Reviews

Fundamentals of human embryonic growth *in vitro* and the selection of high-quality embryos for transfer

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Dr Irene Boiso was born in Buenos Aires, Argentina. She received her BSc degree in Biological Sciences from the Universidad Central de Venezuela. In 1993 she was awarded a 3-year fellowship from the Spanish Instituto de Cooperación Iberoamericana and moved to Barcelona, Spain. She wrote her thesis on the effect of cryopreservation on the structure of the meiotic spindle of mature and immature human oocytes, and received a PhD degree (cum laude) from the Universidad Autónoma de Barcelona. She joined the team of the Servicio de Medicina de la Reproducción from the Institut Universitari Dexeus in Barcelona as an embryologist in 1994. She has been a researcher at the Fundación Santiago Dexeus Font since 1999. Her areas of interest include assisted reproductive technologies, early embryonic development, oocyte and ovarian tissue cryopreservation and oocyte in-vitro maturation.

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Abstract

Knowledge of the nature of embryo growth, and the handling and scoring of quality in human embryos are significant aspects for embryologists in IVF clinics. This review describes the formation, growth and maturation of human oocytes, many aspects of fertilization *in vitro*, embryonic transcription during preimplantation stages, and the formation of polarities, timing controls, role of mitochondria and functions of endocrine and paracrine systems. Modern concepts are fully discussed, together with their significance in the practice of IVF. This knowledge is essential for the correct clinical care of human embryos growing *in vitro*, especially in view of their uncharacteristic tendency to vary widely in implantation potential. Underlying causes of such variation have not been identified. Stringent tests must be enforced to ensure human embryos develop under optimal conditions, and are scored for quality using the most advanced techniques. Optimal methods of culture are described, including methods such as co-culture introduced to improve embryo quality but less important today. Detailed attention is given to quality as assessed from embryonic characteristics determined by timers, polarities, disturbed embryo growth and anomalous cell cycles. Methods for classification are described. Approaches to single embryo transfers are described, including the use of sequential media to produce high-quality blastocysts. These approaches, and others involved in surgical methods to remove fragments, transfer ooplasm or utilize newer approaches such as preimplantation diagnosis of chromosomal complements in embryos are covered. New outlooks in this field are summarized.

Keywords: *embryo surgery, embryonic regulation, human embryos, pregnancy rates, selection for implantation potential*

Introduction

Two interrelated challenges face embryologists in IVF clinics. A current clinical need is to find out why human embryos have such low implantation rates that several treatment cycles or multiple embryo transfers are needed for assisted reproduction. Finding the cause demands a deeper scientific understanding of the regulation of the formation and growth of oocytes and embryo development, and knowledge on these topics forms the first part of this manuscript.

Clinical practice aimed at improving implantation rates will then be described in the second section of this paper. Initially, investigators believed human embryos had implantation potentials typical of other mammals, i.e. 80–90% per embryo in many species. Instead, these rates are approximately 20% even in couples wishing to achieve conception, and similar to implantation rates achieved with single embryo transfers during assisted human conception.

Detailed attention will therefore be given to the culture of human embryos and selection for those embryos with high implantation potential. This quality is not studied in any animal species, and it is apparently entirely a clinical problem.

This review will also be published in a Spanish textbook entitled *Modern Assisted Conception.*

Why some embryos implant, whereas most do not, will be a common theme running through both sections of this manuscript.

Part 1. The human embryo in the laboratory

Developmental systems discussed in this section include cell structure, transcription, maternal and embryonic gene expression, polarity, timing and integration, mitochondria and embryonic cytokines. Understanding these factors from earliest stages of oocyte formation in humans depends partially on analysing homologous systems in flies, nematodes and amphibians. Clear gene homologies have been identified between these disparate organisms (Edwards, 2001).

Formation, growth and maturation of the oocyte

Follicle formation and growth

Differentiating primordial germ cells produce gametogenic cells of both sexes in mammals. Multiplying mitotically in both sexes, they diverge and differentiate into male and female germline cells. Female germ cells form oogonia, and then oocytes as they enter their first meiotic division. This step is delayed in male embryos until the testis has formed. In most mammals, meiosis in primordial oocytes arrests at the diplotene stage of meiosis 1, when a germinal vesicle forms. Several ovarian cells, probably arising from rete tubules, enclose each oocyte to form the primary follicle.

All human follicles are formed before birth to form a pool, which is utilized progressively from the moment of their formation, through puberty and during successive reproductive cycles in the adult (Kably and Barroso, 2000). The end of reproductive life in women, and in certain mouse strains, is signified as the pool declines to very few or no remaining follicles.

Follicles migrate from the pool at a constant rate over much of the reproductive lifespan in women, with a sudden increase during perimenopausal years (Faddy *et al.*, 1992). They develop at astonishingly regular rates through the stages of folliculogenesis. The great majority of growing follicles and oocytes never attain ovulation or fertilization, many becoming atretic during each stage of differentiation. Follicles continuing to grow develop an antral cavity and become sensitive to gonadotrophins as FSH and, later, LH receptors differentiate on theca and granulosa cells. Pituitary gonadotrophins are now essential, their high levels during ovarian stimulation preventing atresia so that many follicles continue to grow (Kably and Barroso, 2000).

In natural cycles, one follicle becomes dominant. Internal paracrine systems and external secretions from the ovary to the hypothalamus co-operate in selecting the dominant follicle, which then impairs challenger follicles (Baker and Spears, 1999; Mikkelsen *et al.*, 2001). This significant phase of follicle growth occurs as follicles reach >10 mm diameter and the dominant follicle withstands slight decreases in FSH concentrations typical of the late follicular phase of the menstrual cycle. It is also important if the aspiration of immature oocytes is desired, since a dominant follicle and oestradiol output improves their chances of maturing *in vitro* and establishing pregnancy (Mikkelsen *et al.*, 2001). Ovarian stimulation also overcomes atresia, so many follicles do develop to ovulation (Risquez, 2001; Ulloa-Aguirre *et al.*, 2000).

Atresia at all stages of follicle growth is largely due to apoptosis involving proto-oncogenes of the Bcl-2 family (Hsueh *et al.*, 1994). In mouse follicles, genes such as *bcl-2* and *bclxs*, *c-myc*, *bax* and the *caspases* regulate apoptosis although *c-myc* may not have a major role (Nandekar and Dharma, 2001). Apoptosis leads to a dearth of follicles and the menopause, the primary limit to female conception. Analyses of apoptotic pathways as a cause of atresia have opened new concepts on delaying the menopause, by neutralizing apoptotic agents at various stages in the apoptotic pathway. Sphingosine-1-phosphate, for example, acts early in the apoptotic chain, and prevents atresia in mouse follicles, so many survive to old ages (**Table 1**) (Morita *et al.* 2000). In contrast, neutralizing compounds with a later action, for example caspases, may convert apoptosis to a form necrosis. Distant clinical treatments based on these principles may enable the fertile period in women to be extended beyond the menopause.

Mitochondrial inefficiency, restricting ATP generation in follicle cells and oocytes during dictyotene, may be another cause of follicle death. It might also cause the 'oopause', the sterile period before the menopause. Mitochondrial inefficiency might be due to accumulating mutations in mtDNA, although older women with many point mutations can be fertile, indicating that defective respiration is not a significant cause of female infertility (Shoubridge, 2000). The significance of pathogenic point mutations in mitochondria as a cause of infertility is also questioned since carrier women conceive normal children (Schon *et al.*, 2000).

Oocyte growth and maturation

These stages are crucial to female fertility (Brugo-Olmeda *et al.*, 2001). Oocytes expand in early growth stages, as they synthesize RNAs and many proteins. Large stores of RNA, including ribosomal, transfer and messenger RNAs, are deposited in ooplasm. Later, they carry newly fertilized eggs through their early growth stages, as part of the 'maternal' phase of early cleavage. Specific oocyte proteins include the well-known zona pellucida proteins ZP1, ZP2 and ZP3. Many oligopeptides are synthesized and locate at specific polarized positions within ooplasm, discussed below.

Table 1. Increased oocyte numbers after disruption of SMPD1 activity in mice (Morita *et al.*, 2000).

Follicle type	*Control*	*SMPD1 disruption*
Primordial	19,210 ± 602	30,480 ± 2397[b]
Primary	707 ± 93	1573 ± 141[b]
Preantral	13 ± 13	160 ± 46[a]

SPMD1 = sphingomyelin phosphodiesterase 1.
[a]$P < 0.05$; [b]$P < 0.01$.

During their growth stages, oocytes co-operate with granulosa cells, and might control most if not all aspects of follicular metabolism. A 'production line' in the formation and migration of primordial follicles from the pool may also be oocyte-regulated. Expanding oocytes develop a cytoarchitecture and synthesize various proteins concerned with metabolic processes and structural features. Preparations for their maturation phase are indicated as the germinal vesicle migrates from the centre of the oocyte to the cortex. Maturation divisions are triggered by the LH surge, meiosis resuming at diakinesis and the second meiotic division progress until an arrest at metaphase II and an extruded first polar body. Meiotic resumption in maturing oocytes is regulated by the genes MPF (maturation promoting factor) and *c-mos* which encodes mos protein. MPF is a protein-serine/threonine kinase formed through the association of cyclin B2 and cdc2, its activity being regulated by phosphorylation. Metaphase II is reached at 37 h after the onset of the LH surge in women, and signifies the moment of ovulation and entry of oocytes into the ampulla of the oviduct.

Maturation phases of meiosis II have been described in detail (Edwards and Brody, 1995). Chromosome misalignment on the meiotic spindle, premature centromere separation, and the presence of translocations can disturb their normal segregation into oocyte and first polar body. This can lead to monosomy, trisomy and aneuploidy in resulting embryos. Defects in cytoplasm during maturation are an equally common cause of impaired development as chromosomal anomalies (Eppig *et al.*, 1994).

Mitochondria are important in follicle, oocyte and embryonic growth. They reproduce asexually, according to their own cycle of multiplication and death. They display high mutation rates perhaps driven by their own free radicals. Each somatic cell possesses between 1 and 10,000 mtDNA copies, all usually identical (homoplasmic), although two or more types arise in some cells (heteroplasmy). Primordial germ cells in yolk sac each contain perhaps <10 mitochondria, and numbers increase as these cells migrate to genital ridge, to reach 200 in oogonia at week 9 (Cummins, 2002). Mitochondria associate with nuclear pores and cytoplasmic bridges linking neighbouring oogonia, which may permit some form of cytoplasmic flow (Motta *et al.*, 1997). In growing oocytes, they cluster with organelles close to the nucleus to form Balbiani's body and nuage (Motta *et al.*, 1997). Nuage, a matrix of ribosomes, mitochondria and tubules, may represent germplasm, and local mitochondrial stores of ATP might sustain a need for high metabolic activity. Mitochondria become inert in fully grown and in maturing human oocytes, possess a dense matrix and few transverse cristae, and are usually absent from cortical cytoplasm. They translocate along the outer surface of the nuclear membrane during germinal vesicle breakdown and early meiotic prophase, and associate with microtubule organizing centres in long arrays (**Figure 1**). Those associated with pronuclei segregate into individual blastomeres less stringently than peripheral mitochondria. Mitochondrial inefficiency and low ATP concentrations may influence follicle growth and embryonic development, especially in older women, and may be one cause chromosomal non-disjunction (Van Blerkom *et al.*, 1997). The amount of oxygen dissolved in follicular fluid may depend on blood supply to individual follicles, and could be a major determinant in the quality of human embryos for implantation.

Table 2. Some homologies between human, mouse and *Drosophila* genes in early embryos. Based on Edwards (2001).

Human	Mouse	*Drosophila*
Premature ovarian failure	Formins	*diaphanous*
HS6ST	–	*egalitarian*
staufen	–	*staufen*
Oct-4	*Oct-4*	–
α integrins	*α integrins*	–

Oocytes in many if not all vertebrates establish their anterior/posterior (A/P) and dorso–ventral (D/V) axes even as they are formed. This step seems to be essential in all species from earliest formative stages. It presumably forms a scaffold for numerous transcripts and proteins synthesized during successive stages of oocyte growth. Numerous genes determine axes in oocytes and surrounding follicle cells, including *gurken*, *EGF* and *staufen* in *Drosophila*. Their actions are very well known, even to fine details of RNA:protein interactions. Several of them share sequence homologies with mammalian genes or regulate conserved systems in mammals, e.g. ooplasmic rotation at fertilization (**Table 2**) (Edwards, 2001). Little is known about these early oocyte stages in mammals although close parallels are identified in lower animals. The mammalian oocyte is certainly polarized during its growth phases with well-defined animal and vegetal poles (Antczak and Van Blerkom, 1997), the animal pole in mature oocytes being marked to some degree by the first polar body.

Fertilization and the fertilized egg

Sperm binding to the zona pellucida and oolemma

Fertilization is a highly significant moment in conception. Virtually impossible to measure *in vivo*, its failure could be a major cause of human infertility. Knowledge on its components has accumulated from studies *in vitro* (e.g. Edwards and Brody, 1995). Spermatozoa ascending the female reproductive tract may briefly attach to oviductal epithelium, then be liberated in groups ready for fertilization. Good sperm motility is essential for their passage through cumulus cells and the zona pellucida. Abnormal forms of spermatozoa can bind to the zona pellucida but may be incapable of passing through it. Spermatozoa binding to the zona pellucida have undergone the acrosome reaction, when the outer acrosomal membrane fuses with the outer sperm membrane. This change is induced as spermatozoa bind to ZP3, and later to ZP2, in the zona pellucida They are activated to release intracellular calcium, leading to the formation of multiple vesiculations in the acrosomal vesicle, and the release of its contents including hyaluronidase, acrosin, arylsulphatase and other enzymes. These enzymes, and the characteristic sperm hyperactivity at this stage might assist spermatozoa to pass through the zona pellucida to the perivitelline space surrounding the oocyte (Croxatto, 2002).

Spermatozoa rapidly migrate through the zona pellucida, although in human eggs many remain trapped there,

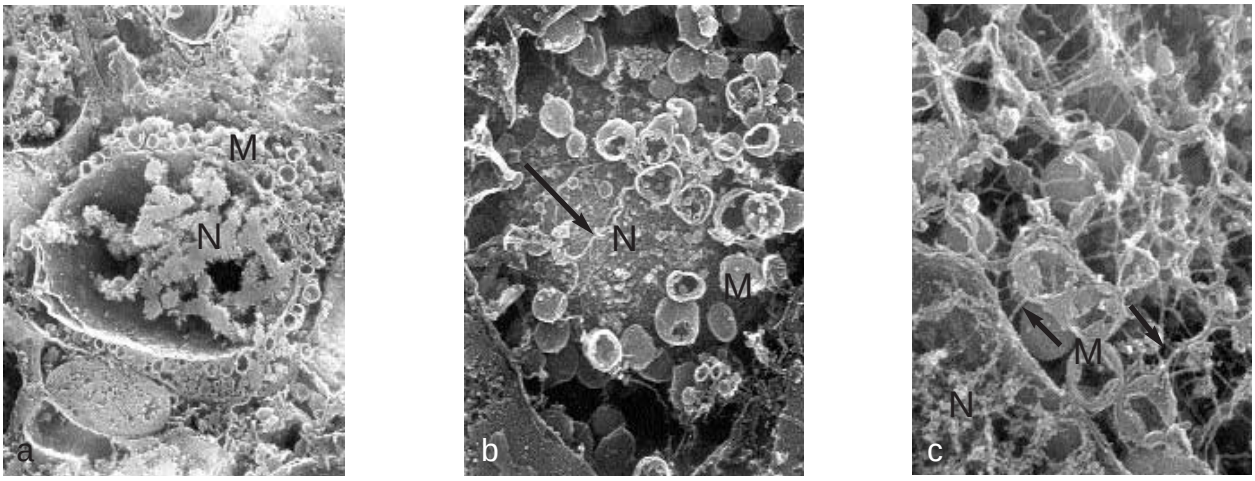


Figure 1. Successive stages of oocyte formation in the fetal human ovary. (**a**) A primary meiotic oocyte in a sexual cord at 14 weeks gestation. Its large spherical nucleus (N) contains chromosomes in the prophase of meiosis and is surrounded by many mitochondria (M). Original magnification SEM ×7500. (**b**) Primary meiotic oocyte in a 14-week ovary. Mitochondria (M) are assembled around the nucleus (N) and are associated with microtubules (arrow). Spherical mitochondria contain sparse cristae. N indicates the outer surface of the nuclear envelope. Original magnification SEM ×15,000. (**c**) A human fetal ovary at 22 weeks of gestation showing mitochondria (M) intermingled with microtubules (arrows). Mitochondria are present in a cytoplasmic area closely associated with the nucleus (N). Original magnification SEM ×12,000. (**d**) A primary oocyte examined by transmission electron microscopy and showing mitochondria (M) in close apposition with the nuclear membrane. N, nucleus. Original magnification ×18,000. Reproduced by courtesy of PM Motta and S Makabe, Rome and Tokyo Universities.

apparently unable, or too late, to bind to the oolemma. The fertilizing spermatozoon can bind instantly to receptors in oolemma, via its posterior acrosomal membrane, even as its tail still protrudes outside the zona (**Figure 2**). The entire spermatozoon is ultimately drawn into ooplasm. The oocyte is now activated, and discharges the contents of cortical granules into perivitelline space. A 'tanning' effect of these discharges on inner layers of the zona pellucida apparently stops other spermatozoa from further penetration of the zona, even if already half-way through it. Nevertheless, two or more spermatozoa do occasionally penetrate into human oocytes.

Very few sperm mitochondria enter fertilized eggs (approximately 75), and may be overlooked among the 100,000 or more in the oocyte. They are unlikely to have a long-term role, since even a small paternal leakage is not compatible with detailed characteristics of mitochondrial inheritance. Sperm mitochondria are probably degraded by ubiquitin mediated systems or segregated to trophectoderm and its derivatives (Ludwig *et al.*, 2001).

Oocyte responses to sperm binding

Sperm attachment activates the oocyte, stimulating the completion of meiosis II through anaphase and telophase, and extrusion of the first polar body. This process is facilitated as oocytes produce calpain, which inactivates mos protein and MPF and so removes the metaphase II block. Somewhat surprisingly, human oocytes are also activated during intracytoplasmic sperm injection (ICSI). This contrasts with oocytes of several animals which require an electrical or some other stimulus for activation. Morphologically abnormal spermatozoa can be used successfully in ICSI, without apparent harm to resulting embryos (Ludwig *et al.*, 2001). Mos protein can be inactivated experimentally in maturing mouse oocytes by targeting it using RNA interference or by knocking-out the gene in transgenic mice. Removal of this metaphase II block permits maturing oocytes to pass through metaphase II, anaphase and telophase to spontaneously expel their second polar body. They form rudimentary parthenogenetic embryos, without any form of sperm involvement (Tavernakis *et al.*, 2000).

Rotations of the meiotic spindle during anaphase and telophase expel half of the meiotic chromosomes into the second polar body, leaving the remainder in ooplasm. Rotation is easily impaired by colchicine or similar agents and in certain clinical conditions such as recurrent hydatidiform mole. This condition disturbs normal spindle rotation, so it moves entire into ooplasm or polar body (Edwards and Brody, 1995). Either no chromosomes or a diploid number remain in oocytes, so fertilization then results in androgenetic haploid or triploid embryos.

Oocytes display other morphogenetic movements, which could offer potential markers of embryo quality. Ooplasm rotates clockwise several times between the completion of maturation and sperm entry. For unknown reasons, the polar axis also undergoes rotation, perhaps controlled by the sperm aster, which may bring the oocyte axis into line with the fertilizing sperm head in some oocytes (Edwards and Beard, 1997; Payne *et al.*, 1997). This event may accommodate the pre-existing polarity in the oocyte to the position of the sperm head, and it could a significant measure of embryo quality. Unfortunately, it can be measured *in vitro* only with some difficulty.

Two membrane-bound pronuclei form, a larger paternal pronucleus initially then a smaller maternal pronucleus located near the polar body which marks the animal segment of the oocyte. The paternal pronucleus may move adjacent to vegetal cortex during ooplasmic rotation in some eggs (Edwards and Beard, 1997; Payne *et al.*, 1997). Pronuclei enlarge and move together (apposition) as nucleolar-like bodies form within each of them and begin their distinct cycle (**Figure 3**). Initially small, and often formed in similar numbers in both pronuclei, nucleoli enlarge and distribute randomly in pronuclear nucleoplasm. They move together within pronuclei during apposition, to become polarized adjacent to the apposing pronuclear membranes. Towards the end of the 1-cell stage, pronuclei decondense ready for approaching syngamy and the first cleavage division. The first cleavage occurs about 24 h post-fertilization in human eggs, after the sperm centriole has organized the mitotic spindle.

Even though pronuclei rotate soon after ICSI in some human eggs, rotation occurs much later in others. Some even retain pronuclei at right angles to the polar bodies until syngamy. The whole spindle then seems to rotate as shown in videos of two human embryos (Edwards and Beard, 1999). All these morphogenetic changes in pronuclei and their rotation may enable embryos capable of implantation to be selected.

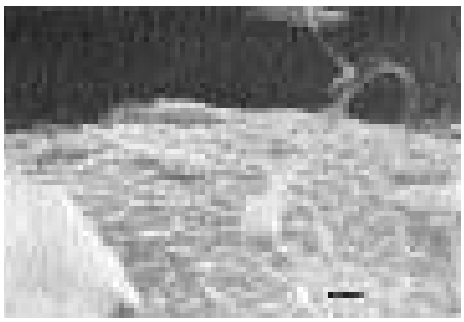
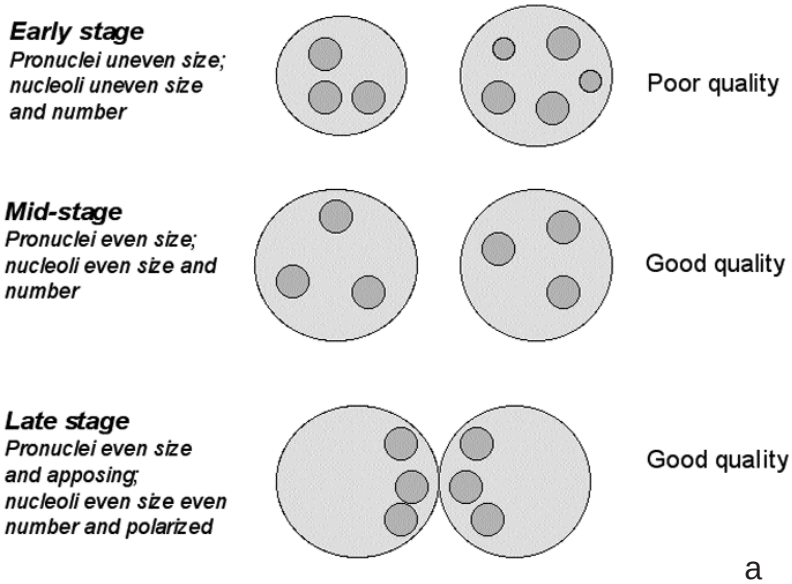


Figure 2. Scanning electron microscopy of an early stage of human fertilization *in vitro*, showing some spermatozoa attached by their heads to the zona pellucida, and other now a sperm tail beating outside the zona pellucida. Scale bar = 1 μm. Image supplied courtesy of Dr Montse Boada.

Minor and major transcription

Sperm entry into oocytes invokes rapid biochemical changes in the paternal gamete as it transforms into a pronucleus. Chromatin packaging is reorganized, protamine replaced by histones, and paternal pronuclei outcompete maternal pronuclei for hyperacetylated histone H4 pool until close to syngamy. Promoter and transcriptional activities are also higher than in maternal pronuclei and zygotic nuclei throughout virtually all 1-cell stages in mice, a difference probably reflected in variations in chromatin structure (Nothias *et al.*, 1995). Core histones such as somatic linker histone H1 regulate metabolism, and promoters and enhancers are active in pronucleate eggs and cleaving embryos, illustrated by injecting the luciferase gene into mouse 1-cell eggs.

Initially, development in 1-cell and cleaving mammalian embryos is regulated by maternal genes and proteins inherited from the oocyte. The *oct* genes, a good example, have



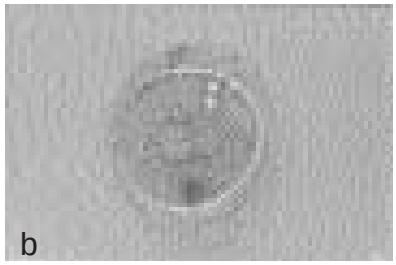


Figure 3. (**a**) Sketch showing successive stages of the polarization of pronuclei and nucleoli in 1-cell human embryos. (**b**) Human pronucleate egg displaying the characteristics of polarization in pronuclei and nucleoli. Note the pronuclei are fully apposed and nucleoli are approaching polarity especially in the pronucleus on the left.

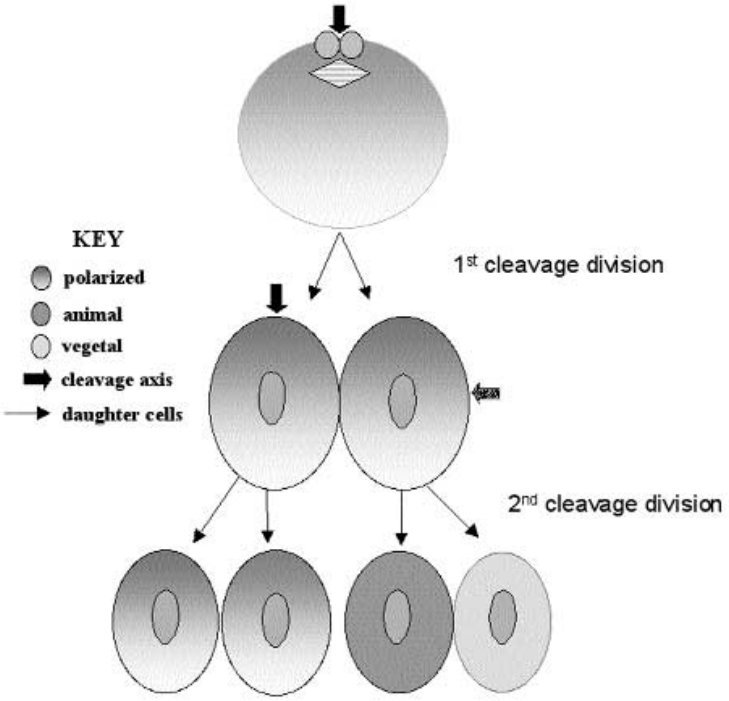


Figure 4. Sketch showing possible animal (darker shading) and vegetal (light) poles, forming an axis across the unfertilized egg. Note the meridional first cleavage plane bisects the oocyte from the metaphase II spindle (black arrow), so its 2-cell daughter blastomeres have a polarity resembling the oocyte. In 2-cell embryos, one blastomere cleaves meridionally again (black arrow), producing two similar daughters in the 4-cell stage. The other 2-cell blastomere cleaves transversely (speckled arrow), so one daughter inherits mostly animal and the other mostly vegetal cytoplasm. Based on Edwards (2001).

numerous functions in cleaving embryos and blastocysts, interacting with transcription factors and regulatory elements. Maternal effects become less evident as development proceeds. Many such situations are discussed below, one specific example being maternal expression of SCF (stem cell factor) and tumour necrosis factor (TNF) α until 4–6 cell stages in mice (Sharkey *et al.*, 1995). Some zygotic genes are expressed transiently in late 1-cell and 2-cell stages, known as the period of 'minor' embryonic transcription. Examples include the translation initiation factor e1F-4C, transcription requiring complex (TRC), some sex-determining genes carried on the Y chromosome and the major class 1 *H-2D* gene (Edwards and Beard, 1997). Major zygotic transcription begins later, in the 2–4 cell stage.

Shape, size and topography of mitochondria change after sperm entry. Some concentrate in ooplasm near the sperm aster, then in peri-pronuclear regions as they are depleted from cortex. This may cause the 'halo' effect, valuable in scoring human embryos for quality. Those around pronuclei or in ooplasm may associate with ooplasmic architecture, especially microtubules (Motta *et al.*, 1997), and display a clear polarized distribution on outfacing surfaces of apposed pronuclei. In later pronuclear stages, mitochondria surround pronuclei in a concentrated elliptical array, perhaps producing ATP in association with microtubules (Van Blerkom *et al.*, 2000). ATP is generated by oxidative phosphorylation in cleaving embryos, shifting to glycolysis at compaction. In human eggs with pronuclei and a halo, together with polarizing nucleoli, numerous mitochondria concentrate in perinuclear ooplasm, and few are present in regions of cytoplasmic flare. Areas devoid of mitochondria in ooplasm found in some oocytes can be transmitted to blastomeres (Van Blerkom *et al.*, 2000).

Cleavage stages, morulae and blastocysts

Patterns of cell division

Early blastomeres are relatively undifferentiated, although sizes of 2-cell blastomeres differ slightly. Four-cell embryos are products of a meridional division in one 2-cell blastomere, and a transverse division in the other (**Figure 4**). Three cleavage divisions by 2.5 days post-fertilization produce 8-cell human embryos in their initial stages of differentiation. Many blastomeres have a normal morphology. Others display fragments, some adjacent to the site of cytokinesis, and possibly caused by membrane ruffling. Large fragments characterize some blastomeres, perhaps indicative of cell death, discussed in detail below. Apoptosis in oocytes, polar bodies and blastomeres might impair growth and cause blastomere fragmentation and programmed cell death as measured by terminal deoxynucleotidyl transferase-mediated dUDP nick-end labelling (TUNEL)-positive staining (Takase *et al.*, 1995). Cell death is widespread in preimplantation human embryos, arresting embryos with various degrees of apoptosis, including cytoplasmic, nuclear and DNA fragmentation. Scattered cells in morulae and blastocysts display apoptosis, which may eliminate abnormal cells (Hardy, 1997).

Cellular polarity, as opposed to embryonic polarity, becomes important from 8-cell stages. Gap junctions and zonular adherents-type junctions form in membranes of 8-cell or older embryos. Established and maintained by microfilaments and microtubules in outer cells of 16-cell mouse embryos, it involves a specific outer location of carbohydrates, alkaline phosphate and 5-nucleotidase, actin and myosin (Ziomek and Johnson, 1980).

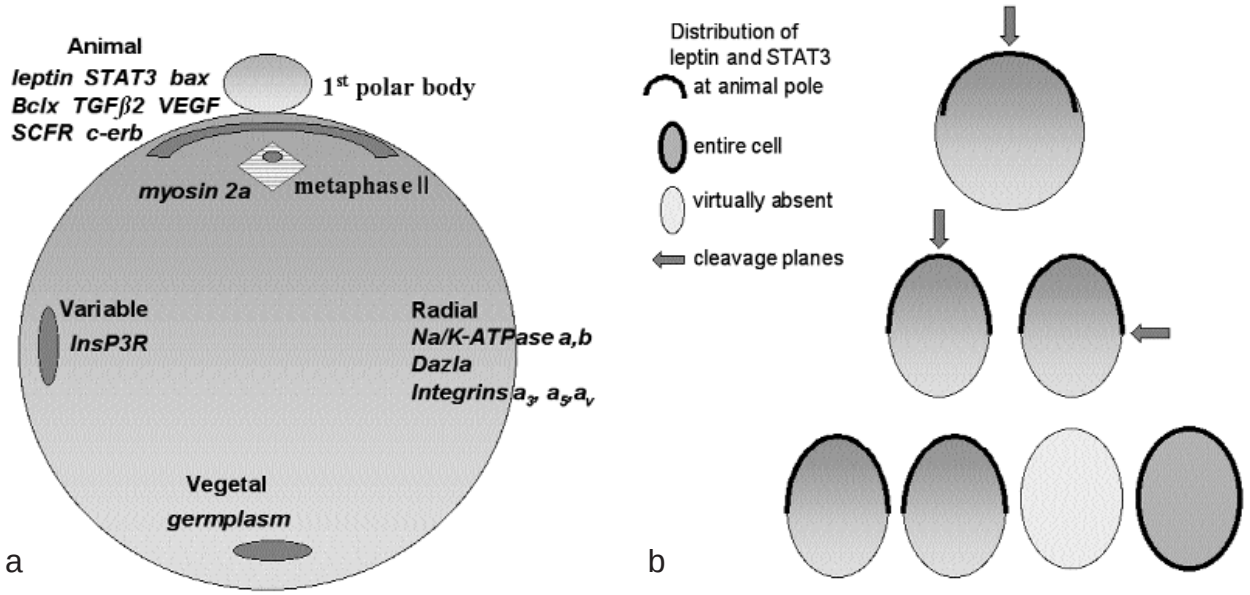


Figure 5. (**a**) Diagram showing the distribution of various genes in animal and vegetal poles of fertilized mouse and human eggs, one gene with a variable equatorial distribution and several expressed radially. (**b**) Model indicating the cortical location of leptin and STAT3 in unfertilized oocytes, fertilized eggs, and cleaving embryos. Notice how cleavage planes shown in **Figure 4** distribute these maternal proteins unequally by the 4-cell stage. Shading represents animal–vegetal gradient. Modified from Edwards (2001).

Morphologically distinct populations of inner cells (inner cell mass precursors) and outer cells (trophoblast precursors) segregate at the 16 cell stage, as compaction occurs, morulae are formed and these cell lineages now begin their own gene activity. Morulae and blastocysts have typical structures, the former arising after compaction and the latter within a day or so. The inner cell mass is located eccentrically, a hint of polarity, its embryonic/abembryonic axis being typical of earlier polarities in the embryo as revealed from the permanent attachment of a polar body in its original position in cleaving embryos and blastocysts (Gardner, 1997). Inner cell mass contains numerous stem cells already allocated to various tissues. Outer cells of morulae and blastocysts produce trophoblast, the first tissue to differentiate ready for embryo attachment to uterine epithelium at implantation.

Tightly packed gap junctions and microvilli characterize the outer surface of trophectoderm, intermittent punctate regions forming between adjacent cells in human blastocyts (Hardy, 1997). Trophectoderm divides into polar (overlying inner cells mass) and mural regions. Its growth is tightly regulated. Cells in central polar trophectoderm displace murally in expanding mouse blastocysts whereas periperhal cells move either way (Gardner, 1996). A subset of trophectoderm differentiates into giant cells expressing cyclin B1, which is inhibited in non-giant cell precursors (Palazon *et al.*, 1998).

Many early twentieth century investigators believed in axes passing transversely across the mammalian embryo, just as in lower animal forms. These have now been identified, especially that extending from the metaphase II spindle (the animal pole) to the opposite segment of oolemma (vegetal pole). Direct evidence arose in studies fixing polar bodies fixed in one position, marking the point of sperm entry, and the distribution of ooplasmic proteins in oocytes, fertilized and cleaving embryos and blastocysts (**Table 3**) (Edwards and Beard, 1997; Gardner, 1997). Polarity is apparent long before ovulation, even in oocytes with 1–2 layers of follicle cells (Antczak and van Blerkom, 1997). It is also shown by the movement of germinal vesicles to cortex in growing oocytes as maturation approaches. A leaflet of a unique myosin A attaches the second metaphase spindle to oolemma at the point of this axis, its only known function in the entire lifespan (Hewitson *et al.*, 1999).

Oocyte polarity apparently rotates after sperm entry, to accommodate it with the position of the fertilizing spermatozoon (Edwards and Beard, 1997; Payne *et al.*, 1997). Polarities then dominate chromatin structure in pronuclei, movements of nucleoli, pronuclear rotation and the axes of cleavage (**Figure 4**). Pronuclei in some eggs rotate to polarity soon after fertilization, yet in others they persist at right angles to polarity until syngamy. Several genes have been identified at specific positions at the poles, the equator or radially distributed in fertilized eggs (**Figure 5a**). Planes of successive cell divisions relate to this polar axis, distributing maternal proteins such as leptin and STAT3 to specific loci in preimplantation embryos (Antczak and van Blerkom, 1997) (**Figure 5b**). A single 4-cell blastomere with high levels of these proteins could be trophectoderm precursor, where these proteins concentrate in blastocysts. Another blastomere, virtually devoid of these proteins, could be germline precursor (Edwards and Beard, 1999). Other proteins display similar distributions as leptin and STAT3 in oocytes, described above, including bax, Bcl-x, TGF-2, VEGF, c-kit and c-erb (Antczak and Van Blerkom, 1999). In contrast, leptin receptor locates in ooplasm at fertilization, and in pronuclei (Antczak and van Blerkom, 1997) while the α1 and α3 subunits of Na/K-ATPase, and Dazla protein locate radially in cortex of mouse oocytes (Edwards, 2001). The nuclear multipotency factor oct-4 is not polarized. Embryos with high implantation potential may display characteristics typical of an early polarization after fertilization.

Table 3. Polar bodies and blastocyst symmetry (Gardner, 1997).

One polar body usually persists to blastocyst in mice.
Distributed there non-randomly, aligned with bilateral axis.
Second polar body and blastocysts are coupled ionically.
Blastocysts have bilateral not radial symmetry.
Blastocyst bilateral symmetry accords with the embryo A/V axis.
Blastocyst embryonic/abembryonic axis orthogonal to it.

Maternal and embryonic regulation

The typical multipotency of early blastomeres is modified in later cleavage stages and cells specialize to form widely competent stem cells in inner cell mass. Maternal proteins are essential regulators of early differentiation, some persisting briefly into blastocyst and later stages as they are replaced by embryonic transcripts. Their early role from the 2-cell stages is reflected in the axes typical of early cleavage divisions. In 2-cell embryos, a meridional division in one blastomere, and a transverse division in the other, distribute polarized maternal proteins unevenly into particular blastomeres of 4-cell embryos as just described (**Figure 5b**). Leptin and STAT3 thus segregate highly unevenly into 4-cell blastomeres so that one of them inherits virtually none of these proteins. Similar patterns in 8-cell stages result in two or more blastomeres being devoid, while others contain high levels of these proteins (Antcak and van Blerkom, 1997).

Minor embryonic transcription begins in 1-cell stages, to become much greater (major) in late 2-cell stages. Numerous embryologically-transcribed genes are active among all blastomeres in 4–16 stages. Gene expression during these stages is regulated by chromatin structure, DNA accessibility and feedback systems. Some genes are expressed transiently, e.g. the α subunit of F1 ATP synthase in 2-cell stages. c-DNA libraries reveal how numerous genes are transcribed, most very briefly throughout all stages from late 1-cell to blastocysts. Allied with chip technology, cDNA extracted from embryos between these stages identified thousands of potentially active genes, most persisting for a day or so and being activated in groups on the genome map on successive days (**Table 4**) (Ko *et al.*, 2000). Some embryonic genes are demethylated and imprinted, which imposes new patterns of expression. The mouse gene *U2afbp-rs*, is transcriptionally active from 2-cell stages especially in male embryos, when it is already imprinted. Many genes are silenced, imprinted or inactivated in preimplantation embryos, especially towards blastocyst stages when large amounts of the genome are affected and one X chromosome is inactivated randomly in female embryos.

Original papers should be consulted for details of gene expression in mammalian embryos. One example includes the galectins, expressed at specific blastocyst locations. *Gal-1* distributes uniformly around mouse/human blastocysts on day 3, and *Gal-1* and *Gal-3* co-express in mouse trophectoderm after hatching (Poirier and Kimber, 1997). Another involves leptin and STAT3, which down-regulate in the inner cell mass, and persist in trophectoderm and in syncytiotrophoblast. It is essential to note that they help to identify trophectodermal differentiation from earliest growth stages. They do not regulate axes and their major physiological role may arise in uterine angiogenesis, reacting with long-isoform leptin receptors in endometrium at mid-luteal phases in women (Alfer *et al.*, 2000). This isoform activates STAT transcription factor.

Table 4. Approximately 10,000 genes active from 1-cell to blastocyst (Ko *et al.*, 2000). Grouped cDNAs from 1, 2, 4, 8, 16-cell, morulae and blastocysts were prepared. Stage-specific expression (% of all RNAs).

1-cell[a]	2.5	*8-cell*	3.6
2-cell	2.9	*Morulae*	4.0
4-cell	2.6	*Blastocysts*	1.7

Approximately 800 were identified on the mouse genetic map.
[a]Fertilized eggs.

Numerous receptors expressed on trophectoderm in the newly formed, expanding and hatched blastocyst must react with ligands on uterine epithelium (Edwards and Brody, 1995). The typical polarity of trophectodermal cells, allied with a similar system in uterine epithelium, is probably an essential aspect of implantation. Polarization remains a characteristic feature of embryos after implantation and in their early growth stages (Gardner, 1997).

Mitochondria in preimplantation mammalian embryos

Mitochondria in early embryos are quiescent, and their replication may begin at day 6.5 post-fertilization in mice (Piko and Matsumoto, 1976). Mitochondrial numbers per blastomere decline during successive cleavages to 10^3 copies in blastocyst cells as in most somatic cells (Cummins, 2002). Synthesis, perhaps at gastrulation, increases numbers to 10^4 per cell in the fetus, and to 10^5 in growing oocytes in adult ovaries. This phenomenal increase after yolk sac stages far exceeds germ cell multiplication. Round or oval mitochondria with a dense matrix and few arched cristae transform in cleaving embryos to elongated forms with less dense matrix and transverse cristae (Sathananthan *et al.*, 1993; Motta *et al.*, 1997). They distribute evenly between newly forming daughter 2-cell blastomeres, becoming distributed in cytoplasm in the apical edges of dividing blastomeres, then return to perinuclear regions during intermitotic stages (Van Blerkom *et al.*, 2000).

Elongated mitochondria with numerous cristae characterize 4-cell blastomeres, and their scarcity in occasional blastomeres may cause death by preoteolysis (Van Blerkom *et al.*, 2000). They associate with microtubular arrays. Mitochondria remain apical and polarized, especially if orientated in this manner in 1- and 2-cell stages. Similar patterns persist in 8-cell stages. Pale 8-cell blastomeres contain scarcely developed mitochondria and elongated forms with numerous transverse cristae. Blastocysts are characterized by less electron-dense and elongating forms with transverse cristae. Mitochondrial patterns presumably characterize the amounts of ATP per μm^3 in individual embryos. Amounts in 8-cell embryos with good cleavage range from 7.2–9.2 amol, versus 1.1–10.4 in slow-cleaving embryos.

Endocrine and paracrine systems in preimplantation embryos

An astonishing group of endocrine and paracrine factors are active in early mammalian embryos, sources of autocrine and paracrine systems and preparing for implantation. Their complex interactions are essential in establishing embryo quality. Some systems resemble those in the hypothalamic/pituitary axis, e.g. the synthesis of transcripts of gonadotrophin releasing hormone (GnRH) and GnRH receptor in 8-cell, morulae and expanded blastocysts (Casalfi *et al.*, 1999), and for human chorionic gonadotrophin (HCG) in 2-cell stage in human embryos and then in trophectoderm and trophoblast (Juriscova *et al.*, 1999), while the hormone itself is first released from day 7–8 human embryos (Fishel *et al.*, 1984).

Embryonic cytokines include leukaemia inhibitory factor (LIF), integrins, epidermal growth factor (EGF), transforming growth factor (TGF), platelet-derived growth factor (PDGF) and insulin-like growth factor (IGF). Many display an initial maternal inheritance. LIF is produced in human trophectoderm and in endometrium during the mid- and late secretory phases, and its receptor LIF-R in inner cell mass. LIF-Rβ and gp130 are characteristically heterodimerized between maternal and embryonic proteins from 8-cell stages to provide high affinity cell signalling perhaps involved in maintaining pluripotency in inner cell mass and ES cells (Charnock-Jones *et al.*, 1994). Gene knockout showed how the maternal expression of LIF was essential for human implantation (Stewart *et al.*, 1992).

Several integrins are active during the implantation phase, some inherited maternally (Campbell *et al.*, 1995). Human oocytes express two α subunits, α_3 and α_v, and three β subunits, β_1, β_3 and β_4, with β_1 located in cortex displaying a marked polarity and still being weakly expressed in mouse blastocysts. Integrins $\alpha_5\beta_1$, $\alpha_6\beta_1$, $\alpha_5\beta_3$ are produced continuously, while $\alpha_v\beta_3$, and $\alpha_1\beta_1$ are regulated developmentally (Sutherland *et al.*, 1993).

c-kit and its ligand, Steel factor (Sl, or stem cell factor, SCF) co-express with EGF and TGFα and EGF receptor (EGFR), their common receptor in cleaving human embryos. A small variant of SCF, of 821 bp lacking exon 6, displays maternal and later embryonic expression in 2-cell human embryos and morulae (Sharkey *et al.*, 1995). Smaller and larger variant forms with additional exons characterize cleaving embryos. The relative significance of these isoforms is unknown.

EGF, TGF and their common receptor EGFR co-express in human preimplantation embryos (Chia *et al.*, 1995). Homologous with *Drosophila* Notch, EGF is involved in intracellular signalling in follicles, expressed during morula/blastocyst transition, then restricted to trophectoderm. It may have growth factor activity at 8-cell stages and promotes HCG secretion by syncytiotrophoblast (Smotrich *et al.*, 1996). The extracellular domain of EGFR, expressed weakly in 2–4 cell mouse but not human embryos, localizes in apical cell surface membranes in blastomeres. Maternal TGFα persists to late cleavage stages, promotes blastocyst expansion in mice if added to culture medium and binds to EGFR. TGFβ, a homologue of activin A and a mesodermal inducer, shifts in expression from maternal to embryonic in blastocysts and early stem cells (Rappolee *et al.*, 1988). Its isoforms, TGFβ1 β2 β3, are transcribed in 4-cell mouse embryos, inner cell mass and trophectoderm. A and B subunits of PDGF, initially maternal, form dimeric AA, AB and BB disulphide-bonded polypeptide chains which bind to receptors. Its receptors PDGFRα and PDGFRβ are expressed from 4–8 cell stages.

Maternal forms of insulin, IGF-II and their receptors persist until 2-cell stages and reappear in inner cell mass and trophectoderm (Liu *et al.*, 1997). Released into and sensitive to ionic changes in culture medium, it may provide a marker of embryo quality. Its synthesis in 8-cell human embryos is enhanced by platelet activating factor (PAF) or by culturing embryos in groups. IGF-1R is expressed moderately in human 4-cell embryos. IGF-IIR is synthesized throughout preimplantation phases and may protect against apoptosis.

Among other cytokines, TNFα is released from 2- to 4-cell mouse and human embryos, and its receptor, TNFRp60, might form in 6-cell and older human embryos. PAF might assist embryonic growth, but PAF-R is not expressed (Sharkey *et al.*, 1995). PAF may sustain IGF-II synthesis and induce calcium oscillations at 2-cell stages. Cytokines secreted in later stages include interleukins IL-6 and IL-6R. *fgf4*, a growth factor in inner cell mass, is a target gene for *Sox 2* and *oct4*.

The implantation process must be understood to appreciate the nature of embryo-uterine interactions. Brief mention can only be made here on the endometrium. Essential aspects of attachment and invasion of the embryo involve detailed interactions of markers of uterine activity including LIF, integrins, and many of the cytokines discussed above (Lessey, 2001). Some integrins date the endometrium through its various stages, but may be insufficient to provide exact markers. Mucins, uteroglobins and pinopods may also provide good indications of implantation potential, although much more knowledge is needed.

Timing and integration in developmental processes

Close timing and integration characterize morphogenetic movements in mammalian oocytes and early embryos as described earlier. Integrative systems are essential from earliest stages of oocyte formation, to regulate oocyte differentiation, polarities, microtubule activity and other aspects of cellular architecture, movement and mitochondrial organization. These essential regulators coordinate many aspects of growth, cleavage divisions and morphogenetic movements such as blastulation, and could provide markers of normal growth.

Several timing systems in embryos cannot be switched off once started. Individual cells contain several clocks, some described above, affecting various cellular processes. Clocks active in fertilized eggs and embryos continue relentlessly throughout cleavage, blastulation and possibly implantation. Clear examples are found in mammals, e.g. exact timings of successive maturational stages in oocytes resulting in the exact 37 h interval between an HCG injection and ovulation in all women treated for IVF (Steptoe and Edwards, 1970). Cytokines also regulate timing, e.g. granulocyte–macrophage colony-stimulating factor (GMCSF) promotes growth of

human embryos to blastocysts, perhaps by enhancing cell division (Sjoblom *et al.*, 1999).

While highly regular, cleavage times can be influenced by external agents, shown by the rapid cleavage of male mouse, cow and human embryos, although this concept has been challenged. The *Ped* (preimplantation embryo development) gene, located in the MHC complex in mice, influences timing of preimplantation growth. Transcribed embryonically from 2-cell stages, the protein locates to inner cell mass and trophectoderm (Warner *et al.*, 1998). Its fast-cleaving (*Qa-2+*) allele stimulates embryos to produce more inner cell mass and trophectoderm cells than those with the slow-cleaving allele (*Qa-2–*). *Ped* may be homologous with, or closely linked to human *HLA-G* (Warner *et al.*, 1998), although this gene was not identified active in cDNA libraries prepared from individual human embryos (Verlinsky *et al.*, 1998).

Circadian rhythms in reproductive cycles are well known to regulate the master clock in the hypothalamic suprachiasmatic nucleus. Similar local systems in individual cells organize their own clock. It depends on light stimuli from retina reaching the suprachiasmatic nucleus. Peripheral clocks run with a few hours delay. Feedback systems in *Drosophila* regulate the *mPer* genes, i.e. *mPer1*, *2* and *3*, and two cryptochrome genes *mCry1* and *mCry2*. Similar genetic systems in mammals include suprachiasmatic signals involving the *Per* and *Cry* genes, and immortalized embryonic fibroblasts utilize the same system if they do not carry *Cry1* and *Cry2* mutants. Master clocks tick constantly; peripheral clocks damp down after some days (Yagita *et al.*, 2001). Diurnal zygotic clocks are illustrated by the biphasic transcription of RNA polymerase I and III, so expression of this enzyme can be time related and stage-dependent (Nothias *et al.*, 1996). Perhaps intra- or intercellular conflicts in timing between individual blastomeres influences embryonic growth when blastomeres are highly independent.

Timers integrate with other developmental controls, operative through multifunctional regulatory genes throughout preimplantation stages. Present in multipotential cells throughout cleavage stages, the *Oct 4* gene, a transcriptional regulator, down-regulates in trophectoderm, continues expression in inner cell mass, but is finally restricted to primordial germ cells (Ovitt and Scholer, 1998). Its expression relates to an undifferentiated cellular phenotype, and one function is to silence *HCGβ* gene in inner cell mass (Liu and Roberts, 1996).

Classic examples of integration include the *Par* genes, well known in establishing and integrating polarities and germline in *C. elegans* oocytes. Several interact when axes and cell fate are determined. Six or more distinct oocyte proteins distribute unequally among successive blastomeres to help impose their distinct embryonic fates (Guo and Kemphues, 1996). *par-1* and *par-4* are primary organizers of protein and transcript localization in cytoplasm; *par-2, 3, 5* influence spindle orientation. *Par*-1 characterizes their actions, encoding a serine-threonine protein kinase to interact with a protein kinase (PKC) and Par-2 at restricted sites over particular time periods. Similar systems exist in *Drosophila* and mammals, but are known only in outline (Edwards, 2001).

Telomeres locate on terminal regions of homologues chromosomes, and may measure longevity. They are expressed in immortal cell lines and many tumours. Their length is maintained by telomerase, and shortening is characteristic of ageing cells. Composed of repeated DNA sequences, telomerase replenishes them by adding to DNA synthesized during the cell cycle. This enzyme reaches high levels in fetal, newborn and adult gonads, but not in oocytes or spermatozoa (Wright *et al.*, 1996). Resynthesized in early embryos, high levels are attained in blastocysts but then decline in differentiating tissues (Eisenhauer *et al.*, 1997). Telomeres may stabilize chromosome ends to prevent potential recombinations. Shortened telomeres in Dolly the sheep indicated premature ageing as compared with normal sheep of the same age.

Part 2. The human embryo in the clinic

Introduction

Human embryos have typically low implantation rates whether grown *in vivo* or *in vitro*. Overall clinical pregnancy rates of <30% with two or more replaced embryos decline further with increasing maternal age, certain aetiologies of infertility and with various clinical parameters. Establishing singleton pregnancies has proved to be an elusive target, compromised by the need to replace several embryos to attain sufficiently high pregnancy rates. Improved implantation rates will only be achieved by selecting for transfer those embryos with high implantation potential. Selection will depend on current knowledge on fundamentals of embryonic growth, yet if successful it should enable single transfers of selected embryos between 1–8 cells or blastocysts. Either approach could transform the infertility treatment and the diagnosis of genetic diseases in preimplantation stages. Transferring cleaving embryos avoids prolonged culture periods and the death *in vitro* of many cleaving embryos capable of implantation unless left too long in culture. Transferring blastocysts demands all the skills of the IVF clinic but those that survive have passed a stringent test of quality. It is essential that users of either approach relate numbers of pregnancies to numbers of transferred and started embryos, the only verifiable parameters of success.

General principles of embryo culture and transfer

Laboratory conditions

The IVF laboratory must provide a non-toxic, pathogen free and stable environment, with suitable equipment and techniques. Suboptimal culture conditions can be expressed as embryonic death *in vitro*, or loss of viability after implantation. Environmental pollutants must be monitored to avoid the contamination of media.

The importance of good culture methods, and highly suitable media cannot be overstressed. Acidity is maintained around 7.4, usually with bicarbonate buffer under 5% CO_2 in air, a physiologically based system of low toxicity. CO_2 in liquid phases must be equilibrated with gas phases, often achieved by placing media in CO_2 incubators before use. Some laboratories

use 5% O_2, 5% CO_2 and 90% N_2. HEPES buffer maintains pH stability in the bicarbonate buffer system when working outside the CO_2 incubator, e.g. during oocyte recovery.

Osmotic pressure of ~285 mOsm/kg must be maintained stringently, and needs close care since it can shift with time, temperature and atmospheric humidity. Evaporation is a serious problem, demanding constant air humidity in incubators. Open culture systems include small plastic tubes containing 1 ml of medium, now largely replaced by wells in plastic dishes. One closed system utilizes microdroplets of medium under paraffin or mineral oil. It protects oocytes and embryos from contamination, and from swings in pH, temperature and gas phases. This overlayer also counteracts evaporation, provides a physical barrier to infections and separate droplets permit rapid and easy examination of single embryos *in vitro*. Paraffin oil must be constantly equilibrated with CO_2, with steps to maintain it when working outside the incubator.

Embryo transfer to the mother is the final stage of culture. Several transfer catheters each offer different benefits. Good transfer technique is crucial to IVF success, since otherwise implantation is jeopardized, to literally destroy all that went on before. Nurses have replaced doctors for transfers in some clinics, with similar success. Catheter technology, a significant element in embryo replacement, was assessed by Edwards and Brody (1995).

Media

Media used for oocyte retrieval, oocyte recovery and embryo culture must meet minimal nutritional requirements, provide stable pH through effecting buffering, and have an appropriate osmolarity. Quality control must ensure they are endotoxin free, especially with media prepared in house. Pre-tested commercial media are suitable, the majority having similar quality standards as media made within the clinic.

Many media are based on well-known physiological salt solutions, such as Krebs, Waymouth's, and Earle's, fortified with human albumin, serum or its synthetic substitutes. Animal proteins must be excluded. More complex media include Ham's F10 and T6, widely used in IVF although less so recently. Ménézo's B2 medium contains amino acids, vitamins, nucleic acid precursors and saline, but includes bovine serum albumin. HTF is a balanced salt solution based on human tubal fluid, free of amino acids, with a higher K^+ content than other media and an ionic environment similar to oviduct (Quinn *et al.*, 1985).

Sequential media offer changing requirements for embryos in different developmental stages. They have been used since IVF was introduced 30 years ago when a physiological saline, e.g. Earle's or Krebs' fortified by 8% v/v of serum or human albumin, sustained fertilization, while complex media, e.g. Ham's F10 fortified with higher serum or albumin levels and sometimes with glutamine, sustained after pronucleate stages. A recent, widely used system began as three distinct media designed for successive growth stages. The first, with little glucose, sustained for fertilization. The second contained lactate, and nonessential amino acids to culture cleaving embryos. Amino acids promote the development of human blastocysts *in vitro* (Devreker *et al.*, 2001). Pyruvate was included and glucose was omitted, being considered toxic for embryos aged days 1 to 3. Care was needed with amino acids, which might spontaneously break down to ammonium at 37ºC, and harm embryo growth. The third medium for growth to blastocysts contained glucose, essential and non-essential amino acids, vitamins and nucleic acids precursors among other components (Gardner and Lane, 1997). These were the forerunners of G1 and G2 media, widely used today, and of other less-used sequential media. Merits of sequential media and harmful effects of glucose have been questioned in favour of one medium, KSOM, over the 5-day period (Biggers, 2000).

Longer-term cultures using sequential media, e.g. G1 and G2, produce high-quality blastocysts, so few are transferred and so multiple pregnancies are rare (Gardner and Schoolcraft, 1998; Gardner *et al.*, 2000). Only one-half of embryos reach blastocysts, others dying between days 3 and 5, which is not very different to media available in the 1980s (Fishel *et al.*, 1985).

Embryo selection and transfer on days 2–3 is also widely practised. Fortunately, in humans if not in animals, the uterine environment tolerates early embryos and transfer procedures. Three or even more embryos have been transferred, risking high multiple pregnancies. Most clinics abhor low pregnancy rates, often associated with few replaced embryos, so models of embryo quality became paramount. These were devised to select the best single embryos for transfer and so avoid multiple births (**Table 5**) (Steer *et al.*, 1992; Staessen *et al.*, 1993). Today, embryos are selected between 1–8 cell stages for key characteristics, such as early polarization, timing of cleavage divisions, the degree of blastomere fragmentation and good morphology, hopefully several times daily. Earlier selection equates with early transfer, shorter culture periods and the use of cryopreservation to store remaining high-quality embryos.

Co-culture

Co-culture has been widely popular in some clinics. Feeder cell monolayers may remove heavy metal divalent cations and metabolic inhibitors, reduce high oxygen levels, and provide small embryotrophic metabolites and growth factors. However, exact mechanisms are not understood. Initially applied 1965, then in the 1990s, cellular feeder layers combined with sequential media were used for longer-term cultures to blastocysts. Today, IVF clinics utilize Vero cells (kidney epithelial cells from African monkeys), oviductal epithelial cells of different species (Ménézo *et al.*, 1986;

Table 5. Cumulative embryo score predicting optimal numbers of embryos to replace (Steer *et al.*, 1992).

Initial classification
Grade 1: Total fragmentation or embryos still pronucleate.
Grade 2: Serious blastomere fragmentation of 10–50%.
Grade 3: Uneven blastomeres with <10% fragmentation.
Grade 4: Equal-sized symmetrical blastomeres.
Multiply grade by numbers of blastomeres to produce a quality score.

Bongso *et al.*, 1989), bovine fetal uterine fibroblasts (Wiemer *et al.*, 1994), autologous granulosa cells (Plachot *et al.*, 1993) and homologous endometrial cells. Beneficial effects are neither species-specific nor specific to the female reproductive tract, which raised concern about their value (Edwards and Brody, 1995). Cell lines are grown under standard cell culture methods with specific modifications. Vero cells, commercially available in frozen cryotubes, are checked for bacteria, viruses and other possible contaminants.

Co-cultures were credited with sustaining more embryo cells and better inner cell masses (Ménézo *et al.*, 1998). Initially, co-cultured human embryos produced 61% blastocyst versus 3% in controls. Such low rates in controls implied defects in culture methods. Co-cultures with Vero cells led to slightly higher pregnancy rates than in controls (23% co-culture versus 16% in controls), as clinical trials yielded 57% blastocysts and 44% pregnancies among 62 patients given a mean of 1.65 blastocysts (Ménézo *et al.*, 1992). Wide variations between co-cultures and controls were reported (Plachot *et al.*, 1993; Wiemer *et al.*, 1994), and some patients failed to reach transfer. Claimed benefits included reduced blastomere fragmentation and more pregnancies. Other investigators demurred. A prospective randomized study using Vero cells produced no significant advantages for growth to blastocysts (Van Blerkom, 1993), nor did benefits accrue in numbers of pregnancies for first-time IVF patients. Many co-culture studies were not controlled statistically, or involved comparisons with previous transfers in the same patient. Some implantation rates in controls were desperately low even though patients were subfertile.

Co-cultures offer a restricted future, and are now largely replaced by sequential media. Some experience with co-cultures has nevertheless been positive for patients with previous failed transfers or advanced age. Our own work (Boiso and Vega, unpublished) produced two-thirds of embryos reaching blastocysts. Pregnancy rates were not high at 12.5% pregnancies per transfer, but many of the patients suffered from a low pregnancy potential.

Cryopreservation

Many human embryos are cryopreserved after those selected for high quality have been replaced. Many thousands now lie in store. Slow-freezing, slow-thawing systems using glycerol, dimethyl sulphoxide, propanediol and others have been used over many years (Edwards and Brody, 1995). In some clinics, pregnancy rates with thawed embryos were only just below those gained using fresh embryos. Some clinics produced very poor results, a risk to be considered when assessing the value of cryopreservation.

Slow methods are now being replaced with faster methods such as vitrification or combinations of high concentrations of several cryopreservative agents (Rall and Fahy, 1985). Overall, several investigators produced results equivalent to those gained with well-established slow forms of cryopreservation. Improvements in oocyte quality are claimed with better straw technology, and human births have been reported using vitrified embryos (Kuleshova *et al.*, 1999). This better approach to cryopreservation cannot be long delayed.

Evaluation of oocytes and 1-cell embryos

Oocyte quality is essential in determining embryo viability. It is measured non-invasively in oocytes first polar bodies, and zonae pellucidae by scoring visible characteristics during IVF and ICSI procedures. Properties of granulosa cells may also be helpful.

The freshly collected oocyte

Follicular fluids and oocytes arriving in the laboratory should be quickly scanned and examined for maturity such as an expanded cumulus mass, coronal cells surrounding the oocyte and its nuclear status. A visible polar body indicates the oocyte is in metaphase II. Granulosa cells form large viscous masses surrounding oocytes. One classification of oocytes according to their maturity is germinal vesicle stage (prophase I), metaphase I, metaphase II, very mature or post-mature metaphase II, and atretic or degenerative (Veeck, 1999).

Morphology

Cumulus and coronal cells obscure oocytes during conventional IVF. More information is gained during micromanipulative techniques such as ICSI, which require their complete removal. Nuclear maturation and cytoplasmic morphology can then be assessed and handling facilitated. Morphological defects in oocytes include an enlarged perivitelline space, cytoplasmic granularity and inclusions. Variations are not uncommon in oocyte morphology granularity, such as areas of necrosis, organelle clustering, vacuoles, clustering of smooth endoplasmic reticulum, anomalies in the first polar body and zona pellucida, and non-spherical shape of the oocytes (**Figure 6**), and have been linked with aneuploidy, abnormal fertilization, poor embryo quality, and low pregnancy and implantation rates (reviewed by Alikani *et al.* 1995). Results are controversial. Aneuploidy in oocytes and embryos may be linked to several dysmorphic forms arising before metaphase I, perhaps up to one-half of cases (Van Blerkom and Henry, 1992). Embryos originating from dysmorphic oocytes suffered higher rates of pregnancy loss (Alikani *et al.*, 1995). Similar conclusions were drawn in other studies, but without effects on fertilization rates and embryo quality. Persistent cytoplasmic granulation in oocytes of patients during repeated treatment cycles did not affect fertilization and pregnancy rates, except for large, deep granular zones which were linked with aneuploidy and abortion (Kahraman *et al.*, 2000).

First polar bodies have received much attention, with similar disagreements about their predictive value. Positive examples include low fertilization rates and embryo quality when it is fragmented and the perivitelline space is enlarged (Xia, 1997). Fragmented or huge polar bodies may also signify disordered fertilization rates. Many negative reports have been published.

More controlled trials are needed to confirm all these findings on oocytes. Many parameters are subjective. Attention should be given to the aspiration of atretic or endometriotic follicles, which may produce oocytes with many of the various dysmorphisms.

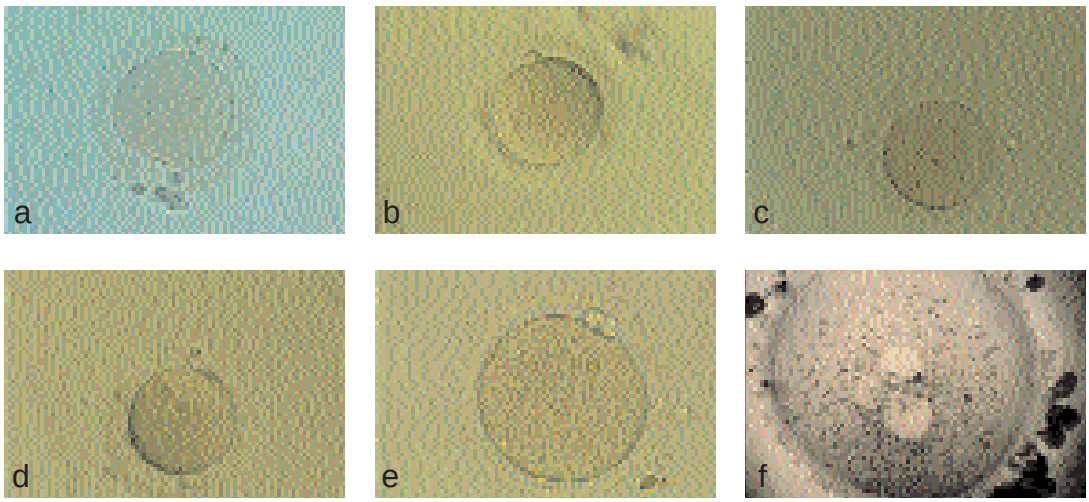


Figure 6. Anomalies in human oocytes. (**a**) Non-spherical shape. (**b**) Cytoplasmic granularity. (**c**) Cytoplasmic inclusions. (**d**) Vacuolated oocyte. (**e**) Fragmented polar body. (**f**) Clustering of organelles in the perinuclear region in a fertilized egg with three pronuclei; note that all three pronuclei are apposed (image supplied courtesy of Dr Montse Boada).

Normal and abnormal pronuclei

Cytoplasm in 1-cell embryos can be scored visibly alterations for the presence of 'haloes' around cortical regions or surrounding pronuclei (**Figure 7**). These may be invoked during mitochondrial aggregation in perinuclear regions, and could be associated with good quality embryos.

Two pronuclei found at 16–18 h post-insemination is usually taken as evidence of fertilization. They should enlarge, and their membranes should be polarized at membranal sites of pronuclear apposition (Payne *et al.*, 1997). Pronuclei differ in their form of growth, paternal pronuclei being larger until close to syngamy. Pronuclear diameters differing by at least 4 microns are associated with more developmental arrest than smaller differences.

Unusual numbers of pronuclei may form after fertilization, i.e. none, one, three or more. If neither pronuclei nor one large polar body is observed, the meiotic spindle might have passed outside the egg as occurs in patients with recurrent hydatidiform mole (Edwards and Brody, 1995). The presence of two polar bodies only does not necessarily indicate fertilization failure, since pronuclear formation may have failed after sperm entry. This condition arises after experimental treatments on oocytes, e.g. heavy X-irradiation, but is very rare.

Some eggs possess a single pronucleus. It can have a varied origin. It may be due to parthenogenetic activation, perhaps the cause in 25–30% of cases (Staessen *et al.*, 1993), and more common after ICSI (Munné and Cohen, 1998). Uniform haploid embryos can also arise and proceed normally through a few cleavages. Delayed formation of one pronucleus, or the fusion of two pronuclei, are alternative causes (Nagy *et al.*, 1994). Meiotic errors in chromosomal segregation could have the same consequence. Sometimes, two pronuclei seem to fuse, to produce one pronucleus. If correctly identified, these embryos can probably be transferred safely, perhaps after scoring their growth to blastocysts.

More than two pronuclei imply polyspermic penetration or retention of the second polar in the oocyte. Fertilization involving two spermatozoa (dispermy) produces eggs with three pronuclei and two polar bodies. This frequent anomaly in human eggs can result in the formation of two sperm asters and tripolar spindles at syngamy, leading to complex segregational anomalies (**Figure 8**). The worst cases presumably arrest after irregular cleavage and fragmentation. Others develop as chromosome mosaics, and a few as pure triploids (Munné and Cohen, 1998). While eggs with three or more pronuclei are usually not transferred, it is still possible to excise the extra male pronucleus and restore diploidy to the embryo.

Digyny arises when the second polar body is retained in the egg. This results in three pronuclei, two being maternal. This condition (monospermic digyny) affects 4% or fewer fertilized eggs. Most develop as uniform triploids, indicating that syngamy involved three pronuclei with a single bipolar spindle.

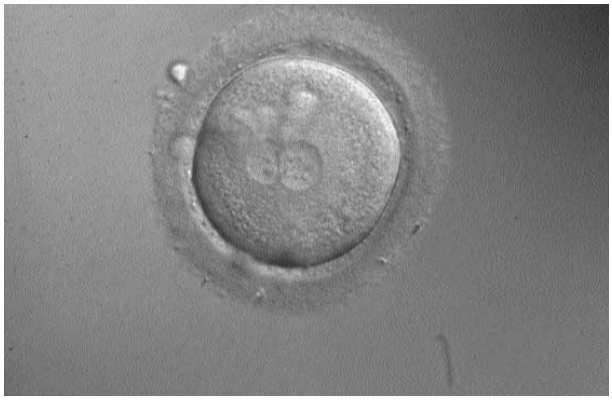


Figure 7. Presence of a local halo in ooplasm of a human oocyte. Notice the pronuclei are conjoined and nucleoli in one pronucleus are apposing.

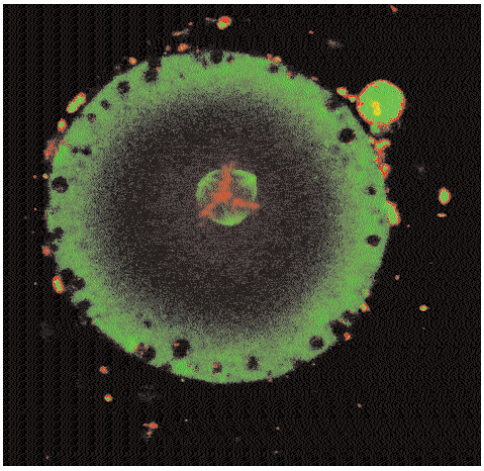


Figure 8. Formation of a tripolar spindle in a pronucleate human egg that had earlier contained three pronuclei. Notice the three sets of chromosomes, stained red, and the three distinct poles of the spindle apparatus.

IVF and ICSI

Benefits of ICSI and IVF have been widely explored. ICSI is clearly far superior for severe forms of male infertility, but more IVF embryos develop to blastocysts (Schröder *et al.*, 2001). Perhaps IVF is more 'natural', with sperm entry stimulating normal responses of oocyte activation with its major consequences such as induced Ca^{2+} oscillations, cortical granule discharge, decondensation of the sperm head and natural forms of ooplasmic rotation. The delayed dissolution of the acrosomal vesicle in monkey oocytes after ICSI traps areas of DNA in early forms of decondensation (Hewitson *et al.*, 1999). Incomplete sperm injection, oolemmal breakage, apparent incomplete oocyte activation and rapid formation of pronuclei are included among other consequences of ICSI, although effects on embryonic growth do not seem to be significant.

Curiosity about ICSI, and the existence of polarities in mammalian eggs, has led to analyses on embryo quality when spermatozoa are placed at specific points in oocytes (reviewed by Ludwig 2001). Despite many reports, there is no overall agreement about an optimal site. The significance of placing spermatozoa at widely differing sites may be nullified through the rotation of ooplasm to accommodate to sperm entry from any site.

Polarities and selection in 1-cell stages

Polarity characteristics in human oocytes and embryos offer some of the best chances of deciding embryo quality. They are measured using various parameters. These include pronuclear rotation at intervals after sperm entry, pronuclear apposition and chromatin coarsening, nucleolar growth and polarization in pronuclei, and an early first cleavage division.

In this context, Garello *et al.* (1999) measured angles between the pronuclear axis and the axis of pronuclei with polar bodies, to calculate the degree of rotation. Wider angles were associated with low embryo quality, and a low angular variation with higher implantation rates. Care is needed with this form of analysis because first polar bodies can be displaced from their original position.

Nuclear chromatin also displays polarity characteristics, coarsening at points of pronuclear apposition. This parameter has not been used to score embryos. Nucleolar migration and alignment, and pronuclear apposition, are clear markers of growth, and best achieved before human embryos are cryopreserved (Wright *et al.*, 1990). These data were among the earliest on polarities. Structure, size and position of nucleoli were employed by Tesarik *et al.* (2000) to classify fertilized human eggs, measuring discordance between the two pronuclei, well scattered nucleoli, and small and large nucleoli polarized adjacent to pronuclear apposition (**Figure 3**). Chosen embryos were selected again later from their day 3 morphology. Implantation rates reached 50% with one embryo having optimal patterns, this estimate being revised downwards later but still above 30% (**Tables 6, 7**) (Tesarik *et al.*, 2000). Greater precision might be gained if eggs are scored several times daily. Detailed trials are needed to confirm these conclusions.

A scoring system based on pronuclear size and the alignment and number, size and distribution of nucleoli within the pronuclei produced four embryo categories (**Table 6**) (Scott *et al.*, 2000). High quality embryos were identified (**Table 7**), scores on nucleoli being more effective than those on morphology. Implantation rates of 31% agree closely with 30% found by Tesarik *et al.* (2000). They challenge the higher rates obtained with blastocysts, descibed below, which are obtained after many embryos capable of implantation die between days 3 and 5.

The cleaving embryo

Embryo selection based on multinucleated and fragmented blastomeres

Most embryos have successive and timely blastomeres of approximately even size during early cleavages. Multiple nuclei (MNB) in blastomeres are not infrequent in human embryos (**Figure 9**). They are important signals of reduced embryo viability, used widely, and among the best parameters for scoring embryo quality. Multinucleation and mosaicism in embryos are more frequent with dysmorphic pronuclei (Sadowy *et al.*, 1998).

Two-thirds of multinucleated blastomeres involve defective cleavage in a mononucleated parent cell, such as flawed chromosome migration, nuclear fragmentation, failure to divide, and/or errors in cell packaging (Pickering *et al.*, 1995). Multinucleation in early cleavage stages, but not at 8-cell and later, compromises implantation and diploidy (Hardy, 1997; Alikani *et al.*, 2000; Sandalinas *et al.*, 2001). Disordered chromosome separation at anaphase and telophase results in mosaic embryos (Kligman *et al.*, 1996), although progeny of multinucleated blastomeres are not always abnormal (Staessen *et al.*, 1998). This characteristic is usually included with others when constructing models of embryo quality.

Blastomere fragmentation is very frequent, and may be among the most significant defect in cleaving embryos whether innate

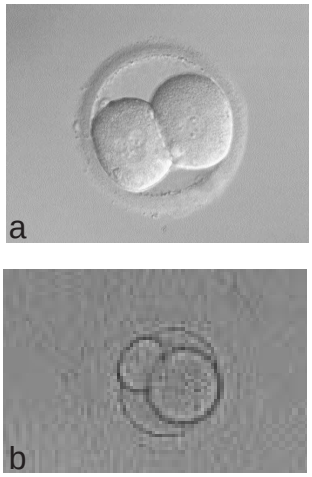


Figure 9. (**a**) Presence of three multiple nuclei in one blastomere of a human 2-cell embryo. (**b**) Uneven-sized blastomeres in a 4-cell human embryo.

or due to external factors. It has been studied over many years (Plachot and Mandelbaum, 1990). Classifications are expressed as the percentage of embryo volume occupied by fragments. Spindle errors may result in lagging or broken chromosomes, which are expelled into fragments. Sophisticated analyses have largely replaced the earlier habit of simply classifying embryos as free of fragments or otherwise (Alikani *et al.*, 1999). Loss of polarized proteins into fragments could affect blastomeres unequally and impair developmental potential in embryos, particularly 1–2 cell stages (Antczak and Van Blerkom, 1999). The percentage of fragmentation is positively correlated with mosaicism, but not to aneuploidy and fragmentation (Munné and Cohen, 1998). Fragments can be excised surgically, using micromanipulation, to improve embryo quality.

Such sophisticated analyses now classify fragments into five groups according to size and distribution (**Table 8**; **Figure 10**) (Alikani *et al.*, 1999). This model assesses the degree and pattern of fragmentation on day 3 and reliably predicts implantation rates. Type IV is serious, even in minor quantities, shown in transfers consisting uniquely of these embryos (Alikani *et al.*, 1999). Exceptions occur, e.g. the 5% of embryos which implant despite fragments sized between one-tenth and one-half of embryo volume at day 2. Overall, blastocyst formation is impaired with 15% fragmentation (Alikani *et al.*, 2000). Not all patterns are detrimental, except for loss of large cytoplasmic volumes.

Table 6. Numbers, size and polarization of human nucleoli in pronucleate eggs (Scott *et al.*, 2000).

Z-1: equal numbers and size of nucleoli (between 3 and 7 per nucleus) aligned at pronuclear junction.
Z-2: equal numbers of nucleoli not yet aligned at pronuclear junction.
Z-3: unequal sized and numbers of nucleoli, unequal alignment at pronuclear junction.
Z-4: grossly abnormal embryos, different sized nuclei, nuclei not aligned in central ooplasm, small misplaced nuclei.

Examples of data gained with transfers including fragmented embryos are given in **Table 9**. This parameter is often included with others in more complex models, as shown below. Scoring and selecting embryos can be done without difficulty, which adds to its wide use as a method for scoring human embryos in so many clinics.

Embryo selection based on timing and polarities

Knowledge on timing and polarity in human oocytes and eggs has produced models designed to measure the ability of individual human embryos to achieve implantation (Edwards and Beard, 1999). This widely used model for scoring embryo quality is often combined in complex models. Embryos with early first and second cleavages have implantation rates well above 30% per embryo (Edwards *et al.*, 1984) and many form blastocysts (Sakkas *et al.*, 1998). Several groups have included this marker as a major parameter helping to identify high-quality embryos (e.g. Scott *et al.*, 2000). Selecting single embryos with early cleavage and other characteristics might establish pregnancy rates surpassing 30%, compared with 20% if only one embryo was available for transfer (Vilska *et al.*, 1999).

Early cleavage is also measured by counting number of blastomeres at specific times after insemination or ICSI. This method is also in wide use in numerous models.

Table 7. Results with selection for polarized nucleoli and morphology.

A. Data of Tesarik *et al.* (2000). Embryos were selected for nucleoli and good morphology.

	Polarized	*Non-polarized*
Transfers	98	77
No. embryos transferred	189	161
Mean no. transferred	1.9	2.1
Pregnancies (%)	44 (45)	17 (22)
Embryo sacs	57	18
Implantation rate	30	11

B. Data of Scott *et al.* (2000). Results in relation to type of classification and day of transfer.

	Nucleoli		*Plus morphology*	
	Day 3	***Day 5***	***Day 3***	***Day 5***
No. transfers	94	117	134	87
Clinical pregnancies (%)	57	73	33	58
Mean no. transferred embryos	2.8	1.9	3.3	2.2
Implantation rate (%)	31	52	19	39

Growth to day 5 blastocysts according to classification: Z-1 = 50%; Z-2 = 25%; Z-3 = 15%; Z-4 = 15%.

Measuring multiple criteria in embryos

It is now time to combine several parameters of quality in 1-cell and cleaving embryos to select embryos of very high implantation potential. This process began when models predicting the relative importance of embryo and uterus were proposed 20 years ago.

One model involved an embryo score, based on blastomere numbers and fragmentation to achieve a cumulative embryo score, and moderate scores gave modest implantation rates and few multiple pregnancies (**Table 5**; **Figure 11**) (Steer *et al.*, 1992). Weighting coefficients were then applied to alignments of pronuclei and nucleoli, presence of an ooplasmic halo, early first cleavage and good morphology, all being summed to score individual embryos (Scott *et al.*, 2000). Embryos with high scores implanted in far greater numbers than the others, more than 90% of embryos that implanted having high scores. High embryo scores were also associated with steadily rising oestrogens during follicular phases, implying that high embryo quality is a consequence of good follicle physiology.

'Strict embryo criteria' were formulated and applied to day 2 or day 3 cleavage stage embryos to select single embryos with high implantation potential (Gerris *et al.*, 1999; Van Royen *et al.*, 1999). The 'top' quality embryo was characterized examining retrospectively all 23 double transfers resulting in ongoing twins. Top-quality embryos possessed no multinucleated blastomeres, four or five blastomeres on day 2 and seven or more on day 3, with <20% anucleate fragments. Initial studies utilized this data to begin a policy of elective single embryo transfers (SET) in a prospective randomized trial comparing single and double embryo transfers. Single transfers of selected embryos had implantation rates of 42.3% with ongoing pregnancy rates of 38.5%. Double elected transfers produced similar implantation rates (48%) and ongoing pregnancy rates of 74.1% (Gerris *et al.*, 1999). Extension of this trial involved group cultures of embryos with two clear pronuclei on day 1, scoring each one for multiple nuclei. Blastomere numbers and fragments were counted on day 2. A final selection on day 3 chose embryos which had 4–5 blastomeres on day 2, and 7 or more on day 3, combined with <20% fragments and total lack of multinucleated blastomeres on days 2 and 3. Implantation rates with single selected embryos reached 50%, with ongoing pregnancies of 33% (**Table 9**) (Gerris *et al.*, 2001). Multiple pregnancies were virtually absent in the group of 118 pregnant patients after single embryo transfers.

Table 8. Model for classifying blastomere fragmentation (Alikani *et al.*, 1999).

Type I: minimal volume, fragments associated with one blastomere.
Type II: fragments localized and predominantly in perivitelline space.
Type III: small scattered fragments, between blastomeres, peripheral or both.
Type IV: large fragments, resembling whole blastomeres, distributed randomly, associated with uneven blastomeres.
Type V: necrotic fragments, characteristic granularity and cytoplasmic contraction.
Other types are grouped as having no distinct pattern.

Implantation rates for Types I–IV declined from 37.9 to 18.2%. Selective hatching was applied to all transferred embryos.

The blastocyst

Embryo quality and culture in defined medium

Blastocyst transfer depends on advanced technologies and considerable skill in laboratories. It may represent the strictest form of embryo selection. The first clinical pregnancy arose from a transferred blastocyst, and initial analyses of embryo growth *in vitro* showed how 50% of human embryos developed to blastocyst stages in fortified Ham's F10 (Fishel *et al*., 1985). Transfers of cryopreserved blastocysts were only moderately successful, but in this study the best-quality embryos had previously been used for transfer (Fehilly *et al.* 1985; Hartshorne *et al.*, 1991). Disappointing results using a single medium to culture blastocysts led to the introduction of co-cultures, producing 50% blastocyst formation and implantation rates of 25% with many multiple pregnancies (Ménézo *et al.*, 1998). Blastocyst transfer was also utilized for patients with repeated implantation failures (Veiga *et al.*, 1995, 1999a).

Sequential media were then applied in detail to blastocyst culture. It gave similar rates of success as co-culture (Ménézo

Table 9. Elective single embryo transfers (SET) on day 3 (Gerris *et al.*, 2001).

All ages	*SET/TOP*	*SET/nonTOP*	*All non-SET*	*Total*
No. of transfers	207	56	810	1073
No. of conceptions (%)	102 (49)	16 (29)	399 (49)	517 (48)
Biochemical pregnancy	18	4		
Clinical miscarriages	10	1		
Ongoing pregnancies (%)	71 (34)	11 (20)	279 (34)	361 (34)
Singleton pregnancies	70	11	174	255
Ectopic pregnancy	3	0		
Multiple pregnancies	1	0	105	106

SET/TOP: single top-quality embryo transfer.
SET/nonTOP: single non-top-quality embryo transfer.

Table 10. Results with blastocyst transfers (Gardner *et al.*, 1998; Schoolcraft and Gardner, 2000).

A. Transfers on day 3 versus day 5.

	Day 3	Day 5
Stage	7.9 cells[a]	Blastocysts[b]
Mean no. fertilized eggs	10.9	11.6
Mean no. transferred	3.7	2.2
Mean no. cryopreserved	3.0	3.2
Implantation rate (%)	37.0	55.4
Pregnancy rate (%)	66	71

[a]Grown in Ham's F10, assisted hatching.
[b]Grown in sequential media.

B. Number of blastocyst transferred on day 5.

Stage	Blastocysts	Blastocysts
Mean no. fertilized eggs	10.9	11.6
Mean no. transferred	2	3
Implantation rate (%)	52	49
Pregnancy rate (%)	68	87

C. Pregnancies according number of good quality blastocysts on day 5.

No. good quality blastocysts	2	1	0
Patients	64	21	15
Implantation rate	70	50	28
Pregnancy rate	87	70	44
Twins	61	50	29

All values in percentages.

et al., 1998). High implantation rates using sequential media arose with selected groups of patients responding well to ovarian stimulation, with sufficient high-quality embryos on day 3 or with previous IVF cycles or pregnancies (**Table 10**) (Gardner *et al.*, 2000; Schoolcraft and Gardner, 2000). Comparisons between day 3 embryos grown in fortified Ham's F10 (implantation rates of 37%) and those grown in sequential media (55%) did not seem to be very different, considering the degree of embryonic death between days 3 and 5 is 33% (Sandalinas *et al.*, 2001). Implantation rates of highly selected embryos reached 70%, and multiple pregnancies were reduced. Prolonged culture has not helped patients in their first IVF cycle. Randomized studies are needed to establish the use of blastocyst transfers in relation to types of patients who would benefit.

Timing of blastocyst formation is significant. Expanded blastocysts frozen on day 5 or 6 provided almost 40% of pregnancies when thawed, versus 6.2% of those frozen on day 7. The best blastocysts are those formed on day 5 (Shoukir *et al.*, 1998).

Scoring blastocyst morphologies

Wide variation in morphology arises in long-term blastocyst cultures. Blastocyst quality was measured in terms of inner cell mass size, areas of necrosis and other factors (Fehilly *et al.*, 1985; Hartshorne *et al.*, 1991). Compactness and volume

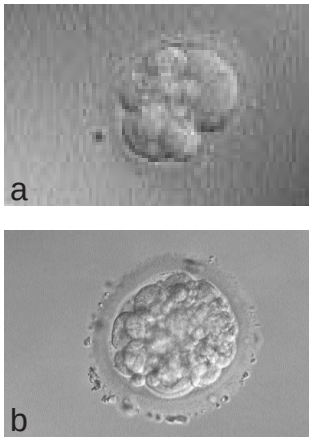


Figure 10. Two examples of fragmentation in a human embryo. (**a**) Fragments in a cleaving embryo; note the fragments are different sizes. (**b**) A totally fragmented embryo.

of inner cell mass was also used to evaluate blastocyst quality (Ménézo *et al.*, 1998). Variations are found with compaction and blastulation, optimal values involving compaction by day 4, cavitation on days 5–7 and hatching on days 7–8. Optimal mean cell numbers in blastocysts are 50–100 on day 5 and 100–200 on day 6 (**Figure 11**).

Blastocyst scoring by a numerical score has been based on blastocoel expansion, development of inner cell mass, development of trophectoderm and state of the zona pellucida (**Table 11**) (Gardner *et al.*, 2000). A novel scoring system classified embryos with numbers, and inner cell mass and trophectoderm by letters.

A top-scoring blastocyst is thereby classified ≥3AA. Applying this score pregnancy outcome gave high implantation rates with two high scoring blastocysts (**Table 10**). If only one high scoring blastocyst was available, and a low-scorer was added, transfers produced lower implantation rates. Low-scoring blastocysts gave low implantation rates (Gardner *et al.*, 2000).

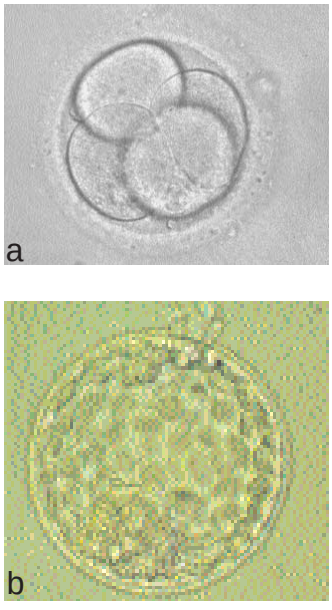


Figure 11. Top-quality human embryos. (**a**) 4-cell. (**b**) blastocyst.

Table 11. Model for grading of human blastocysts *in vitro* (Gardner and Schoolcraft, 1999; Gardner *et al.*, 2000).

Blastocyst
Grade 1: early blastocyst, blastocoel less than half embryo volume.
Grade 2: blastocoel size half or more of embryo volume.
Grade 3: full blastocyst, blastocoel completely fills embryo volume.
Grade 4: expanded blastocyst, blastocoel volume larger than in early embryo, thinning zona.
Grade 5: hatching, trophectoderm herniating through zona.
Grade 6: hatched blastocyst completely escaped from zona.
Inner cell mass scoring for embryos graded 3–6
Grade A: tightly packed, many cells.
Grade B: loosely grouped, several cells.
Grade C: very few cells.
Trophectoderm scoring for embryos graded 3–6
Grade A: many cells forming a cohesive epithelium.
Grade B: few cells forming a loose epithelium.
Grade C: very few, large cells.

Other approaches to improving embryo quality

Surgery on embryos

New research often brings new ideas to alleviate infertility. Some are perhaps too complicated for large-scale benefits. IVF is complex enough for most couples, and invasive methods interfering with embryos make further large demands on resources. They may be welcome, even essential, for specific cases or conditions. The recent use of somatic cells in their G1 phase of the cell cycle is a case in point; it may bring unexpected benefits to some families where both parents lack gametes. So far, it has encountered similar difficulties to cloning, in that embryos develop very poorly. Nevertheless, clinical methods of establishing fertilization (if that is the correct term) are now available (Lacham-Kaplan *et al.*, 2001; Tesarik *et al.*, 2001).

Zona drilling has also had a somewhat chequered history. It offers a clear basis for research, since hatching might be a problem for some blastocysts. Some earlier studies recommended it for older mothers, or those embryos with a poor prognosis of implantation (Cohen *et al.*, 1992). Moves to non-contact infrared laser systems simplified the process, but prospective controlled studies have been rare. One has now been reported, utilizing two 1.48 IR based systems, focusing the laser beam through microscopic optics to the plane of the specimen (Malter *et al.*, 2001). Despite technical difficulties, and a weaker growth in some treated embryos using one system, clinical pregnancy rates of 42–49% were obtained with three replaced embryos, itself not an improvement on standard acid drilling.

Other visible characteristics typical of polarity may also be helpful in identifying high-quality embryos including mitochondrial clouds, cortical granules, Balbiani's body, microgranules, an opaque rim of ooplasm, thickness of the zona pellucida and perhaps even germ plasma. Other characteristics may be revealed using more invasive methods, e.g. video recordings every few hours or staining or using special microscopical methods to check for the distribution of gene products.

Mitochondrial and ooplasmic transfers

Mitochondrial mutations in individual oocytes may indicate reproductive senescence, and impair transcription and regulation of mtDNA in early embryos. Mitochondrial transfer used among different primates revealed congruences between mitochondrial and nuclear genes, indicating that care will be needed about mitochondrial transfer techniques (Nagao *et al.*, 1998).

Accumulating mtDNA rearrangements might reduce oxidative phosphorylation, especially if age-related mtDNA rearrangements accumulate in ovarian oocytes. In one study, the frequency of point mutation at T414G located in the mtDNA control region increased from 4.4% to 39.5% in oocytes of older women, i.e. from one oocyte among 11 patients aged 26–36 compared with 17 among ten women aged 37–42 years (Barritt *et al.*, 2000). Donating ooplasm from young to older oocytes may improve cytoplasmic and mitochondrial function in recipient embryos, and a human child has been born carrying donor mitochondria (Barritt *et al.*, 2001).

Preimplantation genetic diagnosis and embryo quality

Recent developments in preimplantation genetic diagnosis (PGD) have led to human embryos being classified for their chromosomal content. It is clear that enormous numbers of preimplantation embryos suffer from various chromosomal disorders, some arising through malsegregation, and others through cellular and embryological systems (**Table 12**) (Márquez *et al.*, 2000). Detailed knowledge now being gained through fluorescence in-situ hybridization (FISH) and other methods has provided information on numerous forms of chromosomal disorder.

PGD enables many of these errors to be identified, using several chromosomes to score embryos. The major disadvantage of this approach is the need to remove one or two blastomeres from embryos for diagnosis, which must underestimate values obtained if all blastocysts could be assessed, and might compromise embryonic growth. Early developmental blocks characterize embryos with chromosomal abnormalities, illustrated by data from PGD revealing more abnormal cells in arrested embryos (54%) as compared with those reaching blastocysts (17.1%) (Veiga *et al.*, 1999b). Nor does extended culture modify the proportion of blastocysts carrying numerical chromosomal abnormalities (Sandalinas *et al.*, 2001).

Combining PGD with chromosomal analysis now helps to clarify exact survival rates in embryos with varying chromosomal inheritance (**Table 12**). Some forms of damage

Table 12. Frequency of chromosomal anomalies in human embryos *in vitro*, and the proportions deveoping to specific preimplantation stages Márquez *et al.*, 2000; Sandalinas *et al.*, 2001).

A. Chromosome analyses on 363 cleaving embryos (Márquez *et al.*, 2000).

Condition	*Maternal age (years)*			
	20–34	35–39	40–47	All
Aneuploid for XY, 13, 16, 18, 21	13.5	13.9	25.5	15.9
Mosaic, haploid, polyploid	32.6	32.6	37.3	33.9
Total abnormal	43.3	44.7	53.3	46.3

B. Proportions of selected groups of embryos arresting at various developmental stages until blastocyst (Sandalinas *et al.*, 2001).

	Number	*Arrest*		*Live blastocysts (%)*
		Day 3	***Day 4***	
Normal				
Uniform	9		3	
Mosaic 2n/4n	23	3	2	18 (78)
Total		9	2	21 (66)
Aneuploids				
Monosomies	23	14	7	2 (9)
Trisomies	35	15	7	13 (37)
Diploid mosaics				
2N/4N	9	3	3	3 (33)
2N/chaotic	36	36	8	6 (12)
Polyploids	14	11	0	3 (21)
Haploids	5	4	1	0

are compatible over several cleavages, but on the other hand, embryos classified as normal succumb in large numbers before reaching blastocysts. Even so, improved possibilities of pregnancy are emerging. A study in women older than 40 years involved 23 patients in whom 17 cycles with one chromosomally normal embryo was transferred. Six established ongoing pregnancies (35%), although some patients had no normal embryos (Obasaju *et al.*, 2001). Pregnancies arose from single normal embryo transfers in six others.

Conclusions

Numerous regulatory systems control embryonic development. Some have been well studied, including polarities, transcription, metabolism and mitochondria. Others are still under-analysed, e.g. timing, integration, and cell cycle systems. Benefits could accrue from simple systems, such as avoiding apoptosis, measuring HLA antigens, and paying much more attention to timing systems. Several clocks exist in single cells, and the possibilities for disturbing embryonic growth would seem to be considerable. It is even possible that errors known in cytokinesis or fragmentation involve timing errors causing conflicts within and between specific blastomeres. Other emerging characteristics of human embryos seem to be far more difficult to utilize simply. Analysing gene systems, histones or maternal proteins seems to be far too difficult. There is no need in a sense to analyse zona proteins any further from a clinical standpoint in view of the effectiveness of ICSI.

Selecting quality in human embryos is also leading IVF into new social issues. If relatively few embryos can implant, then many patients may not have any high-quality embryos for transfer. This has already arisen in some clinics (Gerris *et al.*, 2001). Perhaps a model will be devised where one first class, two second class embryos, and more are transferred with progressive declines in embryo quality. It certainly seems ethically wooden to pass laws restricting transfers to one embryo in fertile women, and two if the patient is older or subfertile. Several embryos may have to be transferred, even in younger women, if all their embryos have a poor prognosis for implantation.

Finally, all major efforts at improving IVF success have been aimed at producing more follicles or better media for growing oocytes and embryos. Much debate concerns the advantages of transferring embryos on day 3 or day 5 (**Table 13**). Yet it seems that most human oocytes are already of low quality before they were even aspirated for IVF. Knowledge on polarities places new approaches in a different framework. Axes are almost certainly formed in newly forming oocytes in mammals, as with other organisms. This almost certainly occurs in the fetal ovary, and be inaccessible to study, or in resting primordial human follicles or those about to leave the pool. If the latter is the case, they might just be available for analysis on polarities,

Table 13. Advantages of day 3 versus day 5 selection.

Day 3	*Day 5*
Easier culture	Longer culture
Embryos	
More for transfer 30–35% implantation rate More cryopreserved	Fewer for transfer 50% implantation rate Fewer cryopreserved
Pregnancies	
More? Replace 1–2 45–50% pregnancy rate	Fewer? Replace 1–2 50–65% pregnancy rate

gene expression and other factors. Such approaches may be essential since modern gonadotrophins, for all their expense, merely forestall apoptosis in fully-grown oocytes and have little effect on oocyte quality. Different media or manipulations on embryos try to improve embryos whose quality was lost months earlier. Similar questions might arise with spermatogenesis, where aberrant axes may cause the desperately low sperm quality typical of most men.

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