCl_2.6H_2O$ | 0.096 | 203.30 | 0.47 | BDH 10149 |
| | $Na_2HPO_4.12H_2O$ | 0.129 | 358.14 | 0.36 | BDH 10248 |
| | Na Lactate | 4.651 of 60% syrup | 112.1 | 24.89 | Sigma L-1375 |
| | Glucose | 1.000 | 180.16 | 5.56 | BDH 10117 |
| | Penicillin | 0.060 | 1625 U ml$^{-1}$ | 97.5 U ml$^{-1}$ | Sigma PEN-K |
| | Streptomycin | 0.050 | 750 U ml$^{-1}$ | 37.5 U ml$^{-1}$ | Sigma S-6501 |
| ***B1*** | | *g 100ml$^{-1}$* | | | |
| (10×) | $NaHCO_3$ | 2.101 | 84.01 | 20.01* 5.00‡ | BDH 10247 |
| | Phenol Red | 0.010 | | | BDH 20091 |
| ***B2*** | | *g 100ml$^{-1}$* | | | |
| (10×) | NaCl | 1.461 | 58.44 | 5.00* 10.00‡ | BDH 10241 |
| | Phenol Red | 0.010 | | | BDH 20091 |
| ***C*** | | *g 10ml$^{-1}$* | | | |
| (100×) | Na Pyruvate | 0.052 | 110.0 | 0.47 | Sigma P2256 |
| ***D*** | | *g 10ml$^{-1}$* | | | |
| (100×) | $CaCl_2.2H_2O$ | 0.262 | 147.02 | 1.78 | BDH 10070 |
| ***E*** | | *g 100ml$^{-1}$* | | | |
| (10×) | HEPES§ | 5.958 | 238.3 | 10.00‡ | Calbiochem 391338 |
| | Phenol Red | 0.010 | | | BDH 20091 |

* Culture medium, ‡ Handling and flushing medium, § pH adjusted to 7.3 with N/5 NaOH before making up to 100ml. Stocks prepared with Boot's 'Water for Injections' B.P. throughout, filter sterilized as Table 8.1 & stored in Falcon tissue culture flasks (3024F) at 4°C. A, B2, D & E are made every 3 months; B1 every 2 weeks and C every week.

**Table 8.4**: *Composition of concentrated stocks for preparation of Earle's Balanced Salt Solution used for human IVF and embryo culture*

| Stock (concentration) | Component | Amount | Molecular weight | Final media mM | Supplier & catalogue number |
|---|---|---|---|---|---|
| ***EBSS*** | | *g L$^{-1}$* | | | Gibco 042-4050 |
| (10×) | NaCl | 68.00 | 58.44 | 116.36 | |
| | KCl | 4.0 | 74.55 | 5.37 | |
| | $MgSO_4.7H_2O$ | 2.0 | 246.47 | 0.81 | |
| | $NaH_2PO_4.2H_2O$ | 1.58 | 156.01 | 1.01 | |
| | $CaCl_2.2H_2O$ | 2.64 | 147.02 | 1.80 | |
| | Glucose | 10.00 | 180.16 | 5.55 | |
| ***A*** | | *g 100ml$^{-1}$* | | | |
| (10×) | $NaHCO_3$ | 2.101 | 84.01 | 25.00* 4.01‡ | BDH 10247 |
| | Phenol Red | 0.010 | | | BDH 20091 |
| ***B*** | | *g 10ml$^{-1}$* | | | |
| | Penicillin | 0.060 | 1625 U ml$^{-1}$ | 97.5 U ml$^{-1}$ | Sigma PEN-K |
| | Streptomycin | 0.050 | 750 U ml$^{-1}$ | 37.5 U ml$^{-1}$ | Sigma S-6501 |
| ***C*** | | *g 10ml$^{-1}$* | | | |
| (100×) | Na Pyruvate | 0.052 | 110.0 | 0.47 | Sigma P2256 |
| ***E*** | | *g 100ml$^{-1}$* | | | |
| (10×) | HEPES§ | 5.958 | 238.3 | 21.00 | Calbiochem 391338 |

* Culture medium, ‡ Handling and Flushing medium
§ pH adjusted to 7.3 with 0.4N NaOH before making up to 100ml.

**Table 8.5**: *Preparation of T6 human embryo handling and culture medium from concentrated stocks.*

| | Handling medium (100ml) | Culture medium (100ml) |
|---|---|---|
| Stock | ml | ml |
| A | 10 | 10 |
| B1 | 2 | 8 |
| B2 | 4 | 2 |
| C | 1 | 1 |
| D | 1 | 1 |
| E | 4 | 0 |
| $H_2O$ | 78 | 78 |

Osmolality 282-284
Media are filter-sterilized by passing through a 0.22μ filter unit (Falcon, Becton Dickinson, U.K. 7103 or, if >1L, Gelman Sciences 12158) and stored in tissue culture flasks (Falcon, 3024) at 4°C.

**Table 8.6**: *Preparation of Earle's human embryo handling and culture medium from concentrated stocks*

| | Handling medium (1L) | Culture medium (1L) |
|---|---|---|
| Stock | ml | ml |
| EBSS | 100 | 100 |
| A | 16 | 100 |
| B | 10 | 10 |
| C | 0 | 10 |
| E | 84 | 0 |
| $H_2O$ | 790 | 780 |

Osmolality 282-284
Media are filter-sterilized by passing through a 0.22μ filter unit (Falcon, Becton Dickinson, U.K. 7103 or, if >7L, Gelman Sciences 12158) and stored in tissue culture flasks (Falcon, 3024) at 4°C.

## §8.2 Source and recovery of embryos

### §8.2.1 Mouse embryos

$F_1$ (C57BL/6 × CBA/Ca) female mice (3-4 weeks old) were superovulated by intraperitoneal injection of 7.5 I.U. pregnant mares' serum gonadotrophin (PMS:

Folligon; Intervet, Cambridge UK) between 13.00 and 18.00 followed 48h later by 7.5 I.U. human chorionic gonadotrophin (hCG: Chorulon; Intervet, Cambridge UK), paired with males of the same strain, and examined the following morning for copulation plugs (day 1 of pregnancy). Two-cell embryos were flushed from the oviducts mid-day 2 (44 ± 0.5h post-hCG) in medium M2 (Table 8.2), supplemented with 4mg $ml^{-1}$ bovine serum albumin (BSA) (Quinn *et al* 1982) and cultured in droplets of Medium M16 (Table 8.2; Whittingham, 1971) supplemented with 4mg $ml^{-1}$ BSA, under pre-equilibriated paraffin oil (Light Liquid Paraffin Oil, BDH, 29436) at 37°C in a humidified atmosphere of 5% $CO_2$ in air.

### *§8.2.2 Human preimplantation embryos*

Work involving human embryos has been approved by both the local ethical committee of the Royal Postgraduate Medical School, Hammersmith Hospital and the Interim Licensing Authority for Human in Vitro Fertilization and Embryology of the Medical Research Council and the Royal College of Obstetricians and Gynaecologists.

Surplus human embryos were obtained from patients undergoing *in vitro* fertilization (IVF). Formerly, superovulation was achieved using concurrent clomiphene citrate (Clomid, Pregnyl) and human menopausal gonadotrophin (hMG; Pergonal, Serono) (Hiller *et al*, 1985). More recently, patients undergo pituitary downregulation with a luteinising hormone releasing hormone (LHRH) agonist (Buserelin, Hoechst) prior to and during superovulation with hMG (Rutherford *et al*, 1988). Under both regimens, 10,000 I.U. human chorionic gonadotrophin (hCG; Pregnyl, Organon) is administered when a minimum of three follicles have attained a mean diameter of 17mm and serum oestradiol levels are greater than 3,500 pmol $L^{-1}$. Oocyte retrieval is performed 34h later.

Oocyte-cumulus complexes are preincubated for 4 - 7h in 1ml preequilibriated culture medium (Earle's or T6; §8.1.2) in 4ml Falcon tubes (Becton Dickinson) at 37°C inside a Flow "modular incubator chamber" in a 5% $CO_2$-in-air incubator. The module itself is flushed with a 5% $CO_2$, 5% $O_2$, 90% $N_2$ gas mixture and sealed during use. A humidified atmosphere is maintained by the presence of an open petri dish containing distilled water. After the preincubation period, oocyte-cumulus complexes are transferred to insemination tubes (4ml, Falcon, Becton Dickinson) containing 1ml culture medium and approximately $1 \times 10^5$ motile sperm (insemination; day 0). 15-20h later (morning of day 1) the oocytes are checked for the development of two pronuclei, to confirm normal

fertilization. To inspect the embryos, each is transferred to the central well of an organ culture dish (Falcon, 3057) containing 1 - 2ml handling medium (§8.1.2). The cumulus cells are removed by aspiration with a finely drawn mouth-controlled Pasteur pipette, enabling pronuclei to be visualized under a stereo dissecting microscope. Oocytes are replaced in fresh preequilibriated culture tubes (4ml, Falcon, Becton Dickinson) containing 1ml culture medium. On Day 2, each fertilized embryo is examined under the stereo microscpe without removal from the tube, and up to 3 are selected on the basis of their morphology and rate of development for embryo transfer, on days 2 or 3. After confirming patients consent, surplus embryos are allowed to develop *in vitro*, in a 5% $CO_2$-in-air incubator, either in the original 1ml of medium or in 20µl drops of the same medium under pre-equilibriated paraffin oil (Light Liquid Paraffin Oil, BDH, 29436) in a 60mm petri dish (Falcon, Becton Dickinson, 3002) to facilitate scoring.

### *§8.2.3 Classification and grading of human embryos*

Oocytes and embryos are initially classified according to the number of pronuclei visible on day 1 (16h after insemination) as follows: (i) fertilized embryos (with two pronuclei); (ii) polyspermic embryos (with three or more pronuclei); and (iii) unfertilized oocytes (with one or no pronucleus). Later on day 2, fertilized embryos are graded according to eveness of blastomeres, fragmentation and presence of cellular debris from perfectly symmetrical embryos with no fragmentation (grade I) to embryos having one intact blastomere with gross fragmentation (grade IV) or being totally degenerate (grade V) (Dawson *et al*, 1987). Also, unfertilized oocytes which cleave over this period are reclassified as parthenogenetic embryos.

## §8.3 Embryo manipulation

### §8.3.1 Removal of the zona pellucida

**Table 8.7:** *Acid Tyrodes solution ( Nicolson et al, 1975) for the removal of the zona pellucida of mouse and human preimplantation embryos*

| Component | Amount (g 100ml$^{-1}$) | Molecular weight | Final mM | Supplier (catalogue number) |
|---|---|---|---|---|
| NaCl | 0.800 | 58.44 | 136.89 | BDH10241 |
| KCl | 0.020 | 74.55 | 2.68 | BDH 10198 |
| $CaCl_2.2H_2O$ | 0.024 | 147.02 | 1.63 | BDH 10070 |
| $MgCl_2.6H_2O$ | 0.010 | 203.30 | 0.49 | BDH 10149 |
| Glucose | 0.100 | 180.16 | 5.55 | BDH 10117 |
| PVP* | 0.4 | 40,000 | 4 mg ml$^{-1}$ | Calbiochem 5295 |

* PVP: Polyvinylpyrrolidone, dialysed against excess distilled water and freeze-dried.

The salts were dissolved in 50ml "Water for Injections" B.P. (Boots), the PVP sprinkled on the surface of the liquid and allowed to dissolve, and the pH adjusted to between 2.0 and 2.4 with N/5 HCl before making up to final volume of 100ml.

Embryos were incubated in acid Tyrodes solution for <30s until their zonae were completely dissolved, and then washed thoroughly in M2 + BSA.

### §8.3.2 Bisection of mouse embryos

10ml of 10× Hank's Basic Salt Solution without calcium and magnesium (HBSS-$Ca^{2+}$-$Mg^{2+}$) (Imperial Laboratories, U.K.; 2-036-07) was diluted with 40ml "Water for Injections" B.P. (Boots) and supplemented with 0.6mg Bovine Serum Albumin (BSA; Crystallized Albumin, ICN Flow, 81-001). The pH was adjusted to 7.4 with 1N NaOH and the volume made up to 100ml. The medium was filter-sterilized with a 0.22μ Millex-GS disposable Millipore filter (SL GS 025 BS) and stored in 2ml aliquots in 6ml Falcon tubes (Becton Dickinson) at -20°C.

Zona-free (§8.3.1) 8-cell embryos were incubated in pre-warmed (37°C) HBSS-$Ca^{2+}$-$Mg^{2+}$ for up to 30min. Half embryos were produced by gentle pipetting with a flame polished pipette of internal diameter just smaller than the diameter of the embryo. If one or two cells were dislodged from a half embryo during this process, they were reaggregated after incubating the half embryo and

dislodged cells for 30s in 25μg $ml^{-1}$ phytohemagglutinin (PHA) ( Erythroagglutinin Type III; Sigma, L8629) in M2 + BSA, causing the cells to adhere to each other. Half embryos were then thoroughly washed in M2 + BSA and M16 + BSA (§8.1.1) before further incubation.

### §8.3.3 Aggregation of mouse embryos

Aggregation of zona-free (§8.3.1) 8-cell embryos was assisted by incubation for 30s in 25μg $ml^{-1}$ phytohemagglutinin (PHA) ( Erythroagglutinin Type III; Sigma, L8629) in M2 + BSA. This caused the embryos to adhere closely to each other. Single control embryos were also incubated for the same length of time in PHA. Aggregates and control embryos were thoroughly washed in M2 + BSA and M16 + BSA (§8.1.1) before further incubation.

## §8.4 Labelling of the cell surface and nuclei of embryos

### §8.4.1 Surface labelling of mouse embryos

Mouse embryos were incubated at room temperature for 10min in heat-inactivated rabbit anti-mouse spleen-cell antiserum diluted 1:10 in M2 + BSA, washed through three washes of M2 + BSA and transferred to a 1:10 dilution of FITC goat anti-rabbit IgG (H + L) antibody (ICN Flow 65-173-2) in M2 + BSA for 30min. Embryos were briefly washed in M2 + BSA before viewing under UV light with a fluorescent microscope (§8.5.1).

### §8.4.2 Differential labelling of the trophectoderm and inner cell mass nuclei of mouse blastocysts

Zona-free (§8.3.1) mouse blastocysts were incubated at room temperature for 10 min in heat-inactivated (56°C for 30min) rabbit anti-mouse spleen-cell antiserum diluted 1:10 with M2 + BSA, before thorough washing in M2 + BSA. Embryos were transferred to a 1:10 dilution of guinea pig complement serum (Sigma, S1639, purchased lyophilized, reconstituted in ice-cold distilled water as per instructions supplied and stored in aliquots at -70°C) in M2 + BSA containing 0.01 mg $ml^{-1}$ propidium iodide (Sigma, P4170) at 37°C for 10-15 min. Embryos were briefly washed in phosphate-buffered saline without $Ca^{2+}$ and $Mg^{2+}$ before fixing and labelling in 0.05 mM-bisbenzimide (Hoechst 33258; Sigma, B2883) in absolute alcohol overnight at 4°C. Labelled blastocysts were washed for between 1 and 24h in absolute alcohol before examination (§8.5.2).

### §8.4.3 Differential labelling of the trophectoderm and inner cell mass nuclei of human blastocysts

Zona-free (§8.3.1) human blastocysts were incubated in 10mM trinitrobenzenesulphonic acid (Sigma, P2297) in ungassed M16 supplemented with 4mg $ml^{-1}$ polyvinylpyrrolidone (Calbiochem 5295) on ice for 10 min., washed three times in M2 + BSA, and incubated in 0.1 mg $ml^{-1}$ anti-dinitrophenol-BSA (anti-DNP-BSA; ICN Flow, 61-006-1) in M2 + BSA at 37°C for 10 min. After further washing in M2 + BSA, blastocysts were finally incubated in a 1:10 dilution of guinea pig complement serum (Sigma, S1639, §8.4.2) in M2 + BSA containing 0.01mg $ml^{-1}$ propidium iodide (Sigma, P4170) at 37°C for 10-15 min. The degree and evenness of lysis of the outer TE cells were assessed by examination under a stereo dissecting microscope and the blastocysts were briefly washed in phosphate buffered saline without $Ca^{2+}$ and $Mg^{2+}$ before fixing and labelling in 0.05mM bisbenzimide (Hoechst 33258; Sigma, B2883) in absolute alcohol overnight at 4°C. Labelled blastocysts were washed for between 1 and 24h in absolute alcohol before examination (§8.5.2).

## §8.5 Fluorescence microscopy and photography

Labelled embryos were examined using a Leitz Laborlux D fluorescence microscope (Leica, U.K.). The microscope was fitted with either of two filter blocks, or a combination of both (Table 8.8).

**Table 8.8:** *Characteristics of filter blocks for Leitz Laborlux D fluorescence microscope*

| Filter block | Excitation filter | Emission filter |
|---|---|---|
| Leitz A2 | Band pass* 270 - 380 nm | Band pass* 410 - 580 nm |
| Leitz I2 | Band pass* 450 - 490 nm | Long-wave pass‡ >515 nm |

* A band pass filter allows through light of wavelengths between the values stated
‡A long-wave pass filter allows through light of longer wavelengths than the value stated, although the cut-off is not sharp, and some light of slightly shorter wavelengths will be allowed through.

**Table 8.9:** *Colours of light of different wavelengths*

| Wavelength (nm) | Colour |
|---|---|
| <390 | U.V. |
| 390 - 455 | Violet |
| 455 - 492 | Blue |
| 492 - 577 | Green |
| 577 - 597 | Yellow |
| 597 - 622 | Orange |
| 622 - 780 | Red |
| > 780 | Infrared |

Embryos were observed with a 10×, 20× or 63× objective and were photographed with an Olympus OM2 35mm single lens reflex camera using Kodak Ektachrome 35mm 400ASA daylight colour slide film.

**Table 8.10:** *Characteristics of fluorochromes used to fluorescently label either the cell surface or the nuclei of embryos*

| Fluorochrome | Excitation peak (nm) | Emission peak (nm) | Emission colour |
|---|---|---|---|
| FITC | 480 | 525 | Green |
| Bisbenzimide (Hoechst 33258) | 350 | 505 | Green |
| Propidium Iodide | 287 | 602 | Orange |

### §8.5.1 Surface labelled embryos

FITC-labelled embryos were mounted in M2 + BSA in a glass chamber. The glass chamber was constructed by raising a 22mm$^2$ glass coverslip above the surface of a glass slide using small blobs of Blue-Tack at each corner (Fig. 8.1).

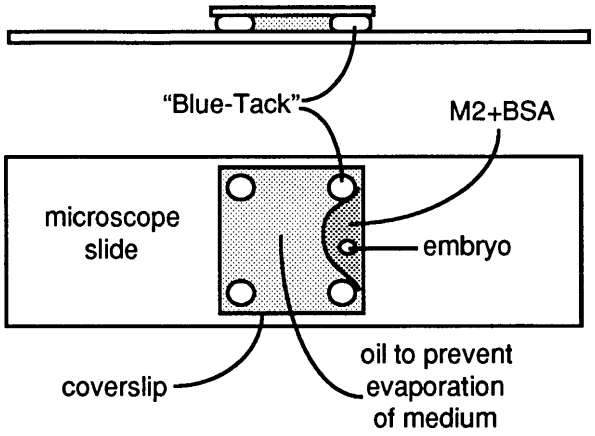



**Figure 8.1:** *Diagram of chamber for observing surface labelled mouse embryos with the fluorescence microscope*

Labelled embryos were examined using a Leitz Laborlux D fluorescence microscope (Leica, U.K.) fitted with an I2 filter set (Table 8.8). The surface of the embryo, labelled with FITC (Table 8.10), appears green/yellow (Table 8.9), with more intense labelling in the cleavage furrows. Individual labelled embryos were viewed with a 20× PHACO 1 objective and were photographed (§8.5) using an exposure time of between 30 - 60sec. To facilitate calibration of the photographic slides, the engraved squares on an Advanced Neubauer haemocytometer were photographed at the same magnification.

Photographic slides were viewed using a Wotan Diastar 320 slide viewer. Cleavage furrows were clearly visible, enabling the outline of the apical surface of exposed cells to be traced on to tracing paper. Only fully visible ("face-on") cells were traced, cells on the edge of the embryo were ignored. In addition, the diameter of the embryo was measured. Tracings of the cell outlines were then transferred to graph paper and the surface area of each exposed surface calculated by counting squares.

### *§8.5.2 Differentially labelled embryos*

Embryos with labelled nuclei were individually mounted on microscope slides in glycerol underneath a coverslip and initial examination was carried out in whole mount. Using a combination of two filter sets (Table 8.8), ie. the Leitz A2 excitation band pass filter (Leica, U.K.) with the Leitz I2 emission filter (Leica, U.K.), ICM nuclei labelled with bisbenzimide appear green and TE nuclei labelled with a combination of bisbenzimide and propidium iodide appear orange (Fig. 8.2, Tables 8.9 & 8.10). Embryos were observed with a 63× EF Leitz objective (Leica,

U.K.) and photographed (§8.5) using an exposure time of 1/4 or 1/2 sec. After photography and description of the staining pattern, the embryo was carefully squashed and disaggregated by gently applying pressure with a pencil eraser to the coverslip, and examined again using a 20× PHACO 1 objective for counting of nuclei.

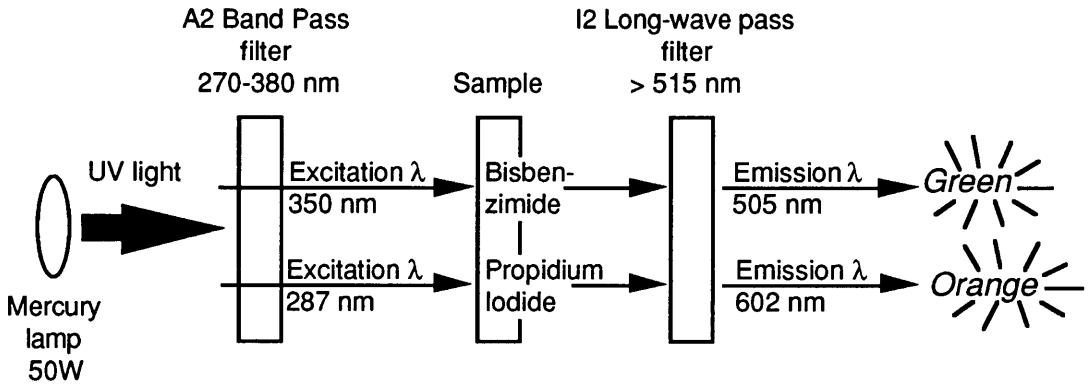



**Fig. 8.2:** *Schematic representation of excitation of bisbenzimide and propidium iodide labelled specimens by U.V. light with the resulting colours emitted*

Normal nuclei had a distinct nuclear outline, brightly staining nucleoli and even shape. Cells at the metaphase stage of mitosis (where the condensed, labelled chromosomes were clearly visible) were counted as single cells. Estimates of the number of dead cells were based on the presence of degenerating nuclei characterized by discrete clusters of labelled nuclear fragments. These dead cells were not included in the overall total of cell numbers. Mitotic and dead cell indices were calculated as follows:

$$\text{Mitotic cell index} = \frac{\text{No. of metaphases}}{\text{Total no. of cells}} \times 100$$

$$\text{Dead cell index} = \frac{\text{No. of dead cells}}{\text{Total no. of cells + no. of dead cells}} \times 100$$

## §8.6 Non-invasive measurement of nutrient uptake by human and mouse embryos

In order to assess the uptake of substrates, embryos are incubated for a known period of time in a medium containing defined concentrations of glucose, pyruvate

and lactate. The T6 medium used for the culture of human embryos (Table 8.3) has been modified so that the concentrations of substrates are 1mM glucose, 0.47mM pyruvate and 5mM lactate. The medium is supplemented with 4 mg $ml^{-1}$ BSA (Crystallized Albumin, ICN Flow, 81-001).

Embryos were washed thoroughly through several changes HLT6 (Table 8.12), and incubated individually (human embryos) or in groups of up to ten (mouse embryos) in 3 or 4µl droplets of HLT6 under pre-equilibriated paraffin oil (Light Liquid Paraffin Oil, BDH, 29436), with a gas phase of 5% $CO_2$ in air. At daily intervals, embryos were removed from the droplets in the tip of an oil-filled drawn Pasteur pipette with the minimum of medium, washed through three changes of fresh HLT6 and transferred to new pre-equilibriated droplets for the next incubation period. Droplets of medium alone incubated adjacent to the droplets containing the embryo(s) were used as controls. Incubation and control droplets were taken up individually in 10µl microcaps (Drummond: Philip Harris Scientific, P46-452) which were sealed with "Blue-Tack" and stored at -70°C prior to transport on dry ice to the Department of Biology, University of York, for assay (§8.6.1). By analysing the difference between substrate levels in control and incubation droplets, measurements of the uptake of pyruvate and glucose by single or groups of embryos could be made.

**Table 8.11**: *Composition of concentrated stocks for HLT6 medium used for assessment of substrate uptake by human embryos ( Based on modified T6, Hillier et al, 1984; Table 8.2)*

| Stock (concⁿ.) | Component | Amount | Molecular weight | Final media mM | Supplier & catalogue number |
|---|---|---|---|---|---|
| ***A*** | | *g 100ml⁻¹* | | | |
| (10 x) | NaCl | 7.13 | 58.44 | 122.01 | BDH 10241 |
| | KCl | 0.106 | 74.55 | 1.42 | BDH 10198 |
| | $MgCl_2.6H_2O$ | 0.096 | 203.30 | 0.47 | BDH 10149 |
| | $Na_2HPO_4.12H_2O$ | 0.129 | 358.14 | 0.36 | BDH 10248 |
| | Na Lactate | 0.93 of 60% syrup | 112.1 | 5.0 | Sigma L-1375 |
| | Glucose | 0.18 | 180.16 | 1.0 | BDH 10117 |
| | Penicillin | 0.060 | 1625 U $ml^{-1}$ | 97.5 U $ml^{-1}$ | Sigma PEN-K |
| | Streptomycin | 0.050 | 750 U $ml^{-1}$ | 37.5 U $ml^{-1}$ | Sigma S-6501 |
| ***B*** | | *g 100ml⁻¹* | | | |
| (10 x) | $NaHCO_3$ | 2.101 | 84.01 | 20.01 | BDH 10247 |
| | Phenol Red | 0.010 | | | BDH 20091 |
| ***C*** | | *g 10ml⁻¹* | | | |
| (100 x) | Na Pyruvate | 0.052 | 110.0 | 0.47 | Sigma P2256 |
| ***D*** | | *g 10ml⁻¹* | | | |
| (100 x) | $CaCl_2.2H_2O$ | 0.262 | 147.02 | 1.78 | BDH 10070 |

Stocks prepared with Boots "Water for Injections" B.P. throughout; filter-sterilized by passing through a 0.22μ Millex-GS disposable Millipore filter (SL GS 025 BS) using a 20ml Monoject disposable syringe; stored in 6ml and 14ml Falcon (Becton Dickinson, U.K.) tubes at 4°C. Stocks A, D & E are kept for 3 months, B & C for 2 weeks.

**Table 8.12:** *Preparation of HLT6 culture medium for assessing nutrient uptake by human embryos (100ml 1×)*

| Stock | ml |
|---|---|
| A | 10 |
| B | 10 |
| C | 1 |
| D | 1 |
| $H_2O$ | 78 |

Osmolality: ≈290.
Media are filter-sterilized by passing through a 0.22μ Millex-GS disposable Millipore filter (SL GS 025 BS) using a 20ml Monoject disposable syringe; stored in 6ml and 14ml Falcon (Becton Dickinson, U.K.) tubes at 4°C

### §8.6.1 Ultramicrofluorescence assays

Samples of medium were coded before assay to disguise their identity. The analyses were carried out in microdrops of reaction mixture (§8.6.2, §8.6.3) under heavy paraffin oil (Sigma) in siliconized glass wells of 75 × 25 × 5 mm. The glass wells were siliconized by immersion in dimethyldichlorosilane solution (BDH) for a minimum of 30s, air dried, rinsed in distilled water and air dried again. The glass well containing the microdroplets of reaction mixture was placed on the stage of a Leitz Diavert Fluorescence microscope with photomultiplier and digital microphotometer attachments, and the microdrops of reaction mixture observed with a Leitz 10× objective (Leica, UK). The edge of the microdrop was brought into sharp focus, before centering in the field of view. Each droplet of reaction mixture was exposed to 0.55s of UV light of between 340 - 380nm. NADH or NADPH are excited at 340nm and, upon excitation, emit fluorescence at 459nm and above. The emitted fluorescence is amplified by the photomultiplier and the intensity displayed on the digital photometer. The amount of fluorescence emitted is proportional to the amount of NADH or NADPH in the reaction droplet. As the generation and consumption of NAD(P)H is directly coupled to glucose and pyruvate consumption respectively (§8.6.2 and §8.6.3), it is possible, by comparison with glucose and pyruvate standard curves, to calculate the amount of these substrates in the sample medium and therefore the amount consumed by the embryo per hour. Background readings of the drops of reaction mix were taken before addition of the sample

medium. 2 - 3min after addition of 0.5µl of sample medium a further reading was taken, which was subtracted from the background value.

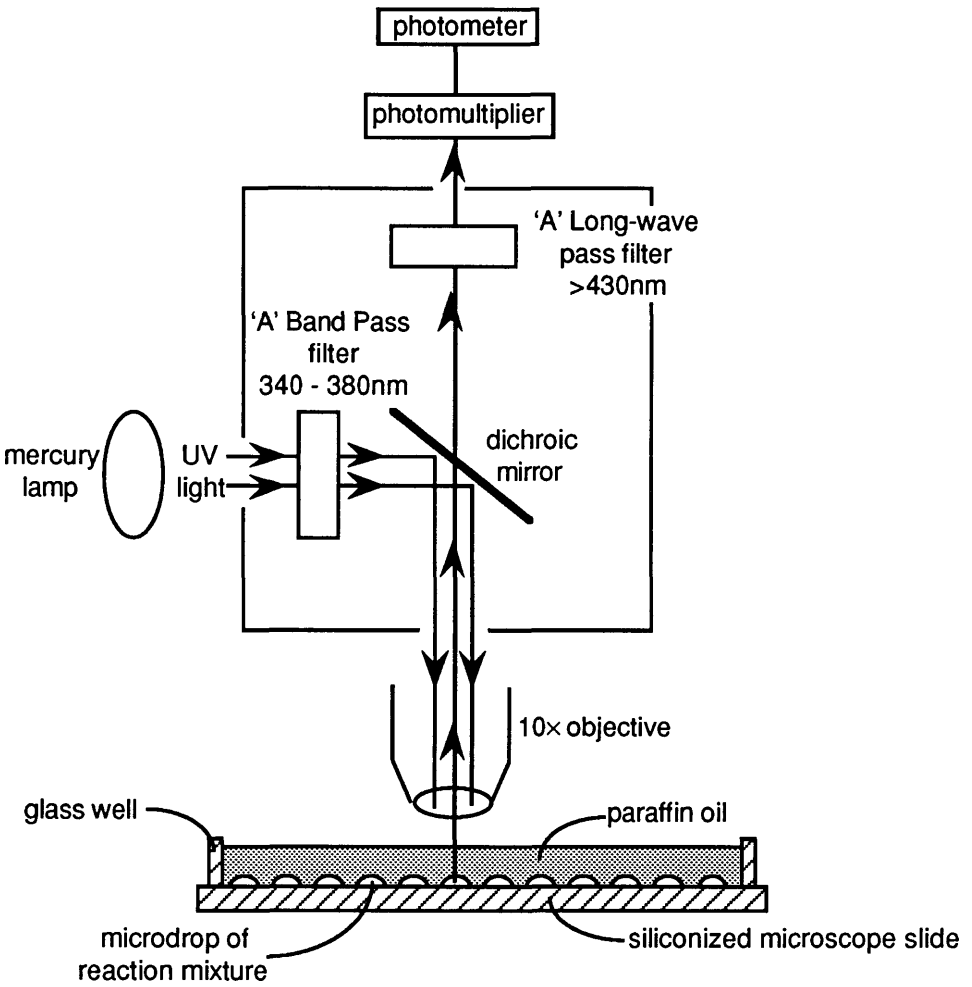



**Figure 8.3:** *Schematic representation of ultramicrofluorescence assay*

### *§8.6.2 Pyruvate assay.*

**Table 8.13**: *Pyruvate assay reaction mix*

| Compound | Amount (ml) | Stock solution (mM) | Supplier & catalogue no. |
|---|---|---|---|
| EPPS Buffer* | 14 | 2.52g $200ml^{-1}$ | Sigma E9502 |
| NADH | 0.3 | 17.74mg $5ml^{-1}$ (5mM) | BM‡ 128023 |
| LDH | 0.4 | | BM 107042 |

* pH adjusted to 8.0 with 1N NaOH before making up to 200ml. Supplemented with 5mg $100ml^{-1}$ Penicillin and Streptomycin. ‡Boehringer Mannheim.

0.5 µl of medium was added to 5µl of assay mixture containing 0.1 mM NADH and 41 U $ml^{-1}$ of Lactate Dehydrogenase (LDH) in EPPS buffer, pH 8.0 (Leese and Barton, 1984) (Table 8.13).

$$\text{Pyruvate} + \text{NADH} + H^+ \xleftrightarrow{\text{LDH}} \text{Lactate} + NAD^+$$

Decreases in fluorescence due to oxidation of NADH were monitored. Duplicate readings for each sample of medium were taken and these were calibrated against a series of pyruvate standards (0-0.5 mM).

### §8.6.3 Glucose assay.

**Table 8.14:** *Glucose assay reaction mix*

| Compound | Amount (ml) | Stock solution (mM) | Supplier & catalogue number |
|---|---|---|---|
| EPPS Buffer* | 18.75 | 2.52g $200ml^{-1}$ | Sigma |
| Dithiothreitol | 2.5 | 7.715mg $10ml^{-1}$ (5mM) | Sigma |
| $MgSO_4.7H_2O$ | 2.5 | 91.2mg $10ml^{-1}$ (37mM) | BDH |
| ATP | 1.25 | 30.26mg $5ml^{-1}$ (10mM) | BM‡ |
| Hexokinase/G6PDH | 1.25 | 15mg $5ml^{-1}$ | BM |
| NADP | 3.75 | 39.37mg $5ml^{-1}$ (10mM) | BM |

* pH adjusted to 8.0 with 1N NaOH before making up to 200ml. Supplemented with 5mg $100ml^{-1}$ Penicillin and Streptomycin. ‡ Boehringer Mannheim

0.5 µl of medium was added to 5 µl of assay mixture containing 0.42 mM dithiothreitol (Sigma), 3 mM $MgSO_4$ (BDH), 0.42 mM ATP, 1.25 mM NADP and 18 U $ml^{-1}$ Hexokinase/Glucose-6-phosphate Dehydrogenase (G6PDH) (12 U $ml^{-1}$ Hexokinase, 6 U $ml^{-1}$ G6PDH) in EPPS buffer at pH 8.0. (Table 8.14).

$$\text{Glucose} + \text{ATP} \xrightarrow{\text{HK}} \text{Glucose-6-phosphate} + \text{ADP}$$

$$\text{Glucose-6-phosphate} + \text{NADP} \xleftrightarrow{\text{G6PDH}} \text{6-phosphogluconate} + \text{NADPH} + H^+$$

The increase in fluorescence caused by the formation of NADPH was measured as for the pyruvate assay. Duplicate readings were calibrated against a series of glucose standards (0-1 mM glucose).

### §8.7 Statistical analysis

Examination of the variation in blastocyst cell number in blastocysts from the same patient and between different patients was carried out by Analysis of Variance. Differences in total cell number and the numbers of TE and ICM cells in blastocysts between days 5 and 7 were analyzed for significance using the Student's *t* test. Differences between distributions of cell numbers in the TE and ICM lineages were analysed using the Wilcoxon Rank-Sum (Mann-Whitney) Test.

# §9 Appendix

## §9.1 Appendix 1: Sphere Geometry

If r is the radius of a sphere,

Surface area of a sphere = $4\pi r^2$

Volume of a sphere = $\frac{4}{3}$ $(\pi r^3)$

Surface area is proportional to volume$^{2/3}$

**Table 9.1**: *Relationship between volume and surface area of a sphere in arbitrary units*

| Volume | Surface area |
|---|---|
| 0.5 | 0.63 |
| 1 | 1 |
| 2 | 1.59 |
| 3 | 2.08 |
| 4 | 2.52 |

## §9.2 Appendix 2: Human IVF outcome data

At the Hammersmith Hospital IVF Unit, between January 1990 and March 1991, 656 patients had between one and three embryos transferred.

**Table 9.2:** *Summary of fertilization rates in 656 patients who had embryos transferred between January 1990 and March 1991*

| | Number | Mean per patient ± standard error |
|---|---|---|
| Total no. of patients having embryos transferred | 656 | |
| No. of oocytes retrieved | 6374 | 9.7 ± 0.2 |
| No. of oocytes normally fertilized | 3597 (56.43%) | 5.5 ± 0.1 |
| No. of embryos transferred | 1427 | 2.2 ± 0.02 |
| No. of pregnant patients as determined by raised hCG levels on day 14 post oocyte retrieval, including biochemical and ectopic pregnancies | 292 (45%) | |

**Table 9.3:** *Pregnancy outcome of patients who had between one and three embryos transferred between January 1990 and March 1991*

| | Number of patients (% of total no.) | No. of embryos transferred (mean ± sem) | No. of fetal sacs* (% of ‡) | No of fetal hearts* (% of ‡) |
|---|---|---|---|---|
| Total patients | 656 | 1427 | | |
| Non pregnant | 364 (55%) | 776 (2.1 ± 0.03) | - | - |
| Pregnant (as determined by the presence of a fetal sac) | 241* (37%) | 542‡ (2.2 ± 0.03) | 346 (64%) | 309 (57%) |
| Pregnant (as determined by the presence of a fetal heart) | 222 (34%) | | | |
| Biochemical pregnancy | 43 (7%) | 91 (2.1 ± 0.08) | - | - |
| Ectopic pregnancy | 8 (1%) | 18 (2.3 ± 0.16) | - | - |

* One patient, who had two embryos transferred, was lost to follow-up and the number of sacs and fetal hearts is unknown

### §9.2.1 Implantation rate

Of the 656 patients who had embryos transferred, 292 patients became pregnant, as determined by raised hCG levels. In 43 (15% of patients with raised hCG) patients the pregnancy did not progress, and was defined as a biochemical pregnancy. In a further 8 patients (3%) the pregnancies terminated in an ectopic pregnancy. In total, 1427 embryos were transferred. In each of the 43 patients who had a biochemical pregnancy and the 8 patients who had an ectopic pregnancy, at least one embryo must have implanted. In addition, 346 embryos reached the fetal sac stage, therefore, a total of at least 397 embryos must have implanted, giving an overall minimium implantation rate of 28%. In some cases where more than one embryo implants it is possible that not all of them develop to the fetal sac stage, therefore the implantation rate could be higher. If the serum hCG levels on day 14 post oocyte retrieval are compared with mean values for singleton, twin and triplet pregnancies (C. Packham, personal communication), 27 patients showed evidence, from higher hCG levels, of more embryos implanting than developed into fetal sacs. This results

in a maximum of 427 implantations (including biochemical and ectopic pregnancies), giving a maximum implantation rate of 30%.

### §9.2.2 Fetal development rate

309 embryos gave rise to a fetus (as determined by the presence of a fetal heart) giving an overall fetal development rate of 22%.

In summary, after transfer, an embryo has a 28-30% chance of implanting and a 22% chance of developing to a fetus.

### §9.2.3 Pregnancy rate and development of surplus embryos

The development *in vitro* of surplus embryos was closely monitored in a total of 67 patients who had embryos transferred. 33 of these patients became pregnant, the remaining 34 did not. In those patients who did not become pregnant, 24 (71%) had one or more surplus embryos which reached the blastocyst stage. In the patients who did become pregnant, 27 (82%) had surplus embryos which reached the blastocyst stage.

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