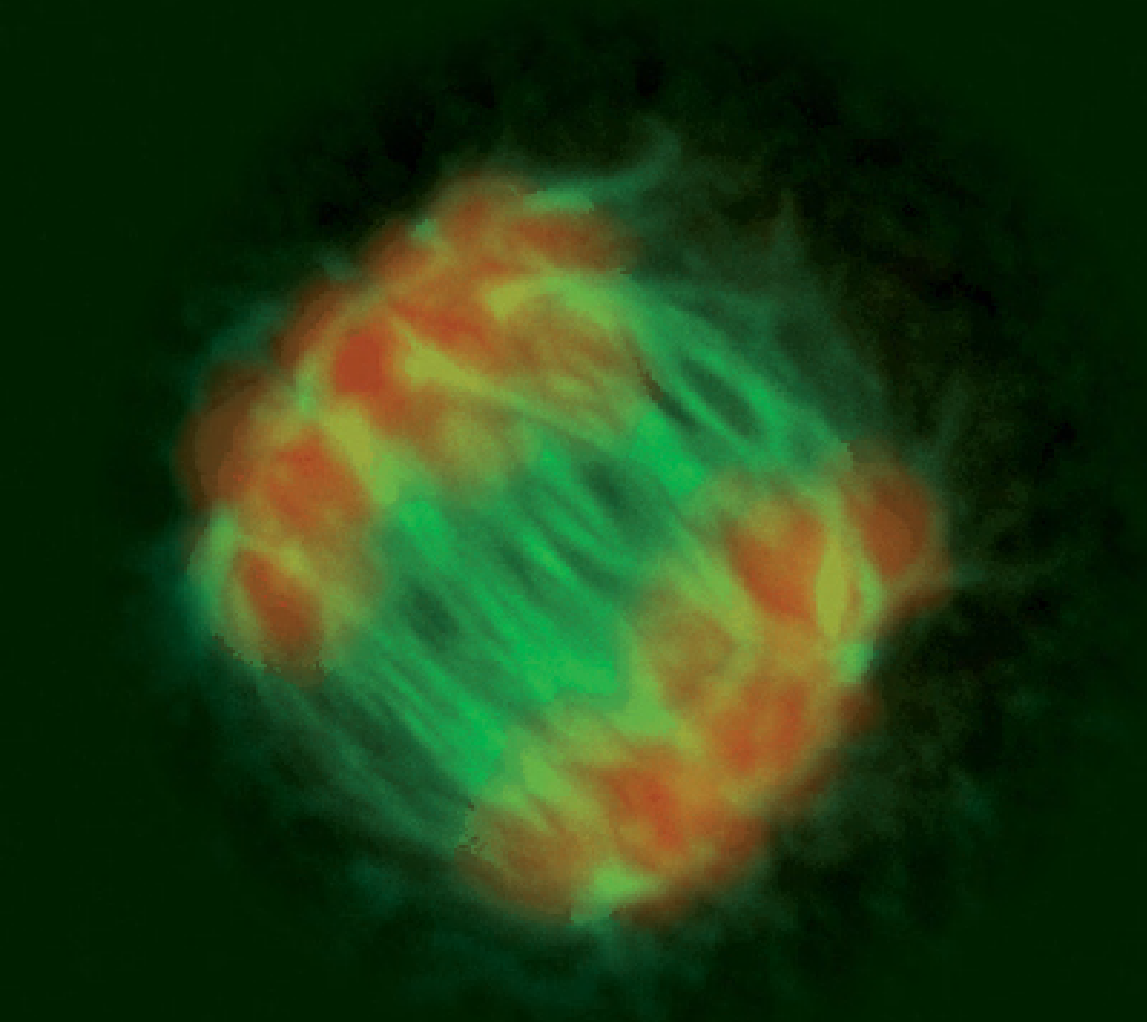
In-Vitro Fertilization

Kay Elder and Brian Dale

THIRD EDITION



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Preface

The union of male and female gametes during the process of fertilization marks the creation of a completely new individual, a unique event that ensures genetic immortality by transferring information from one generation to the next. It also creates variation, which introduces the effects of evolutionary forces. During the first half of the nineteenth century, fertilization and the creation of early embryos was studied in a variety of marine, amphibian and mammalian species, and by the early 1960s had been successfully achieved in rabbits (Chang, 1959), the golden hamster (Yanagimachi and Chang, 1964) and mice (Whittingham, 1968). Following a decade of extensive research in mouse, rat and rabbit reproductive biology and genetics, Robert Edwards began to study in-vitro maturation of human oocytes in the early 1960s (Edwards, 1965). On February 15, 1969, the journal *Nature* published a paper authored by R.G. Edwards, B.D. Bavister and P.C. Steptoe: "Early stages of fertilization in vitro of human oocytes matured in vitro" (Edwards *et al.*, 1969). The paper scandalized the international community – reporters and camera crews from all around the world fought to gain entry to the Physiological Laboratory in Cambridge, where Edwards and his team were based. It drew fierce criticism from Nobel Laureates and much of the scientific, medical and religious establishment of the UK and elsewhere, being regarded as tampering with the beginning of a human life: religious, ethical and moral implications were numerous. IVF is now accepted completely as a clinical procedure; in the quest for improvements via new technology we should not be disheartened or surprised by irrational criticism, but draw courage from the pioneering work of Bob Edwards and his colleagues, whose brave perseverance opened up an entirely new field of interdisciplinary study, embracing science, medicine, ethics, the law and social anthropology.

Half a century later, the creation of new life via human IVF continues to attract debate and discussion, prompting many governments to define "the beginning of a human life" in formulating legislation surrounding assisted reproductive technologies (ART). Not surprisingly, these definitions vary from country to country and often reflect the theological beliefs of the nations involved. Scientifically, a number of basic facts regarding fertilization and embryo development must be considered in defining the "Beginning of Life". Both in vivo and in vitro, gametes and preimplantation embryos are produced in great excess, with only a tiny proportion surviving to implant and produce offspring; human gametes are certainly error-prone, and the majority are never destined to begin a new life. Some female gametes may undergo fertilization, but subsequently fail to support further development due to deficiencies in the process of oogenesis. Once gametes are selected, their successful interaction is probably one of the most difficult steps on the way to the formation of a new life. At this stage the two genomes have not yet mixed, and numerous developmental errors can still occur, with failures in oocyte activation, sperm decondensation, or in the patterns of signals that are necessary for the transition to early stages of embryo development. A fertilized ovum is a totipotent cell that initially divides into a few cells that are equally totipotent, but for a brief period of time these cells can give rise to **one** (a normal pregnancy), **none** (a blighted ovum or anembryonic vesicular mole), or even **several** (monozygotic twinning) individuals. Although fertilization is necessary for the life of a being, it is not the only critical event, as preimplantation embryo development can be interrupted at any stage by lethal processes or simple mistakes in the developmental program. A series of elegantly programmed events begins at gametogenesis and continues through to parturition, involving a myriad of synchronized interdependent mechanisms, choreographed such that each must function at the right time during embryogenesis. Combinations of both physiological and chromosomal factors result in a continuous reduction, or "selection" of conception products throughout the stages that lead to the

potential implantation of an embryo in the uterus. Preimplantation embryogenesis might be described as a type of Darwinian filter where only the fittest embryos survive, and the survival of these is initially determined during gametogenesis.

It may be argued that the task of elaborating and defining the concept of a "new individual" belongs to philosophers and moralists. For some, the beginning of human life coincides with the formation of a diploid body in which the male and female chromosomes are brought together. For others, true human life only occurs after implantation of the embryo in the uterine mucosa. Many believe that a new individual is formed only after differentiation of the neural tube, whilst others believe that life begins when a fetus can live outside the uterus. In its most extreme form, some philosophers consider the acquisition of self-awareness of the newborn to define a new life. Most scientists would probably agree that life is a continuous cyclical process, with the gametes merely bridging the gap between adult stages. Science, one of the bases of human intellect and curiosity, is generally impartial and often embraces international and religious boundaries; ethicists, philosophers and theologians cannot proceed without taking into account the new information and realities that are continuously generated in the fields of biology and embryology. Advances in the expanding range and sensitivity of molecular biology techniques, in particular genomics, epigenomics and proteomics continue to further our understanding of reproductive biology, at the same time adding further levels of complexity to this remarkable process of creating a new life.

In the decade since the previous edition of this book was published, the field of human IVF has undergone significant transformation in many different ways. Further scientific knowledge gained from use of sophisticated technology is one of them; management of patients and treatment cycles has also been influenced by commercial pressures as well as legislative issues. The rapid expansion in both numbers of cycles and range of treatments offered has introduced a need for more rigorous control and discipline in the IVF laboratory routine, and it is especially important that IVF laboratory personnel have a good basic understanding of the science that underpins our attempts to create the potential beginning of a new life.

IVF is practiced in most countries of the world, and the number of babies born is estimated to be in the order of at least 10 million; a vast and comprehensive collection of published literature covers clinical and scientific procedures and protocols, as well as information gained from modern molecular biology techniques. Many books are now available that cover every chapter (and in some cases individual paragraphs) of this edition. Unlike 10 years ago, a wide range and variety of media, equipment and supplies is available specifically for use in human IVF, each with its own instructions and protocols for use. IVF is successfully carried out with numerous adaptations in individual labs, and specific detailed protocols are no longer appropriate. Our aim in preparing this third edition was to try to distill large bodies of information relevant to human IVF into a comprehensive background of physiological, biochemical and physical principles that provide the scientific foundation for well-established protocols in current use.

This book is dedicated to Bob Edwards, who embraces and inspires all who are blessed with the experience of knowing him … we salute and honor his infinite vision and endless optimism:

> *There wasn't any limit, no boundary at all to the future … and it would be so that a man wouldn't have room to store such happiness….*
>
> *(James Dickey, American poet and novelist, 1923–1997)*

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Chapter 1

Review of cell and molecular biology

Gametogenesis, embryo development, implantation and in-vitro culture involve numerous complex pathways and interactions at the cellular and molecular level; a true understanding of their significance requires secure fundamental knowledge of the underlying principles. This chapter therefore provides a condensed overview and review of basic terminology and definitions, with particular emphasis on aspects relevant to reproductive biology and in-vitro fertilization.

Mammalian cell biology

In 1839, two German scientists, Matthias Jakob Schleiden and Theodor Schwann, introduced the "cell theory," the proposal that all higher organisms are made up of a single fundamental unit as a building block. In 1855, Rudolf Virchow extended this cell theory with a suggestion that was highly controversial at the time: "Omnis cellulae e celula" (all living cells arise from pre-existing cells). This statement has become known as the "biogenic law." The cell theory is now accepted to include a number of principles:

1. All known living things are made up of cells.
2. The cell is the structural and functional unit of all living things.
3. All cells come from pre-existing cells by division (spontaneous generation does not occur).
4. Cells contain hereditary information that is transmitted from cell to cell during cell division.
5. The chemical composition of all cells is basically the same.
6. The energy flow (metabolism and biochemistry) of life occurs within cells.

Although these features are common to all cells, the expression and repression of genes dictates individual variation, resulting in a large number of different types of variegated but highly organized cells, with

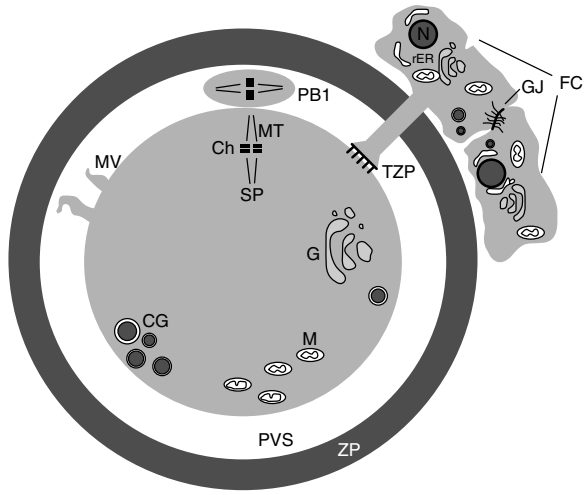

Figure 1.1 Schematic diagram of oocyte ultrastructure showing the zona pellucida (ZP) and the perivitelline space (PVS), first polar body (PB1), microvilli (MV), rough endoplasmic reticulum (rER), chromosomes (Ch) on the spindle (SP), Golgi complex (G), cortical granules (CG), two follicle cells (FC) attached to the oocyte and to each other via gap junctions (GJ). TZP = transzonal process, MT = Microtubules, M= Mitochondria.

convoluted intracellular structures and interconnected elements. The average size of a somatic cell is around 20 μm; the oocyte is the largest cell in the body, with a diameter of approximately 120 μm in its final stages of growth (Figure 1.1). The basic elements and organelles in an individual cell vary in distribution and number according to the cell type. Bacterial cells differ from mammalian cells in that they have no distinct nucleus, mitochondria or endoplasmic reticulum. Their cell membrane has numerous attachments, and their ribosomes are scattered throughout the cytoplasm.

Cell **membranes** are made up of a bimolecular layer of polar lipids, coated on both sides with protein films. Some proteins are buried in the matrix, others float independently of each other in or on the membrane surface,

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forming a fluid mosaic of different functional units that are highly selective and specialized in different cells. Cells contain many different types of membrane, and each one encloses a space that defines an organelle, or a part of an organelle. The function of each organelle is determined largely by the types of protein in the membranes and the contents of the enclosed space. Membranes are important in the control of selective permeability, active and passive transport of ions and nutrients, contractile properties of the cell, and recognition of/association with other cells.

Cellular membranes always arise from pre-existing membranes, and the process of assembling new membranes is carried out by the endoplasmic reticulum (ER, see below). The synthesis and metabolism of fatty acids and cholesterol is important in membrane composition, and fatty acid oxidation (e.g., by the action of reactive oxygen species, ROS) can cause the membranes to lose their fluidity, as well as have an effect on transport mechanisms.

Microvilli are extensions of the plasma membrane that increase the cell surface area; they are abundant in cells with a highly absorptive capacity, such as the brush border of the intestinal lumen. Microvilli are present on the surface of oocytes, zygotes and early cleavage stage embryos in many species, and in some species (but not humans) their distribution is thought to be important in determining the site of sperm entry.

Cell **cytoplasm** is a fluid space, containing water, enzymes, nutrients and macromolecules; the cytoplasm is permeated by the cell's architectural support, the cytoskeleton.

Microtubules are hollow polymer tubes made up of alpha-beta dimers of the protein tubulin. They are part of the cytoskeletal structure, and are involved in intracellular transport, for example, the movement of mitochondria. Specialized structures such as centrioles, basal bodies, cilia and flagella are made up of microtubules. During prophase of mitosis or meiosis, microtubules form the **spindle** for chromosome attachment and movement.

Microfilaments are threads of actin protein, usually found in bundles just beneath the cell surface; they play a role in cell motility, and in endo- and exocytosis.

Centrioles are a pair of hollow tubes at right angles to each other, just outside the nucleus. These structures organize the nuclear spindle in preparation for the separation of chromatids during nuclear division. When the cell is about to divide, one of the centrioles migrates to the other side of the nucleus so that one lies at each end. The microtubule fibers in the spindle are contractile, and they pull the chromosomes apart during cell division.

The **nucleus** of each cell is surrounded by a layered membrane, with a thickness of 7.5 nm. The outer layer of this membrane is connected to the ER, and the outer and inner layers are connected by "press studs," creating pores in the nuclear membrane that allow the passage of ions, RNA and other macromolecules between the nucleus and the cell cytoplasm. These pores have an active role in the regulation of DNA synthesis, since they control the passage of DNA precursors and thus allow only a single duplication of the pre-existing DNA during each cell cycle. The inner surface of the membrane has nuclear lamina, a regular network of three proteins that separate the membrane from peripheral chromatin. DNA is distributed throughout the nucleoplasm wound around spherical clusters of histones to form nucleosomes, which are strung along the DNA like beads. These are then further aggregated into the chromatin fibers of approximately 30 nm diameter. The nucleosomes are supercoiled within the fibers in a cylindrical or solenoidal structure to form chromatin, and the nuclear lamina provide anchoring points for chromosomes during interphase (Figure 1.2):

- Active chromatin = euchromatin – less condensed
- Inactive (turned off) = heterochromatin – more condensed
- Before and during cell division, chromatin becomes organized into chromosomes.

Three types of cell lose their nuclei as part of normal differentiation, and their nuclear contents are broken down and recycled:

- Red blood cells (RBCs)
- Squamous epithelial cells
- Platelets.

Other cells may be multinuclear: syncytia in muscle and giant cells (macrophages), syncytiotrophoblast.

Nuclear RNA is concentrated in **nucleoli**, which form dense, spherical particles within the nucleoplasm (Figure 1.3); these are the sites where ribosome subunits, ribosomal RNA and transfer RNA are manufactured. RNA polymerase I rapidly transcribes the genes for ribosomal RNA from large loops of DNA, and the product is packed in situ with ribosomal proteins to generate new ribosomes (RNP: ribonucleoprotein particles).

Mitochondria are the site of aerobic respiration. Each cell contains 40–1000 mitochondria, and they are

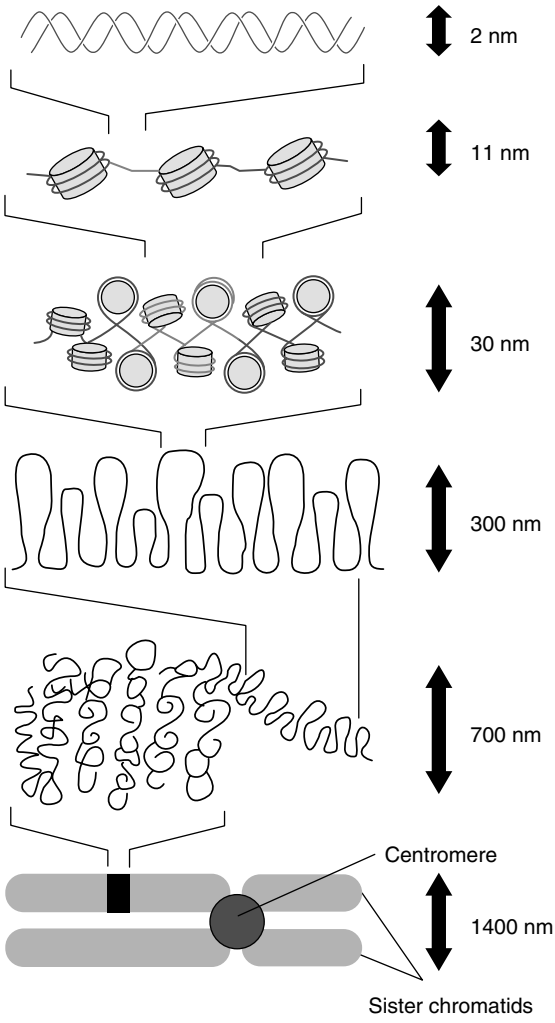

Figure 1.2 Levels of chromatin packaging. From the top: DNA double helix, nucleosome "beads on a string," chromatin fiber of packed nucleosomes, section of extended chromosome, condensed chromosome and finally the entire chromosome.

most abundant in cells that are physically and metabolically active. They are elliptical, 0.5–1 μm in size, with a smooth outer membrane, an intermembranous space, and a highly organized inner membrane which forms cristae (crests) with elementary particles attached to them, "F1-F0 lollipops," which act as molecular dynamos. The cristae are packed with proteins, some in large complexes: the more active the tissue, the more cristae in the mitochondria. Cristae are the site of intracellular energy production and transduction, via the Krebs (TCA) cycle, as well as processes of oxidation, dehydrogenation, fatty acid oxidation, peroxidation, electron transport chains and oxidative phosphorylation. They

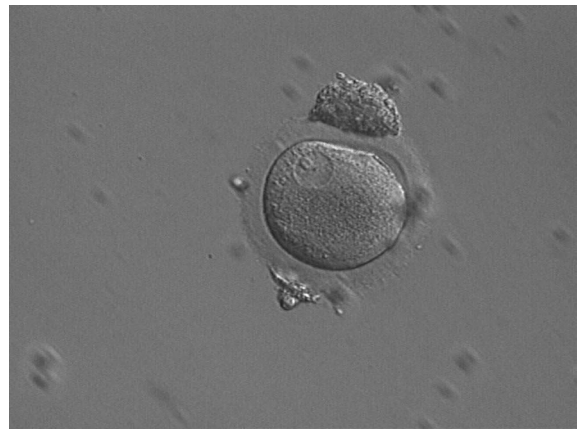

Figure 1.3 Human oocyte at germinal vesicle stage, showing prominent nucleolus.

also act as a Ca^{2+} store, and are important in calcium regulation. Mitochondria contain their own double-stranded DNA that can replicate independently of the cell, but the information for their assembly is coded for by nuclear genes that direct the synthesis of mitochondrial constituents in the cytoplasm. These are transported into the mitochondria for integration into its structures.

A number of rare diseases are caused by mutations in mitochondrial DNA, and the tissues primarily affected are those that most rely on respiration, i.e., the brain and nervous system, muscles, kidneys and the liver. All the mitochondria in the developing human embryo come from the oocyte, and therefore all mitochondrial diseases are maternally inherited, transmitted exclusively from mother to child. In the sperm, mitochondria are located in the midpiece, providing the metabolic energy required for motility; there are no mitochondria in the sperm head.

- Oocytes contain 100 000–1 000 000 mitochondria.
- Sperm contain 70–100 mitochondria, in the midpiece of each sperm. These are incorporated into the oocyte cytoplasm, but do not contribute to the zygote mitochondrial population – they are eliminated at the four- to eight-cell stage.
- All of the mitochondria of an individual are descendants of the mitochondria of the zygote, which contains mainly oocyte mitochondria.

The human mitochondrial genome

The sequence of human mitochondrial DNA was published by Fred Sanger in 1981, who shared the 1980 Nobel Prize in Chemistry with Paul Berg and Walter

Gilbert, "for their contributions concerning the determination of base sequences in nucleic acids." The mitochondrial genome has:

- Small double-stranded circular DNA molecule (mtDNA) 16 568 bp in length
- 37 genes that code for:
  2 ribosomal RNAs
  22–23 tRNAs
  10–13 proteins associated with the inner mitochondrial membrane, involved in energy production
- Other mitochondrial proteins are encoded by nuclear DNA and specifically transported to the mitochondria.
- Mitochondrial DNA is much less tightly packed and protected than nuclear DNA, and is therefore more susceptible to ROS damage that can cause mutations.
- As it is inherited only through the maternal line, mutations can be clearly followed through generations and are used as "markers" in forensic science and archaeology, as well as in tracking different human populations and ethnic groups.

Mitochondria can be seen in different distributions during early development (Figure 1.4); they do not begin to replicate until the blastocyst stage, and therefore an adequate store of active mitochondria in the mature oocyte is a prerequisite for early development.

- Germinal vesicle oocyte: homogeneous clusters associated with endoplasmic reticulum (ER)
- Metaphase I oocyte: polarized towards the spindle
- Metaphase II oocyte: perinuclear ring and polar body
- Embryos at 1c, 2c, 4c stages: perinuclear ring
- Cytoplasmic fragments in cleavage stage embryos contain large amounts of active mitochondria

The **endoplasmic reticulum (ER)** is an interconnected lipoprotein membrane network of tubules, vesicles and flattened sacs that extends from the nuclear membrane outwards to the plasma membrane, held together by the cytoskeleton. The ER itself is a membrane-enclosed organelle that carries out complex biosynthetic processes, producing proteins, lipids and polysaccharides. As new lipids and proteins are made, they are inserted into the existing ER membrane and the space enclosed by it. **Smooth ER (sER)** is involved in metabolic processes, including synthesis and metabolism of lipids, steroids and carbohydrates, as well as regulation of calcium levels. The surface of **rough ER (rER)** is studded with ribosomes, the units of protein synthesis machinery. Membrane-bound vesicles shuttle proteins between the rER and the Golgi apparatus, another part of the membrane system. The **Golgi apparatus** is important in modifying, sorting and packaging macromolecules for secretion from the cell; it is also involved in transporting lipids around the cell, and in making lysosomes.

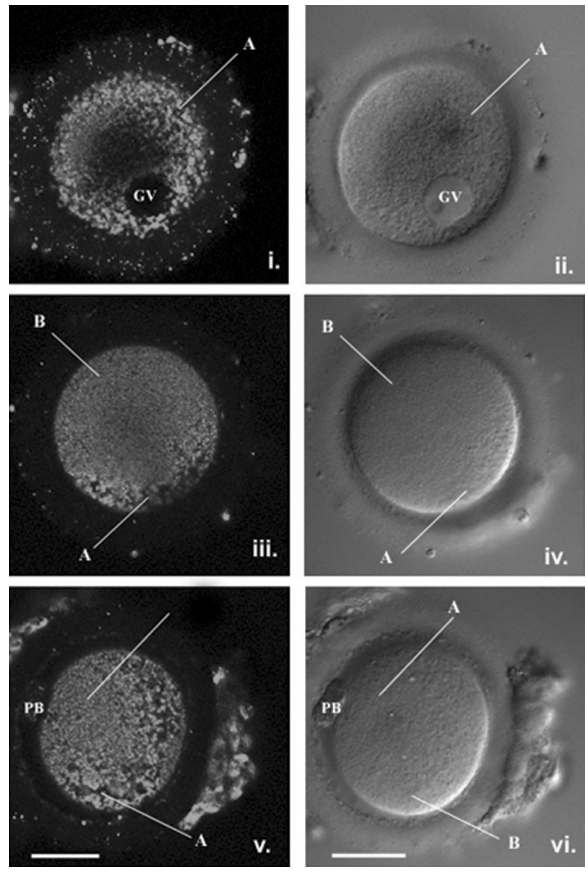

Figure 1.4 Mitochondrial aggregation patterns in a germinal vesicle (GV) oocyte (top), a metaphase I oocyte (center) and a metaphase II oocyte (bottom). Frames to the left are in fluorescence using the potential sensitive dye JC-1 to show the mitochondria, frames on the right are transmitted light images. The two mitochondrial patterns: A (granular-clumped) and B (smooth) are shown. PB = polar body. Scale bars = 50 µm. (With permission from Wilding *et al.*, 2001, *Human Reproduction* 16, pp. 909–917.) See color plate section.

rER

- Has attached 80 s ribonucleoprotein particles, the ribosomes (bacterial ribosomes are 70 s), which

are made in the nucleus and then travel out to the cytoplasm through nuclear pores.
- Ribosomes are composed of two subunits: 40 s and 60 s (bacteria: 30 s and 50 s); the association between the subunits is controlled by Mg^{2+} concentration.
- Polysomes = several ribosomes which move along a single strand of mRNA creating several copies of the same protein.

sER

- A series of flattened sacs and sheets, site of lipid and steroid synthesis.
- Cells that make large amounts of steroids have extensive sER.

The **Golgi apparatus** was first observed by Camillo Golgi in 1898, using a novel silver staining technique to observe cellular structures under the light microscope; he was awarded the 1906 Nobel Prize in Physiology or Medicine for his studies on the structure of the nervous system. The Golgi apparatus consists of a fine, compact network of tubules near the cell nucleus, a collection of closely associated compartments with stacked arrays of smooth sacs and variable numbers of cisternae, vesicles or vacuoles. It is connected to rER, linked to vacuoles that can develop into secretory granules, which contain and store the proteins produced by the rER. All of the proteins exported from the ER are funneled through the Golgi apparatus, and every protein passes in a strict sequence through each of the compartments (cis, tubules, trans). This process consists of three stages:

1. "Misdirected mail" – sends back misdirected proteins (cis).
2. "Addressing" – stacks of cisternae that modify lipid and sugar moieties, giving them "tags" for subsequent sorting.
3. "Sorting and delivering" (trans): proteins and lipids are identified, sorted and sent to their proper destination.

Transport occurs via vesicles, which bud from one compartment and fuse with the next. The Golgi apparatus will move to different parts of the cell according to the ongoing metabolic processes at the time – it is very well developed in secretory cells (e.g., in the pancreas).

The Golgi apparatus also makes **lysosomes**, which contain hydrolytic enzymes that digest worn-out organelles and foreign particles, acting as "rubbish bins" and providing a recycling apparatus for intracellular digestion; they contain at least 50 different enzymes, and "leaky" lysosomes can cause damage and kill cells. Macromolecules inside the cell are transported to lysosomes, those from outside the cell reach them by pinocytosis or phagocytosis; phagocytosis only occurs in specialized cells (e.g., white blood cells).

Peroxisomes are microbody vesicles that contain oxidative enzymes such as catalase; they dispose of toxic hydrogen peroxide, and are important in cell aging.

Metabolism in the mammalian cell

Four basic factors influence the metabolic activity of a cell:

1. Spatial: compartmentation, permeability, transport, interactions.
2. Temporal: products become substrates, positive and negative feedback.
3. Intensity/concentrations: precise amounts of reactants/substrates/products.
4. Determinants that specify the structure of enzymes and direct their formation/activation.

Molecules that are important in the biology/metabolism of the cell include carbohydrates, fats and lipids, and proteins.

Carbohydrates

Carbohydrates are made up of carbon (C), hydrogen (H) and oxygen (O), with the molecular ratio $C_x(H_2O)_y$.

- Monosaccharides: pentose – 5 C's (ribose, deoxyribose); hexose – 6 C's (glucose, fructose)
- Disaccharides: two monosaccharides (sucrose, maltose, lactose)
- Oligosaccharides: combine with proteins and lipids to form glycoproteins and glycolipids, important in cell–cell recognition and the immune response
- Polysaccharides: polymers, insoluble, normally contain 12 to 10 000 monosaccharides (starch, cellulose, glycogen)
  - Also form complexes with lipids and phosphate.

Fats and lipids

Fatty acids (FAs) have a long hydrocarbon chain ending in a carboxyl group:

- Saturated FAs have single bonds between carbon atoms.

- Unsaturated FAs have some double bonds between carbon atoms.

Lipids are made up of FAs plus water:

- Phospholipids are important in membranes.
- Glycolipids are important in receptors.

Proteins

The *primary structure* of a protein is a sequence of amino acids with peptide bonds:

–CONH–

Amino acids have at least one amino and one carboxyl group; they are amphoteric, and form dipolar zwitterions in solution. Proteins have *secondary structures*; they can be folded into a helix, or form beta sheets that are held together by hydrogen bonds:

- Alpha helix – tends to be soluble (most enzymes).
- Beta sheets – insoluble – fibrous tissue.

Proteins also have a three-dimensional *tertiary structure*, which is formed by folding of the secondary structure, held in place by different types of bond to form a more rigid structure: disulfide bonds, ionic bonds, intermolecular bonds (van der Waals – non-polar side chains attracted to each other).

High temperatures and extremes of pH denature proteins, destroying their tertiary structure and their functional activity.

Some proteins have a *quaternary structure*, with several tertiary structures fitted together; e.g., collagen consists of a triple stranded helix.

Enzymes are proteins that catalyze a large number of biologically important actions, including anabolic and catabolic processes, and transfer of groups (e.g., methylase, kinase, hydroxylase, dehydrogenase). Some enzymes are isolated in organelles, others are free in the cytoplasm; there are more than 5000 enzymes in a typical mammalian cell.

- Kinases: add a phosphate group, key enzymes in many activation pathways.
- Methylases: add a methyl group. DNA methylation is important in modifications that are involved in imprinting, lipid methylation is important for membrane stability, and proteins are also stabilized by methylation.

Most enzymes are conjugated proteins, with an active site that has a definite shape; a substrate fits into the active site, or may induce a change of shape so that it can fit.

- The rate of an enzymatic reaction is affected by temperature, pH, substrate concentration, enzyme concentration.
- Enzymes can be activated by removal of a blocking peptide, maintaining the S-H groups, or by the presence of a cofactor.
- The active site of an enzyme is often linked to the presence of an amino acid OH^- group (serine, threonine). Mutations at this level render the enzymes inactive.

Enzyme inhibitors can be:

- Competitive – structurally similar
- Noncompetitive – no similarity, form an enzyme/inhibitor complex that changes the shape of the protein so that the active site is distorted
- Irreversible: heavy metal ions combine with -SH causing the protein to precipitate. Lead (Pb^{2+}) and cadmium (Cd^{2+}) are the most hazardous; these cations can also replace zinc (Zn^{2+}), which is usually a stabilizer of tertiary structures.

Allosteric enzymes are regulated by compounds that are not their substrate, but which bind to the enzyme away from the active site in order to modify activity. The compounds can be activators or inhibitors, increasing or decreasing the affinity of the enzyme for the substrate. These interactions help to regulate metabolism by end-product inhibition/feedback mechanisms.

For example, phosphofructokinase (PFK): high ATP inhibits, low ATP activates.

Km is the substrate concentration that sustains half the maximum rate of reaction. Two or more enzymes may catalyze the same substrate, but in different reactions; if the reserves of substrate are low, then the enzyme with the lowest Km will claim more of the substrate.

Cytokines

- Cytokines are proteins, peptides or peptidoglycan molecules that are involved in signaling pathways. They represent a large and diverse family of regulatory molecules that are produced by many different types of cell, and are used extensively in cellular communication:
  - Colony stimulating factors
  - Growth and differentiation factors

- Immunoregulatory and proinflammatory cytokines function in the immune system (interferon, interleukins, tumor necrosis factors).

- Each cytokine has a unique cell surface receptor that conducts a cascade of intracellular signaling that may include upregulation and/or downregulation of genes and their transcription factors.
- They can amplify or inhibit their own expression via feedback mechanisms:
  - Type 1 cytokines enhance cellular immune responses:
    - Interleukin-2 (IL-2), gamma interferon (IFN-γ), TGF-β, TNF-β, etc.
  - Type 2 favor antibody responses:
    - IL-4, IL-5, IL-6, IL-10, IL-13, etc.
  - Type 1 and type 2 cytokines can regulate each other.

Metabolic pathways

Each metabolic pathway is a series of reactions, organized such that the products of one reaction become substrates for the next (Figure 1.5). The reactants in a pathway may be modified in a series of small steps, so that energy is released in controlled amounts, or minor adjustments can be made to the structure of molecules.

Anabolic pathways require energy to synthesize complex molecules from smaller units.

Catabolic pathways break molecules up into smaller units which can then be used to generate energy.

Each step in a pathway is catalyzed by a specific enzyme, and each enzyme represents a point for control of the overall pathway. The steps of the pathway

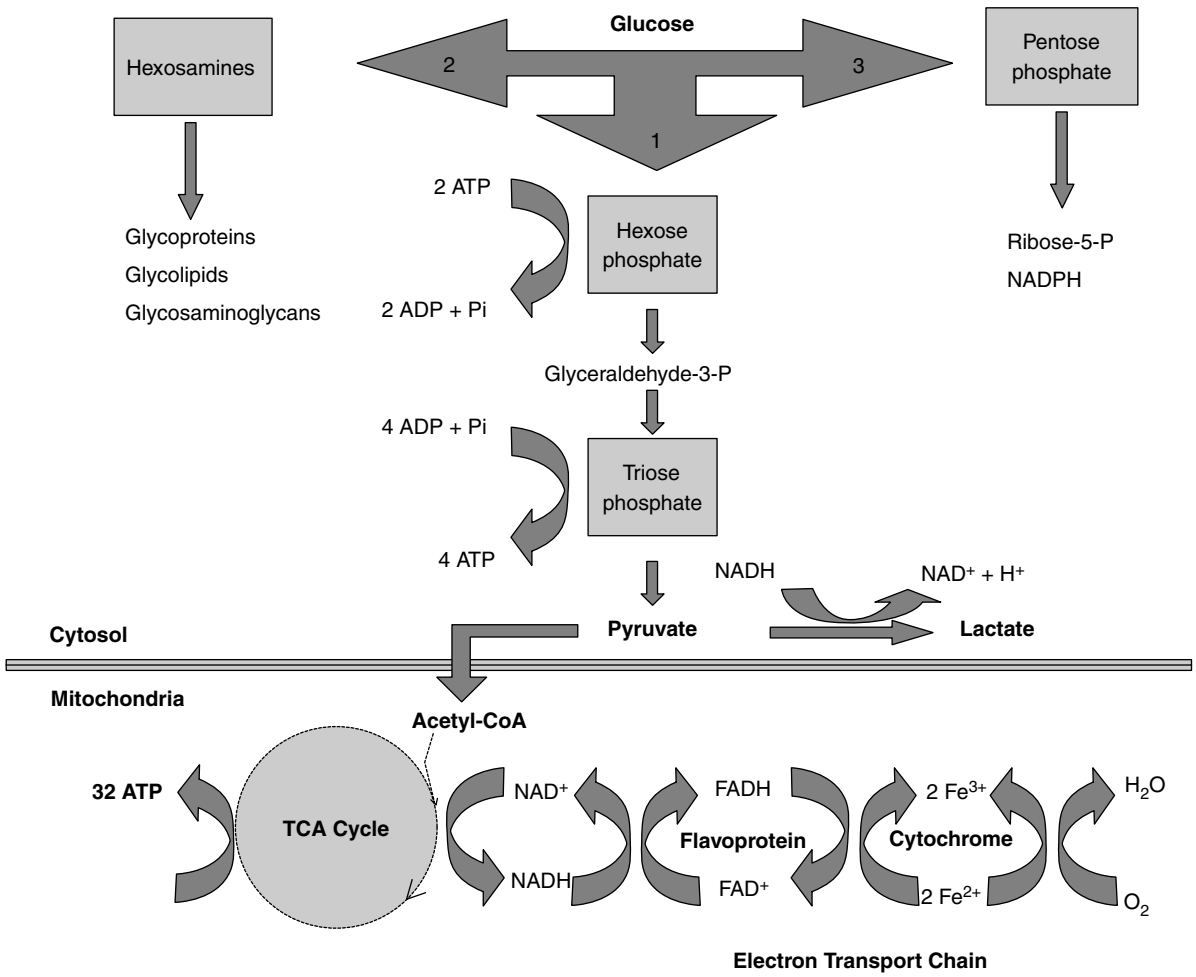

Figure 1.5 Pathways that metabolize glucose in a mammalian cell.

may be spatially arranged, so that the product of one reaction is in the right place to become the substrate of the next enzyme. This allows high local concentrations of substrate molecules to build up, and biochemical reactions to proceed rapidly. A pathway arranged in this manner may be catalyzed by a multienzyme complex.

- **Glycolysis**, which breaks glucose down into pyruvate, takes place in the cell cytoplasm; pyruvate enters mitochondria to be further metabolized.
- **Fatty acid oxidation** and the **Krebs cycle** (TCA or Citric acid cycle) take place in the mitochondrial matrix.

The Krebs cycle is part of a metabolic pathway that converts carbohydrates, fats and proteins into CO_2 and ATP, which is generated by a process of oxidative phosphorylation. ATP is exported from the mitochondria for use in protein synthesis, DNA replication, etc.: all energy-requiring processes of life are coupled to the cleavage of ATP:

$$ATP \Leftrightarrow ADP + Phosphate + Energy$$

ATP is exported from the mitochondria in exchange for ADP arising from the ATP that has been broken down to drive cellular metabolism.

Redox reactions: oxidation and reduction are electron-transfer processes, involving NAD-NADH.

- NADP(H) is generally used for anabolic reactions.
- NAD(H) is used for catabolic reactions.

These reactions need ubiquinone and cytochrome C, cytochrome oxidase (inhibited by cyanide).

- Oxidation: loss of electrons; reduction: gain of electrons.
- An oxidizing agent removes electrons and is itself reduced.
- A reducing agent gains electrons and is itself oxidized.

Reactive oxygen species (ROS, oxygen radicals)

ROS are molecules that contain the oxygen ion or peroxide; the presence of unpaired valence electrons makes them highly reactive. They are formed as a by-product of oxygen metabolism, and have an important role in cell signaling mechanisms. However, high levels of ROS (i.e., oxidative stress) can cause oxidative damage to nucleic acids, proteins and lipids, as well as inactivate enzymes by oxidation of cofactors.

Antioxidants such as ascorbic acid (vitamin C), tocopherol (vitamin E), glutathione, hypotaurine, pyruvic acid, uric acid and albumin are important in cellular defense mechanisms against ROS damage (Figure 1.6).

Superoxide dismutase (SOD)

SOD enzymes catalyze the dismutation of superoxide into oxygen and hydrogen peroxide, an important defense against potential ROS damage. Three SOD enzymes are present in mammalian cells:

- SOD1: dimer, present in the cytoplasm, contains Cu^{2+} and Zn^{2+}
- SOD2: tetramer, mitochondrial enzyme, contains Mn^{2+}
- SOD3: tetramer, extracellular, contains Cu^{2+} and Zn^{2+}

ROS can cause damage to DNA in oocytes, sperm and embryos, with important consequences for fertilization and embryo development (Guerin *et al.*, 2001). Oocytes are particularly susceptible during the final stages of follicular growth, and ROS damage to sperm DNA has been strongly linked to male infertility (Sakkas *et al.*, 1998).

Fundamental principles of molecular biology

The **nucleic acids, DNA** (deoxyribose nucleic acid; Figure 1.7) **and RNA** (ribose nucleic acid), are made up of:

1. Nucleotides: organic compounds containing a nitrogenous base
2. Sugar: deoxyribose in DNA, ribose in RNA
3. Phosphate group.

Nucleotides are purines and pyrimidines, determined by the structure of the nitrogenous base.

	DNA	RNA
Purines	Adenine (A)	Adenine (A)
(double ring)	Guanine (G)	Guanine (G)
Pyrimidines	Cytosine (C)	Cytosine (C)
(single ring)	Thymine (T = methylated U)	Uracil (U)

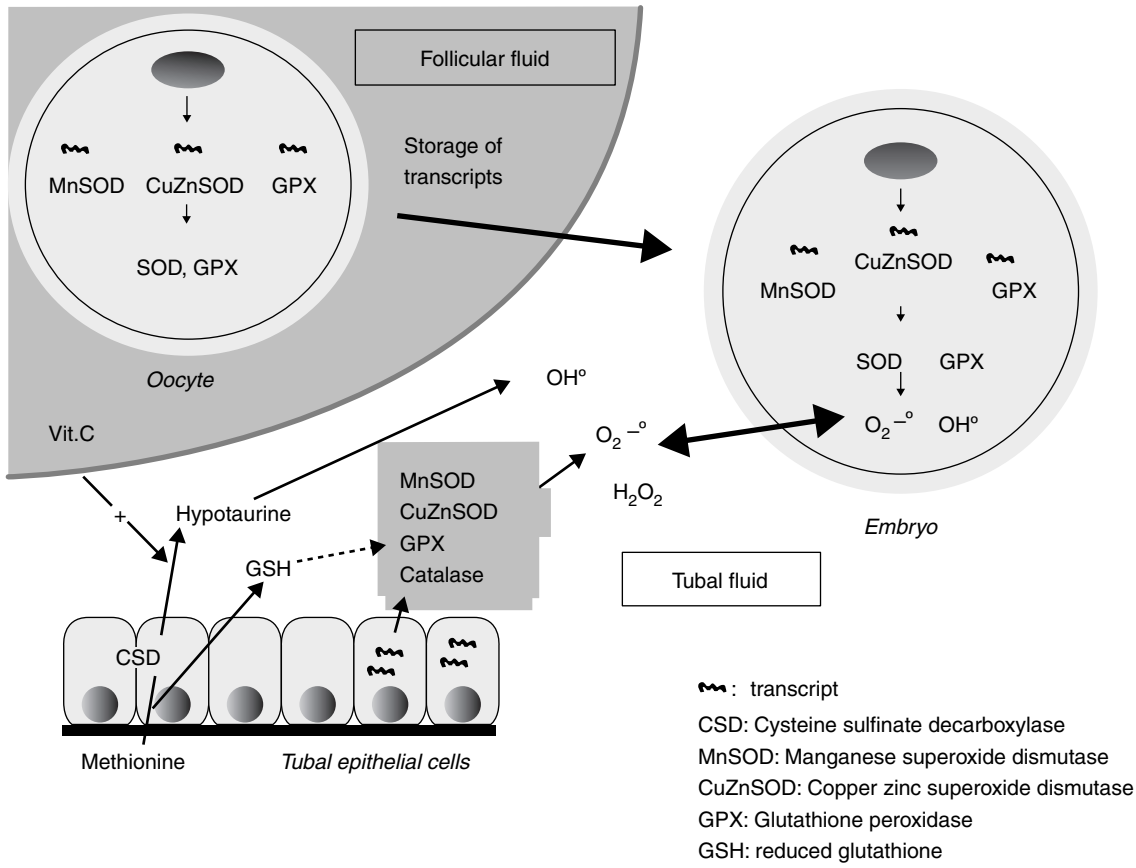

Figure 1.6 Mechanisms that protect oocytes and embryos from ROS damage (with thanks to Y. Ménézo).

Methylation of cytosine is important in gene silencing and imprinting processes.

Nucleotides also function as important cofactors in cell signaling and metabolism: coenzyme A (CoA), flavin adenine dinucleotide (FAD), flavin mononucleotide, adenosine triphosphate (ATP), nicotinamide adenine dinucleotide phosphate (NADP).

DNA

- Double-stranded helix with paired bases to form complementary strands
- G=C or A=T
- Pentose deoxyribose – phosphate backbone
- Stabilized by H bonds between purines and pyrimidines, on the inside of the helix
- Each pitch of the double helix has 10 base pairs.

DNA replication

DNA copies itself by semi-conservative replication: each strand acts as a template for synthesis of a complementary strand.

1. Free nucleotides are made in the cytoplasm, and are present in the nucleoplasm before replication begins.
2. The double helix unwinds, and hydrogen bonds, holding the two DNA strands together, break. This leaves unpaired bases exposed on each strand.
3. The sequence of unpaired bases serves as a template on which to arrange the free nucleotides from the nucleoplasm.
4. DNA polymerase moves along the unwound parts of the DNA, pairing complementary nucleotides from the nucleoplasm with each exposed base.

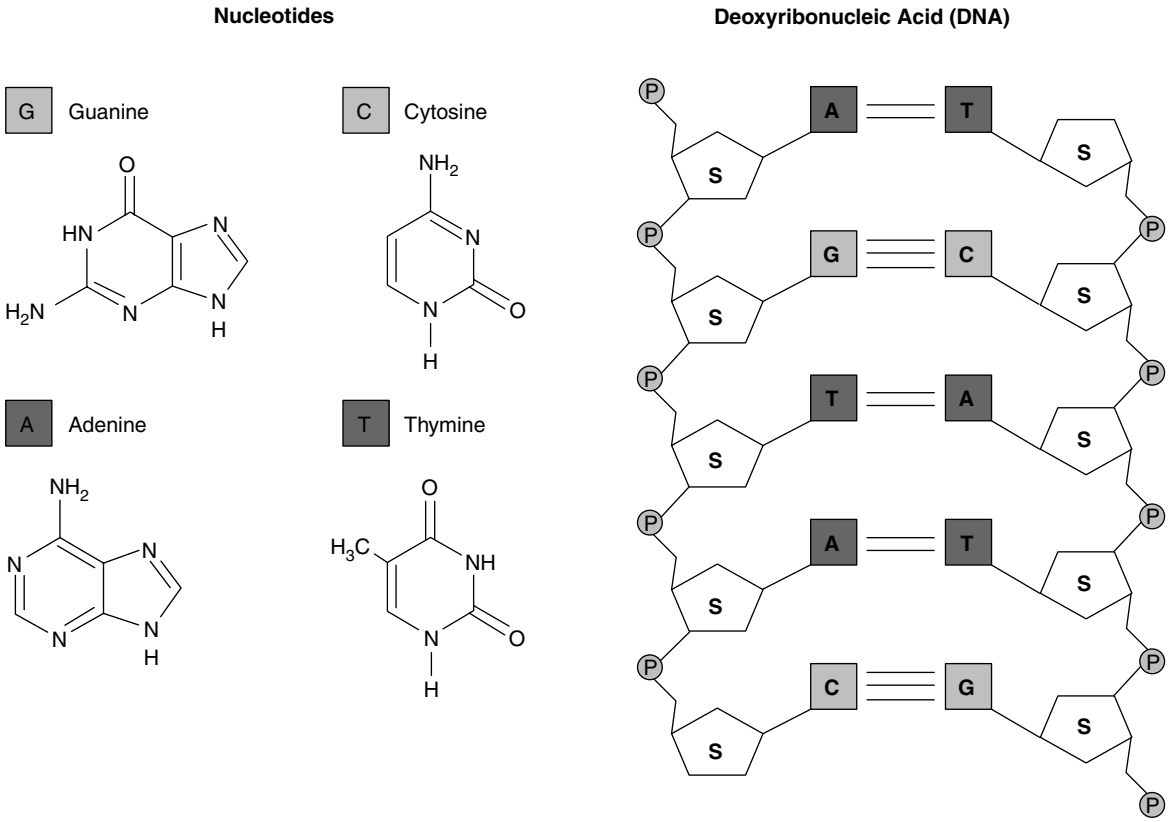

Figure 1.7 Structure of DNA. Complementary base pairs form the DNA double helix; two hydrogen bonds form between A and T, three hydrogen bonds form between G and C. The two polynucleotide chains must be antiparallel to each other to allow pairing. S = sugar, P = phosphate group.

5. The same enzyme connects the nucleotides together to form a new strand of DNA, hydrogen bonded to the old strand:
   - DNA polymerase forms new hydrogen bonds on the 5′3′ strand
   - DNA ligase acts on the 3′5′ strand
   - Several replication points appear along the strand, which eventually join.

6. DNA is then mounted on "scaffolding proteins," histones – and this is then wrapped around non-histones to form chromatin. Histones are basic proteins that bind to nuclear DNA and package it into nucleosomes; the regulation of gene expression involves histone acetylation and deacetylation. There are two ATP-dependent remodeling complexes and acetyltransferases that preferentially bind activated states and fix chromatin configurations:
   - Histone acetyltransferase coactivator complex
   - Histone deacetylase corepressor complex.

Methylation of protamines and histones is a crucial component of imprinting processes: an association has been found between Beckwith–Wiedemann syndrome and epigenetic alterations of LitI and H19 during in-vitro fertilization (DeBaum *et al.*, 2003).

Each mammalian cell contains around 1.8 m of DNA, of which only 10% is converted into specific proteins; the noncoding part of the DNA still carries genetic information, and probably functions in regulatory control mechanisms.

- Genes = chief functional unit of DNA
- Exons – contain information for the amino acid sequence of a protein (coding sequence)
- Introns = non-coding regions in between exons
- Codon = a group of three nucleotide bases which code for one amino acid.

The genetic code: the biochemical basis of heredity

In 1968, the Nobel Prize in Physiology or Medicine was awarded to Robert Holland, Ghobind Khorana and Marshall Nirenberg "for their interpretation of the genetic code and its function in protein synthesis":

- DNA transfers information to mRNA in the form of a code defined by a sequence of nucleotides bases.
- The code is triplet, unpunctuated and non-overlapping.
- Three bases are required to specify each amino acid, there are no gaps between codons, and codons do not overlap.
- Since RNA is made up of four types of nucleotides (A, C, G, U), the number of triplet sequences (codons) that are possible = 4 × 4 × 4 = 64; three of these are "stop codons" that signal the termination of a polypeptide chain.
- The remaining 61 codons can specify 20 different amino acids, and more than one codon can specify the same amino acid (only Met and Trp are specified by a single codon).
  - Since the genetic code thus has more information than it needs, it is said to be "degenerate."
- A mutation in a single base can alter the coding for an amino acid, resulting in an error in protein synthesis: translated RNA will incorporate a different amino acid into the protein, which may then be defective in function (sickle cell anemia, phenylketonuria are examples of single gene defects).

Regulation of transcription

- A **homeobox** is a DNA sequence that codes for a 60 amino acid protein domain known as the **homeodomain**.
- A **homeodomain** acts as a switch that controls gene transcription.
- **Homeobox genes**, first discovered in 1983, are a highly conserved family of transcription factors that switch on cascades of other genes:
  - Involved in the regulation of embryonic development of virtually all multicellular animals, playing a crucial role from the earliest steps in embryogenesis to the latest stages of differentiation
  - Are arranged in clusters in the genome.
- **POU factors** are a class of transcriptional regulators, required for high affinity DNA binding, that are important in tissue-specific gene regulation; they are named after three proteins in the group: **P**it-I (also known as GHF-1), **O**ct-I and **U**nc-86.

RNA

- Paired bases are G–C and A–U.
- Pentose sugar = ribose.
- Basic structure in mammalian cells is single-stranded, but most biologically active forms contain self-complementary sequences that allow parts of the RNA to fold and pair with itself to form double helices, creating a specific tertiary structure.
- RNA molecules have a negative charge, and metal ions such as Mg^{2+} and Zn^{2+} are needed to stabilize many secondary and tertiary structures.
- Hydroxyl groups on the deoxyribose ring make RNA less stable than DNA because it is more prone to hydrolysis.

There are many different types of RNA, each with a different function:

- Transcription, translation/protein synthesis: mRNA, rRNA, tRNA
- Post-transcriptional modification or DNA replication: small nuclear RNA (snRNA), small nucleolar RNA (snoRNA), guide RNA (gRNA), ribonuclease P, ribonuclease MRP, etc.
- Gene regulation: microRNA (miRNA), small interfering RNA (siRNA), etc.
  - microRNAs are increasingly recognized as "master regulators" of gene expression, regulating large networks of genes by chopping up or inhibiting the expression of protein coding transcripts.

Ribosomal RNA: 80% of total RNA

- Made in the nucleolus, then moved out into the nucleoplasm and then to the cytoplasm to be incorporated into ribosomes.

Transfer RNA (4S RNA): 10-15% of total RNA

- Single strand, 75–90 nucleotides wound into a clover leaf shape; each tRNA molecule transfers an amino acid to a growing polypeptide chain during translation.

A three-base anticodon sequence on the "tail" is complementary to a codon on mRNA; an amino acid is attached at the 3′ terminal site of the molecule, via a covalent link that is catalyzed by an aminoacyl tRNA synthetase. Each type of tRNA molecule can be attached to only one type of amino acid; however, multiple

codons in DNA can specify the same amino acid, and therefore the same amino acid can be carried by tRNA molecules that have different three-base anticodons.

- Methioninyl tRNA has a critical function, required for the initiation of protein synthesis.

Messenger RNA: 3-5% of total cellular RNA (exception: sperm cells contain approx. 40% mRNA, and very little rRNA)

mRNA molecules are single-stranded, complementary to one strand of DNA (coding strand) and identical to the other. DNA is **transcribed** into mRNA molecules, which carry coding information to the ribosomes for translation into proteins.

Transcription

- DNA information is transcribed to mRNA in the nucleus, starting from a promoter sequence on the DNA at the 5′ end, and finishing at the 3′ end.
- All of the exons and introns in the DNA are transcribed – stop and start sequences are encoded in the gene.
- The product, nuclear mRNA precursor (HnRNA, heterogeneous nuclear RNA) is processed into mature cytoplasmic mRNA by splicing at defined base pairs to remove the introns and join the exons together.
- A cap of 7-methylG is added at the 5′ end.
- A string of polyA is added at 3′ end = polyA tail, 50–300 residues.
- Polyadenylated mRNA molecules are very susceptible to degradation by ribonuclease (RNAse) after their release from the nucleolus

A **promoter** is a specific DNA sequence that signals the site for RNA polymerase to initiate transcription; this process needs an orchestrated interaction between proteins binding to specific DNA sequences, as well as protein–protein interactions. DNA methylation is involved in the regulation of transcription. Gene sequences that lie 5′ to the promoter sequence bind specific proteins that influence the rate of transcription from a promoter:

- A **TATA box** aligns RNA polymerase II with DNA by interacting with transcription initiation factors (TFs).
- Proteins that bind to a CAAT box determine the rate at which transcription is initiated, bringing RNA polymerase II into the area of the start site in order to assemble the transcriptional machinery.

The tertiary structure of the DNA (bends and folds) is important in making sure that all components are correctly aligned.

- Enhancers, silencers, hormone response elements (steroid receptors) are important in determining the tissue-specific expression or physiological regulation of a gene; these factors respond to signals such as cAMP levels.

Transcription in oocytes

- Transcription takes place during oocyte growth, and stops before ovulation.
- mRNA turnover begins before ovulation: mRNA molecules must be protected from premature translation.
- Oocytes contain mechanisms that remove histone H1 from condensed chromatin.
- Differential acetylation profiles of core histone H4 and H3 for parental genomes during the first G1 phase may be important in establishing early zygotic "memory."
- The timing of transcriptional events during the first zygotic cell cycle will have an effect on further developmental potential.

Translation (protein synthesis)

During protein synthesis, ribosomes move along the mRNA molecule and "read" its sequence three nucleotides at a time, from the 5′ end to the 3′ end. Each amino acid is specified by the mRNA's codon, and then pairs with a sequence of three complementary nucleotides carried by a particular tRNA (anticodon). The translation of mRNA into polypeptide chains involves three phases: initiation, elongation and termination. Messenger RNA binds to the small (40 s) subunit of the ribosome on rER in the cytoplasm, and six bases at a time are exposed to the large (60 s) subunit. The endpoint is specified by a "stop" codon: UAA, UAG or UGA.

1. *Initiation*: The first three bases (codon) are always AUG, and the initiation complex locates this codon at the 5′ end of the mRNA molecule. A methionyl-tRNA molecule with UAC on its coding site forms hydrogen bonds with AUG, and the complex associates with the small ribosome subunit (methionine is often removed after translation, so that not every protein has methionine as its first amino acid). Some mRNAs contain a supernumerary AUG and associated short coding region upstream and independent

of the main AUG coding region; these upstream open reading frames (uORFs) can regulate the translation of the downstream gene.

The large ribosome subunit has a P site which binds to the growing peptide chain, and an A site which binds to the incoming aa-tRNA.

2. *Elongation*: the unbound tRNA may now leave the P site, and the ribosome moves along the mRNA by one codon.
   - A peptide bond is formed, and the aa-tRNA bond is hydrolyzed to release the free tRNA.
   - A second tRNA molecule, bringing another amino acid, bonds with the next three exposed bases. The two amino acids are held closely together, and peptidyl transferase in the small ribosomal subunit forms a peptide bond between them.
   - The ribosome moves along the mRNA, exposing the next three bases on the ribosome, and a third tRNA molecule brings a third amino acid, which joins to the second one.
3. *Termination*: The polypeptide chain continues to grow until a stop codon (UAA, UAC or UGA) is exposed on the ribosome. The stop codon codes for a releasing factor instead of another aa-tRNA; the completed peptide is released, and components of the translation complex are disassembled.

Cellular replication

Replication of individual cells is the fundamental basis of growth and reproduction for all living organisms: each cell grows and then divides to produce daughter cells. Cells can divide by using one of two different mechanisms – mitosis or meiosis. During mitosis, the cellular DNA that represents the cell's entire diploid genome is replicated into two copies, and the two cells produced after division each contain this same diploid genome. Meiosis, or "reduction division," splits the DNA in the diploid genome in half, so that the daughter cells are haploid, with a single copy of each gene. This is the process that leads to the generation of the germ cells, sperm and oocyte.

The sequence of growth, replication and division that produces a new cell is known as the **cell cycle**; in human cells, the mitotic cell cycle takes from 8 to 24 hours to complete. The cell cycle is divided into phases: G1, S, G2 (interphase) and M (Figure 1.8):

- G1: cell growth, a period of high metabolic activity when new proteins are synthesized; rRNA, mRNA, tRNA are produced in the nucleolus, and new organelles are formed.
- S: synthesis of DNA and duplication of the centrosome. Each new DNA double helix is surrounded by histones to form chromatids, which are held together at the centromere.
- G2: centrioles replicate, and the cell prepares for mitosis.
- M: mitosis has four phases, during which the replicated DNA is distributed to the two new daughter cells:
  - prophase, metaphase, anaphase, telophase.

    (i) Early prophase: the two replicated chromosomes condense, the nuclear

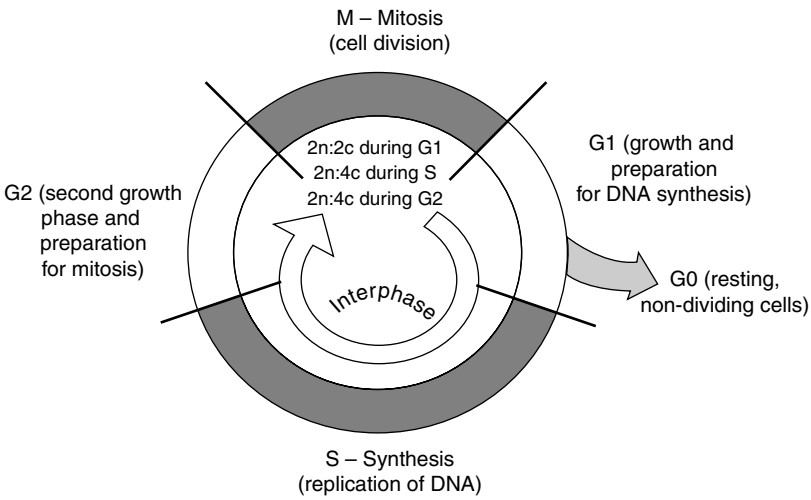

Figure 1.8 Phases of the cell cycle.
n = chromosome number
c = chromatid (DNA copy) number

membrane dissociates, centrioles divide and migrate to different poles, microtubules form an aster shape around the centrioles, and form the spindle. Spindle fibers attach to the centromeres at the kinetochore.

(ii) Metaphase: chromosomes are lined up on the equatorial spindle plate; centromeres start to divide at the end of metaphase.

(iii) Anaphase: chromatids separate and are pulled by the spindle fibers to opposite poles.

(iv) Telophase: chromatids have reached opposite poles, and a nuclear membrane forms around them. The chromosomes uncoil to become indistinct, spindle fibers disintegrate, and the nucleolus reforms.

During **cytokinesis**, the cytoplasm divides to form two daughter cells. A ring of microfilaments develops around the cell, usually near the equator. These microfilaments are attached to the cell membrane and contract when division takes place to pull the cell in (like a drawstring), creating a division furrow.

G0: a cell can leave the cell cycle, temporarily or permanently during G1, and enter a "resting" phase known as G0, where they will continue to carry out their designated function (e.g., secretion, immune functions). Some G0 cells may be terminally differentiated, and will never re-enter the cell cycle to continue dividing. Others can be stimulated to re-enter at G1 and proceed to new rounds of cell division (e.g., lymphocytes encountering a new antigen). A G0 phase requires active repression of the genes required for mitosis – cancer cells cannot enter G0 and continue to divide indefinitely.

The cell cycle is regulated by a sophisticated and carefully orchestrated series of control mechanisms, involving a number of proteins and cofactors whose levels fluctuate according to the phase/stage: the major regulatory proteins include cyclins, cyclin-dependent kinases (CdKs) and anaphase-promoting complex (APC, or cyclosome).

Definitions

Chromosomes: the repository of genetic information, molecules of DNA complexed with specific proteins.
Chromatin: the protein/DNA complex that makes up the chromosome.
Chromatids: pairs of identical DNA molecules formed after DNA replication, joined at the centromere.
Centromere: constricted region in a chromosome, which divides it into two "arms." It serves as an attachment site for sister chromatids and spindle fibers, allowing chromosomes to be pulled to different poles. Normally located centrally, but in some species found near the end (pericentric), at the end (telocentric) or spread all over the chromosomes (holocentric).
Kinetochore: structure that forms at the centromere to bind microtubules during mitosis.
Diploid: two pairs of each chromosome in a cell.
Haploid: one of each pair of chromosomes in a cell.
Aneuploid: incorrect number of chromosomes; e.g., trisomy (three copies), monosomy (one copy).

Reproduction is based upon transmission of half of each parent's chromosomes to the next generation. This is carried out by setting aside a special population of germ cells that are destined to form the gametes, i.e., spermatozoa and oocytes. Successful completion of meiosis, a specialized form of cell division, is a fundamental part of gametogenesis, and a detailed understanding of this process is a crucial background.

Mitosis and meiosis

Meiosis differs from mitosis in a number of ways, as summarized below (see also Figure 1.9).

Mitosis

- Occurs in all tissues.
- Involves one round of DNA replication for each cell division.
- Produces genetically identical diploid somatic (body) cells.
- Is a rapid process.
- The daughter cells are genetically identical to the parent cell and have the same number of chromosomes.
- This type of cell division takes place during growth of an organism (e.g., embryonic growth), healing, the development of new cells, and is also important for maintaining populations of cells, replacing those that die.

Meiosis

- Occurs only in the ovary (oocytes) and the testis (spermatozoa).
- Involves one round of DNA replication and two cell divisions, thus generating haploid products.

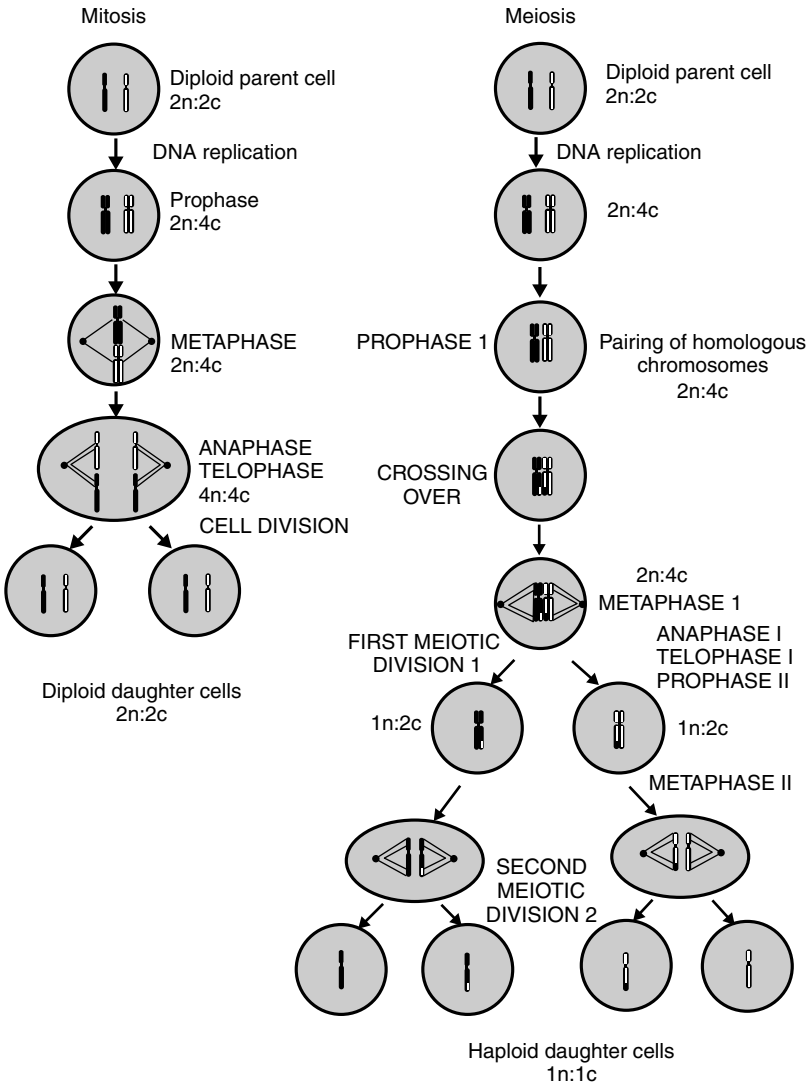

Figure 1.9 Comparison of mitosis and meiosis; mitosis generates two identical diploid daughter cells, and meiosis generates four chromosomally unique haploid cells from each diploid cell.

- Involves pairing of specific chromosome homologues that then exchange pieces of DNA (genetic recombination), which results in daughter cells that are genetically different from the original germ cells.
- Completion of this cell division may take years.

Principles of meiosis

In humans, meiosis is initiated during the first trimester of gestation in females, and following puberty in males. Meiosis allows the exchange of DNA between overlapping sister chromatids, with subsequent recombination into two "new" chromatids – new, but related, gene combinations can be created, facilitating genetic diversity. This occurs during the pachytene/diplotene stages of meiotic prophase, as illustrated in Figure 1.10. In the middle diagram, the sister chromatids cross over at a single crossover point. In the diagram on the bottom, this leads to an equal and reciprocal exchange of chromatin.

The crossover point occurs between two DNA duplexes that contain four DNA strands (see Figure 1.10). The strands in fact switch their pairing at the joining point to form a crossed-strand junction, a mechanism that was first proposed by Robin Holliday in 1964 and is known as a Holliday structure/junction. Heteroduplexes are regions on recombinant DNA molecules where the two strands are not exactly

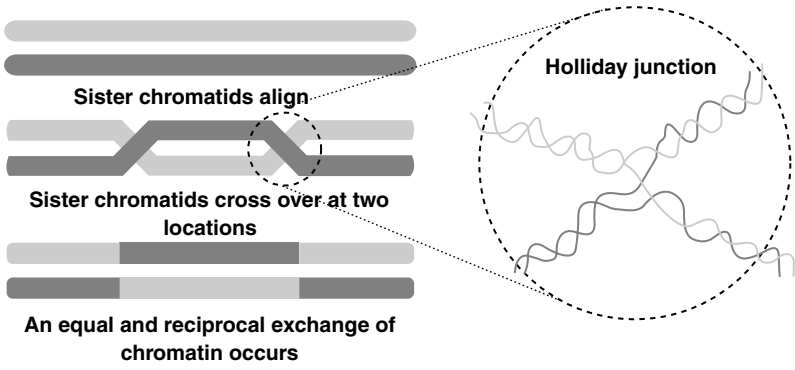

Figure 1.10 Exchange of genetic material during chromosomal crossing over; a cross-over point is magnified to illustrate the Holliday junction.

complementary, and Holliday carried out experiments that detected heteroduplex regions in both strands of recombining DNA. In Figure 1.10, the junction is magnified to reveal its structure.

Meiosis differs from mitosis in terms of:

1. Checkpoint controls
2. DNA replication
3. Dependence on external stimuli
4. Regulation of cell cycle control proteins.

Cell cycle checkpoints control the order and timing of cell cycle transition, ensuring that critical events such as DNA replication and chromosome separation are completed correctly – one process must be completed before another starts. During meiotic division, recombination must be completed before the beginning of cell division so that a correct segregation of homologous chromosomes is obtained. Several genes have been identified in yeast that are responsible for blocking meiosis when double-strand DNA breaks are not repaired. A checkpoint specific to meiotic cells ensures that anaphase I does not begin until paired chromosomes are correctly attached to the spindle. This control resembles the spindle-assembly checkpoint of mitotic cells. Fulka *et al.* (1998) carried out a series of experiments to test DNA-responsive cell cycle checkpoints in bovine and mouse oocytes, using ultraviolet (UV) irradiation to induce DNA damage or chemical treatment to prevent chromosome condensation. Their results suggest that replication-dependent checkpoints may be either inactive or highly attenuated in fully grown mammalian oocytes; this should be borne in mind when considering the effects of endocrine or in-vitro manipulations carried out during assisted reproduction cycles, or the in-vitro maturation of oocytes. Although resumption of meiosis apparently has no cell cycle checkpoint, the first cell cycle does, as does each embryonic cell cycle. Micromanipulation experiments in spermatocytes show that tension on the spindle generated by attached homologues acts as a checkpoint. If this tension is eliminated by experimental manipulation, anaphase is prevented. A major difference between the mitotic and meiotic cell cycles lies in the fact that during meiosis the oocyte can be blocked at precise phases of the cell cycle, until a specific stimulus (e.g., hormone or sperm) removes the block. In somatic cells, a state of quiescence or cell cycle block in response to a specific physiological state of the cell is described as the G0 phase of the cell cycle. However, G0 differs from meiotic blocks in terms of cell cycle regulation and the activity of the key kinases that maintain the arrest.

In oocytes, progression from the first to the second meiotic arrest is usually referred to as **oocyte maturation**, and the oocyte is now ready to be ovulated, i.e., expelled from the ovary. Shortly after ovulation, fertilization occurs; removal of the second meiotic block at fertilization is called **oocyte activation**.

- In the female, only one functional cell is produced from the two meiotic divisions. The three remaining smaller cells are called polar bodies.
- In the male, four functional spermatozoa are produced from each primary cell (Figure 1.11).

Errors in meiosis

The process of crossing over at diplotene in the female lasts for many years (from birth until ovulation), and the chromosomes may become sticky, become knotted together at diakinesis, and fail to separate correctly. Failure of chromosomes to separate during first or second meiotic divisions results in aneuploidy. With autosomes this is usually lethal; sex chromosome aneuploidy can lead to anomalies of sexual development.

Sometimes pairs of chromosomes fail to rejoin after crossing over at diplotene and are lost from the gamete – they may become attached to another chromosome to produce a partial trisomy, or a balanced translocation.

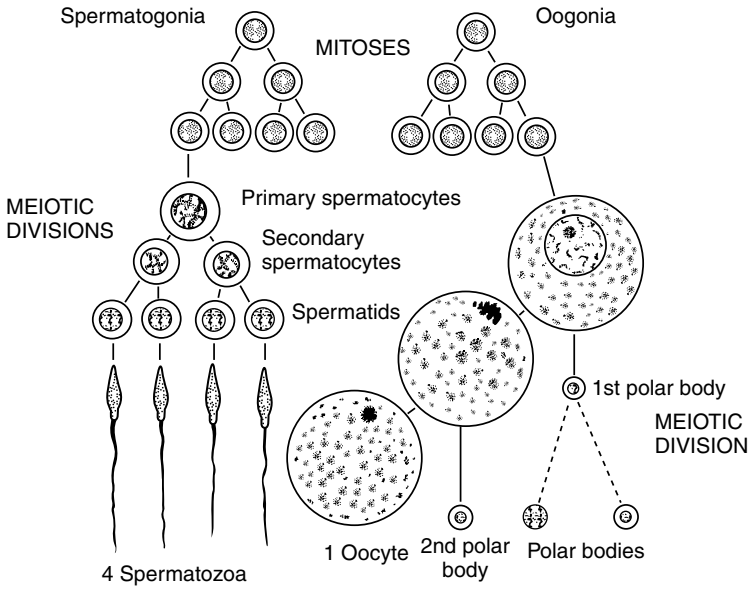

Figure 1.11 Gametogenesis. Gametogenesis in the male gives rise to four functional spermatozoa; in the female only one of the four daughter cells becomes a functional oocyte. Modified from Dale (1983).

HeLa cells

The majority of our knowledge about fundamental principles of cell and molecular biology has been gained from model systems, particularly in yeast and bacteria, as well as human cell lines maintained in tissue culture. The first human cell line to be propagated and grown continuously in culture as a permanent cell line is the HeLa cell, an immortal epithelial line: knowledge of almost every process that takes place in human cells has been obtained through the use of HeLa cells, and the many other cell lines that have since been isolated.

The cells were cultured from biopsy of a cervical cancer taken from Henrietta Lacks, a 31-year-old African American woman from Baltimore, in 1951. George Gey, the head of the cell culture laboratory at Johns Hopkins Hospital, cultivated and propagated the cells; Henrietta died from her cancer 8 months later. Gey and his wife Margaret continued to propagate the cells, and sent them to colleagues in other laboratories. In 1954, Jonas Salk used HeLa cells to develop the first vaccine for polio, and they have been used continually since then for research into cancer, AIDS, gene mapping, toxicity testing, and numerous other research areas – they even went up in the first space missions to see what would happen to cells in zero gravity.

HeLa cells attained "immortality" because they have an active version of the enzyme telomerase, which prevents telomere shortening that is associated with aging and eventual cell death. They adapt readily to different growth conditions in culture, and can be difficult to control: their growth is so aggressive that slight contamination by these cells can take over and overwhelm other cell cultures. Many other in-vitro cell lines used in research (estimates range from 1-10% of established cell lines) have been shown to have HeLa cell contamination. Twenty-five years after Henrietta's death, many cell cultures thought to be from other tissue types, including breast and prostate cells, were discovered to be in fact HeLa cells, a finding that unleashed a huge controversy and led to questions about published research findings. Further investigation revealed that HeLa cells could float on dust particles in the air and travel on unwashed hands to contaminate other cultures.

The cells were established in culture without the knowledge of her family, who discovered their "fame" accidentally 24 years after her death – they were contacted for DNA samples that could be used to map Henrietta's genes in order to resolve the contamination problem (Skoot, 2010).

Further reading

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Chapter 2

Endocrine control of reproduction: Controlled ovarian hyperstimulation for ART

Introduction

Synchrony is essential for gametogenesis and correct embryo development, and a basic knowledge of reproductive endocrinology is fundamental to understanding synchrony in reproductive physiology. Although sexual arousal, erection and ejaculation in the male are obviously under cerebral control, it is less obvious that the ovarian and testicular cycles are also coordinated by the brain. For many years after the discovery of the gonadotropic hormones follicle stimulating hormone (FSH) and luteinizing hormone (LH), the anterior pituitary gland was considered to be an autonomous organ, until animal experiments in which lesions were induced in the hypothalamus clearly demonstrated that reproductive processes were mediated by the nervous system. The hypothalamus is a small inconspicuous part of the brain lying between the midbrain and the forebrain; unlike any other region of the brain, it not only receives sensory inputs from almost every other part of the central nervous system (CNS), but also sends nervous impulses to several endocrine glands and to pathways governing the activity of skeletal muscle, the heart and smooth muscle (Figure 2.1). Via a sophisticated network of neural signals and hormone release, the hypothalamus controls sexual cycles, growth, pregnancy, lactation and a wide range of other basic and emotional reactions. Each hypothalamic function is associated with one or more small areas that consist of aggregations of neurons called hypothalamic nuclei. In the context of reproduction, several groups of hypothalamic nuclei are connected to the underlying pituitary gland by neural and vascular connections.

Functions of the hypothalamus in reproduction

The hypothalamus links the nervous system to the pituitary gland by receiving signals from:

- The central nervous system (CNS)
- Neurons from other parts of the brain, including the amygdala (involved in emotional response), the visual cortex, and the olfactory cortex
- Endocrine factors from the testis, ovary, and other endocrine glands.

It then releases factors into the hypothalamic-hypophyseal portal veins that stimulate or inhibit synthesis and release of hormones by the pituitary, including (but not only):

- GnRH: FSH and LH
- TRH: Thyroid stimulating hormone (TSH)
- CRF: (CRH) Adrenocorticotropic hormone (ACTH)
- GHRH: Growth hormone (GH).

Gonadotropin hormone releasing hormone (GnRH) is secreted by groups of hypothalamic nuclei and transported to the anterior pituitary through the portal vessels. GnRH, a decapeptide with the structure (Pyr)-Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2, is the most important mediator of reproduction by the CNS. Any abnormality in its synthesis, storage, release or action will cause partial or complete failure of gonadal function. GnRH is secreted in pulses, and binds to specific receptors on the plasma membrane of the gonadotroph cells in the pituitary, triggering the inositol triphosphate second messenger system within these cells. This signal induces the movement of secretory granules towards the plasma membrane and eventually stimulates the pulsatile secretion of LH and FSH. Alterations in the output of LH and FSH may be achieved by changing the amplitude or frequency of GnRH, or by modulating the response of the gonadotroph cells.

The anterior pituitary secretes LH, FSH and TSH, which are heterodimeric glycoprotein molecules that share a common alpha-subunit (also shared by human

In-Vitro Fertilization: Third Edition, ed. Kay Elder and Brian Dale. Published by Cambridge University Press.

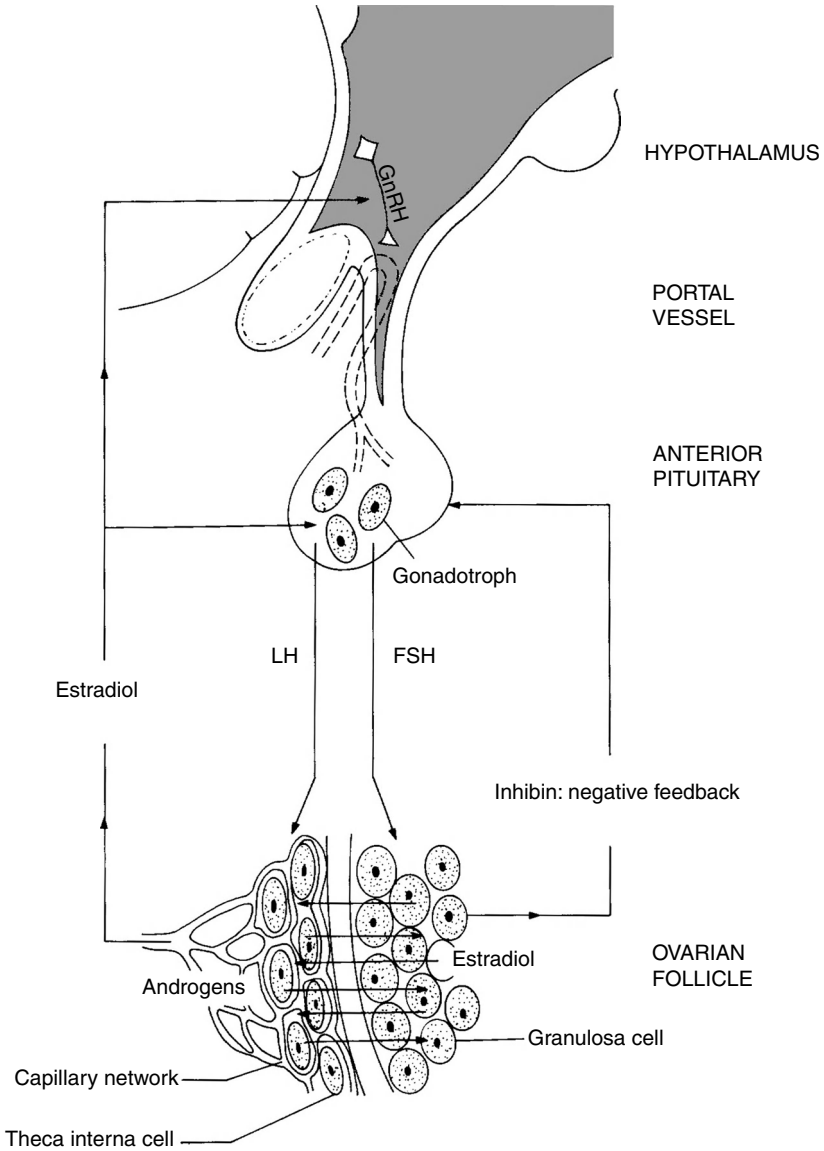

Figure 2.1 HPG axis. Schematic summary of the endocrine control of reproduction in mammals. From Johnson (2007).

chorionic gonadotropin: hCG), and differ by their unique beta-subunit. Only the heterodimers have biological activity. The specific functions of FSH have been studied with targeted deletions in "knock-out" mice: FSH-deficient male mice have a decrease in testicular size and epididymal sperm count, but remain fertile. In contrast, homozygous females with the mutation are infertile: their ovaries show normal primordial and primary follicles, with no abnormalities in oocytes, granulosa or theca cells, but the follicles fail to develop beyond the preantral stage. The ovaries also lack corpora lutea, and serum progesterone levels were decreased by 50% compared with normal mice. These studies indicate that, in mice, whereas spermatogenesis can continue in the absence of FSH, follicle maturation beyond the preantral stage is FSH-dependent.

At the onset of puberty, increased activity of the GnRH pulse generator induces maturation of the pituitary-gonadal axis. A progressive increase in the release of gonadotropins from the pituitary stimulates a subsequent rise in gonadal steroid hormones. In males, FSH released in response to the GnRH pulses acts on Sertoli–Leydig cells to initiate spermatogenesis, and LH acts on the Leydig cells to stimulate testosterone

production. In females, FSH initiates follicular maturation and estrogen production, and LH stimulates theca cell steroidogenesis and triggers ovulation.

Male reproductive endocrinology

The neuroendocrine mechanisms that regulate testicular function are fundamentally similar to those that regulate ovarian activity. The male hypothalamo–pituitary unit is responsible for the secretion of gonadotropins that regulate the endocrine and spermatogenic activities of the testis, and this gonadotropin secretion is subject to feedback regulation. A major difference between male and female reproductive endocrinology is the fact that gamete and steroid hormone production in the male is a continuous process after puberty, and not cyclical as in the female. This is reflected in the absence of a cyclical positive feedback control of gonadotropin release by testicular hormones. The hypothalamus integrates all of these signals, and relays a response via the release of the peptide hormone GnRH. The hormone is released in pulses, with peaks every 90–120 minutes, and travels to the anterior pituitary gland, where it stimulates the synthesis and episodic release of gonadotropic hormones to regulate sperm production in the testes. Interestingly and paradoxically, after the pituitary is initially stimulated to produce these gonadotropins, exposure to constant GnRH (or a GnRH agonist) occupies the receptors so that the signaling pathway is disrupted, and inhibits gonadotropin release.

The process of spermatogenesis is a "two-cell" process, dependent on cross-talk between the Leydig (equivalent to ovarian theca) and Sertoli (equivalent to granulosa) cells via their respective gonadotropin receptors, LH and FSH. Sertoli cells respond to pituitary FSH, and secrete androgen binding proteins (ABPs). Pituitary LH stimulates the interstitial cells of Leydig to produce testosterone, which combines with ABP in the seminiferous tubules, and testosterone controls LH secretion by negative feedback to the hypothalamus, maintaining the high intratesticular testosterone that is appropriate for normal spermatogenesis. Sertoli cells also produce inhibin, which exerts a negative feedback effect on pituitary FSH secretion. It probably also has a minor controlling influence on the secretion of LH.

Although less clearly than in the female, inhibin-like molecules in the male have also been found in testicular extracts, which presumably also regulate FSH secretion. In humans, failure of spermatogenesis is correlated with elevated serum FSH levels, perhaps through reduced inhibin secretion by the testis. In the testis, LH acts on Leydig cells, and FSH on Sertoli cells; males are very sensitive to changes in activity of LH and are relatively resistant to changes in FSH activity.

Gonadotropins in the male

1. Follicle stimulating hormone (FSH): acts on the germinal epithelium to initiate spermatogenesis; receptors are found on Sertoli cells.
   - Sertoli cells secrete inhibin, which regulates FSH secretion.
2. Luteinizing hormone (LH): stimulates the Leydig (interstitial) cells to produce testosterone.

Gonadotropic stimulation of the testes regulates the release of hormones (androgens) that are required for the development of puberty, and then to initiate and maintain male reproductive function and spermatogenesis. Testosterone is the major secretory product of the testes, responsible for male sexual characteristics such as facial hair growth, distribution of body fat/muscle and other "masculine" features. Testosterone is metabolized in peripheral tissue to the potent androgen dihydrotestosterone, or to the potent estrogen estradiol. These androgens and estrogens act independently to modulate LH secretion. In the testis, the androgen receptor (AR) is found on Sertoli cells, Leydig and peritubular myoid cells. AR ablation inhibits spermatogenesis.

Feedback mechanisms are an important part of the reproductive axis; testosterone inhibits LH secretion, while inhibin (secreted by Sertoli cells in the testes) regulates FSH secretion. If negative feedback is reduced, the pituitary responds by increasing its FSH secretion, similar to the situation in women reaching the menopause. Serum FSH levels in the male therefore act as an indicator of testicular germinal epithelial function – i.e., they are broadly correlated with spermatogenesis. Testosterone levels indicate Leydig cell function, and reflect the presence of "masculine" characteristics:

- Low levels in boys and castrates (4 nm/L).
- Varies throughout the day in adult males – highest in the morning.
- Levels decrease in older men.

Serum LH level is difficult to assess, because it is released in pulses. Prolactin also interacts with LH and FSH in a complex manner, via inhibition of GnRH release from the hypothalamus. In males with hyperprolactinemia, inhibition of GnRH decreases LH secretion and testosterone production; elevated prolactin levels may also have a direct effect on the CNS.

Male sexual maturity and reproductive function depends upon appropriate secretion of hormones:

- Gonadotropin releasing hormone (GnRH)
- Luteinizing hormone (LH)
- Follicle stimulating hormone (FSH)
- Testosterone (T)
- Inhibin

Serum endocrinology in the male

An endocrine profile should be performed in men with oligospermia, or if there are signs or symptoms to suggest androgen deficiency or endocrine disease.

1. Testosterone: normal range is 10–35 nmol/L, but serum levels undergo diurnal variation, with highest levels in the morning. Therefore the time of the test is important, and borderline levels should be compared with other measurements taken on different days.
2. FSH and LH: normal serum levels of both are <10 IU/L (but normal ranges for specific laboratories should be noted).
   - Azoospermia with normal T, FSH and LH indicates mechanical obstruction to the passage of sperm.
   - Elevated FSH indicates germinal cell insufficiency.
   - Elevated FSH and LH, low T indicates primary testicular failure.
   - Low FSH, LH and T indicates hypothalamic or pituitary insufficiency.

Female reproductive endocrinology

In females, gonadotropin output is regulated by the ovary.

1. Low circulating levels of estradiol exert a negative feedback control on LH and FSH secretion, and high maintained levels of estradiol exert a positive feedback effect.
2. High plasma levels of progesterone enhance the negative feedback effects of estradiol and keep FSH and LH secretion down to a low level.
3. The secretion of FSH, but not LH, is also regulated by nonsteroidal high molecular weight (around 30 000 kDa) proteins called inhibins found in follicular fluid: inhibin is found at high levels in late follicular phase plasma of fertile women, and although it has been used as a relative marker of ovarian reserve, the direct correlation is not clear.

In an ovulatory cycle, FSH stimulates the growth of follicles and increases the rate of granulosa cell production, the aromatization of androgenic precursors, and the appearance of receptors for LH/hCG on granulosa cells. Estrogens enhance the action of LH on the follicle, and also exert a negative feedback effect on the hypothalamus and pituitary to inhibit further release of gonadotropins. The levels of estrogen produced by the granulosa cells increase throughout the follicular phase until a threshold is reached where a separate positive feedback mechanism elicits the LH surge and overrides the negative feedback on gonadotropin secretion – this is the trigger for ovulation.

Inhibin is also secreted by the ovary, and reduces pituitary FSH secretion.

LH stimulates theca cells to secrete androgens, which are converted to estrogens by the granulosa cells, and also influences granulosa cell differentiation, i.e., limits their replication and causes luteinization. LH stimulates the formation of progesterone directly from cholesterol in both granulosa and theca cells, and after ovulation the follicle changes rapidly from estrogen to progesterone dominance within a short period of time.

Two-cell two-gonadotropin theory

The "two-cell two-gonadotropin" theory suggests that both FSH and LH are required for estrogen biosynthesis, and this requires the cooperation of both theca and granulosa cells, with coordinated expression of a cytochrome P450 enzyme triad: cholesterol side-chain cleavage cytochrome P450, 17-alpha hydroxylase cytochrome P450 and aromatase cytochrome P450.

1. The surface of theca interna cells express LH receptors, and LH binding to these receptors initiates a G-protein cell signaling pathway that leads to production of cAMP. cAMP in turn stimulates the synthesis of androgens from acetate and cholesterol precursors.
2. Androstenedione is released from the thecal cell, and enters the mural granulosa cells, where P450 aromatase converts it to estradiol (E2), which is released into the follicular fluid and the bloodstream. This reaction is enhanced by FSH.

The secreted estradiol is required for follicle maturation and subsequent follicle survival, and these follicles are referred to as "gonadotropin-dependent," since they require both LH and FSH in order to carry out their steroid biosynthesis (Figure 2.2).

Serum endocrinology in the female

A baseline endocrine profile should be performed during the first 3 days of the menstrual cycle; if the patient has amenorrhea or oligomenorrhea, a random blood sample must be used for the profile.

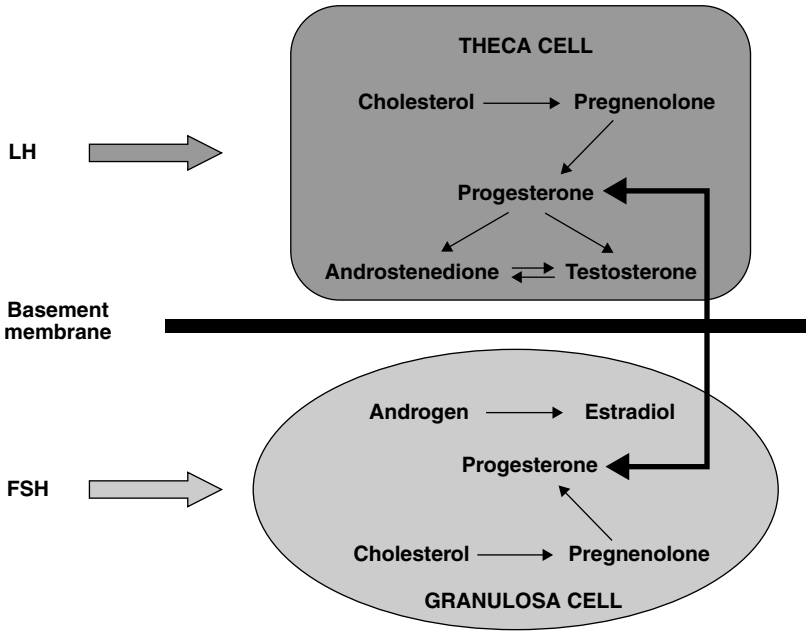

Figure 2.2 Two-cell two-gonadotropin hypothesis.

Hormone	Normal range
Follicle stimulating hormone (FSH)	<8 IU/L
Luteinizing hormone (LH)	<10 IU/L
Estradiol: early follicular	50–200 pmol/L
Mid-cycle	50–1000 pmol/L
Luteal	250–1000 pmol/L
Progesterone mid-luteal	>30 nmol/L
Testosterone	0.5–3.0 nmol/L
Sex hormone binding globulin (SHBG)	16–119 nmol/L
Free androgen index (FAI) (T × 100)/SHBG	<5
TSH	0.5–5.5 mU/L
Total thyroxine	60–160 nmol/L
Prolactin	<600 mIU/L

- Serum FSH levels >15 IU/L indicates poor ovarian activity, and levels >25 IU/L suggest menopause or premature ovarian failure.
- Elevated serum LH suggests the presence of polycystic ovarian disease.
- A serum level of progesterone >30 nmol/L is indicative of ovulation.
- Amenorrhea with very low levels of FSH and LH (2 IU/L) suggests pituitary failure or hypogonadotropic hypogonadism.
- The most usual cause of elevated serum testosterone is polycystic ovary syndrome (PCOS), but levels >5 nmol/L should be investigated to exclude other causes (congenital adrenal hyperplasia, Cushing's syndrome, androgen secreting tumors).
- Mild elevations in serum prolactin are associated with stress, and can occur simply as a result of having blood taken.

Controlled ovarian hyperstimulation (COH) for ART

During controlled ovarian hyperstimulation for assisted reproduction, GnRH agonists are used to suppress pituitary release of both LH and FSH. Follicle growth and development can be achieved by the administration of pure FSH alone, in the absence of exogenous LH. However, in women with hypogonadotropic hypogonadism, who lack both LH and FSH, administration of FSH alone promotes follicular growth, but the oocytes apparently lack developmental competence. The difference between these two patient populations has been attributed to the fact that downregulation with a GnRH agonist leaves sufficient residual LH secretion to support FSH-induced follicular development. The response to FSH in downregulated ART patients is independent of serum LH levels at the time of starting FSH administration. Granulosa cells synthesize estradiol in response to FSH and LH, and estradiol levels per retrieved oocyte appear to

be correlated to developmental competence of the oocytes. Follicular fluid contains high levels of steroids and enzymes, and aspiration of follicles during ART procedures removes this milieu from its natural environment after follicle rupture in vivo; in addition, follicle flushing removes the cells which would have been incorporated in the new corpus luteum. It is possible that this artificial separation leads to luteal insufficiency or other subtle consequences on ovarian physiology which are not at present evident, and progesterone is usually administered to support the luteal phase in downregulated cycles.

GnRH analogues have been used in COH protocols since the mid-1980s, when high tonic levels of LH during the follicular phase were found to be detrimental to oocyte competence, decreasing fertilization and pregnancy rates (Howles *et al*., 1986). GnRH agonists were used to suppress and control the LH surge, and thus the timing of ovulation could be regulated. Their use then led to the development of programmed COH protocols, which provide a convenient and effective means of scheduling and organizing a clinical IVF program: oocyte retrievals can be scheduled for specific days of the week, or in "batches." GnRH analogues have substitutions in their peptide sequence that increase their bioavailability over that of native GnRH, and they bind to receptors on the anterior pituitary so that the receptors are fully occupied, blocking release of FSH and LH. Two types of analogues are currently used in ART protocols:

1. **GnRH agonists**: Continuous administration initially causes LH and FSH hypersecretion (flare-up), and after a period of about 10 days the pituitary store of gonadotropins is depleted. The pituitary is desensitized so that secretion of LH and FSH is suppressed, preventing ovarian steroidogenesis and follicular growth, creating an artificial but reversible menopausal state. Different GnRH agonist preparations can be administered by depot injection (Decapeptyl, Zoladex), daily subcutaneous injection (buserelin), or daily intranasal sniff (nafarelin, Synarel).
2. **GnRH antagonists** bind to and immediately block receptors on the pituitary; there is no initial hypersecretion of gonadotropins, but their release is instead immediately and rapidly suppressed. A third generation of these compounds (cetrorelix, ganirelix) is now used to suppress LH secretion after follicular growth has been first stimulated by gonadotropin administration on Day 1 or 2 of a menstrual cycle (or withdrawal bleed after oral contraceptive pill administration). The antagonist is administered by daily subcutaneous injection from approximately Day 6 of stimulation, or when the largest follicle size reaches 14 mm, and continued until the day of hCG administration. Due to the fact that LH suppression is more complete than with agonist administration, some protocols advocate compensating by simultaneously "adding back" recombinant LH (Luveris) during the period of antagonist treatment.

Several protocols using GnRH analogues have been devised, and individual ART programs apply the same strategy with a variety of different drugs and schedules. Downregulation with a GnRH agonist may begin either in the luteal or the follicular phase ("long protocol') of the previous menstrual cycle, and can be administered with any preparation of choice. It may also be administered from Day 1 of the treatment cycle and continued until ovulation induction with hCG ("short protocol," sometimes also known as "flare-up protocol"). The "ultrashort protocol" uses only three doses of the agonist, on Days 2, 3 and 4 of the treatment cycle. Treatment cycles can also be scheduled by programming menstruation using an oral contraceptive preparation such as norethisterone 5 mg three times a day, and inducing a withdrawal bleed. The "standard" protocols are not always suitable for every patient, and every treatment regimen should be tailored according to the patient's medical history and response to any previous ovarian stimulation. Patients with suspected polycystic ovarian disease (PCO) and those with limited ovarian reserve ("poor responders") require careful management and individualized treatment regimens.

The use of GnRH antagonists is felt to be more physiological ("natural"), since there is no initial suppression of pituitary FSH; however, antagonist cycles require more meticulous monitoring of the cycle in order to prevent a premature LH surge.

Original protocols for ovarian stimulation involved oral administration of clomifene citrate (Clomid), which acts as both an agonist and an antagonist by competitively binding to receptors on the hypothalamus

and pituitary. It displaces endogenous estrogen and eliminates feedback inhibition, which stimulates FSH release from the pituitary. Clomid is administered in a dose of 50–100 mg twice daily for 5 days from Days 2–5 of the menstrual cycle, and FSH injections are commenced on Day 3. This protocol has the disadvantage that it does not block the LH surge, and therefore the cycle must be carefully monitored in order to detect and intercede before an LH surge induces ovulation. However, it may sometimes be useful for patients who have failed to respond to agonist or antagonist protocols.

With any of the COH protocols, a baseline assessment is normally conducted prior to starting gonadotropin stimulation, in order to ensure that the ovaries are quiescent and the endometrial lining has been shed, as well as to exclude any pathologies that might jeopardize the treatment cycle.

Stimulation protocols for IVF

1. Clomifene citrate (CC)/Gonadotropins

- CC 100 mg from Day 2, for 5 days.
- Gonadotropin stimulation from Day 4 to day of hCG.

2. Long GnRH agonist protocols

a. Luteal phase start (7 days after presumed ovulation, approx. Day 21)

- GnRH agonist from Day 21 until menses.
- Start gonadotropins after baseline assessment, continue to day of hCG.
- Reduce GnRH agonist dose after start of stimulation, continue until day of hCG.

b. Follicular phase start

- Start GnRH agonist Day 2 after menses.
- Continue until downregulation, usually at least 14 days.
- Start gonadotropin stimulation.
- Reduce GnRH dose after start of stimulation, continue to day of hCG.

3. Short GnRH agonist protocol

- Start GnRH agonist on Day 2 after menses, continue to day of hCG.
- Gonadotropin stimulation from Day 3 to day of hCG.

4. Ultrashort GnRH agonist protocol

- Start GnRH agonist on Day 2 after menses, continue for 3 days.
- Gonadotropin stimulation from Day 3 to day of hCG.

5. GnRH antagonist protocol

- Gonadotropin stimulation from Day 2 until day of hCG.
- Start antagonist after 6 days of stimulation, or when largest follicle size = 14 mm.
- Continue antagonist until day of hCG.

Baseline assessment: ultrasound

- Ovaries: size, position
- Shape, texture
- Cysts
- Evidence of PCO
- Uterus: endometrial size, shape, texture and thickness
- Fibroids
- Congenital or other anomalies/abnormalities
- Hydrosalpinges, loculated fluid

Baseline assessment: endocrinology

- Estradiol: < 50 pg/mL
- LH: < 5 IU/L
- Progesterone: < 2 ng/mL

If any values are elevated:

- continue GnRH agonist treatment
- withhold stimulation
- reassess 3–7 days later.

If LH remains elevated:

- withhold stimulation
- increase dose of GnRH agonist.

(FSH: < 10 IU/L without downregulation.)

Ovarian stimulation

Pure FSH (Gonal F, Puregon, Menopur) by subcutaneous self-injection. Starting dose according to age and/or history:

- age 35 or younger: 150 IU/day
- age over 35: 225 IU/day
- depending on previous response, up to 300–450 IU daily.

Begin monitoring after 7 days of stimulation *(adjusted according to history and baseline assessment*).

Cycle monitoring

Assess follicular growth after 6–8 days of gonadotropin stimulation.

- Ultrasound assessment

  - Follicle size 14 mm or less: review in 2 or 3 days
  - Follicle size 16 mm or greater: review daily
- Plasma estradiol
- Plasma LH
  - Review as necessary.

Induction of ovulation

hCG (Profasi) 10 000 IU or r-hCG (Ovitrelle/Ovidrel 6500 IU) by subcutaneous injection when:

- Leading follicle is at least 17–18 mm in diameter
- Two or more follicles >14 mm in diameter
- Endometrium: at least 8 mm in thickness with trilaminar "halo" appearance
- Estradiol levels approx. 100–150 pg/mL per large follicle
- Oocyte retrieval scheduled for 34–36 hours post-hCG.

Oocyte retrieval (OCR) procedures are performed by vacuum aspirating follicles under vaginal ultrasound guidance using disposable OCR needles, collecting aspirates into heated 15 mL Falcon tubes. The procedure can be safely carried out as an outpatient procedure, using either paracervical block for local anesthesia, intravenous sedation or light general anesthesia. An experienced operator can collect an average number of oocytes (i.e., 8–12) in a 5–10 minute time period, and the patient can usually be discharged within 2–3 hours of a routine oocyte collection

Luteal phase support

Hormonal support of the luteal phase is felt to be necessary following pituitary downregulation with GnRH agonist treatment, and is usually also used in antagonist cycles. Progesterone supplementation may be introduced on the evening following oocyte retrieval:

- Cyclogest pessaries per vaginam, 200–400 mg twice a day
- Utrogestan capsules per vaginam, 100–200 mg three times a day
- Crinone gel per vaginam, once daily application
- Gestone 50–100 mg daily by intramuscular injection.

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Chapter 3

Gametes and gametogenesis

Gamete precursor cells: primordial germ cells

After a blastocyst has implanted in the uterus and begins to differentiate into the three primary germ layers, a special population of cells develop as primordial germ cells (PGCs). These are destined to become the gametes of the new individual: future reproduction of the organism is absolutely dependent upon the correct development of these unique populations of cells. They originate immediately behind the primitive streak in the extraembryonic mesoderm of the yolk sac; towards the end of gastrulation they move into the embryo via the allantois, and temporarily settle in mesoderm and endoderm of the primitive streak. In humans, PGCs can be identified at about 3 weeks of gestation, close to the yolk sac endoderm at the root of the allantois. These cells have many special properties in terms of morphology, behavior and gene expression, and undergo a number of unique biological processes:

- Lengthy migration through the developing embryo to the gonadal ridge
- Erasure of epigenetic information from the previous generation
- Reactivation of the X chromosome that has been silenced (Barr body) in XX cells.

Migration of primordial germ cells

PGCs proliferate by mitosis, and begin to migrate through the embryonic tissue, completing approximately six mitotic divisions by the time they colonize the future gonad (Figure 3.1).

Proliferation and migration continue for 3–4 weeks in humans, and during their migration the germ cells and the somatic cells interact together via a number of different types of signals. The PGCs move to the inside of the embryo along with the gut to embed in the connective tissue of the hindgut, migrate through the dorsal mesentery along the hindgut a few days later, and then finally populate the gonadal ridge to form the embryonic gonad. The tissue of the gonadal ridge makes up the somatic (non-gamete) part of the gonads. In humans, PGCs can be seen in the region of the developing kidneys (the mesonephros) approximately 4 weeks after fertilization (gestational age 5–6 weeks), and their migration is completed by 6 weeks of gestation. Primordial gonads can be identified on either side of the central dorsal aorta between 37 and 42 days after fertilization (gestational age 8–9 weeks), as a medial thickening of the mesodermal epithelium that lines the coelom (body cavity).

PGC in the mouse

- During their migration in the mouse, the population of PGCs increases from around 100 cells to 25 000 cells by stage 13.5 of embryonic development (E13.5).
- The genital ridges may secrete a chemotactic substance (probably SDF-1, stromal cell derived factor 1 and its receptor CXCR4) that attracts the PGCs: primordial gonadal tissue grafted into abnormal sites within a mouse embryo attracts germ cells to colonize it.
- Experiments using gene knock-out animal models have identified a number of genes involved in regulating the establishment and migration of PGCs, the signals involved in their movement, and their renewal properties (see MacLaren, 2003).

Gonadogenesis: from primordial germ cells to gametes

The primordial gonadal ridges develop on the posterior wall of the lower thoracic and upper lumbar regions, and in both sexes this undifferentiated mesenchymal

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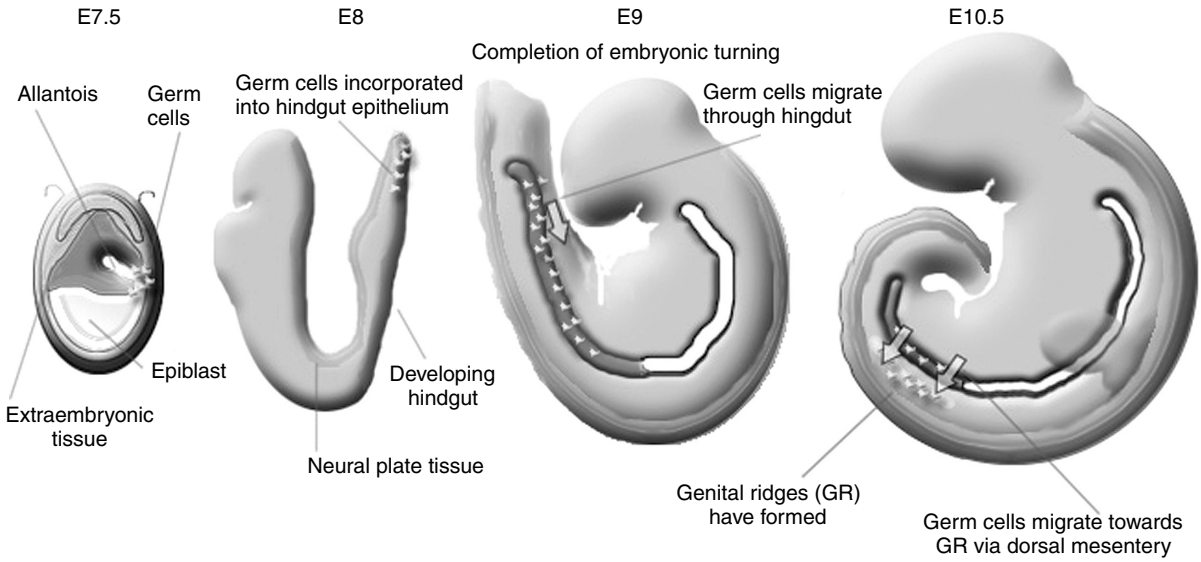


Figure 3.1 Migration of primordial germ cells in the mouse. (Adapted diagram courtesy of J. Huntriss, University of Leeds. Modified from Starz-Gaiano, M. and Lehmann, R. 2001. Moving towards the next generation. *Mechanisms of Development* 105: 1–2, with permission from Elsevier Ltd.) PGCs arise at the start of gastrulation, around embryonic Days 7–7.25 (E7–7.5) at the border of the extraembryonic tissue and the epiblast, at the root of the allantois. The PGCs can be identified and distinguished from the surrounding somatic cells by their positive staining for alkaline phosphatase. Expression of the OCT4 transcription factor becomes restricted to PGCs around day E8 and is used as a PGC marker. See color plate section.

tissue forms the basic matrices of the testes and ovaries. At approximately 6 weeks of gestation the developing gonad appears identical in male and female embryos, and remains sexually undifferentiated for a period of 7–10 days. During this period, groups of cells derived from the columnar coelomic (germinal) epithelium surrounding the genital ridge migrate into the undifferentiated tissue as columns to colonize the gonad – these are known as the primitive sex cords. Key morphological changes then start to take place in the gonads, which depend upon whether or not the Sex determining Region Y (SRY) gene on a Y chromosome is expressed in the cells of the sex cords. These morphological changes result in the formation of:

1. Genital ducts
   - Wolffian duct = male, precursor of the vas deferens and epididymis
   - Müllerian duct = female, precursor of the uterus, the upper parts of the vagina and the oviducts.
2. Urogenital sinus.

Knock-out mouse technology has identified a number of genes involved in these early stages of gonadogenesis, some of which are outlined in Figure 3.2, and summarized here:

1. SRY expression is upregulated by only one isoform of Wilms' tumor gene product WT1(–KTS).
2. The WT1(–KTS) isoform also upregulates DAX1 expression (which antagonizes development of Sertoli cells). The WT1(–KTS) isoform is therefore considered essential for development of both the male and the female gonad.
3. The WT1(+KTS) isoform increases the number of SRY transcripts, and is required for formation of the male gonad.
4. WNT4 acts to repress migration of mesonephric endothelial and steroidogenic cells in the XX gonad, preventing the formation of a male-specific coelomic blood vessel and the production of steroids. WNT4 expression is downregulated after sex determination in the XY gonad.
5. DAX1 may inhibit SRY indirectly by inhibiting expression of male-specific genes that are activated by SF1.
6. SRY upregulates expression of a related transcription factor, SOX9. SOX9 is required for activation of anti-Müllerian hormone (AMH)/Müllerian inhibitory substance (MIS), which causes regression of the female Müllerian duct.

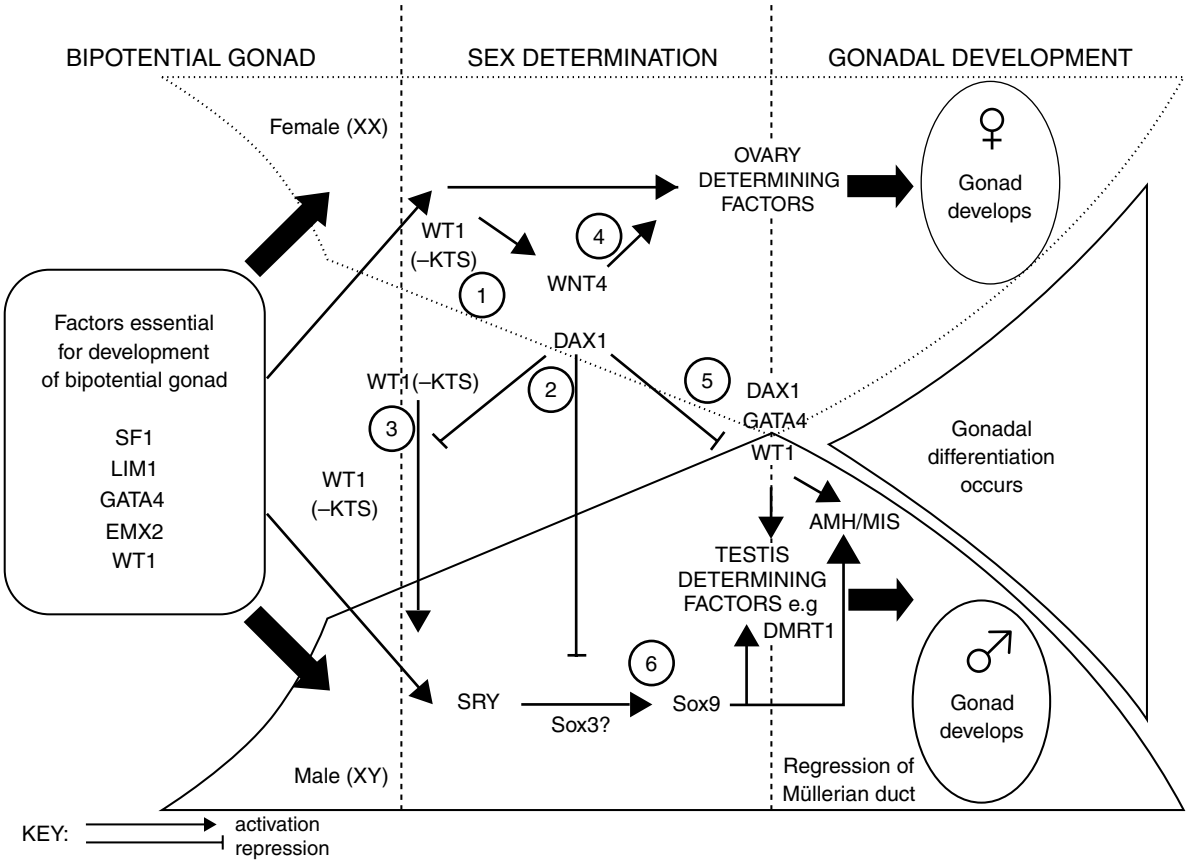


Figure 3.2 Genes involved in gonadogenesis; key points are circled, outlining the complex regulatory molecular pathways involved (Adapted from Clarkson MJ, Harley VR, 2002)

7. Dmrt1 is thought to interfere with the action of SOX9. Dmrt1 is a candidate sex-determining gene in birds, carried on their Z chromosome.

Sex determination

A classic experiment by Alfred Jost in the 1940s demonstrated that mammalian embryos castrated prior to differentiation of the testis appear to develop phenotypically as females. This established that the female route of sexual development is the default differentiation pathway, and led the authors to propose the existence of a testis-determining factor (TDF). This has now been established by experiments on early embryos, and by molecular experiments. Genetic studies also suggest that ovarian differentiation and development may be regulated by certain "anti-testis" factors:

- In XY humans who carry a duplication of part of the small arm of the X chromosome (Xp21) (and in XY mice of certain genetic backgrounds), overexpression of the DAX1 gene causes sex reversal; i.e., these human or mouse individuals develop as females. Therefore, DAX1 can apparently antagonize SRY in a dosage-sensitive manner to cause sex reversal.
- Wnt4 is also required for female development. Genetic studies in the mouse show that:
  - Wnt4 is initially required for the formation of the Müllerian duct in both sexes
  - In the developing ovary, Wnt4 suppresses the development of Leydig cells.
  - In Wnt4 mutants, the Müllerian duct is absent, and the Wolffian duct develops further.

Wnt4-mutant females activate testosterone biosynthesis and become masculinized.

Knock-outs of SF1, Lim-9 and Wnt1 genes develop as phenotypic females, in support of Jost's proposal.

Development of the testis

After the mesonephros has been populated with primordial germ cells to form the genital ridge, the coelomic

epithelium proliferates at a faster rate in male than in female gonads, and the cells penetrate deep into the medullary mesenchyme to form the testis cords. Two different testicular compartments are formed: the testicular cords, and the interstitial region. Expression of the SRY gene initiates differentiation of Sertoli cells, and the developing Sertoli cells produce a growth factor, fibroblast growth factor 9 (FGF-9) that is necessary for formation of the testicular cords. At 7–8 weeks of gestation, the testicular cords (precursor of the seminiferous tubules) can be seen in histological sections as protrusions of the cortical epithelium into the medulla; animal experiments indicate that germ cells are not involved in this process.

- Sertoli cells cluster around the germ cells; peritubular myoid cells surround the clusters, and deposit the basal lamina.
- Sertoli cells secrete AMH/MIS, which suppresses the default pathway that would develop Müllerian ducts as precursors of female sexual anatomy. The Sertoli cells continue to secrete AMH throughout fetal and postnatal life until the time of puberty, when the levels drop sharply.
- Leydig cells remain in the interstitial region, close to blood and lymphatic systems, and they actively secrete androgens from at least 8–10 weeks of gestation. This capacity to secrete testosterone is essential for continued testicular development, and, ultimately, for the establishment of the male phenotype.
- Testosterone causes growth and differentiation of the Wolffian duct structures (precursor of the male sexual anatomy).
- Dihydrotestosterone (a metabolite of testosterone) induces virilization of the urogenital sinus and the external genitalia.

The Müllerian duct then regresses, and the Wolffian duct develops further.

By 16–20 weeks of fetal life, Sertoli cells and relatively quiescent prospermatogonial cells lie on a basement membrane within seminiferous cords; these are within a vascularized stroma that also contains condensed Leydig cells, and the entire structure is enclosed within a fibrous capsule, the tunica albuginea. The testes gradually increase in size until the time of puberty, and with the onset of puberty they begin to rapidly enlarge:

- The solid cords canalize to give rise to tubules, which eventually connect to the rete testis, the vasa efferentia, and then the epididymis.
- Leydig cells significantly increase their endocrine secretion, and intratubular Sertoli cells also increase in size and activity.
- Prospermatogonial cells in the cords now line the seminiferous tubules as spermatogenic epithelium, and begin to divide by mitosis.

Testicular descent

The testes develop initially in the upper lumbar region of the embryo, and gradually migrate during fetal life through the abdominal cavity and over the pelvic brim. This descent is influenced by hormones secreted by the Leydig cells, and involves two ligamentous structures: the suspensory ligament at the superior pole, and the gubernaculum at the inferior pole of the testis. As the fetal body grows in size, the suspensory ligament elongates and the gubernaculum does not, so that the position of the testis becomes localized to the pelvis. Between weeks 25 and 28 of pregnancy, the testes migrate over the pubic bone, and reach the scrotum via the inguinal canal by weeks 35–40. As a result of their external position outside the body cavity, the temperature of the testes is approximately 2°C below body temperature, optimal for spermatogenesis.

Genetic control of testis development

- SRY, SOX9, WT1, XH2, SF1, and DAX1 are known to be involved in the control of testis determination. Many of these genes have been identified through analysis of cases of sex reversal.
- The SRY gene is a key switch in male sexual differentiation; it acts only briefly in male embryos, to initiate differentiation of the Sertoli cells in the somatic cells of the genital ridge.
- SRY may function either:
  - As a transcriptional repressor to repress activation of the genes that cause differentiation of the ovary
  - As a repressor of the factors that repress testis development
  - Synergistically with SF1 to activate SOX9.

The adult testes contain approximately 200 m of seminiferous tubule, forming the bulk of the volume of the testis. These tubules are the site of spermatogenesis. The round tubules are separated from each other by a small amount of connective tissue that contains, in addition to blood vessels, a few lymphocytes, plasma cells and clumps of interstitial Leydig cells. The tubules are lined by spermatogenic epithelium, which

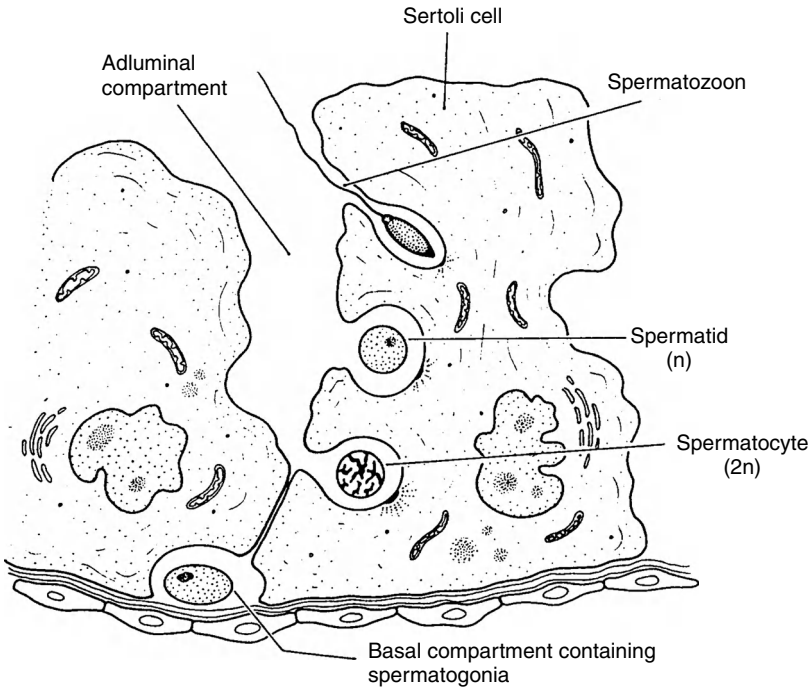


Figure 3.3 Spermatogenesis in the mammal. Maturation and modeling of the male gamete is regulated by the Sertoli cell. Modified after Johnson (2007).

is made up of spermatogonia at different stages of maturation – a cross-section of any normal seminiferous tubule reveals four or five distinct generations of germ cells. The younger generation cells are on the basement membrane, and the more differentiated cells approach the lumen of the tubule. This growth pattern has a wavelike cycle with intermingling of different stages that lie close to each other – any single cross-section of the tubule does not always reveal all generations of spermatogenesis. The tubules rest on a delicate anuclear basement membrane that in turn lies on a connective tissue layer, the tunica propria. The supporting Sertoli cells, which are believed to nourish the germ cells, form a continuous layer connected by tight junctions. These large polymorphous cells have large, pale nuclei, and abundant cytoplasm that extends from the periphery of the tubule to the lumen, stretching through the layers of developing germ cells. Mature spermatozoa can be seen attached to and surrounding the Sertoli cells prior to their release (Figure 3.3). A wave of spermatogenesis passes along the tubule, and the process of development from spermatogonium to spermatozoon takes approximately 65 days. A transverse cross-section through the human testis shows tubules containing cells at many different stages in spermatogenesis (in contrast to the rat testis, where every tubule has cells at the same stage). In humans, many seminiferous tubules can be seen that are apparently without spermatocytes and spermatids, a phenomenon that may contribute to the relatively poor efficiency of spermatogenesis.

Sperm released into the lumen of the seminiferous tubules pass via the rete testis through the ductuli efferentia into the caput epididymis. They traverse the epididymis over a period of 2–14 days, undergoing a number of changes in preparation for fertilization, and are then stored sequentially in the cauda, vas, seminal vesicles and ampullae prior to ejaculation. The seminal vesicles, prostate and urethral glands add glandular secretions to the sperm at the time of ejaculation. Figure 3.4 illustrates the anatomy of the adult mammalian testis.

Spermatogenesis

The process of spermatogenesis can be divided into three phases, and each phase is associated with a specific type of germ cell:

1. Proliferation (spermatocytogenesis): spermatogonia
2. Reduction division (meiosis): spermatocytes
3. Differentiation (spermiogenesis): spermatids

In the fetal testis, primordial germ cells differentiate into spermatozoal stem cells, type A (A0) spermatogonia.

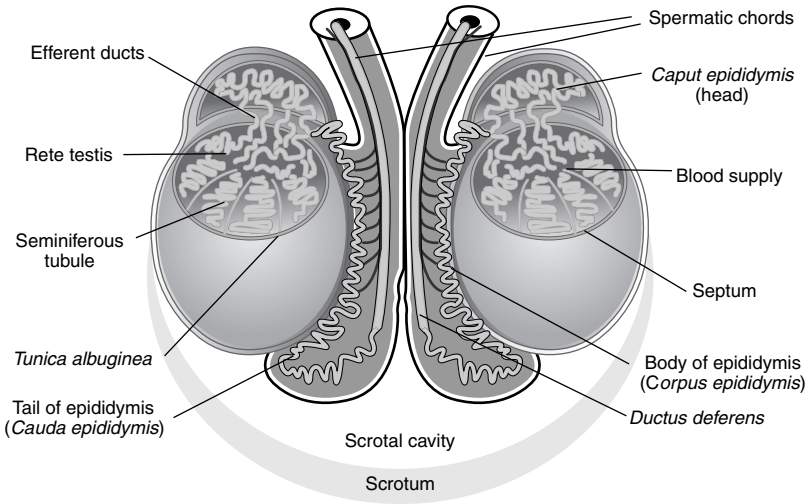


Figure 3.4 Anatomy of the adult mammalian testis. (Drawing adapted from a number of sources.)

These stem cells line the basement membrane of the seminiferous tubules, have large spherical or oval nuclei, and are connected to each other via intercellular bridges to form a germ cell syncytium. They are in contact with Sertoli cells, which extend from the epithelium into the lumen of the tubules. At puberty, the spermatogonia start to proliferate by mitoses; this is followed by meiosis and a gradual reorganization of cellular components and a loss of cytoplasm.

Spermatocytogenesis

- At intervals after puberty, stem cells in the germinal epithelium of the seminiferous tubules (*type A spermatogonia*) replicate their DNA and divide by mitosis.
- Each mitotic division produces two cells: another type A spermatogonium, and a second cell, *type B spermatogonia*; these move into the adluminal compartment and start their differentiation by entering into meiosis.

Meiosis

In the adluminal compartment, the cells undergo two meiotic divisions to form two daughter secondary spermatocytes initially, and eventually four early spermatids. Through a series of different phases, meiosis (reduction division) converts diploid stem cells (spermatogonia) containing 46 chromosomes into haploid gametes, with 23 chromosomes. In the first phase of meiosis, type B spermatogonia (2n:2c) become primary spermatocytes (1n:2c). These cells divide again, to become secondary spermatocytes (1n:1c). The cells go through this stage quickly, and complete the second meiotic division. After the second meiotic division, the cells are known as spermatids. These cells must now go through a process of maturation (spermiogenesis) in order to finally emerge as mature spermatozoa (1n:1c).

Spermiogenesis

Spermatid differentiation occurs in four stages (Figure 3.5):

1. Golgi phase
2. Cap phase
3. Acrosomal phase
4. Maturation phase.

Spermatid nuclei now contain haploid sets of chromosomes. Their autosomes continue to direct the synthesis of low levels of rRNA, mRNA and proteins as they enter into their prolonged phase of terminal differentiation, spermiogenesis. During this phase, round spermatid cells are converted into elongated sperm cells with a condensed nucleus and a flagellum. They do not divide again, either by mitosis or meiosis, but must differentiate to acquire functions that will allow them to traverse the female tract and fertilize an oocyte. This differentiation process takes approximately 2 weeks in most species, and follows well-defined stages:

1. Spermatid DNA becomes highly condensed, and somatic histones are replaced with protamines.
2. The acrosome, a sac containing enzymes necessary for oocyte penetration, is constructed from Golgi membranes.

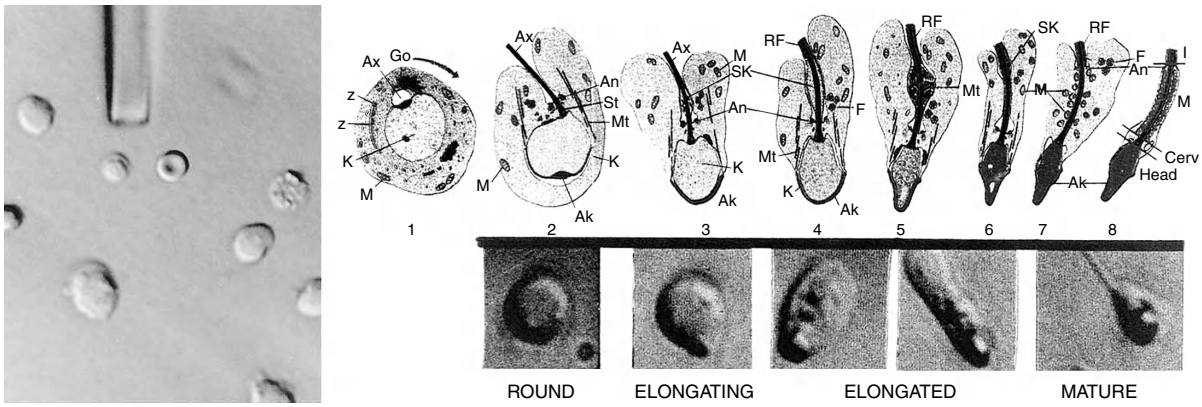


Figure 3.5 Developing sperm. (Courtesy of M. Nijs and P. Vanderzwalmen, Belgium.) A = acrosome; An = annulus; Ax = axoneme; C = centriole; F = flowerlike structures; Fs = flagellar substructures; M = mitochondria; Mp = middle piece; Mt = manchette; Ne = neck; PP = principal piece; R = ring fibers; Sb = spindle-shaped body.

3. Cytoplasmic reorganization gives rise to the midpiece, which contains mitochondria and associated control mechanisms necessary for motility.
4. The flagellar apparatus (tail) is formed, which will make the cells motile.
5. A residual body casts off excess cytoplasm.

Sperm modeling is probably regulated by the Sertoli cells, and the cells are moved to the center of the tubular lumen as spermatogenesis proceeds. The rate of progression of cells through spermatogenesis is constant, and is not affected by external factors such as hormones. The timing of stored mRNA translation is a major point of control: for example, the protamine1 gene is transcribed in round spermatids, and the resulting mRNA is stored for up to 1 week before it is translated in elongating spermatids. Other mRNAs are stored for only hours or a few days, indicating that there must be a defined temporal program of translational control.

- Sertoli cells are joined by tight junctions, and act as nurse cells during spermatogenesis.
- Tight junctions restrict the passage of substances from the blood to the lumen of the seminiferous tubules and therefore form a blood–testis barrier. This barrier protects sperm from antibodies circulating in the bloodstream.

Molecular features of spermatogenesis

- The trigger that determines which spermatogonia become committed to meiosis is not known.
- DNA is transcribed from both diploid (proliferative type A (A0) spermatogonia) and haploid (committed type B [A1] spermatogonia) genomes throughout the process.
- Only type B spermatogonia undergo differentiation into spermatozoa, and the vast majority of the germ cell cytoplasm is lost during the terminal stages of differentiation, when the spermatids condense into spermatozoa.
- The reduction process that generates haploid sperm cells takes at least 65 days in humans, and involves six successive stages over four consecutive spermatogenic cycles.
- Note the nomenclature relating to chromosome complement (n) and DNA copy number (c). 1c is the DNA copy number of a haploid gamete, and no gamete is ever tetraploid.

Epididymal maturation

Mammalian spermatozoa leaving the testis are not capable of fertilizing oocytes. They gain this ability while passing down the epididymis, a process known as epididymal maturation. The epididymis is divided into different regions: the caput is the upper third, formed by efferent ductules that are lined by pseudostratified columnar ciliated epithelium (such as is found in nasal and bronchial passages – patients with upper epididymal obstruction often have associated nasal or respiratory problems, as in mucoviscidosis, Young's syndrome). The vasa efferentia tubules unite to form the single coiled tubule of the corpus, which has flatter, non-ciliated epithelium and microvilli on the luminal surface. It starts to form a muscular wall towards the cauda, where the lumen is wider, and spermatozoa can be stored prior to ejaculation.

During its journey through the different regions of the epididymis, the head of the spermatozoon acquires the ability to interact with the zona pellucida, with an increase in net negative charge. Many antigens with a demonstrable role in oocyte binding and fusion are synthesized in the testis as precursors, and then activated at some point in the epididymis either through direct biochemical modification, through changing their cellular localization, or both. Examples of such antigen processing include a membrane-bound hyaluronidase, fertilin, proacrosin, 1,4-galactosyltransferase (GalTase) and putative zona ligands sp56 and p95. The terminal saccharide residues of membrane glycoproteins and lipids also undergo changes in their physical and chemical composition.

Although all the necessary morphological structures for flagellar activity are assembled during spermiogenesis, testicular spermatozoa are essentially immotile, even when washed and placed in a physiological solution. Spermatozoa from the caput epididymis begin to display motility, and by the time they reach the cauda they are capable of full progressive forward motility. Demembranation and exposure to ATP, cAMP and Mg^{2+} triggers movement, which suggests that the ability to move is probably regulated at the level of the plasma membrane. Transfer of a forward motility protein and carnitine from the epididymal fluid are believed to be important for the development of sperm motility. Since the osmolality and chemical composition of the epididymal fluid varies from one segment to the next, it may be that the sperm plasma membrane is altered stepwise as it progresses down the duct, and motility is controlled by an interplay between cAMP, cytosolic Ca^{2+} and pH. During maturation, the spermatozoa use up endogenous reserves of metabolic substrates, becoming dependent on exogenous sources such as fructose; at this point they shed their cytoplasmic droplet.

Pathology affecting spermatogenesis

- A number of different pathologies can disrupt the orderly pattern of spermatogenesis, causing immature forms, especially spermatocytes to slough into the tubular lumen. Less frequently, maturation may proceed to the spermatid stage and be arrested there.
- Any lesion that causes generalized arrest of maturation, or a mixture of maturation arrest and atrophy to a stage preceding spermiogenesis, will result in azoospermia.
- The tubular epithelium is very sensitive to toxins and to ischemia; damage may result in partial, focal or total obliteration of the spermatogenic epithelium, including the Sertoli cells, while the Leydig cells remain functionally normal.
- In cases of severe injury, the tubules may be totally destroyed and become hyalinized, or may be replaced by fibrous tissue. Since the whole tubule is destroyed, this disorder is associated with a much reduced testis size, with absence of Sertoli cells resulting in raised serum FSH.
- Focal lesions can cause oligospermia of varying severity, and patients with focal lesions may have normal levels of FSH in their serum.

DNA packaging in sperm

The amount of DNA in the sperm nucleus (approximately 1 m in length) has to be packaged into a volume that is typically less than 10% of the volume of a somatic cell nucleus; a different mechanism of packaging is required, as illustrated in Figure 3.6.

- Somatic cell DNA is packaged into nucleosomes by a process of primary compaction that uses histones. A 10 nm fiber is supercoiled into the 30 nm solenoid, and supercoiled again into loops (A–D). These loops are the major structural form of interphase chromatin.
- During spermatogenesis, DNA is initially packaged by histones (as in A–D) but following meiosis, at the secondary spermatocyte stage of spermiogenesis, histones are replaced first by transition proteins and then by protamines (G). The solenoid structure is replaced by torroids (doughnut shapes), which are in turn supercoiled into torroidal loops. This highly compacted structure shuts down transcription during spermiogenesis. The loop domains drawn in E and J represent the chromatin state in the interphase somatic cell nucleus (E) and the sperm nucleus (J). Sperm appear poised for transcription.

Gene expression during spermatogenesis

Gene expression during spermatogenesis can be subdivided into two distinctive phases:

1. Prior to meiosis, all stages up to and including the completion of telophase: diploid cells.
2. During spermiogenesis: late secondary spermatocytes are haploid cells, and the developmental process from this stage onwards is referred to as spermiogenesis.

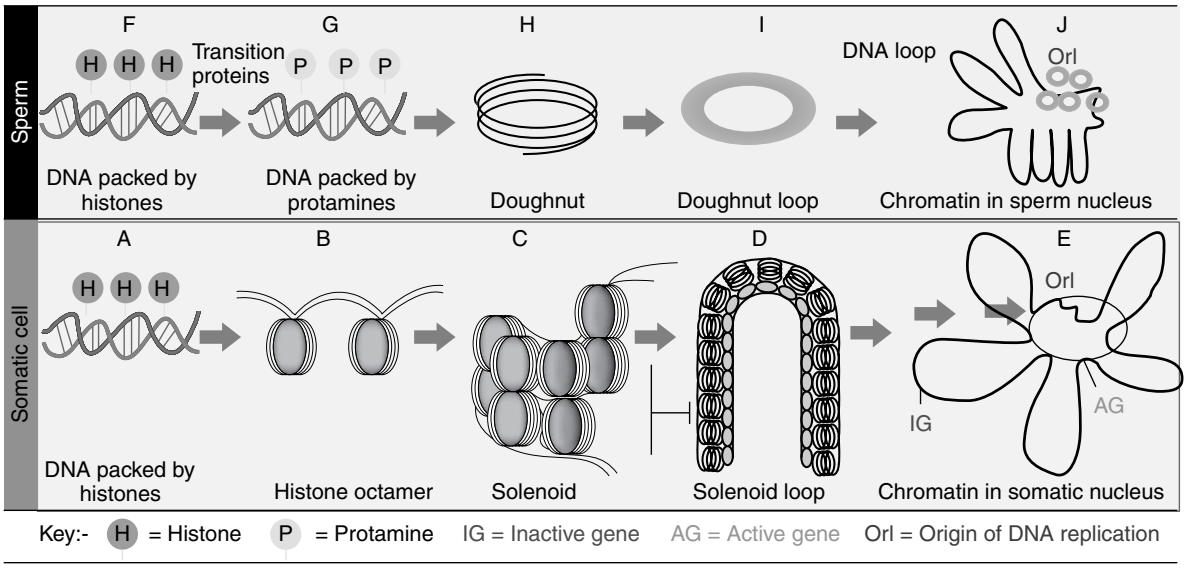


Figure 3.6 DNA packaging in somatic cells and spermatozoa. (Image adapted from Ward, 1993.) See color plate section.

Genes are expressed from both the diploid and the haploid genomes. DNA transcription is often coordinated with mRNA translation into its protein product (e.g., histones, X-linked lactate dehydrogenase). In spermiogenesis, however, transcription may be shut down well before the protein appears, i.e., the mRNA is translated later (e.g., PGK2, acrosin). As spermatogenesis progresses, the transcripts encoding the same protein differ in size, due to alterations in the length of the mRNA polyA tail (a similar phenomenon occurs in oogenesis, as described later).

Control of gene expression during spermatogenesis

- Between primitive spermatogonial and final mature spermatozoa, cellular chromatin is restructured so that certain genes are repressed, potentiated, or potentiated and transcribed.
- Phosphoglycerokinase 1 gene (PGK1), an essential glycolytic enzyme, lies on the X chromosome, and this gene is highly expressed, potentiated and transcribed early in spermatogenesis. As the cells progress into meiosis, the X chromosome becomes progressively inactivated, and during spermiogenesis expression of PGK1 is replaced by expression of an autosomal homologue, PGK2.
- During spermiogenesis (the haploid stages of spermatogenesis), cellular histones are replaced first by testis-specific histones and then by the transition protein TNP2 and the protamines PRM1 and PRM2. This substitution is a prerequisite for the extremely compact packaging of sperm DNA. These genes are located on chromosome 16.
- Not all histones are replaced. Human sperm retain approximately 15–20% of their chromatin in a nucleosomal configuration, and we now know that mature sperm cells retain a complex repertoire of mRNAs that may be involved in embryogenesis.

The ultimate aim of the male reproductive system is to parcel the male genetic package, a set of 23 chromosomes, into the head of a single spermatozoon, and deliver this to the female reproductive tract, in the right place at the right time. However, in order to fertilize the oocyte and initiate embryonic development, the spermatozoon must also contribute two epigenetic factors: an oocyte activating factor and the centrosome or cell division mechanism (see Chapter 4).

Development of the ovary

In female embryos, primordial germ cells containing two X chromosomes migrate from the genital ridge to the primordial gonad, and these are known as oogonia. The sex cords, instead of penetrating deeply into the genital ridge as in males, condense as small clusters around the PGCs, and these clusters of cells initiate the formation of primordial follicles (Figure 3.7). Cells of the cortical sex cords will form the somatic components of the follicle: granulosa, theca, endothelial cells and supporting connective tissue. Once they reach the gonadal ridge (approximately Days 25–30), the oogonia start to replicate by mitosis for a limited period. Cells from the mesonephros invade to form the ovarian medulla, forcing the germ cells towards

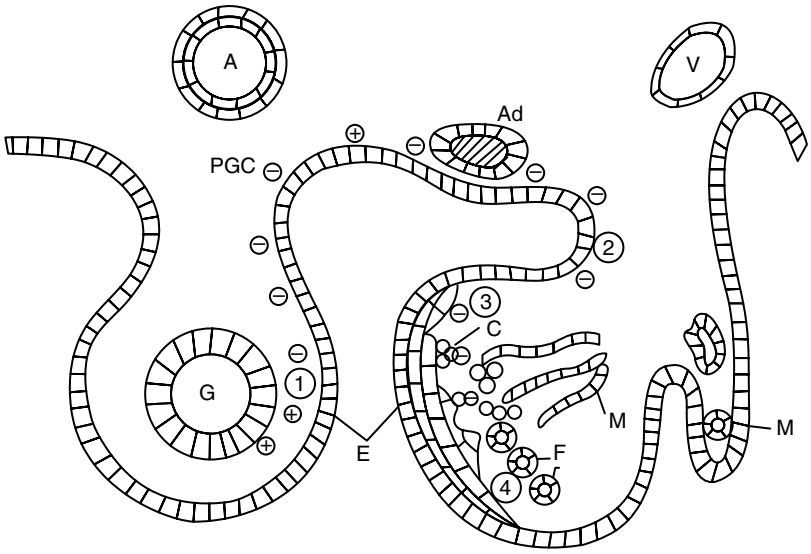
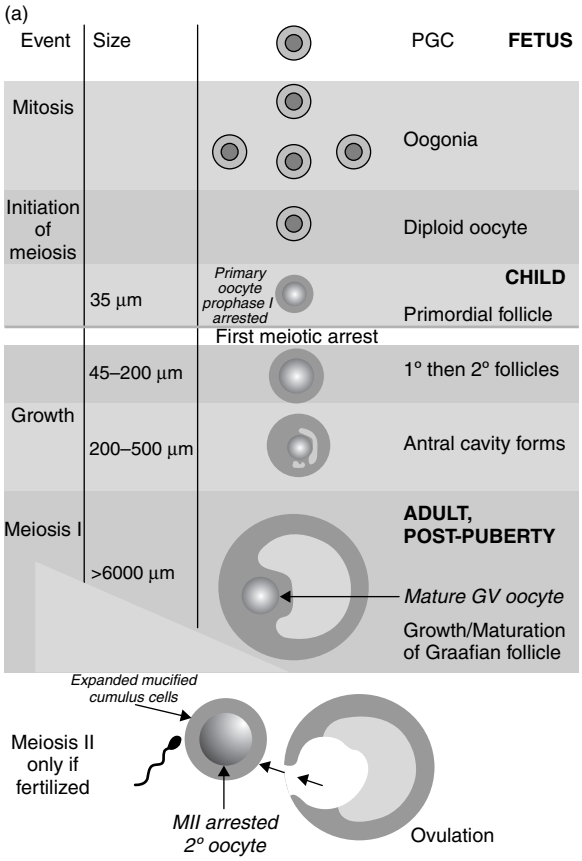


Figure 3.7 Development of the human ovary from PGC. (With permission from Gosden, R. 1995. Ovulation 1: Oocyte development throughout life, in J.G. Grudzinskas, Jurgis Gediminas Grudzinskas, John Yovich (eds.) *Gametes: The Oocyte*. Cambridge: Cambridge University Press.)
PGCs travel along the gut (G) mesentery (1) to the gonadal ridge (2), and after proliferation and migration become associated with cortical cords (C, 3). They begin meiosis and become enveloped within follicles (F, 4). Ad = adrenal gland; A = aorta; V = cardinal vein; E = coelomic epithelium; M = mesonephric tubules and duct.

the ovarian cortex. Whereas in male embryos spermatogonia do not start to enter meiosis until the onset of puberty, in females the oogonia start to enter into their first meiotic division around the twelfth week of gestation, at the end of the first trimester. In humans, the population of oogonia is estimated to increase from a pool of around 600 000 at 8 weeks to a maximum of 6–8 million at 16–20 weeks. At this stage they become primary oocytes, and do not replicate further by mitosis. Oocytes that are not incorporated into follicles degenerate, and thus the number of oocytes is then reduced to around 1–2 million at birth, when the ovary is now populated with its full complement of oocytes.

After the oogonia enter meiosis I, they arrest in the diplotene stage of prophase I, after chromatid exchange and crossing-over (diakinesis) have taken place – the last phase of prophase I (see Chapter 1, Figure 1.9). These arrested oocytes are said to be in the dictyate (germinal vesicle) stage. The chromosomes disperse, and appear as visible chromosomal threads packaged within a large nucleus, the germinal vesicle (GV). The first meiotic prophase stage can be seen at around 9–10 weeks, and diplotene stage chromosomes are apparent around 16 weeks, during the second trimester of pregnancy. The oocytes remain arrested at this stage until the onset of ovulatory cycles at puberty: subsequent developmental stages that lead to the resumption of meiosis are not completed unless the Graafian (antral) follicle is recruited after puberty. The process of oogenesis, from primordial germ cell to pre-ovulatory oocyte, takes a minimum of 11 years; human oocytes complete meiosis only after fertilization.

The oogonia within the embryonic ovary are initially arranged into clusters called syncytia, which are connected by intercellular bridges. Organelles, mitochondria and other cellular factors are probably exchanged through these connections. These syncytia are programmed to break down on a large scale during fetal life, and this is followed by the formation of primordial follicles. A single layer of pre-granulosa cells surrounds a single oogonium; once a complete cell layer has been formed around individual oocytes, the surrounding stromal cells secrete type IV collagen, laminin and fibronectin. These proteins form a thin basement membrane around each cluster of cells, and a discrete population of primordial follicles is formed. Each follicle has an oocyte arrested in prophase I of meiosis, surrounded by a single layer of flattened stromal pre-granulosa cells that are linked to the oocyte by gap junctions and other cellular connections. The primordial follicles become localized to the peripheral region of the ovarian cortex, and remain there in a quiescent state for many years (Figure 3.8a). In this pool of follicles, each will either undergo a phase of growth and development that lasts approximately 6 months, or will become atretic and die. When they resume their growth after puberty, usually only one oocyte matures and is ovulated per month for the remaining 35 or so years of the reproductive lifespan (Figure 3.8b). Oocytes must complete their growth phase and resume meiosis before they can be fertilized.



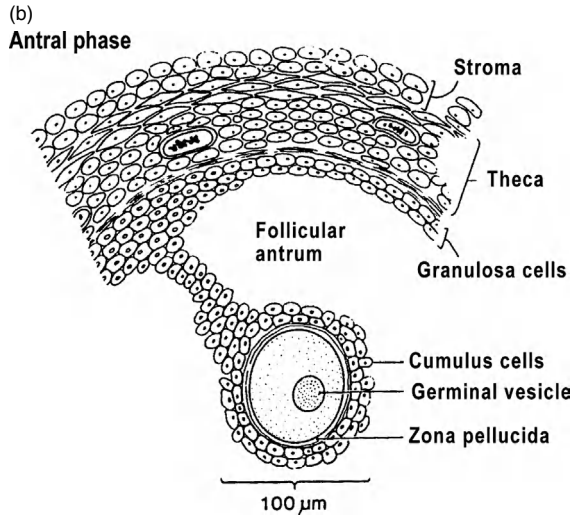


Figure 3.8 (a) Schematic diagram of oocyte and follicular development from prenatal through to adult life. (b) Schematic diagram of preovulatory antral follicle.

Ovarian reserve

Primordial follicles are the most abundant follicle type within the adult ovary, but there is a high rate of wastage. From around Day 100 of fetal life onwards, oocytes that have arrested in meiotic prophase start to undergo atresia, and this continues throughout fetal and neonatal life. This programmed elimination of germ cells may be associated with a redistribution of organelles (e.g., mitochondria) in order to provide optimal function for the remaining oocytes. Pre-granulosa cells may be involved in this degenerative process. In the mouse, around 70% of oogonia are lost by apoptosis upon entry into meiosis; in humans, the population is reduced to approximately 1–2 million by birth. This pool of oocytes, (the ovarian reserve) was traditionally viewed as the finite resource of oocytes that dictates the reproductive lifespan of the individual (Figure 3.9). However, this dogma has recently been challenged (Johnson *et al.*, 2005).

Follicle development

Formation of primordial follicles

In humans, the first primordial follicles can be seen during the fourth month of fetal gestation, and they begin to grow at approximately 20 weeks of fetal life, under the influence of gonadotropins. At this stage the oogonia are still active, before their arrest at the dictyate stage in prophase 1. Their pre-granulosa cells enter a period of quiescence, and cell proliferation will not resume until the primordial follicle begins to grow, often months or years after it was formed. The primordial follicles are first established in the medullary region of the ovary, and they continue their development in the more peripheral parts (cortex). During the growth phase, the follicle develops morphologically, acquiring a theca interna containing steroidogenic cells, and a theca externa of connective tissue cells forming its

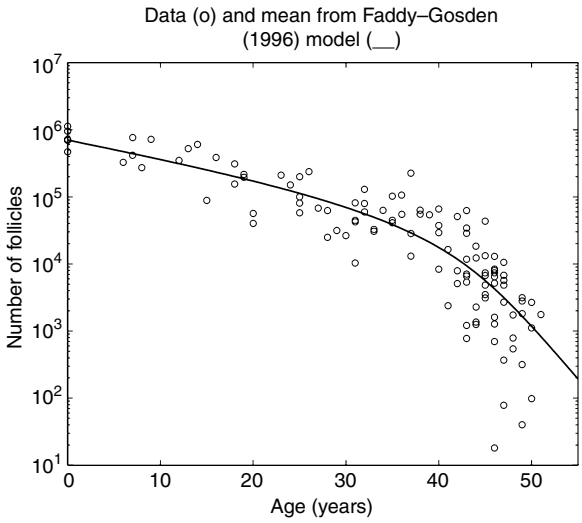


Figure 3.9 Variation in total numbers of follicles in human ovaries from neonatal age to 51 (Reproduced from M.J. Faddy *et al.* 1996 Ovary and ovulation: A model conforming the decline in follicle numbers to the age of menopause in women. *Human Reproduction* 11.7, with permission from Oxford University Press.)

outer layers. The basement membrane around it must either expand or be remodeled to adjust to its increasing size, and it becomes a dynamic system that nurtures the oocyte, responding to endogenous and exogenous influences via autocrine and paracrine effects. Follicular development is regulated by the oocyte itself, in combination with numerous cell interactions. A number of key molecules involved in the regulation have been identified, products of genes that are expressed specifically in oocytes: FIGalpha/FIGLA, GDF-9, BMP-6 and BMP-15, AMD, cKit, Kit ligand, etc.

Primordial follicles remain in their arrested state for up to 50 years, waiting for a signal to resume development. After puberty, a few primordial follicles become recruited to the growth phase every day, and these then go through three phases of development: primary or preantral, secondary or antral (also known as Graafian), and finally, preovulatory (Figure 3.8). As described below, the follicular cells produce growth factors and hormones, and also provide physical support, nutrients (such as pyruvate), metabolic precursors (such as amino acids and nucleotides) and other small molecules that can equilibrate between the two compartments.

Follicular cells

The mass of granulosa cells associated with the oocyte from the antral follicle stage until after fertilization is known as the cumulus oophorus, a complex tissue that is unique to eutherian mammals.

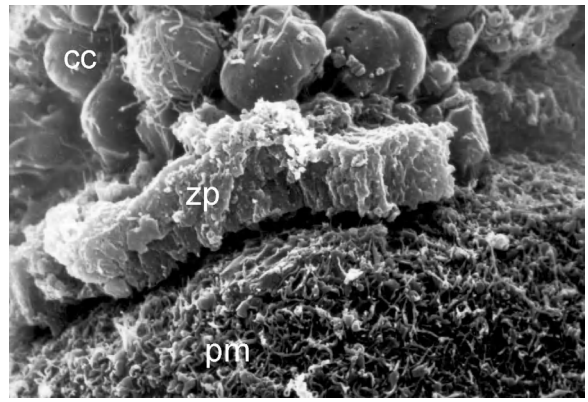


Figure 3.10 Cumulus–cell interactions. Scanning electron micrograph of the surface of an unfertilized metaphase II human oocyte, with the zona pellucida (zp) and cumulus cells (cc) partially dissected to demonstrate the microvillar organization of the plasma membrane (pm). (From Dale 1996)

- Cumulus cells contribute to the intrafollicular environment of the developing oocyte, and the oocyte and its surrounding cells are in close association; electron microscopy shows gap junctions between the apposing membranes.
- In later stages of growth the oocyte plasma membrane increases in surface area and is organized into long microvilli, presumably required for an increase in transmembrane transport. Under the control of the FIGalpha gene, the oocyte secretes a dense fibrillar material that forms a 5–10 μm layer around the female gamete, called the zona pellucida (Figure 3.11). At this stage, follicle cells remain in contact with the oocyte by means of long interdigitating microvilli, which gives a striated image under the light microscope: this is known as the zona radiata.
- Cumulus cells express the ganglioside GM3 which has been implicated in cell recognition, differentiation and signaling. Blocking the gap junctions interferes with transmission or action of molecules such as 2-deoxyglucose, transforming growth factor alpha (TGF-α) and mitogenic agents on the oocyte.
- In many species (but not in humans) physically removing the cumulus cells can inhibit oocyte maturation. Signaling between oocyte and cumulus occurs in both directions, and cumulus cells express growth factor receptors and the mRNA for a number of growth factors. They are also a source of prostaglandins, and express angiogenic factors (vascular endothelial growth factor, VEGF) which may have a role in neovascularization of follicles and angiogenesis at the implantation site of the embryo.

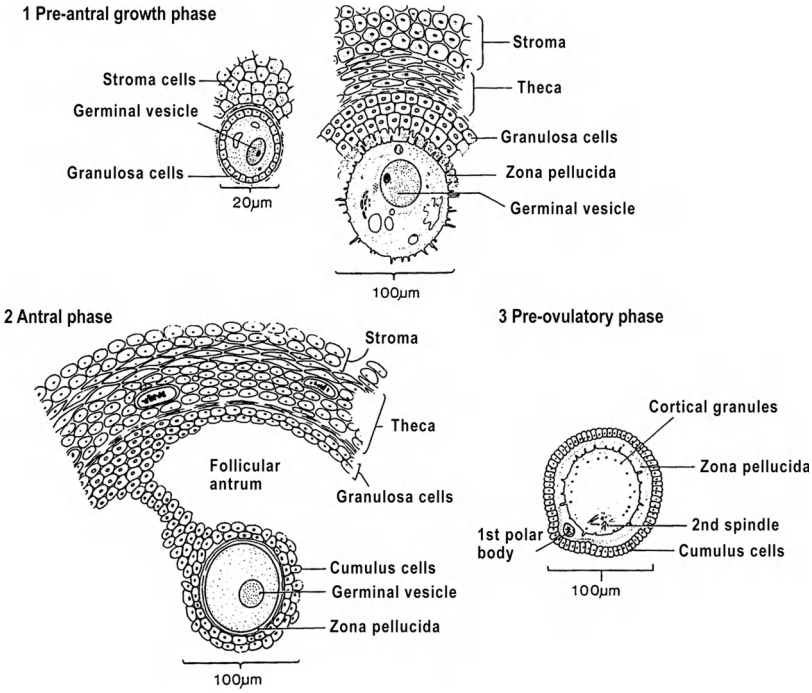


Figure 3.11 Oogenesis in the human. Modified from Johnson (2007).

- The cumulus cells become polarized during oocyte maturation, and begin to secrete a hyaluronic acid extracellular matrix.

Follicular development after puberty

Very small follicles have no independent blood supply; in medium-sized follicles, an anastomotic network of arterioles appears just outside the basement membrane. This network becomes more extensive as the follicle grows, and each ripe preovulatory follicle has its own rich blood supply. Changes in hormone levels during folliculogenesis affect the composition of the follicular fluid, which is probably the source of energy substrates for the developing oocyte. The oocyte plays a fundamental role in follicle development, and controls the differentiation of follicular granulosa cells. In order to acquire its necessary developmental competence, there must be communication between the oocyte and granulosa cells.

Follicle recruitment: from primordial to preantral (primary) follicle

In humans the number of follicles recruited into development at any given time ranges between 2 and 30, related to age. The precise mechanisms that regulate the initiation of primordial follicle growth are not well understood, and two possible mechanisms have been proposed:

(a) Growth initiation is a pre-programmed feature of the ovary: the first primordial follicles to enter the growth phase are those that contain the first oocytes to enter meiosis in the human prenatal ovary (or prenatal/early postnatal mouse ovary (Henderson and Edwards, 1968).
(b) Initiation is regulated by certain growth factors or peptides

Primordial follicles that enter the growth phase show two distinct morphological changes:

1. The oocyte increases in size.
2. Flattened pre-granulosa cells become cuboidal in appearance.

However, more subtle biochemical, physiological and molecular changes occur prior to these events; e.g., increased expression of the proliferating cell nuclear antigen (PCNA) has been correlated with the earliest stages of follicle growth (Oktay *et al.*, 1995). A number of other genes that are expressed exclusively in the oocyte or germline have also been identified, including growth development factor (GDF)-9, required by the granulosa cells and Oct-4 (also known as Oct-3), a POU factor that is also expressed in pluripotential stem

cells of the embryo (Rosner *et al*., 1990). Follicles that have started the growth phase are referred to as early primary follicles.

Follicular destiny

One of the most intriguing mysteries in ovarian physiology is what determines whether one follicle remains quiescent, another begins to develop but later becomes atretic, while still a third matures and ovulates.

- Over 99% of follicles are destined to die rather than ovulate; the degenerative process by which these cells are irrevocably committed to undergo cell death is termed atresia.
- Atretic oocytes show germinal vesicle breakdown, followed by fragmentation and disruption of the oocyte–cumulus complex – granulosa cells from an aspirated atretic follicle show clear signs of fragmentation.
- Despite its critical role during the recruitment of follicles for ovulation, the mechanisms underlying the onset and progression of atresia remain poorly understood. There are four degenerative stages during ovarian development which result in a massive loss of ovarian cells:
  1. During migration of primordial germ cells from the yolk sac to the genital ridge – many of these cells undergo degeneration.
  2. At the time of entry into the first meiotic stage, some germ cells undergo attrition before follicles are formed.
  3. In the later stages of development, early antral follicles either differentiate or undergo atresia.
  4. If ovulatory signals are absent, the mature follicle also may undergo degeneration.

Ovarian stimulation with FSH during ART cycles rescues follicles that were destined for atresia; therefore most atretic follicles are evidently normal, or we would have seen more defects from children conceived by ART.

Preantral to antral (secondary) follicle

The oocyte continues to increase in size, and the granulosa cells proliferate and divide; when one to two layers of granulosa cells surround the oocyte, the follicle reaches a transitional stage. Further growth produces a secondary follicle with multiple layers of granulosa cells, and the follicles become associated with small blood vessels. This is a significant feature, since the preantral development of ovarian follicles depends upon locally acting factors and oocyte–granulosa cell communication events, independent of the gonadotropins that are delivered via the bloodstream. In humans, the theca layer does not form until the follicle contains between three and six layers of granulosa cells.

Antral to preovulatory development

Growth factors and hormones induce further follicular growth, the theca and granulosa cell layers proliferate, and the oocyte expands in size, accumulating water, ions, lipids, RNA and proteins. Follicle stimulating hormone (FSH) is required for the formation of follicular fluid, and several pockets of this fluid, precursor spaces of the antral cavity, begin to form between the granulosa cells. The fluid is derived from the bloodstream, with added glycoproteins secreted by the granulosa cells. When five to eight layers of granulosa cells surround the oocyte, these pockets of fluid merge to form the antral cavity (follicular antrum). As the antral cavity extends, the oocyte takes up an acentric position in the follicle, and is surrounded by two or more layers of granulosa cells (Figure 3.11).

Under the influence of FSH released from the pituitary, the granulosa cells differentiate into two distinct populations:

1. The oocyte and its surrounding follicular cells form a network or syncytium, a close association that is essential for follicular growth to continue. Cumulus granulosa cells surround the oocyte; these cells are mitotically inactive, and do not divide further. The innermost layers of cells become columnar, forming the corona radiata, and these cells communicate with the oocyte via gap junctions linking them with the oolemma.
2. Mural granulosa cells line the follicle wall; these cells stop dividing and also become columnar in appearance. Under the influence of FSH, they express receptors for luteinizing hormone (LH) and steroidogenic enzyme pathways become active within the cells.

Other candidates involved in granulosa cell differentiation include insulin-like growth factors IGF-I and IGF-II; the oocyte itself may also play an important role.

Preovulatory antral follicles

In humans, the oocyte is 120 μm in size at the time of antral cavity formation. The oocyte is now *capable* of resuming meiosis, but only a limited number of oocytes proceed past the antral stage towards ovulation. Around 20 antral follicles are recruited each month from the total

number available, and even fewer are selected to ovulate (this number varies between species). The remaining follicles degenerate through apoptosis and become atretic. The recruited follicles continue to grow, and (in monovular species) one follicle in the cohort is selected to become dominant. This preovulatory follicle then grows rapidly. In humans the size increases from 6.9 mm (±0.5) to a size of 18.8 mm (±0.5) over a period of 10–20 days. The follicular basement membrane must expand by approximately 400 times its original size to accommodate this follicular growth. The granulosa cell population increases from approximately 2–5 million to 50 million, and the mural granulosa cells differentiate further.

Ovulation

1. Within a few hours of the LH surge, the follicle becomes more vascularized and swollen, and expansion of the granulosa/cumulus mass causes the follicle to form a visible bulge on the surface of the ovary. The projecting follicle wall becomes thin, forming the stigma.
2. A combination of tension and the action of a collagenase enzyme causes the follicle wall to rupture, and follicular fluid containing the cumulus–oocyte complex (COC) is released from the antral cavity.
3. The fimbriated mouth of the oviduct scrapes the sticky mass of cumulus-enclosed oocyte off the surface of the ovary; synchronized beating of cilia in the tubal wall move the COC along the oviduct. The oocyte remains viable in the oviduct for as long as 24 hours.

Generation of the corpus luteum

After ovulation, the follicular basement membrane degenerates, and blood vessels populate the remaining structure of the follicle to form the corpus luteum. This structure contains large luteal cells derived from luteinized granulosa cells, and smaller luteal cells derived from the theca interna. Both populations of cells release progesterone. If pregnancy is not established, the corpus luteum remains in the ovary for 2–14 days (varying with species), and then degenerates by luteolysis.

Oocyte maturation and ovulation

Oocyte growth

The environment in the developing follicle provides an essential niche for oocyte survival, nourishment and development, but oocyte growth does not run strictly in parallel with follicular development (see Figure 3.8). The primordial human follicle contains a primary oocyte approximately 35 μm in size, and grows to its final size of 120 μm over a period of around 85 days. During this time, it must acquire competence and the ability to be successfully fertilized and then support early embryo development. This process of oocyte maturation involves the coordination of integrated, but independent nuclear and cytoplasmic events: the nucleus undergoes germinal vesicle breakdown, resumption of meiosis, and completion of the first meiotic division. Cytoplasmic maturation requires relocation of cytoplasmic organelles and establishment of oocyte polarity, with an increase in the number of mitochondria and ribosomes. There are alterations in membrane transport systems, and the developing Golgi apparatus expands and migrates to the periphery. Organelles appear in the cytoplasm that reflect storage and export of materials: membrane-bound vesicles, multivesicular and crystalline bodies, fat droplets and glycogen granules.

During its growth phase, the oocyte prepares and stores reserves that will be needed for the first stages of fertilization and initiation of embryo development: water, lipids, carbohydrates and ions accumulate, and RNA and proteins are synthesized and stored. As the follicle moves from the primordial to the primary stage, the diameter of the oocyte also increases. Although the distribution of organelles and storage materials do not show a definite and obvious polarity, spatial patterning cannot be entirely ruled out in mammalian eggs (Gardner, 1999). A number of features can be seen during these stages:

1. The numbers of mitochondria increase to approximately 10^5, and they become more spherical, with fewer concentrically arched cristae, indicating that they are less active.
2. Under the control of the FIGalpha gene, genes coding for the zona pellucida proteins (ZP1, ZP2, ZP3, ZP4) are expressed coordinately and specifically. The proteins that will make up the zona pellucida (ZP) are secreted, and this forms a 5–10 μm layer around the oocyte. At this stage, the ZP maintains communication between the oocyte and the granulosa cells; ZP3 is the primary sperm receptor.
3. Cytoplasmic organelles become far more abundant, with the notable exception of centrioles,

which disappear and are not found until after fertilization. The numbers of ribosomes multiply fourfold to around 10^8 in the mature oocyte.

4. Large amounts of RNA are synthesized and stored: the human oocyte has an estimated 1500 pg of total RNA, in contrast to 14 fg in the spermatozoon.

Oocyte polarity

Polarization represents a differential distribution of morphological, biochemical, physiological and functional parameters in the cell. The appearance of polarization may be associated with triggering the developmental program. The growing oocyte does not have a homogeneous structure; in particular, many cytoplasmic organelles become segregated to various regions of the oocyte and this regional organization determines some of the basic properties of the embryo. In all animal oocytes, the pole where the nuclear divisions occur (forming a cleavage furrow), resulting in the formation of the polar bodies, is called the animal pole. The opposite pole (opposite to the extruded polar body) is called the vegetal pole, and often contains a high concentration of nutrient reserves. Scanning electron microscopy shows that mouse oocytes have a microvillus-free area on the plasma membrane, adjacent to the first polar body and overlying the meiotic spindle. Human oocytes show no polarity in the distribution and length of the microvilli, either in the animal or the vegetal pole. Studies with fluorescent lectins reveal no signs of polarization in membrane sugar distribution. However, Antczak and Van Blerkom (1997) found that two regulatory proteins involved in signal transduction and transcription activation (leptin and STAT3) are polarized in mouse and human oocytes and preimplantation embryos. They suggest that a subpopulation of follicle cells may be partly responsible for the polarized distribution of these proteins in the oocyte, and that they may be involved in determining its animal pole, and in the establishment of the inner cell mass and trophoblast in the preimplantation embryo.

The intracellular location of mRNAs and protein translation machinery is related to cell cytoskeleton regulation. Several lines of evidence suggest that mammalian ooplasm redistributes after sperm entry during fertilization (Edwards and Beard, 1997). The meiotic spindle that begins to form just before ovulation migrates to the cortex, and its position determines the cleavage plane for extruding the first polar body.

Storing information

The three main classes of RNA (mRNA, tRNA and rRNA) are all involved in the synthesis of protein. The relative amounts of the three types of RNA present during oogenesis varies from species to species, in the order of 60–65% rRNA, 20–25% tRNA, 10–15% mRNA. Whereas in species such as *Xenopus* and *Drosophila* the embryo retains the vast majority of stored mRNA until the blastula stage to direct protein synthesis later in development, mammalian oocytes contain a finite amount of stored RNA, which supports only the very early stages of preimplantation embryo development. The dictyate chromosomes in mammalian oocytes actively synthesize ribosomal and mRNA as the follicle starts to grow; there is a dramatic increase in the size of the nucleolus as RNA accumulates in the nucleus, including a significant proportion of translatable polyadenylated mRNA. Nucleosomes contain DNA packaged within chromatin, which also contains structural proteins such as histones, and careful regulation of transcriptional machinery controls the expression of particular genes at specific times. When the oocyte is fully grown, transcription of new RNA stops almost completely following germinal vesicle breakdown until the time of zygotic genome activation (ZGA) when the new embryonic genome starts to direct further development. During the period prior to ZGA, the oocyte is dependent upon its pool of stored mRNA, which has been processed with elegant mechanisms that control its expression. The stability of mRNA is related to the length of its polyA tail: a long polyA tail is required for translation, but this long tail also makes the message vulnerable to degradation. There are at least two different mechanisms of mRNA adenylation in oocyte cytoplasm – the genes are transcribed with long polyA tails, but some messengers are transcribed and translated during growth, whereas others are "masked" by deadenylation, reducing the tail to less than 40 "A" residues: this prevents their translation, and also protects these messages from degradation. In later stages of maturation, the masked genes can then be activated by selective polyadenylation when their products are required. Both gene types contain a highly conserved specific sequence in their 3′ untranslatable regions (UTR) that signals polyadenylation. The mRNA for the masked transcripts also contains a further sequence 5′ to the polyadenylation signal, known as the cytoplasmic polyadenylation element (CPE) or the adenylation control element (ACE). It seems that this sequence controls the expression of stored mRNAs: ACE-containing mRNAs are masked and protected from degradation, whilst non-ACE-containing mRNAs, available for immediate translation, have long and relatively stable

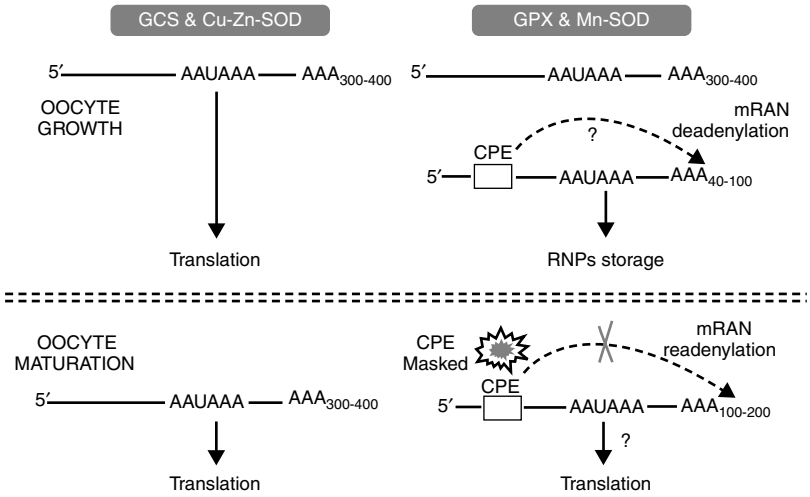


Figure 3.12 mRNA processing during oocyte maturation (with thanks to Y. Ménézo).

polyA tails (Figure 3.12). The mRNAs are also packaged in association with ribonucleoprotein (RNP) particles, and this may represent another control mechanism as part of the complex regulation of transcription and translation. Packaging with RNPs probably plays a part in controlling the access of ribosomes to regulatory elements within the mRNA.

Oocyte reserves

The growing oocyte contains a large amount of information that is masked, but the rest of the protein synthesizing machinery is functional. Many proteins synthesized during oogenesis are stored in the oocyte cytoplasm for later use; for example, the enzymes necessary for DNA synthesis are present in the growing oocyte and yet DNA replication is switched off. Experiments with interspecies nuclear transfer using nuclei from fibroblast cells transferred into bovine, sheep and monkey enucleated oocytes revealed that the first two cell division cycles were regulated by the oocyte cytoplasm; thereafter, the donor nucleus assumed regulatory control, but development arrested after a limited number of cleavage divisions (Fulka *et al.*, 1998). This again demonstrates that the oocyte itself has a large reserve of functional activity, sufficient to sustain initial cell division cycles – but differentiation events in both cytoplasmic and nuclear compartments are essential for continued development.

Stages of oocyte maturation

During the final stages of follicle development, mural granulosa cells increase their estrogen synthesis, and serum estrogen levels are significantly elevated. This exerts a positive feedback on the pituitary to increase its release of gonadotropins, in particular LH, resulting in the preovulatory LH surge. Binding of LH to its receptors on mural granulosa is enhanced, and this activates the pathways that promote oocyte maturation. The increased binding eventually downregulates the steroidogenic pathway that leads to estradiol synthesis, and the cells switch their steroid synthesis to the production of progesterone for the luteal phase. Under the influence of LH, the primary oocyte matures via a complex interplay between the follicular cells and the oocyte, involving numerous metabolic pathways. The final stages that lead to ovulation include:

1. *Nuclear maturation* to allow resumption of meiosis beyond arrest in prophase I. The mid-cycle surge of LH initiates a complex cascade of events, which will be described in detail below.
2. *Cytoplasmic maturation*. During oocyte growth, the Golgi apparatus enlarges and develops into separate units in the cortex; these export glycoproteins to the zona pellucida and form approximately 5×10^3 cortical granules that collect at the surface of the oocyte. The cortical granules contain enzymes that are later released to modify the zona pellucida (see Chapter 4). A fine network of endoplasmic reticulum extends throughout the cytoplasm, with dense patches of membrane that become oriented closer to the periphery of the cortex, where they may be involved in calcium release for cortical granule exocytosis. An important feature of cytoplasmic maturation is the translation of mRNA species that have

accumulated in a stable and dormant form during oogenesis.

(c) *Cumulus expansion/mucification*. The preovulatory hormone surge leads to changes in the morphology of the cumulus granulosa cells, and they secrete hyaluronic acid into the intercellular spaces. Oocytes also produce a soluble factor that initiates production of hyaluronic acid by the cumulus granulosa cells, and the matrix thus formed transforms the granulosa cells from a tightly packed cellular mass to a more diffuse and dispersed mucified mass. The syncytial relationship between the cumulus cells and the oocyte is lost, intercellular communication via gap junctions between the cumulus cells and the oocyte is terminated, and metabolites and informational molecules can no longer pass. These events may act as the trigger for resumption of meiosis.

Nuclear maturation: resumption of meiosis

Nuclear maturation specifically refers to the first resumption of meiosis, with transformation of the fully grown primary oocyte in the antral follicle into the unfertilized secondary oocyte; this process follows the preovulatory surge of FSH and LH, just prior to ovulation. The germinal vesicle membrane breaks down, and the nucleus progresses from the dictyate state of the first meiotic prophase through first meiosis to arrest again at metaphase II. Up to this stage, the primary oocyte has been maintained in meiotic arrest by a complex balance of cell cycle protein activity, intracellular levels of cAMP and other intracellular messengers. The mid-cycle surge of LH causes the level of cAMP in the oocyte to fall below this threshold level, and a cascade of events is initiated that finally leads to breakdown of the germinal vesicle (GVBD) and resumption of meiosis. This cascade involves intracellular ionic messengers, such as Ca^{2+} and H^{+}, and a series of cell cycle complexes that have accumulated within the oocyte, including maturation promoting factor (MPF) and cytostatic factor (CSF). The surge levels of LH shift follicular steroidogenesis from predominately estrogen to progesterone production, and this may actively promote the resumption of meiosis by stimulating the oocyte to produce a signal that induces GVBD. The nucleus of the germinal vesicle usually contains only one nucleolus, which enlarges from 2 to almost 10 μm in diameter; this nucleolus disappears when the germinal vesicle breaks down. A rim of chromatin forms around the nucleolus in large oocytes, and this is a sign that they are capable of resuming meiosis.

Nuclear maturation during the development of the Graafian follicle

1. In response to the preovulatory surge of gonadotropins, the concentration of cAMP within the oocyte falls.
2. Mitochondria increase in volume, are reduced in number, and move to the perinuclear region, an area that requires high concentrations of ATP during the formation of the first meiotic spindle.
3. Chromatin forms a dense ring around the nucleolus; microtubular organizing centers (MTOC) congregate as microtubules in the cytoplasm and reorganize to form a functional spindle apparatus.
4. The chromosomes condense, the germinal vesicle membrane breaks down, and the first meiotic spindle forms.
5. The spindle migrates to the animal pole of the oocyte and changes orientation.
6. The first meiotic division proceeds through metaphase I, and then to telophase I. Asymmetrical cell division gives rise to the large functional oocyte and the smaller first polar body.
7. As soon as meiosis I is complete, the oocyte enters into the second meiotic division, and this secondary oocyte arrests again at metaphase II – ready for fertilization.
8. The trigger for the resumption of the second meiotic arrest is supplied by the fertilizing spermatozoon, and meiosis is completed with the extrusion of the second polar body (in the animal kingdom only coelenterates and echinoderms have completed meiosis before sperm entry).

Timing of events:

LH surge: 0	GV with nucleolus
+15 hours	GVBD
+20 hours	first meiotic metaphase
+35 hours	second meiotic metaphase
+38 hours	ovulation

Control of nuclear maturation

The control of nuclear maturation involves a complex interplay between numerous metabolic pathways in both the somatic granulosa cells and the oocyte. The follicle cells are in direct physical contact with the oocyte, and they either maintain meiotic arrest, or stimulate resumption of meiosis by transferring the appropriate signals. The metabolic pathways within the granulosa cells are in turn regulated by the binding of gonadotropins to

their cell surface receptors. Small molecules including adenosine, uridine, hypoxanthine and their metabolites diffuse between granulosa cells and the oocyte via gap junctions. The precise mechanism for the maintenance of arrest remains to be elucidated, and two mechanisms have been proposed:

1. Adenosine stimulates oocyte adenylate cyclase via a surface receptor on the oolemma, and hypoxanthine prevents hydrolysis of cAMP: high levels of cAMP sustain meiotic arrest.
2. Adenosine could participate directly in meiotic arrest via its conversion to ATP, a substrate for adenyl cyclase within the oocyte. Purines may also participate in cell signaling via G-proteins on the oolemma and plasma membranes of the cumulus cells.

Production of active maturation promoting factor (MPF) in the oocyte cytoplasm mediates nuclear maturation.

1. MPF consists of two components:
   (a) A 34 kDa protein, serine/threonine kinase, that is activated by dephosphorylation; homologous to the product of the cdc2 gene in fission yeast.
   (b) Cyclin B: activated by phosphorylation, and is probably a substrate for the product of the Mos proto-oncogene, p39.
   - MPF activity is low in GV stage oocytes and increases during GVBD.
   - MPF activity is high after the resumption of meiosis, during both metaphase I and metaphase II.
2. The action of phosphokinase A (PKA) prevents GVBD.
   (a) A decrease in intracellular cAMP reduces PKA activity, and this allows cyclin B and p34cdc2 to associate and form MPF.
   (b) p39mos then participates in the activation of MPF, possibly by cyclin B phosphorylation. Synthesis of p39mos is stimulated by progesterone, and this may be a key event in the induction of meiotic maturation.
   (c) By maintaining cyclin B in its phosphorylated (active) form, p39 may stabilize MPF and act as a cytostatic factor (CSF), thereby maintaining metaphase II arrest.

The product of the oncogene Mos is expressed early in oocyte maturation and disappears immediately after fertilization. The Mos protein has the same effects as CSF, arresting mitosis at metaphase with high p34cdc2 activity. It is thought that CSF is, in part or entirely Mos, and that the second meiotic arrest is due to transcription of the Mos oncogene as the oocyte matures.

In summary, the production of a viable oocyte depends on three key processes:

1. The fully grown oocyte must recognize regulatory signals generated by follicular cells.
2. Extensive reprogramming within the oocyte must be induced – this involves activation of appropriate signal transduction mechanisms.
3. Individual molecular changes must be integrated to drive the two parallel but distinct processes involved in meiotic progression and the acquisition of developmental competence.

The oocyte depends on the follicular compartment for direct nutrient support, and for regulatory signals. After the LH surge, new steroid, peptide and protein signals are generated, and alterations to preovulatory steroid profiles can selectively disrupt protein reprogramming and individual components of the fertilization process.

Localized short- and long-lived maternal mRNAs regulate the initial stages of development and differentiation both in the oocyte and in the early embryo.

There is no doubt that the process of oocyte growth and maturation is a highly complex process, involving a three-dimensional series and sequence of regulatory elements at several different molecular levels. The final evolution of a mature oocyte which has the potential for fertilization and further development is dependent upon the correct completion and synchronization of all processes involved: although an overview is emerging, many aspects still remain to be elucidated. Thus it is impossible to assess and gauge the consequences of manipulations during assisted reproduction practice, and it is essential to maintain an awareness of the complexity and sensitivity of this delicate and highly elegant biological system. Our in-vitro attempts to mimic nature will only succeed if they are carried out within this frame of reference.

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Gametogenesis

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Chapter 4

Sperm–oocyte interaction

Gamete interaction

In nature, fertilization occurs only after both the oocyte and the spermatozoon have completed their final stages of cytoplasmic maturation. The male gamete has completed meiosis prior to the moment of fertilization, but final nuclear maturation with the second meiotic division occurs in the female gamete only after fertilization has taken place. Sperm–oocyte interaction is a complex process of cell–cell interaction that requires species-specific recognition and binding of the two cells. While interacting, each gamete triggers a process of physiological activation in its partner. Controlled, synchronous gamete activation is essential for embryonic development; however, the biochemical and cellular processes that ultimately lead to the fusion of the male and female pronuclei are still poorly understood. In human assisted reproductive technology, although the technique of intracytoplasmic injection of spermatozoa (ICSI) essentially bypasses the initial stages of fertilization, including sperm capacitation and the interaction of the gametes, successful fertilization still requires controlled and correct activation of both sperm and oocyte.

Mammalian fertilization is internal and the male gametes must be introduced into the female tract at coitus. Coitus itself ranges from minutes in humans to hours in camels, but is accompanied by many physiological changes. In the human, tactile and psychogenic stimuli can initiate penile erection, caused by decrease in resistance and consequently dilatation in the arteries supplying the penis, with closure of the arterio-venous shunts and venous blood valves. Vasocongestion can increase the volume of the testes by as much as 50%. Sequential contraction of the smooth muscles of the urethra and the striated muscles in the penis results in ejaculation of semen, with mixing of three different components: prostatic liquid rich in acid phosphatase, the vas deferens fraction containing spermatozoa, and the seminal vesicle fraction containing fructose.

In the woman, tactile stimulation of the glans clitoris and vaginal wall leads to engorgement of the vagina and labia majora, and the vagina expands. Orgasm is accompanied by frequent vaginal contractions, with uterine contractions beginning in the fundus and spreading to the lower uterine segment. In man, rabbit, sheep, cow and cat, the semen is ejaculated into the vagina. In the pig, dog and horse, it is deposited directly into the cervix and uterus. In many species, the semen coagulates rapidly after deposition in the female tract, as a result of interaction with an enzyme of prostatic origin. The coagulation may serve to retain spermatozoa in the vagina or to protect them from the acid environment.

In the human, this coagulum is dissolved within one hour by progressive action of a second proenzyme, also of prostatic origin. Within minutes of coitus, spermatozoa may be detected in the cervix or uterus; 99% of the spermatozoa are lost from the vagina, but the few that enter the tract may survive for many hours in the cervical crypts of mucus. In the absence of progesterone, cervical mucus permits sperm penetration into the upper female tract. Although data are inconclusive, it appears that activity of the musculature of the female tract is not required for sperm transport. Spermatozoa probably move through the uterus under their own propulsion, and are transported in currents set up by the action of uterine cilia. The cervical crypts may serve as a reservoir regulating flow of spermatozoa into the tract, while the utero-tubal junction may act as a sphincter. In hamster and rabbit, the earliest appearance of spermatozoa in the oviducts is 47 hours. Therefore, in most animals, although great quantities of spermatozoa are produced, very few reach the oocyte. Those that do must then traverse and interact with the cumulus and coronal cells, which reduce the number of

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spermatozoa that can reach the oocyte to bind to and penetrate the zona pellucida. Polyspermy, a lethal condition where several spermatozoa enter the oocyte, is probably rare in nature.

Sperm–oocyte ratios

In the course of evolution, it seems that great wastage of spermatozoa has been retained as a requirement for the union of one spermatozoon with one oocyte. In most animals, spermatozoa are produced in huge excess, irrespective of whether fertilization occurs externally or internally: in humans, the sperm–oocyte ratio can be as high as 10^9:1. Of the millions of spermatozoa ejaculated and deposited in the female tract, only a few reach the site of fertilization, the ampullae of the fallopian tube in most species. An in-vivo study of fertilization in the mouse showed a 1:1 sperm–oocyte ratio in the ampullae – supernumerary spermatozoa were never observed.

The initial stages of fertilization depend principally on two structures: the acrosome of the spermatozoon and the zona pellucida of the oocyte. Three major events are involved in sperm–oocyte interaction:

1. The spermatozoon attaches to the zona pellucida (ZP).
2. The spermatozoon undergoes the acrosome reaction, releasing digestive enzymes, and exposing the inner acrosomal membrane.
3. This highly fusogenic sperm membrane makes contact with the oocyte plasma membrane and the two membranes fuse together.

Gamete activation

Sperm activation

Before the male gamete can initiate the steps required for successfully fertilizing an oocyte, the spermatozoon must itself be first activated, a process that involves several behavioral, physiological and structural changes. Some of these changes are induced by exposure to environmental signals, and others are induced whilst the spermatozoon is interacting with the oocyte and its extracellular investments. The steps include changes in motility, capacitation, acrosome reaction, penetration of the ZP, binding to the oolemma, and membrane fusion.

Motility

Spermatozoa are maintained in the testis in a quiescent state, with a metabolic suppression that may be due to many factors, such as physical restraint, low pH and low oxygen tension of the seminal fluid. They acquire motility in the process of epididymal maturation, but only become fully motile after ejaculation and capacitation.

Capacitation

Spermatozoa are not capable of fertilization immediately after ejaculation. They develop the capacity to fertilize (capacitate) after a period of time in the female genital tract; since epididymal maturation and capacitation are unique to mammals, this may represent an evolutionary adaptation to internal fertilization. During capacitation, the spermatozoa undergo a series of changes that give them the "capacity" for binding to and penetrating the oocyte. These changes include an increase in membrane fluidity, cholesterol efflux, ion fluxes that alter sperm membrane potential, increased tyrosine phosphorylation of proteins, induction of hyperactive motility, and the acrosome reaction.

The time required for capacitation varies from species to species and ranges from less than 1 hour in the mouse to 6 hours in the human.

Two changes take place: the epididymal and seminal plasma proteins coating the spermatozoa are removed, followed by an alteration in the glycoproteins of the sperm plasma membranes (an antigen on the plasma membrane of the mouse spermatozoon, laid down during epididymal maturation, cannot be removed by repeated washing, but disappears, or is masked during capacitation). The events are regulated by the activation of intracellular signaling pathways, involving cAMP, protein kinase A, receptor tyrosine kinases and non-receptor tyrosine kinases. A number of different molecules regulate these pathways, including calcium, bicarbonate, reactive oxygen species, GABA, progesterone, angiotensin and cytokines. Phosphorylation of sperm proteins is an important part of capacitation, and this has been shown to be associated with the change in the pattern of sperm motility known as hyperactive motility, recognizable by an increase in lateral head displacement. There is also some evidence that spermatozoa can translate some mRNA species during capacitation (Gur and Breitbar, 2006).

In the human, capacitation in vivo probably starts while the spermatozoa are passing through the cervix. Many enzymes and factors from the female tract have been implicated in causing capacitation, such as arylsulfatase, fucosidase and taurine. The factors involved are not species specific, and capacitation may be induced in vitro in the absence of any signals from

Table 4.1 Survival parameters of mammalian gametes in vitro (with permission from Gwatkin, 1974)

	Time required for capacitation (h)	Duration of sperm motility (h)	Duration of sperm fertility (h)	Fertilizable life of oocytes (h)
Mouse	<1	13	6	15
Sheep	1–5	48	30–48	12–15
Rat	2–3	17	14	12
Hamster	2–4	–	–	9–12
Pig	3–6	50	24–48	10
Rabbit	5	43–50	28–36	6–8
Rhesus monkey	5–6	–	–	23
Man	5–6	48–60	24–48	6–24
Dog	–	268	134	24

the female tract. Follicular fluid can also promote capacitation in vitro. A low molecular weight motility factor found in follicular fluid, ovary, uterus and oviduct may increase sperm metabolism (and hence motility) by lowering ATP and increasing cyclic AMP levels within the sperm. Table 4.1 demonstrates the duration of fertility and motility of mammalian spermatozoa within the oviduct, together with the fertilizable life of oocytes.

Chemically defined media with appropriate concentrations of electrolytes, metabolic energy sources and a protein source (serum albumin) will also induce the acrosome reaction in a population of washed sperm. The removal or redistribution of glycoproteins on the sperm cell surface during capacitation exposes receptor sites that can respond to oocyte signals, leading to the acrosome reaction.

- Capacitation is temperature dependent and only occurs at 37–39°C.
- Sperm surface components are removed or altered during capacitation.
- In vitro, the acrosome reaction cannot occur until capacitation is complete.

Acrosome reaction (AR)

The acrosome is a membrane-bound cap that covers the anterior portion of the sperm head; it contains a large array of hydrolytic enzymes including hyaluronidase, acrosin, proacrosin, phosphatase, arylsulfatase, collagenase, phospholipase C and β-galactosidase. The acrosome originates in the Golgi system of the early spermatid, in a series of concentrically arranged membranes around an aggregation of small vesicles. One of the vesicles increases in size and fills with particulate material, and the vesicle grows by fusion of several smaller vesicles. When the future acrosomal vesicle reaches a certain size, it migrates towards the nucleus. The nucleus then starts to elongate, the vesicle loses much of its fluid content and its membrane wraps around the front of the nucleus to form the typical acrosome.

When the capacitated spermatozoon attaches to the ZP, the permeability of the sperm plasma membrane is altered, causing a transient change in the concentration of several intracellular ions. This triggers the acrosome reaction, which is the final prerequisite step in the activation of the spermatozoon before gamete fusion is possible. The reaction consists of three stages:

1. The outer acrosomal membrane fuses with the overlying sperm head plasma membrane, allowing the contents to be released; this can be monitored in vitro using a fluorescent tag in the acrosome reaction to ionophore challenge (ARIC) test.
2. The acrosomal granule breaks down, releasing lysins. These enzymes "dissolve" a pathway through the ZP.
3. When the sperm head plasma membrane contacts the oocyte plasma membrane, the two membranes fuse.

It appears that not all of the bound sperm are able to penetrate the egg, and many are not triggered into the acrosome reaction. In order for sperm to attach,

a specific molecular fit may be required to induce the acrosome reaction. In the human, the membranes start to fuse near the border between the acrosomal cap and its equatorial segment. Once the correct trigger signals have been received, the acrosome reaction is relatively rapid, taking 2–15 minutes in vitro. Gametes collected from the ampullae of mammals after mating show that free-swimming spermatozoa have unreacted acrosomes, and those within the cumulus mass have either reacted acrosomes, or are in the process of reacting. The majority of spermatozoa attached to the surface of the zona pellucida surface have reacted acrosomes.

The acrosome reaction only occurs in the presence of Ca^{2+}: it may be induced artificially by adding Ca ionophore A23187, a chemical that carries Ca^{2+} across cell membranes to the sperm cytoplasm (Figure 4.1), or simply by increasing the external concentration of Ca^{2+}. An artificially high pH of about 9–9.5 will also induce the AR. It appears that the physiological events leading to the AR parallel those leading to activation of the oocyte, including changes in the ion permeability of the plasma membrane, alterations in the intracellular level of free Ca^{2+} and an alkalinization of the cytoplasm. The influx of calcium triggers the fusion of the acrosomal membranes and the exocytosis of the acrosomal contents. The sequence of events leading to exocytosis may involve potential second messenger pathways, including:

- Changes in intracellular calcium
- Activation of cAMP and phosphokinase A pathways
- Phospholipase C generating InsP3 and diacylglycerol (DAG)
- Phospholipase D generating phosphatidic acid
- Activation of phospholipase A2 generating arachidonic acid.

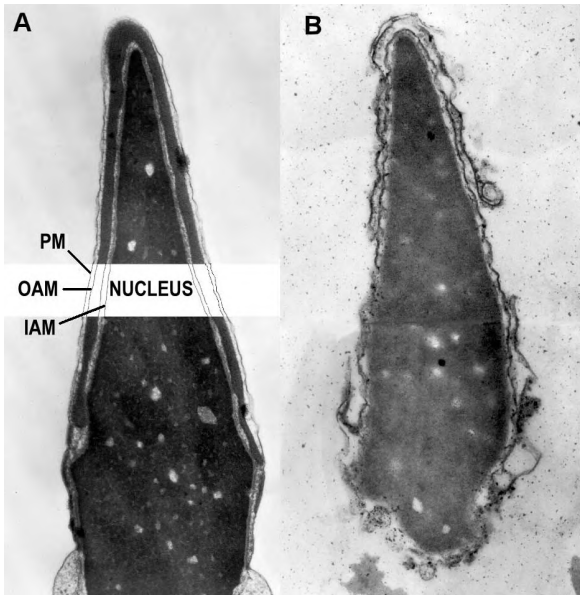

Figure 4.1 Acrosome structure. Transmission electron microscope (TEM) section through a human spermatozoon showing the plasma membrane (PM) and outer (OAM) and inner (IAM) acrosomal membranes. To the right is a TEM of a human spermatozoon after exposure to the calcium ionophore A23187 has triggered the acrosome reaction.

Completion of the acrosome reaction does not necessarily ensure successful fertilization in vitro – enormous variability can be found in a population of spermatozoa surrounding the cumulus mass. Some will acrosome react too soon, others too late: in some the trigger stimulus will be inadequate, perhaps in others the transduction mechanism will fail at some point. The cumulus mass is composed of both cellular and acellular components, and the acellular matrix is made up of proteins and carbohydrates, including hyaluronic acid. In vivo, very few spermatozoa reach the site of fertilization: therefore the idea that large populations of spermatozoa surrounding the oocyte mass dissolve the cumulus matrix, as observed during IVF, is probably incorrect in vivo. Fertilization occurs before the dispersion of the cumulus mass, and in vivo the sperm:oocyte ratio is probably close to 1:1.

Oocyte activation

The zona pellucida (ZP)

The zona pellucida is a glycoprotein sheet several micrometers thick that is secreted by the growing oocyte, providing a protective coat around the oocyte and developing embryo. If we accept the concept that polyspermy prevention is laboratory artifact rather than natural fact, then it probably serves mainly as a protective coat for the developing embryo. Electron microscopy shows the outer surface to have a latticed appearance, and its chemical composition consists of 70% protein, 20% hexose, 3% sialic acid and 2% sulfate. In the mouse oocyte, the zona contains three glycoproteins, ZP1, ZP2 and ZP3, with apparent molecular weights of 200 000, 120 000 and 83 000, respectively; each is heavily glycosylated. Filaments of ZP2 and ZP3 polymers are crosslinked non-covalently, and ZP1 dimers create bridges between them to form a matrix (Wassarman *et al.*, 1996). In the mouse, ZP2 is

distributed throughout the thickness of the zona, and ZP3 binds to primary receptors on capacitated spermatozoa, inducing a cascade of events that lead to the acrosome reaction. Sperm receptors for ZP2, in contrast, are located on the inner acrosomal membrane and therefore are unmasked only after the AR has taken place. Following the AR, ZP2 binds the sperm to the zona, and the sperm penetrate the ZP to fuse with the oocyte plasma membrane.

The ZP gene family has an ancient phylogeny, and the coding sequences of the murine and human ZP genes are 74% identical. However, the zona in many species, including humans, contains a fourth zona protein, ZP4, which is absent in mice. Studies carried out in humans have revealed that ZP2 is modified by cleavage, similar to the mouse model, and sperm penetration through the zona is inhibited after fertilization. After sperm penetration the zona undergoes modifications consistent with its role as a protective device. However, unlike the mouse, the ZP can still bind spermatozoa and induce the AR. Thus, fertilization does not abolish ZP bioactivity. This difference may be linked to the presence of ZP4 – the precise events that describe the role of the zona proteins in gamete interaction require further study in humans (Lefievre *et al.*, 2004; Conner *et al.*, 2005; Patrat *et al.*, 2006).

Cytoplasmic maturation

Oocytes acquire competence for successful fertilization and the ability to sustain early development via cytoplasmic maturation, a process that may be considered a parallel to sperm capacitation. Several milestones in development must be reached before it is capable of being fertilized correctly:

1. M-phase promoting factor (MPF) is expressed at a high level.
2. High levels of other factors are present within the oocyte, including c-mos, mitogen activated protein kinase (MAPK) and active p34cdc2.
3. Progression to the MII stage of meiosis. The first polar body must be extruded to the perivitelline space, between the oolemma and zona pellucida.
4. Virtually all transcription ceases by the time of germinal vesicle breakdown (GBVD). The expression of genes beyond this stage switches to translation of stored mRNA.

These points are summarized in Table 4.2.

The first event of activation in oocytes of most species is an increase in ionic permeability of the plasma membrane. In the human, the spermatozoon induces an outward current in the oocyte plasma membrane by activating calcium-gated potassium channels. In vitro, activation competence in oocytes is continually changing, and is not a stable, prolonged feature of ovulated eggs; therefore timing is critical in the handling of in-vitro manipulations.

Sperm–oocyte fusion

The process of membrane fusion between gametes is temperature, pH and Ca^{2+} dependent, and the two membranes must be in close approximation. Fusion appears to be mediated or facilitated by membrane-associated proteins, but terminal saccharides of glycoproteins are not directly involved in the process. During penetration of the zona, the spermatozoon loses its acrosomal contents and only the inner acrosomal membrane is in direct contact with the zona. In eutherian mammals, the postacrosomal region of the sperm head plasma membrane only attains fusibility after the acrosome reaction; this area apparently fuses with the oocyte plasma membrane, and the two membranes become continuous (Figure 4.2).

The surface of the oocyte membrane is organized into evenly spaced short microvilli that seem to facilitate gamete fusion; these microvilli have a low radius of curvature which may help to overcome opposing electrostatic charges. In mouse and hamster, the area overlying the metaphase spindle is microvillus-free and spermatozoa are not able to, or are less likely to, fuse with this area. The human oocyte, however, has microvilli present over the entire surface, with no obvious polarity at this stage – there may be "hotspots" for sperm entry into the oocyte. The sperm plasma membrane remains fused with the oocyte plasma membrane and indicates the point of "entry." Sperm motility is required for penetration, but not for gamete fusion; the fertilizing spermatozoon continues flagellar movement for around 20 seconds after attachment to the oocyte surface.

In small mammals sperm–oocyte fusion is quite advanced after 3 minutes, the entire incorporation of the sperm head takes 15 minutes, and pronucleus formation takes about 60 minutes. In some mammals (e.g., the Chinese hamster) the tail is not incorporated, while in others it is incorporated by the progressive fusion of the oocyte and spermatozoal plasma membranes. After incorporation, the midpiece mitochondria and axial filament of the tail appear to disintegrate. Immediately after fusion, the sperm plasma membrane

Table 4.2 Expression of factors during late stages of oogenesis in preparation for fertilization

	G2 →M phase					
	First meiotic block			Second meiotic block		
	Germinal vesicle	Germinal vesicle breakdown	Metaphase I	First polar body	Metaphase II	Post fertilization
Phos p34cdc2	++	–	–	–	–	++
Active p34cdc2	–	++	++	++	++	–
MPF	–	++	++	–	++	–
c-mos	+	+	++	++	++	–
MAPK	–	++	++	++	++	–
cAMP	++	–	–	–	–	–

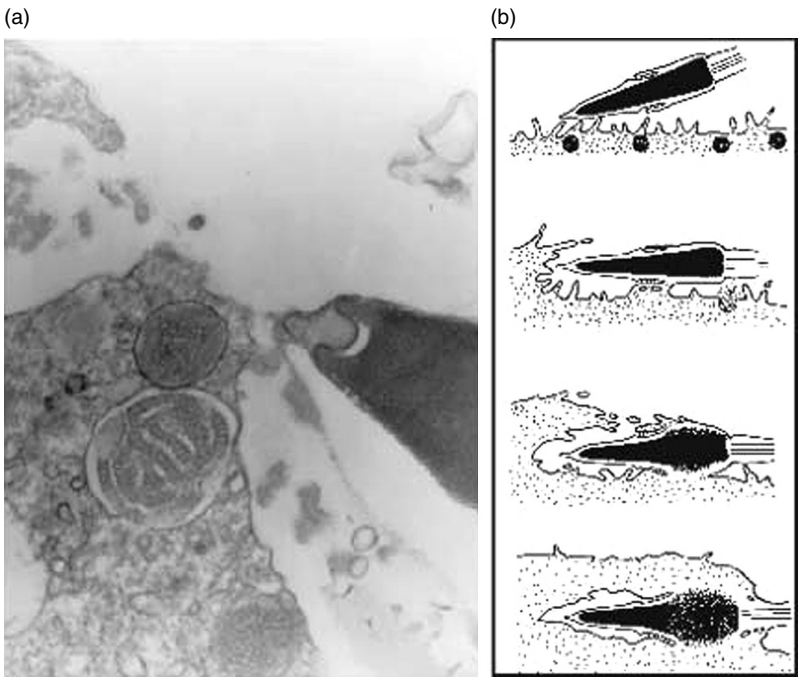

Figure 4.2 (a) Transmission electron micrograph showing the point of sperm–oocyte fusion in the sea urchin. Sperm factor must flow through this cytoplasmic bridge of 0.1 μm diameter. The large granule (1 μm) below the spermatozoon is a cortical granule (from Dale, 1983). (b) Stages in sperm–oocyte fusion in the mammal. Modified from Yanagimachi (1994).

remains localized to the point of entry, integrated into the oocyte plasma membrane, but by the time that the pronuclei have formed, sperm surface antigen has spread all over the surface of the zygote.

The cortical reaction

Cortical granules (CG) are spherical membrane-bound organelles containing enzymes and mucopolysaccharides that lie peripherally in the oocyte (Figure 4.3). Immediately after sperm penetration these granules fuse with the oocyte plasma membrane and release their contents into the perivitelline space by exocytosis – this cortical reaction is the first morphological indication of oocyte activation. The cortical reaction elicits the zona reaction (zona hardening), changing the characteristics of the zona pellucida, and at the same time the egg plasma membrane becomes a mosaic of cortical granule membrane and original plasma membrane. In mammalian oocytes the change is dramatic

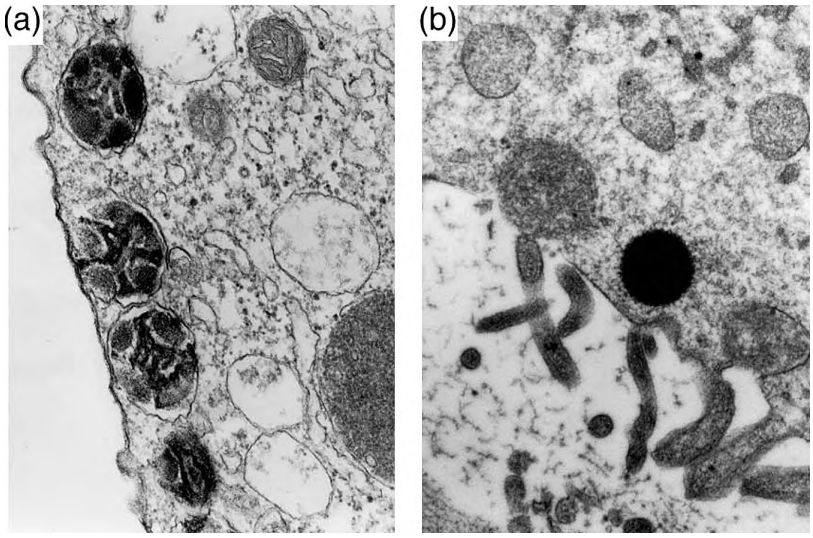

Figure 4.3 Transmission electron micrographs of the surface of unfertilized oocytes of the sea urchin (a) and human (b), showing the cortical granules.

and rapid, does not require any new protein synthesis within the cell, but seems to be related to metabolic de-repression of the oocyte. The resulting transient increase in surface area due to CG/oolemma fusion facilitates the necessary increase in metabolic turnover required by the activated oocyte. In the mouse, the action of proteinases or glycosidases results in hydrolysis of ZP3 receptors, which prevent them from further interaction with sperm – this is known as the vitelline block; the changes that take place via the cortical reaction may have evolved specifically as polyspermy-preventing mechanisms.

The early embryo is a compact mass of cells that are continually dividing, and the embryo is continually changing shape. Such movement would be hindered if the cells were attached to a rigid structure, and the fluid-filled perivitelline space (PVS) acts as flexible boundary that allows movement, while the hardened zona provides a protective structure. The PVS may also serve as a microenvironment, buffering the embryo from changes in its external environment. The hardened ZP keeps the dividing blastomeres of the embryo in close contact, and protects them from potential microbial invasion. The embryo remains in this protective zona coat until hatching, just prior to implantation.

After penetrating the zona and oolemma, the naked sperm nucleus enters the oocyte cortex, moves laterally, rotates approximately 180 degrees, and during the next 10 minutes starts to develop into the male pronucleus. The mitochondria and tail of the spermatozoon also enter the cytoplasm but later degenerate.

Release from meiotic arrest

The universal messenger that triggers reinitiation of meiosis in oocytes at fertilization is an increase in intracellular Ca^{2+} released from intracellular stores in periodic waves or transients (Figure 4.4).

A variety of physical and chemical stimuli can parthenogenetically cause calcium to be released in a similar fashion, but the kinetics of the calcium transients are different, and do not sustain development. There are two hypotheses as to how the spermatozoon triggers intracellular Ca^{2+} release:

The G-protein hypothesis

This model for oocyte activation was extrapolated from the events known about calcium response to hormones in somatic cells. Hormone-receptor binding on the outer surface of the plasma membrane signals through a G-protein in the plasma membrane; this signal triggers the activation of phospholipase C, leading to the formation of inositol 1,4,5-trisphosphate (IP3) and hence calcium release. This model of oocyte activation suggests that the sperm behaves as an "honorary hormone": the attachment of sperm to a sperm receptor triggers IP3 formation through a G-protein linked to this receptor.

The soluble sperm factor hypothesis

The sperm factor hypothesis, proposed by Dale and colleagues in the early 1980s, suggests that intracellular calcium release is triggered by a diffusible messenger(s) in the cytoplasm of the spermatozoon, which enters the

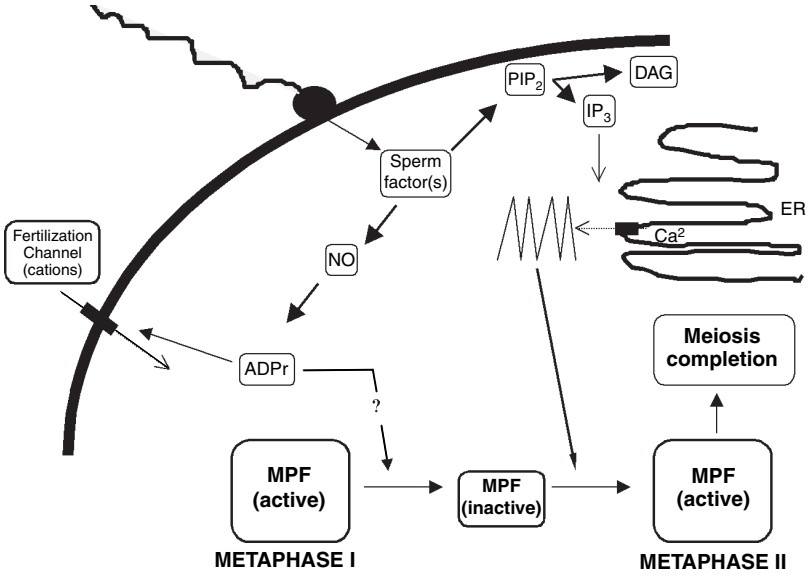

Figure 4.4 Schematic showing the likely mechanisms involved in ascidian oocyte activation after being triggered by the fertilizing sperm. IP3 = inositol 1,4,5-trisphosphate; NO = nitric oxide; DAG = diacylglycerol; PIP2 = phosphatidylinositol-4,5-bisphosphate (from Dale *et al.*, 1999).

oocyte cytoplasm after sperm–oocyte fusion. The first direct evidence for a soluble sperm factor was shown by microinjecting the soluble components from spermatozoa into sea urchin and ascidian oocytes. Several activation events were triggered, including cortical granule exocytosis and gating of plasma membrane currents. The same conclusion was reached when the experiment was repeated in mammals. The soluble sperm factor hypothesis gained further support with the advent of ICSI in the 1990s, where the events of oocyte activation, including calcium release, occur when the surface membrane events are bypassed.

Soluble extracts of spermatozoa can activate oocytes from different phyla as well as different species: mammalian oocytes can be partially activated by microinjecting sea urchin spermatozoa into the cytoplasm. Thus, sperm factors do not appear to be species specific, nor indeed phylum specific. Sperm extracts can also trigger calcium oscillations in somatic cells, suggesting that they may be common calcium-releasing agents rather than sperm-specific molecules. It is possible that both soluble sperm factor and membrane transduction mechanisms interact to trigger oocyte activation.

Spermatozoa contain many calcium-releasing molecules, including adenosine diphosphate ribose (ADPr), IP3, nicotinamide nucleotide metabolites, calcium ions and relatively large proteins. Recently phospholipase C zeta 1, identified in mammalian and human sperm, has been shown to trigger calcium release and lead to oocyte activation. Mammals, together with sea urchins and ascidians, belong to the deuterostome group of animals that show remarkable similarities in gamete physiology. Since changes in plasma membrane conductance, calcium ion release and MPF inactivation are common to all these oocytes at fertilization, the sperm-borne trigger might also be expected to be common. It remains to be seen if phospholipase C zeta 1 is found in the spermatozoa of these other deuterostomes and indeed if this lipase triggers other activation events in the mammalian oocyte such as the ADPr/NO pathway (Dale *et al.*, 2010).

Intracellular calcium release

The pattern of calcium release at fertilization varies from species to species. There are three categories of calcium release mechanisms in oocytes, related to the type of receptor on the intracellular calcium store:

1. Inositol 1,4,5-trisphosphate (IP3)-induced calcium release (IICR). IP3 is produced by the action of phospholipase C on plasma membrane lipid phosphatidylinositol bisphosphate, and IICR is triggered by the binding of IP3 to its receptor on the endoplasmic reticulum.
2. Calcium-induced calcium release (CICR). CICR is triggered by opening the ryanodine receptor on an intracellular store, but can also be triggered in a mechanism involving the IP3 receptor. This can also be triggered by calcium itself, and appears to be modulated by cyclic ADP ribose. Cyclic ADP ribose is, in turn, produced by metabolism of nicotinamide

adenine diphosphate (NAD^+) by ADP ribosyl cyclase or NAD^+ glycohydrolase.
3. $NAADP^+$-induced calcium release. Other calcium-releasing second messengers have been identified, including cATP ribose and $NAADP^+$. Since NAD^+, NADH, $NADP^+$ and NADPH can be metabolized to calcium-releasing second messengers in sea urchin microsomes, it is possible that other calcium-releasing second messengers may be discovered in the nicotinamide nucleotide family.

In mammalian oocytes there is a large increase in the sensitivity to CICR at fertilization, together with a series of repetitive calcium spikes. This again suggests that both CICR and IICR are activated at fertilization.

Centrosomes and centrioles

The centrosome provides the "division center" for the new zygote, forming the basis for the mitotic spindle that guides the chromosomes through their process of duplication and division. In studies on sea urchins and roundworms at the turn of the century, Boveri at the Stazione Zoologica in Naples showed that the male gamete provided this "division center" for the zygote, and predicted that the structure is a cyclical reproducing organ of the cell. The centrosome is paternal in origin, contributed by the sperm – two notable exceptions to this are the mouse and hamster, where it is apparently maternal in origin, lending support to the observation that these rodents are poor model systems for human fertilization. In frogs, however, although the centrosome is contributed by the sperm, it lacks tubulin, which is needed for microtubule nucleation. Tubulin is provided by the oocyte cytoplasm, and therefore the functional frog centrosome is a mosaic of paternal and maternal components.

In somatic cells the centrosome is composed of two structures known as centrioles (Figure 4.5), which are made up of nine triplets of microtubules arranged in a pinwheel shape. These centrioles are placed at right angles to each other, and are surrounded by dense pericentriolar material. During interphase, the centrosome divides to form the poles of the mitotic spindle; after division, this segregates with the chromosomes to each of the daughter cells. In contrast, during oogenesis the centrosome degenerates after meiosis, leaving the oocyte without a "division center" – this is then contributed by the sperm during fertilization. The sperm centrosome has a functional proximal centriole, close to the nucleus, and a degenerate distal centriole.

After the sperm enters the oocyte a small "aster" of microtubules grows out from the centriole, and this aster directs the migration of the sperm pronucleus to the center of the oocyte to make contact with the decondensing maternal pronucleus. This initiates the migration of the maternal pronucleus towards the forming male pronucleus. Microtubules extend from a point in between the now juxtaposed male and female pronuclei, and the centrioles duplicate and migrate to opposite poles during mitotic prophase to set up the first mitotic spindle of the zygote. The zygotic centrosome duplicates and then splits apart during interphase.

Although the centrioles are the main organelles associated with cell division, it is now thought that the pericentriolar material may be principally responsible for organizing the microtubules. In cases of polyspermy, when the oocyte is fertilized by more than one sperm, human oocytes develop multiple asters, each associated with a sperm.

Parthenogenetically activated cattle and human oocytes can organize their microtubules without sperm entry, but this takes place later, and less completely than it does after sperm entry. Defective centrosome function can result in fertilization failure – microtubules are present in the second meiotic spindle of unfertilized oocytes that are arrested in metaphase. During parthenogenesis, where there is no sperm to contribute a centrosome, there is no aster of nucleated microtubules, and the microtubules are found in the cytoplasm throughout the oocyte. In this case, the female centrosome becomes functional, duplicating and forming the mitotic spindle poles.

Six hours post insemination, a small microtubule sperm aster extends from the sperm centrosome, and the activated egg extrudes the second polar body.

Pronucleus formation

During spermatogenesis, gene expression is repressed, DNA replication ceases, and sperm chromatin is tightly packed into a nuclear envelope that lacks pores. The mammalian sperm nucleus is packed with proteins that contain highly charged basic amino acids (protamines) that condense the DNA and repress transcription. Extensive disulfide linking in the protamines make the sperm head rigid, a property that is necessary for penetration of the zona. Protamine crosslinking in the human spermatozoon is regulated by Zn^{2+} present in prostatic gland secretions. When the sperm nucleus enters the oocyte cytoplasm, the process is reversed,

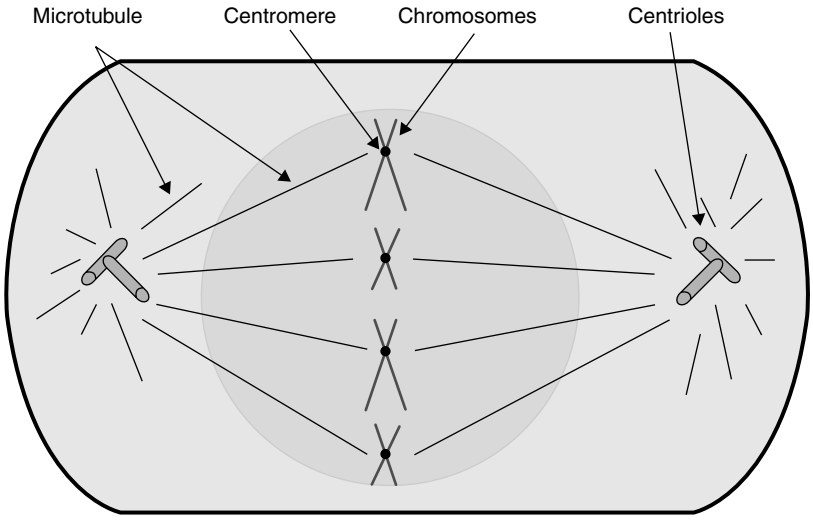

Figure 4.5 Schematic representation of the spindle apparatus, showing centrioles, microtubules and chromosomes with centromeres.

with changes in morphology and biochemical processes. The sperm nuclear membrane breaks down, and under the influence of factors in the oocyte cytoplasm, the highly condensed chromatin starts to swell and become dispersed, so that filaments of chromatin are released into the cytoplasm. The factors that cause decondensation are apparently not species-specific, since human sperm can decondense when microinjected into an amphibian oocyte – a reduced form of glutathione is probably responsible for facilitating the process, by reducing disulfide bonds in sperm nuclear protamines.

The mammalian oocyte is in metaphase II stage at the time of sperm entry; the sperm nuclear envelope dissolves and chromatin starts to decondense while the oocyte transits from metaphase II to telophase II. During telophase II, the sperm chromatin completes its decondensation as the female pronucleus develops. Sperm protamines are replaced by histones, and the male and female pronuclear envelopes then develop synchronously. Recent research, however, suggests that paternal histones delivered to the egg are retained in the male pronucleus and contribute to zygotic chromatin (van der Heijdn *et al.*, 2008). Between 4 and 7 hours after fusion, a new nuclear membrane forms around the decondensed male and female chromatin, creating pronuclei that contain the two sets of haploid chromosomes. Oocyte cytoplasm contains factors that are necessary for the development of the male pronucleus; these factors are again not species-specific, since human spermatozoa can develop into normal pronuclei in hamster oocytes, and form a normal chromosome complement. In hamster oocytes, a maximum of five pronuclei will decondense at any time, indicating that the factors may be present in limited quantities.

In the bovine system, sperm tagged with a mitochondrion-specific vital dye have been used to follow sperm incorporation and the conversion of sperm-derived components within the zygote. The zygotes were then fixed at various times after fertilization for immunochemistry and ultrastructural studies. These experiments showed that complete incorporation of the sperm can be inhibited by the microfilament disrupter cytochalasin B, and therefore this process depends upon the integrity of oocyte microfilaments. After sperm incorporation, the mitochondria were displaced from the sperm midpiece, and the sperm centriole was exposed to egg cytoplasm. The microtubule-based sperm aster then formed, initiating union of male and female pronuclei. The disassembly of the sperm tail occurred as a series of precisely orchestrated events, involving the destruction and transformation of particular sperm structures into zygotic and embryonic components.

Nucleolar precursor bodies and nucleoli

When the two pronuclei are formed in the zygote, they contain structures known as nucleolar precursor bodies (NPBs); the number and pattern displayed by their arrangement became popular as criteria for evaluating the embryonic potential of individual zygotes (Tesarik and Greco, 1999; Scott *et al.*, 2000). Spermatozoal nucleoli are destroyed during spermatogenesis (Schulz

and Leblond, 1990), and recent evidence has shown that oocyte nucleolar material is required for the reassembly of nucleoli in both female and male pronuclei (Ogushi *et al.*, 2008).

Tesarik and colleagues (1986, 1988) described the mechanism of NPB and nucleolar formation in human embryos, a scheme that differs from the process that takes place during the differentiation of adult somatic cells. In somatic cells, nucleoli are periodically reconstituted during mitotic telophase, with first a dense fibrillar component and then a granular component appearing around specific loci on chromosomes bearing rRNA genes, usually located at or close to a secondary constriction. In contrast, rDNA of the mammalian zygote and embryo appears to be incapable of starting transcription and thus triggering the nucleogenetic process unless it is associated with nuclear precursors.

The sequence of events described by Tesarik and colleagues for human embryos involves four stages:

1. Transcriptionally inactive rDNA has not yet penetrated into the homogeneous nuclear precursor (pre-nucleoli).
2. rDNA penetrates into the heterogeneous nuclear precursor and turns on the synthesis of pre-rRNA, whose processing is still inactive.
3. Processing of pre-rRNA is progressively activated in compact fibro-granular nucleoli.
4. Active pre-rRNA synthesis and processing occur in reticulated nucleoli.

This scheme, however, did not include information about the paternal or maternal origin of the components. The oocyte nucleolus is derived from material that is present in the germinal vesicle, and its content may change during the process of oocyte maturation. It is composed of approximately 700 proteins, with roles in many cellular processes, including cell cycle regulation and apoptosis. Ogushi *et al.* (2008) conducted a series of experiments leading to the conclusion that the nucleoli of both male and female pronuclei are maternal in origin, derived exclusively from maternal nucleolar remnants retained at the time of GVBD. Microsurgical removal of murine and pig oocyte nucleoli during prophase I indicated that the nucleoli did not contain the factors necessary for oocyte maturation. However, after fertilization of mature oocytes without nucleoli, no nucleoli were then visible in the resulting zygote pronuclei, and these zygotes could not complete development to blastocyst stage. Transfer of GVs to recipient cytoplasts indicated that material(s) within the mature oocyte GV, showing a ring of condensed chromatin around the nucleolus just prior to GVBD, is essential for full-term development. Although the oocyte nucleolus is clearly involved, the factors have not been identified, and may include precursor molecules required for the assembly of fully functional nucleoli at a later stage in the development of the embryo.

Lefèvre (2008) described and summarized these experiments: "The nucleoli of the two pronuclei are exclusively of maternal origin, and the oocyte nucleolar material is essential for the reassembly of nucleoli in both male and female pronuclei."

Syngamy

After the male and female pronuclei are formed, over the next few hours they gradually migrate to the center of the oocyte, until they are adjacent to each other. During this period DNA is synthesized, in preparation for the first mitotic division. The process of migration has been studied extensively in the sea urchin and the mouse. In the mouse, fluorescein conjugated probes for cytoskeletal elements show a thickened area of microfilaments below the cortex of the polar body region. In addition to the spindle microtubules there are 16 cytoplasmic microtubule organizing centers (MTOCs) or foci, and each centrosomal focus organizes an aster. These foci condense on the surface of the envelope just before the pronuclear membranes disintegrate. The mitotic metaphase spindle then forms, which involves duplication of the proximal centriole contributed by the sperm centrosome to form a pair of polar centrioles, and the chromosomes are aligned along the spindle equator. The plane of cell division is mediated by astral microtubules that extend from the mitotic spindle to the plasma membrane. Between 18 and 24 hours after gamete fusion, the two sets of chromosomes come together in syngamy (Figure 4.6), a cleavage furrow forms as soon as the first mitotic anaphase and telophase are completed, and the zygote becomes a two-cell embryo.

"The sun in the egg"

Leopold Auerbach of Breslau, Germany (1828–1897) described two protoplasmic vacuoles in a newly fertilized egg, as well as a radiating figure between them. In 1876, Oscar Hertwig identified these vacuoles as the male and female pronuclei (Figure 4.7), and he observed their fusion. When the two nuclei merged together in syngamy, he described the figure:

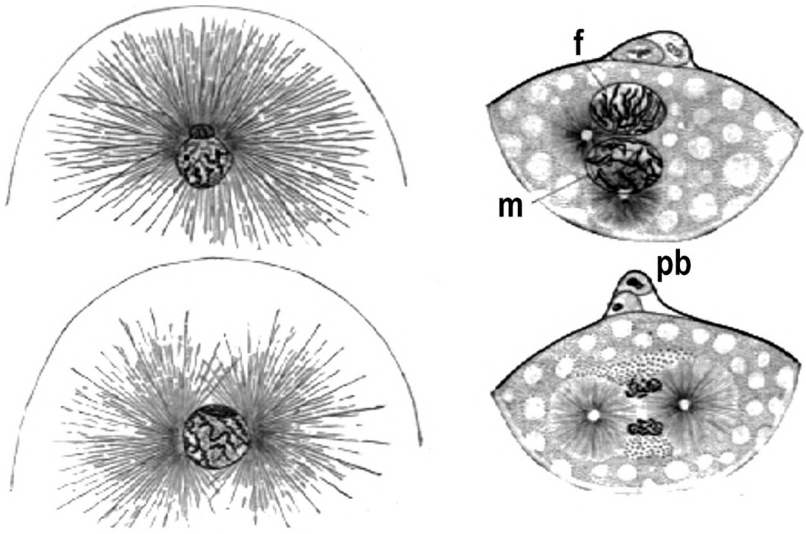

Figure 4.6 A drawing from Wilson (1900) showing (left) the fusion of male (m) and female (f) pronuclei to form the zygote nucleus as in sea urchins. In the majority of animals (and usually in mammals) the pronuclear membranes break down without fusing, allowing the chromosomes to interact in the cytoplasm (frames on the right). pb = polar body.

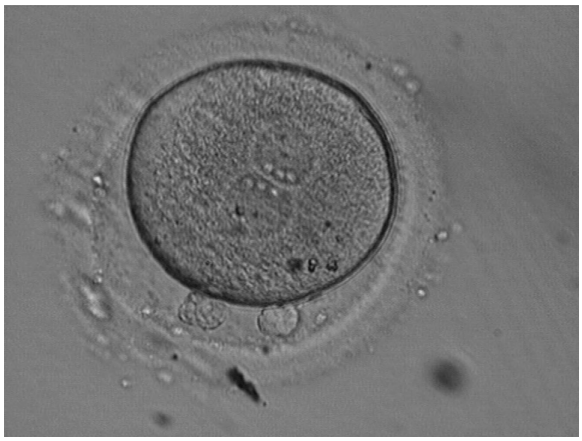

Figure 4.7 Fertilized oocyte with two pronuclei and two polar bodies clearly visible; nucleolar precursor bodies are aligned in the pronuclei.

Es entsteht so vollstandig das Bild einer Sonner im Ei.
It arises to completion like a sun within the egg.

In an analysis of Hertwig's paper, Paul Weindling (1991) proposed, "This vivid image conveyed the discovery of the moment at which a new life was formed. The metaphor expressed awareness that the force of natural powers was greater than the sum of two cells."

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Chapter

5 First stages of development

Preimplantation development

After completing fertilization with fusion of the pronuclei during syngamy, the zygote now has a diploid complement of chromosomes, undergoes its first mitotic division and then continues to divide by mitosis into a number of smaller cells known as blastomeres. In humans, the first few cleavage divisions take place in the oviduct, before the embryo reaches its site of implantation in the uterus (Figure 5.1).

In contrast to oogenesis, where the cell undergoes a period of growth without replication or division, early embryo cleavage involves intense DNA replication and cell division in the absence of growth – the overall size of the embryo does not change as a result of the early cleavage divisions. As cleavage progresses, the embryo polarizes, and differences arise between the blastomeres; this process of differentiation may be regulated by unequal distributions of cytoplasmic components previously laid down in the oocyte during oogenesis, or by changes occurring in the blastomeres as a result of new embryonic gene transcription during development (Figure 5.2). Each blastomere nucleus will be subjected to a different cytoplasmic environment, which, in turn, may differentially influence activity of the genome. As a result, after the onset of zygote gene activation and subsequent differentiation, eventually the blastomeres set off on their own specific program of development. Maternal mRNA encoding developmental information is essential in early differentiation, and has been found to persist during specific patterns of gene expression until gastrulation in some species; embryos in some bats and marsupials can remain in a state of diapause (dormancy) within the uterine cavity for many months. In mammals, maternal mRNA rapidly disappears after the major activation of the genome, i.e., at the two-cell stage in the mouse, four- to six-cell in humans, and eight-cell in sheep and cattle. Localized short- and long-lived maternal mRNAs probably regulate the initial stages of differentiation; there is some evidence to suggest that stores of maternal RNA in the oocytes of older women may be depleted, perhaps due to dysfunction or disruption of the mechanisms that control its storage.

Genome activation

As described in Chapter 3, the developing oocyte accumulates reserves of mRNA, proteins, organelles, etc. which are required to support and direct early development. In vitro, the early embryo shows very little metabolic activity during its first few cleavage divisions. The zygote initially depends on stored maternal mRNA to direct the first two cleavage divisions, and then activation of the new embryonic genome provides novel transcripts and reprograms the pattern of gene expression to direct further development. After the long period of gene suppression in both gametes, the new embryonic cell cycle must be precisely timed and regulated, with a correct timing of DNA synthesis during S-phase. Without appropriate zygotic genome activation (ZGA), the mammalian embryo fails to develop further. This critical transition takes place during the four- to eight-cell stage in humans, and maternal mRNA rapidly disappears whilst the zygote genome gradually increases its expression. However, the transition is not absolute, and a small amount of maternal message is needed almost up until the blastocyst stage – therefore previous failures at any stage of oocyte development, maturation, and handling can affect development even after ZGA. Gene expression involves conformational changes in nucleosome organization (like uncoiling a spring), regulated by interactions between DNA methylation, histone acetylation and messenger RNA polyadenylation patterns. Activation of the embryonic

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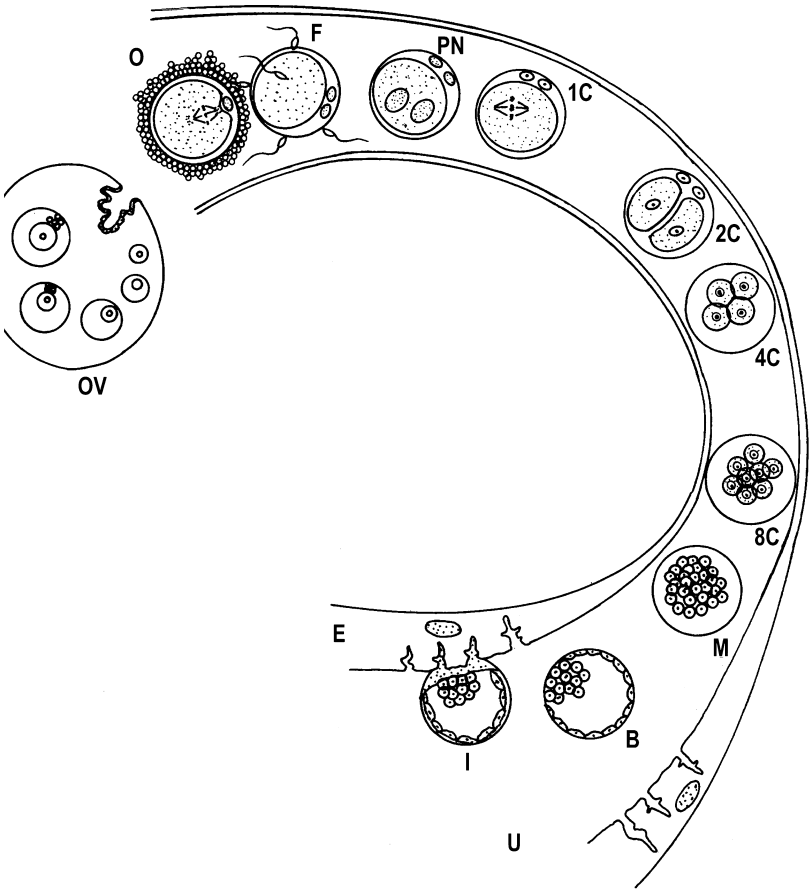

Figure 5.1 Development of the mammalian embryo. The oocytes released from the ovary (OV) enter the ampulla where they are fertilized (F) and then are transported along the fallopian tube, cleaving to generate the morula stage (M). The blastocyst expands, hatches and then implants in the endometrium (E) of the uterus. From Sathananthan *et al.* (1993).

genome follows a series of progressive steps; the timing and coordination of gene expression can be regulated at the level of maternal mRNA translation.

1. Maternal RNA transcripts are depleted, and new embryonic mRNA is transcribed. The replacement of maternal transcripts by those of the zygote occurs at different rates for different genes (Temeles *et al.*, 1994); this may be due to the relative importance of different transcripts for immediate developmental events, or because the protein products differ in their stability, or both.
2. There is a qualitative shift in protein synthesis, and in post-translational modification.
3. A functional nucleosomal structure develops, the nuclear organizing region (NOR). Establishing the precise timing of genome activation is related to the sensitivity of methods available for its detection; new protein synthesis activity has been detected at different stages in different species:

Rabbit	1–2 cells
Mouse	2 cells
Cow	4–8 cells
Human	4 cells
Sheep	8–16 cells
Drosophila	4000 cells
Xenopus	5000 cells

There is evidence to suggest that a minor degree of transcriptional activity does take place prior to the major activation of the genome, with a period of minor gene activation from the paternal pronucleus in the one-cell embryo, followed by a period of gene activation in the two-cell embryo when maternal mRNA and zygotic gene transcripts are handled differently, so that transcription and translation of nascent transcripts is delayed (Wiekowski *et al.*, 1991; Nothias *et al.*, 1995;

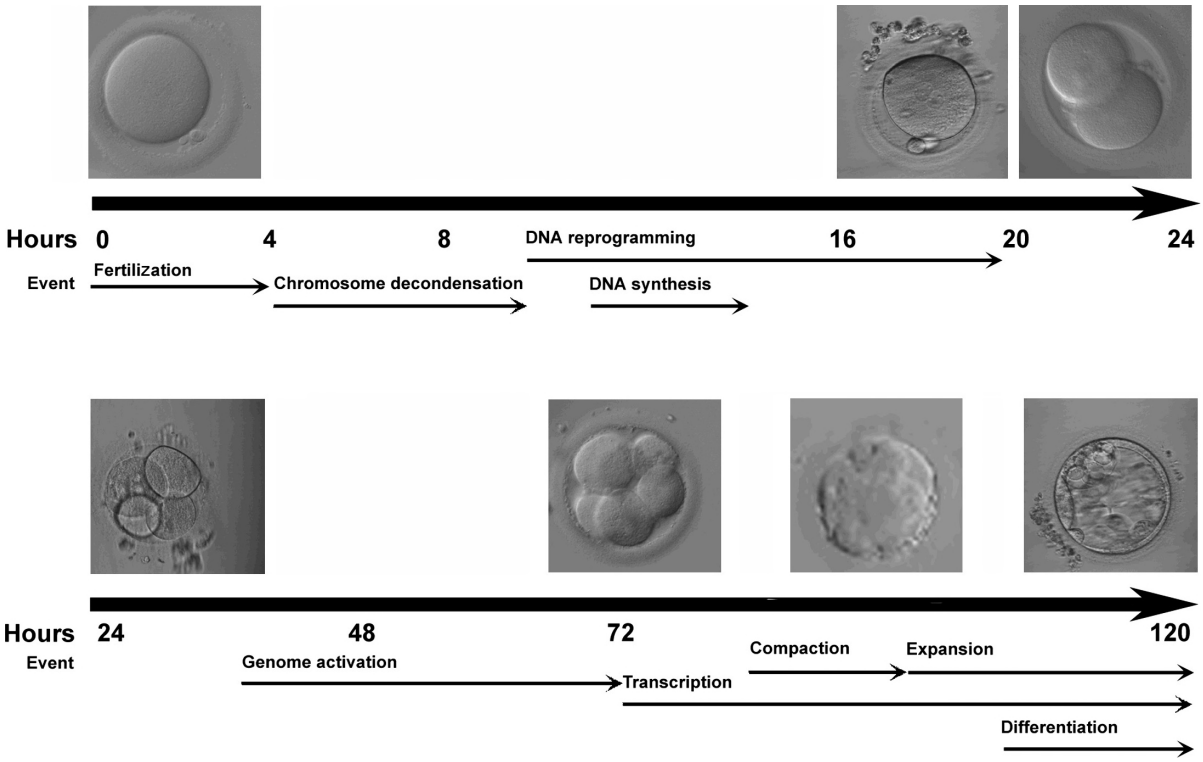

Figure 5.2 Timescale of early human embryo development up to the blastocyst stage, correlating morphological changes with developmental events. Time is in hours.

Schultz, 2002). In human preimplantation embryos, reverse transcriptase-polymerase chain reaction (RT-PCR) detected early transcripts for two paternal Y chromosome genes, *ZFY* and *SRY*. *ZFY* transcripts were detected at the pronucleate stage, 20–24 hours after in-vitro insemination, and at intermediate stages up to the blastocyst stage. *SRY* transcripts were also detected at two-cell to blastocyst stages (Ao *et al.*, 1994).

Cleavage increases the number of nuclei, which amplifies the number of templates that will facilitate the production of specialized proteins needed for the later processes of compaction and differentiation to the blastocyst stage. Changes in chromatin structure, rather than changes in the activity of the transcriptional apparatus, may underlie the timing and basis for ZGA. Transcription factors must be available that can bind to the DNA, and a functional physical structure, the nuclear organizing region (NOR) develops, producing conformational changes in the DNA structure that will allow the binding of promoters and enhancers of transcription. In the mouse and rabbit, there is a general chromatin-mediated repression of promoter activity. Repression factors are inherited by the maternal pronucleus from the oocyte, but are absent in the paternal pronucleus; they become available sometime during the transition from a late one-cell to a two-cell embryo (Henery *et al.*, 1995). This means that paternally inherited genes are exposed to a different environment in fertilized eggs than are maternally inherited genes, a situation that could contribute to genomic imprinting.

The formation of the NOR is related to the nuclear:cytoplasmic ratio. A titratable factor in the cytoplasm, possibly related to cdc25, may be diluted with the increase in nuclear:cytoplasmic ratio, driving maturation promoting factor (MPF) and a kinase cascade that triggers mitosis. The gradual depletion of cdc25 causes a pause in cleavage, which allows time for the nuclear organizing region to develop, and mitosis induces a general repression of promoters prior to initiation of zygotic gene expression. Enhancers then specifically release this repression. A biological clock may delay transcription until both paternal and maternal genomes are replicated; they must then be remodeled from a postmeiotic state to one in which transcription

is repressed by chromatin structure. The chromatin structure must have a configuration that allows specific transcription enhancers to relieve/reverse repression at appropriate times during development. Differential hyperacetylation of histone H4 (particularly on DNA in the male pronucleus) has been implicated in the remodeling of maternal and paternal chromatin, and depletion of maternally derived histones has also been suggested as one of the mechanisms involved in ZGA (Adenot *et al.*, 1997).

Chromatin-mediated repression of promoter activity prior to ZGA is similar to that observed during *Xenopus* embryogenesis; this mechanism ensures that genes are not expressed until the appropriate time in development. When the time is right, positive factors such as enhancers can begin their activity. The mechanism by which enhancers communicate with promoters seems to change during development, and may depend upon the presence of specific co-activators. In the mouse, ZGA occurs during the second cell division cycle (two-cell stage) and seems to be regulated by a "zygotic clock" that measures the time following fertilization rather than progression through the first cell cycle. There is evidence that circadian clock genes may be involved in programming an appropriate timing, so that development is synchronized with endocrine and other local factors to ensure successful implantation. In support of this concept, expression of circadian clock genes has been found in the reproductive tract and conceptus of the mouse during the first four days of pregnancy (Johnson *et al.*, 2002). It may be that in vivo, mammalian ZGA is a time-dependent mechanism that must interact in synchrony with the cell cycle and other physiological events.

Imprinting

During oogenesis and spermatogenesis, the maternal and paternal chromosomes are packaged in a manner that affects subsequent transcription of some of the genes during development. The DNA sequence that specifies the parental genes is not altered, but the way in which it is chemically modified and packaged in chromatin affects the expression of the genes. Because the genetic code itself remains the same, this modification is referred to as an *epigenetic* change, and the phenomenon is known as imprinting. The pattern of epigenetic change is parentally specific, i.e., the genes affected (imprinted) in oocytes differ from those imprinted in sperm – there is differential expression of the two parental alleles of a gene. In terms of function, this means that although the oocyte and the sperm each contribute a complete set of genes, each set on its own is not competent to direct a complete program of development; a fully functional genome requires the combination of both paternal and maternal genes. The process of genomic imprinting is established during gametogenesis, and the nucleus of the zygote has an imprint memory that is retained by the embryo into both prenatal and postnatal life. Imprinting is highly regulated during preimplantation development; the topic is thus highly significant and relevant to in-vitro manipulations and culture, and will be discussed in detail in Chapter 15.

Compaction

The first event that determines the directed development of previously undifferentiated blastomeres is compaction. During the first few cleavage divisions to reach the four- to eight-cell stage, individual blastomeres can be clearly seen in the developing embryo. At about the third cleavage division there is a significant increase in RNA and protein synthesis, a marked change in the patterns of phospholipid synthesis, and the embryo undergoes compaction to form a morula. This process is calcium-dependent, and requires prior transcription of the zygote genome. With compaction, the blastomeres flatten against each other and begin to form junctions between them, so that the boundaries between blastomeres can no longer be distinguished. The cells of the compacted embryo become highly polarized and tightly associated, with redistribution of surface microvilli and other plasma membrane components.

Coordination of these complex developmental processes requires communication between the cells; two types of intercellular junction have been described:

1. Structural tight junctions and desmosomes anchor the cells together and form an impermeable epithelial barrier between cells. Tight junctions are composed of several integral and peripheral proteins, including occludin and cingulin (ZO-1).
2. Low resistance junctions such as gap junctions allow the flow of electrical current and the direct transfer of small molecules, including metabolites and second messengers (cAMP) between blastomeres.

Compaction has been extensively studied in the mouse: the distribution of dense microvillar and amicrovillar regions indicates surface polarity, and

the distribution of endocytotic vesicles and actin filaments, and the location of the cell nucleus demonstrates polarity in the cytoplasm. In the mouse, isolated blastomeres that are decompacted experimentally maintain their polarity, and compaction does not require either a prior round of DNA replication, or protein synthesis (Kidder and McLachlin, 1985). Therefore, the four-cell embryo probably contains some of the proteins required for compaction. Although the factors that trigger the timing of its onset are not known, experimental evidence suggests that this may be regulated by post-translational modification of specific proteins such as E-cadherin. E-cadherin protein (uvomorulin) is expressed in the oocyte, and during all stages of preimplantation development. It is uniformly distributed on the surface of blastomeres and accumulates in the regions of intercellular contact during compaction. E-cadherin phosphorylation can be observed in the mouse eight-cell embryo. Culturing embryos in calcium-free medium inhibits E-cadherin phosphorylation and prevents compaction, but the situation is complex, and precise mechanisms behind the molecular basis for compaction and its timing remain unclear.

In human embryos, tight junctions begin to appear on Day 3, at the 6–10-cell stage, heralding the onset of compaction. The surface morphology of human oocytes and embryos has been studied with scanning electron microscopy (Santella *et al*., 1992; Dale *et al*., 1995; Nikas *et al*., 1996), and this showed that unfertilized oocytes one day after insemination were evenly and densely covered with long microvilli. The length and density of microvilli appeared to decrease in fertilized oocytes, and a further decrease was observed in Day 2 and Day 3 embryos with 2–12 cells. There was no evidence of surface polarity until Day 4, when it was evident in the majority of embryos with 10 or more cells. The microvilli appeared dense again with a polarized distribution over the free surface of the compacted blastomeres.

In the mouse, gap junctions are expressed at the 8-cell stage, and their *de novo* assembly during compaction is a time-dependent event. Inhibition of DNA synthesis during the third and fourth cell cycles has no effect on the establishment of gap junctional coupling during compaction (Valdimarsson and Kidder, 1995), but a delay of 10 hours in DNA synthesis during the second cell cycle results in the failure of gap junctional coupling at the time of compaction. In human embryos, gap junctions are not apparently well developed until the early blastocyst stage, when intercellular communication is clearly seen between inner cell mass (ICM) cells (Figures 5.3 and 5.4; Dale *et al*., 1991).

Following compaction, the developing embryo is described as a morula, seen in the human normally 4 days after fertilization. The embryo now shows a significant increase and change of pattern in RNA, protein and phospholipid synthesis, and this results in a process of differentiation so that cells are now allocated to an ICM, with outer cells forming an epithelial layer of trophectoderm. Whereas early blastomeres are totipotent (as evidenced by experimental embryo splitting and chimera formation), at compaction the cells polarize radially, and differential division across this axis creates different populations of cells, with unequal distributions of organelles:

1. Outer polar cells with surface microvilli and redistribution of other plasma membrane components are restricted to the free outer surface of the embryo, and these cells form the trophectoderm.
2. Inner apolar cells with tight junctions containing basal nuclei, which will form the ICM.

These morphological transitions are thought to be brought about by differential gene expression with corresponding protein expression profiles, but these have not yet been clearly defined at the molecular level. Experimental interference with adhesion between cells during compaction shows that this process is important in determining cell lineages. ICM cells preferentially communicate with each other and not with trophectoderm cells via gap junctions, whereas trophectoderm cells communicate with each other and not with ICM cells. The ratio of trophectoderm to ICM cells can be influenced by culture conditions in vitro, and this might have implications for further embryonic/fetal development.

Cavitation

Between the 16- and 32-cell stage, a second morphological change occurs, known as cavitation. Activation of Na^+, K^+ ATP-ase systems in the trophectoderm cells results in energy-dependent active transport of sodium pumped into the central area of the embryo, followed by osmotically driven passive movement of water to form a fluid-filled cavity, the blastocoele. The

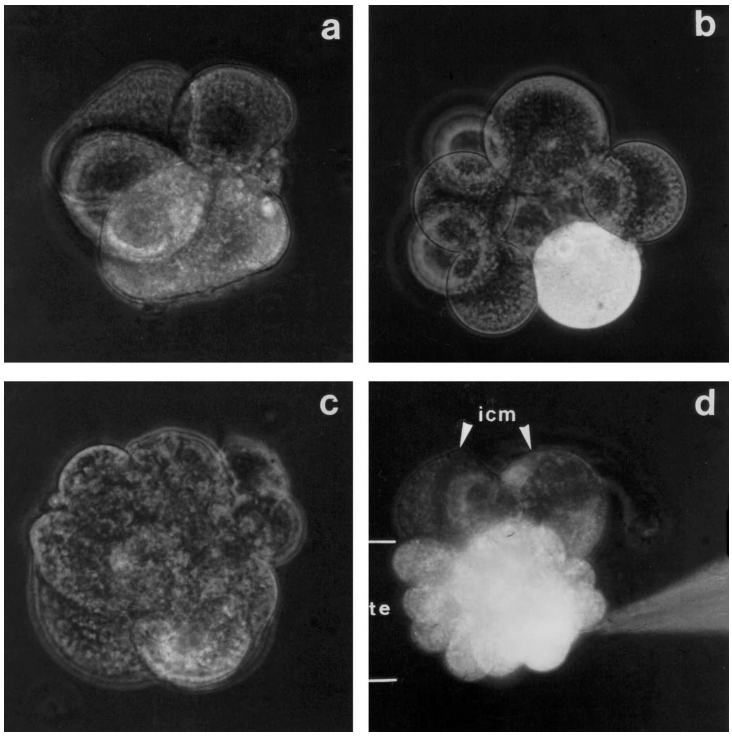

Figure 5.3 Functional expression of gap junctions in the early human embryo shown by microinjection of the low molecular weight tracer lucifer yellow. There is no dye spread in four cells (a), ten cells (b) or morula (c). However, transfer occurs in the blastocyst stage. icm = inner cell mass. From Dale *et al.*, 1991.

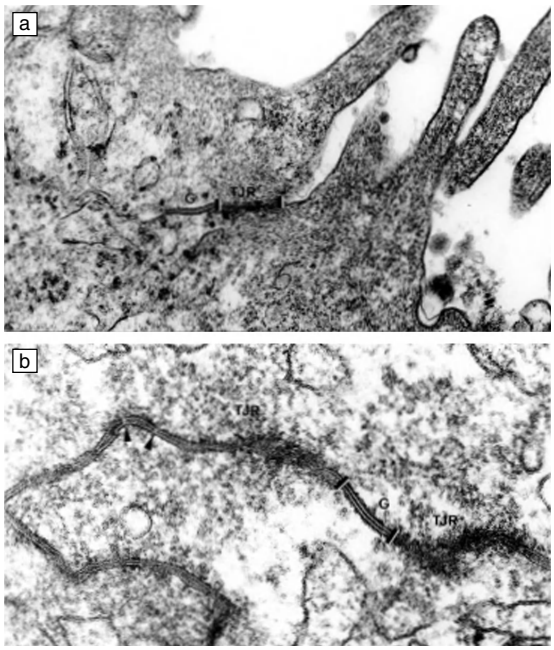

Figure 5.4 Transmission electron micrographs showing tight junctions (TJR) and gap junctions (G) in the human morula. (a) is a section at the apical level between two polar cells at a magnification of ×75 500; (b) shows a typical section between a polar and an apolar cell at ×158 000. Arrowheads show sites of tight membrane contact. From Gualteri *et al.*, 1992.

movement of other ions such as chloride and bicarbonate also contributes to blastocoele formation. Immunohistochemistry shows that the trophectoderm cells are sitting on a basement membrane, and tight junctions form a continuous belt between trophectoderm cells, preventing leakage of small ions in the blastocoelic fluid.

Blastocoele formation and expansion is critical for further development, as it is essential for further differentiation of the ICM. This is now bathed in a specific fluid medium, which may contain factors and proteins that will influence cell proliferation and differentiation. The position of cells within the ICM in relation to the fluid cavity might also contribute to the differentiation of the outer cells into primitive endodermal cells.

Apoptosis can be seen at the blastocyst stage, localized to the inner cell mass: this may represent a mechanism for the elimination of inappropriate or defective cells.

The trophectoderm cells will eventually form the placenta and extra-embryonic tissue. Myxoploidy of trophectoderm cells is a common feature in all animal species, regardless of their implantation mechanisms; mouse and cow, which differ completely in their mechanism of implantation, show this feature, with chromosome complements of 2n, 4n, 8n in their trophectoderm

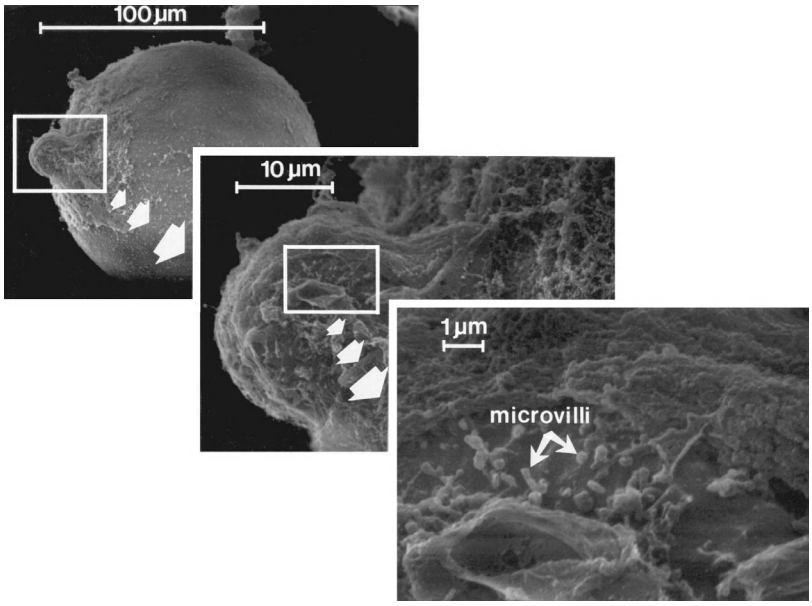

Figure 5.5 Scanning electron micrograph of a hatching human embryo. The microvilli on the surface of the trophectoderm cells are bared owing to internal pressure in the blastocoele and dissolution of the zona pellucida.

cells. However, in humans it could possibly be considered as the initiation of syncytiotrophoblast formation. The regulation of this process, and apparent lack of division in these cells, remains a mystery – but it seems to be related to the appearance of giant cells in the trophectoderm, suggesting that regulation of the nuclear/cytoplasmic ratio is involved. It is interesting to note that there is a counterpart of "giant cells" in the uterus around the time of implantation. A retroviral syncytin envelope gene with cell–cell fusion activity has been identified in mammalian syncytiotrophoblast, and is postulated to be responsible for syncytiotrophoblast formation (Heidmann *et al.*, 2009).

Blastocyst expansion and hatching

In humans, the early blastocyst (Day 4/5) initially shows no increase in size, but it subsequently expands over the next one or two days (Day 5/6) by active accumulation of fluid in the central blastocoelic cavity. Throughout these early stages the embryo is enclosed in the zona pellucida, which keeps the cells together prior to compaction and acts as a protective barrier. If the ICM divides at this early stage, monozygotic (identical) twins may develop. Before the blastocyst can start the process of implantation, it must free itself from the protective zona pellucida, which becomes visibly thinner as the blastocyst expands; in vitro, initiation of the hatching process can be seen as trophectoderm cells "escaping" from the zona pellucida (Figure 5.5). Hatching is completed within a few hours, and the freed blastocyst is separated from its empty zona (see also Chapter 11, Figure 11.8).

Cell fate and cell lineages

At the earliest stages of development the transcriptional machinery that will direct differentiation is not switched on, and transcription is under the control of specific transcriptional regulators, regulatory RNAs and chromatin remodeling machinery, which are in turn influenced by epigenetic marks, cell positional history, cell polarity, and orientation of division. As discussed in Chapter 3, the mammalian oocyte has evidence of polarity, and this has been confirmed in human oocytes. Maternal factors are subsequently important in establishing polarities, regulating cleavage planes, and in allocating specific blastomeres to their eventual fate. By the morula stage, cell fate decisions have been made, and an axis is established with embryonic (ICM) and abembryonic (trophectoderm) poles. Cells positioned on the inside of the morula retain pluripotency, and those on the outside develop into extraembryonic trophectoderm which will support the development of the embryo in the uterus and influence embryonic patterning before gastrulation via signaling mechanisms. The generation of inside cells requires outer cells to divide in an orientation

such that one daughter cell is directed inwards during the 8–16-cell and 16–32-cell stages – these divisions are known as differentiative, in contrast to conservative divisions in which both daughter cells remain on the outside. Because inside and outside cells will follow different fates, differentiative divisions probably distribute cell fate-determining factors asymmetrically between the daughters. Several molecules that influence polarization have been identified: the Ca^{2+} dependent E-cadherin molecule is implicated in generating blastomere polarity, localized geographically at division together with the actin microfilament stabilizing protein ezrin. Homologues of PAR (partitioning defective proteins) also influence the regulation of cell polarization and the control of asymmetric cell divisions via positioning effects on the spindle. The transcription factor Cdx2 is required for the commitment of outer cells to the trophoblast (see Chapter 7).

The molecular basis for the generation and stabilization of polarity in development is not fully understood, but evidence is accumulating to suggest that cell fate may be determined as early as the 4-cell stage in human embryos: mRNAs specific for a trophectoderm lineage (beta-hCG) were identified in a single blastomere of a 4-cell embryo, and not at the 2-cell stage; a single putative trophectodermal precursor appears to emerge during the second cleavage division (Hansis *et al.*, 2002; Edwards and Hansis, 2005). In mouse embryos, lineage tracing experiments using fluorescent tracers and optional sectioning indicate that differences in developmental properties of individual blastomeres may be determined at the 2-cell stage (Piotrowska *et al.*, 2001, 2005). These experiments showed that the first cell to divide to the 4-cell stage contributed preferentially to the embryonic cell lineage, whereas the later-dividing blastomere contributed to the abembryonic (trophectoderm) lineage. By the blastocyst stage, the position of cells within regions of the blastocyst has an influence on their subsequent fate in postimplantation development (Gardner, 2001, 2007).

Causes of early embryo arrest

Cleaved embryos do frequently arrest their development in culture, and a great deal of research has been carried out in animal systems to elucidate possible causes and mechanisms. The longest cell division cycle during development is that during which genome activation takes place, when maternal transcripts are degraded and massive synthesis of embryonic transcripts is initiated. Maternal reserves are normally sufficient until transcription begins, but epigenetic effects of defective sperm can lead to accumulation of delays, with resulting arrested development. Antisperm antibodies can have deleterious effects at this stage, by immunoneutralization of proteins that signal division (CS-1) or regulation (Oct-3). After genome activation, the next critical stage is morula/blastocyst transition. Complex remodeling takes place, and poor sperm quality can compromise this transition (see Ménézo and Janny, 1997).

Embryonic arrest is frequently a result of events surrounding maturation, but can be a result of any metabolic problem. In bovine and pig oocytes, insufficient glutathione inhibits decondensation of the sperm head and polar body formation, and genetic factors regulate the speed of preimplantation development. Genetic factors implicate enzyme deficiencies or dysfunctional regulation, which may have deleterious effects. In domestic animals, as in humans, there is an age-related maternal effect. Maternal age has an effect on embryo quality, especially on blastocyst formation – this may be related to an ATPase dependent Na^+/K^+ pump mechanism, or to a poor stock of mRNA, poor transcriptional and/or post-transcriptional regulation, or accelerated turnover of mRNA.

In clinical IVF, detection of two pronuclei is regarded as evidence that normal fertilization has taken place, and the formation of a normal mitotic spindle following fertilization is critical in order to ensure correct chromosomal alignment. Mistakes at this stage can be lethal, resulting in chromosomal disorders such as aneuploidy. In some cases a first cleavage division takes place when no pronuclei have been detected during the previous 24–28 hours, a phenomenon that has been described as "silent fertilization." The first cell division tends to be asymmetrical, and the embryos arrest during cleavage. Van Blerkom *et al.* (2004) undertook a multiyear study of oocytes and embryos in which silent fertilization was suspected, using scanning laser confocal fluorescence microscopy to study chromosomal and microtubular structures. They were able to visualize maternally and paternally derived spindles in embryos that had shown no sign of pronuclear evolution after multiple, closely-spaced inspections at the 1-cell stage, with maternal and paternal spindles well separated. The authors suggest that the evolution of such embryos may have an unusual pattern of chromosomal segregation, leading to micro- or multinucleation. The mechanisms

involved in silent fertilization could be due to defects in normal calcium signaling, inadequate cytoplasmic maturity, or delayed release of sperm-derived factors that also modulate calcium signaling.

Fluorescent in-situ hybridization (FISH) analysis of cleaved human embryos has confirmed that chromosomal aberrations are found in a significant proportion of embryos which develop with regular cleavage and morphology; this undoubtedly contributes to the high wastage of embryos in human IVF.

Paternal factors

Sperm quality may have an influence on embryogenesis and implantation potential. Increasing paternal age is thought to have an influence on fertility, possibly through increased nondisjunction in the sperm. Damage during spermatogenesis may be induced by reactive oxygen species and defective oxidative phosphorylation, or via inherited dysfunctional mitochondrial DNA. Fertilization by a sperm that is diploid, with incomplete decondensation and DNA activation or inadequate chromatin packaging, may cause aneuploidy or lack of genome competence in the embryo. The quality of condensation and packaging of sperm DNA are important factors for the initiation of human embryo development, even after intracytoplasmic sperm injection (ICSI). The centrosome, involved in microtubular organization, is the first epigenetic contribution of the sperm, and correct and harmonious microtubule arrangement is necessary for chromosome segregation and pronuclear migration. An abnormal sperm carrying an imperfect centrosome can disrupt mitosis, provoking problems at the beginning of embryogenesis with the formation of fragments, abnormal chromosome distribution, and early cleavage arrest. Up to 25% of apparently unfertilized eggs may show signs of having initiated fertilization, but then have anomalies that prevent cell division. In bulls, there is a positive correlation between sperm aster formation at the time of fertilization and the bull's fertility. In the human, paternal Y-linked genes are transcribed as early as the zygote stage, and compromised paternal genetic material could be transcribed at even this early stage, causing fertilization failure or embryonic arrest. Finally, as discussed in Chapter 3, spermatozoa lacking in, or with defective oocyte activating factor may only partially activate oocytes and lead to abortive development.

Metabolic requirements of the early mammalian embryo in vitro

Yves Ménézo

When the oocyte is fertilized and starts the process of transcription, the new embryo must maintain equilibrium between many different parameters:

1. The endogenous pool of metabolites, largely the result of final stages of oocyte maturation
2. Metabolic turnover of RNA messengers and proteins
3. Active uptake of sugars, amino acids and nucleic acid precursors
4. Passive transport, especially of lipids
5. Incorporation of proteins such as albumin, which can bind lipids, peptides and catecholamines.

ATP as an energy source is a basic requirement, and mammalian cells can generate ATP either by aerobic oxidation of substrates to carbon dioxide and water, or by anaerobic glycolysis of glucose to lactic acid. Under in-vitro conditions, oocytes and embryos generate ATP by aerobic oxidative metabolism of pyruvate, lactate, amino acids and possibly lipids. These metabolites have been shown to be important prior to genomic activation; pyruvate can also remove toxic ammonium ions via transamination to alanine.

Pentose phosphate pathway, NADPH and glutathione

An important feature of early embryo metabolism is linked to the activation process that is induced by sperm entry, which increases glycolysis and glucose uptake through transporters. These may provide energy by generating ATP, but upregulation of the pentose phosphate pathway (PPP) is a more significant metabolic parameter during this period. The PPP generates ribose 5-phosphate, a nucleotide precursor for subsequent DNA synthesis and replication. Upregulation of glucose metabolism via the PPP requires a fully grown pronucleus; the activity of the PPP influences the onset of the first S-phase in both male and female pronuclei, and continues to influence embryo development up to the blastocyst stage. The PPP also generates NADPH, which is involved in the majority of anabolic pathways: 1 mole of glucose 6-phosphate generates 1 mole of ribose 5-phosphate plus 2 moles of NADPH. NADPH further allows

methionine to be recycled from homocysteine, with the formation of folic acid via methylene tetrahydrofolate reductase. This pathway influences imprinting processes in the oocyte, and is also involved in thymidine synthesis (5-methyl-uracyl, see section on vitamins, p. 75). NADPH is also required to reduce oxidized glutathione (GSSG). The synthesis of glutathione from cysteine is energy consuming (ATP), and therefore recycling of GSSG is important, reducing energy consumption and decreasing the need for available cysteine.

Glutathione is necessary for sperm head swelling, and the impact of glutathione mobilization on further embryonic development is immediate: an increased blastocyst formation rate is observed, with increased cell number per blastocyst formed. This is probably due to the universal role of glutathione in protection against oxidative stress.

Glucose

It has been suggested that glucose is toxic during in-vitro culture before genomic activation, and that glucose and phosphate together may inhibit early embryo development. The mechanisms proposed include induction of glycolysis at the expense of substrate oxidation, through disrupted mitochondrial function. High levels of glucose can lead to excessive free radical formation, but the toxic effect is dependent upon the overall composition of the media; in some systems the negative effect of glucose may be counterbalanced by the presence of a correct amino acid balance, but not by removing "essential amino acids" (Lane and Gardner, 1997). Amino acids and EDTA suppress glycolysis through different combinations, and act in combination to further suppress glycolysis. After genome activation, glucose becomes a key metabolite, required for lipid, amino acid and nucleic acid synthesis. It is also essential for blastocyst hatching.

Lipids

Lipids can be synthesized (through C-2 condensation reactions), accumulated from the surrounding medium or carried with albumin. Cholesterol synthesis is possible, but slow: there is a rate-limiting step at the level of hMG (3-hydroxy-3-methyl-glutaryl) CoA reductase. If synthesis of cholesterol is experimentally inhibited by chetosterol, the embryos arrest and die.

Electrolytes

The correct balance of electrolytes is always an essential basis for biochemical processes that lead to energy production and cAMP-based regulatory mechanisms. K^+ and HCO_3 are involved in sperm capacitation, and intracellular pH regulation is a vital aspect of correct homeostasis. HCO_3 has a particular role in activation, via an increase in sperm cAMP levels. The carbon from HCO_3 can be incorporated during anabolic processes in the embryo. Iron and copper cations appear to arrest embryo development, encouraging free radical formation – therefore EDTA (or penicillamine) in early stage culture media is beneficial as a chelating agent (but not in fertilization medium, as it chelates calcium which is essential for sperm motility, capacitation and the acrosome reaction). EDTA is also a free radical scavenger. A suggestion that EDTA might be deleterious after genome activation and should be removed in the second phase of culture has been shown to be invalid.

Amino acids

Amino acids are important regulators of embryo development. The concept of "essential and non-essential" amino acids is not valid for preimplantation development, as their role is unlikely to be equivalent to their definition as "essential" in terms of nutrition for the entire individual. The ratio between different amino acids appears to be more important than their actual concentration in culture media, as they compete with each other for membrane transport systems. Differing affinities for transport mechanisms mean that disequilibrium in the external milieu will be reflected in anomalies of the endogenous pool, with potentially deleterious effects on protein synthesis. Active transport mechanisms have been confirmed by the finding that amino acids are found at a higher concentration inside embryos than in the surrounding medium. Sulfur-amino acids play a particularly important role, in that methionine, via *S*-adenosylmethionine, is used for transmethylation reactions involving proteins, phospholipids and nucleic acids, and is probably involved in imprinting through this process. Methylation of nucleic acids is known to modulate gene expression and to participate in the mechanism of genomic imprinting (Figure 5.6). *S*-amino acids also participate in protecting the embryo from free oxygen radicals: cysteine is a precursor

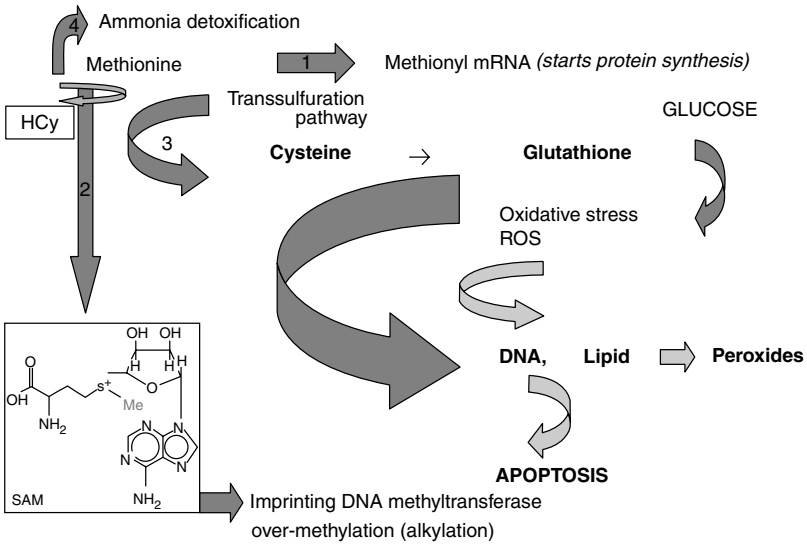

Figure 5.6 Methionine utilization in the preimplantation embryo (with thanks to Y. Ménézo).

for the synthesis of hypotaurine and taurine, and taurine can neutralize toxic aldehyde by-products of peroxidative reactions. Cysteine is a precursor of cysteamine and glutathione, and redox coupling between these amino acids helps the embryo to maintain its redox potential and prevent damage from peroxidative reactions. In the human oocyte, the pathway that recycles homocysteine to methionine via the CBS (cystathionine beta synthase pathway) is poorly expressed or nonexistent (Benkhalifa *et al.*, 2010). Ultimately, the initiation of all protein synthesis requires methionyl t-RNA, and the availability of cysteine and methionine is therefore crucial during early embryogenesis. A shortage of these compounds, linked to either maternal age or transporter deficiency, is detrimental.

Glutamine is also an important source of carbon and energy: in vitro it may be deaminated into glutamic acid, but is also converted to pyroglutamic acid (or pyrollidone carboxylic acid, PCA), which cannot be used for anabolism. However, glutamine is only weakly degraded in slightly alkaline conditions, corresponding to culture media. Introducing Gly-glutamine and Ala-glutamine into culture media to prevent degradation does not help, as they are converted to Gly-PCA and Ala-PCA.

Glycine is an energy source, as it can be deaminated immediately to form glycollate and glyoxylate (C-2 metabolites), and also acts as a precursor for peptides, proteins and nucleic acids. It is a chelating agent for toxic divalent cations, has an in-vitro osmoregulatory role, and is implicated in the regulation of intracellular pH of the embryo.

Deamination reactions are an important part of the biochemical and metabolic processes, and free ammonia is immediately recycled by efficient enzyme systems present in the oviduct (Figure 5.7). In vitro, in the presence of incubator carbon dioxide, free ammonia forms carbonate and ammonium bicarbonate, both unstable compounds. If the embryo cultures are "open," without oil, ammonia is liberated and eliminated by the carbon dioxide atmosphere. However, within microdrops under oil, ammonia can accumulate, which is toxic to the embryo – Gardner and Lane (1993) demonstrated severe teratogenic effects in mouse embryos.

Nucleotides

At a very early stage, embryos can synthesize purine and pyrimidine bases, precursors of RNA and DNA; these are required for DNA repair processes, which are very important during this period of development (Ménézo *et al.*, 2010). Following DNA damage caused by reactive oxygen species (ROS), the oxidized deoxynucleoside triphosphate pool is a significant contributor to genetic instability. These oxidized bases must be removed in order to avoid their reintroduction into DNA. The nucleotide pool sanitization enzymes are the first defenses against mutagenesis, and the human oocyte is well equipped with NUDT (nucleoside diphosphate linked moiety X), the major enzyme involved. However, there is a requirement for ATP,

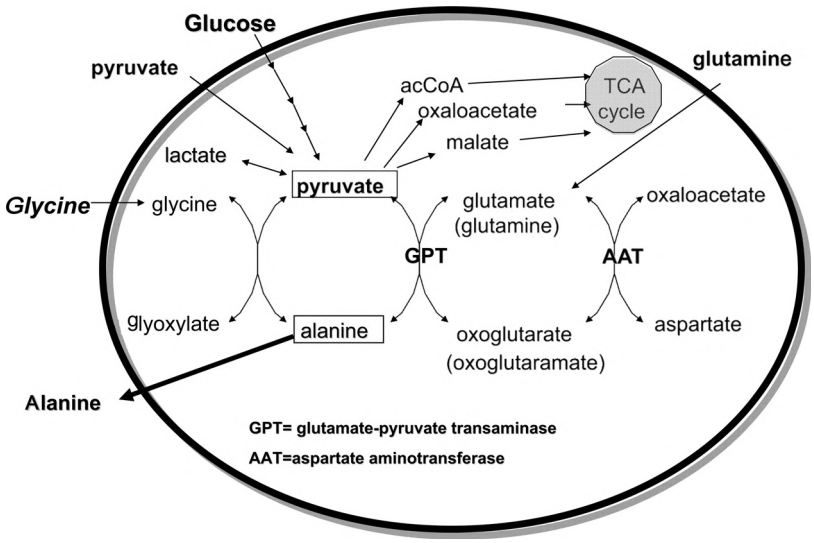

Figure 5.7 Nitrogen recycling in the mammalian preimplantation embryo (with thanks to Y. Ménézo).

CTP, TTP. For example, GTP must replace oxidized G (8-oxoguanosine, the most common DNA decay linked to ROS) in DNA. These bases can also be actively transported from the surroundings; the embryo probably uses this mechanism, as uptake is generally less energy consuming than full synthesis. There is an exponential increase in the accumulation of these precursors during the transition from morula to blastocyst as the number of cells increases.

Vitamins

It is difficult to measure vitamin uptake, but indirectly it is clear that folic acid and vitamin B12, necessary for methylation processes, are mandatory cofactors. Thymine is also known as 5-methyluracil, a pyrimidine nucleobase: inhibition of this methylation process by methotrexate leads to thymidine starvation and developmental arrest (O'Neill, 1998). Other vitamins such as vitamin C and vitamin E may act as redox regulators.

Growth factors

The potential role of growth factors (GF) in culture media remains open. Numerous growth factors are present in the oviduct and uterus, and they clearly play a role in vivo. Co-culture systems demonstrated a positive effect of growth factors on cell number, embryo quality and freeze–thaw tolerance. However, this positive aspect represents the balanced interaction of several GFs such as leukemia inhibitory factor (LIF), growth hormone (GH), mitogenic colony stimulating factor (MCSF), rather than the action of a single one. Some may slow down, and several may accelerate embryonic development: the addition of a single GF in vitro remains questionable at this point.

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Chapter

6 Implantation and early stages of fetal development

Implantation

During the transition from morula to blastocyst the embryo enters the uterus, where it is sustained by oxygen and a rich supply of metabolic substrates in uterine secretions. At the site of implantation the trophectoderm cells produce proteolytic enzymes that digest a passage through the zona pellucida, as the blastocyst "hatches" free of the zona. The uterine environment may also contain proteolytic enzymes, but very little is known about the molecular basis for hatching. The exposed cell layers of the hatched blastocyst make firm physical contact and implantation starts. The process of implantation (Figure 6.1), leading to the successful establishment of a pregnancy, must be carefully coordinated in time and in place. The embryo and the uterus, both highly complex structures, must interact correctly and at the appropriate time in order for a pregnancy to be established; a multitude of different factors can influence their respective unique developmental characteristics. Implantation is initiated via a molecular dialogue between the "free" hatched blastocyst and the endometrium, which allows the embryo to attach to the endometrial epithelium. The blastocyst trophectoderm secretes hCG, which provides direct signaling to the epithelial cells of the endometrium; hCG is detectable in maternal circulation within 3 days of embryo attachment to the endometrial epithelium – i.e., 9–12 days post ovulation. Trophectoderm cells from the blastocyst migrate between the epithelial cells, displace them, and penetrate up to the basement membrane. The process of implantation can be divided into three stages: apposition, adhesion and invasion; the three phases must occur during a period when the uterine endometrium is maximally receptive, a period that is known as the "implantation window."

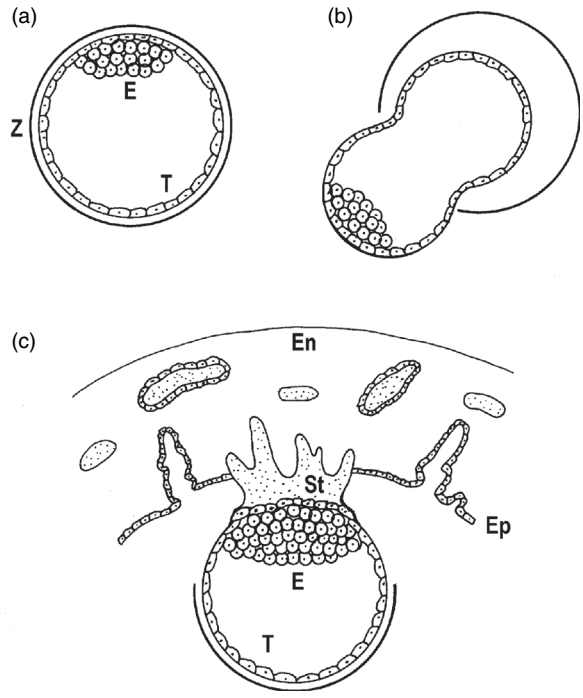

Figure 6.1 Detailed anatomy of implantation in the mammal. (a) Expanded blastocyst showing the flat layer of trophectoderm cells (T) which will become part of the extra embryonic tissue and the inner cell mass (E) from which the embryo derives. (b) Shows hatching, probably due in part to the production of a proteolytic-like enzyme by some of the trophectoderm cells. (c) Invasion of the epithelium (Ep) of the endometrium (En). St: syncytiotrophoblast.

Apposition: the embryo/endometrial dialogue

The embryo enters the uterus 3 or 4 days after ovulation, and hatches from the zona pellucida as an expanded blastocyst on Day 5–6, so that the hatched blastocyst is "apposed" to the uterine endometrium. Apposition

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is facilitated by the transient appearance of specialized epithelial cellular membrane protrusions or "pinopodes," which surround the pits in endometrial glands and actively reabsorb the uterine secretory phase fluid. Their appearance is progesterone-dependent, they are present for only 2–3 days (between Days 5 and 7 post ovulation) and are a morphological marker of the opening of the implantation window (Nikas *et al.*, 1995).

The uterine epithelium is coated with a network of glycoproteins (glycocalyx), which acts as a physical barrier to cell–cell interaction. The embryo must first overcome this "glycocalyx barrier," which contains mucins expressed by endometrial cells, via localized (i.e., embryo driven) enzymatic cleavage of the extracellular glycoproteins, or via binding of receptors expressed by the embryo and subsequent cleavage of the mucins. When apposition is achieved, the adhesion molecules have access to each other and the embryonic cytotrophoblast cells of the trophectoderm become attached to the endometrial cells.

Adhesion

The embryo is thought to attach (adhere) to the endometrium on Day 6–7 post ovulation. Once the embryo and endometrial adhesion molecules have free access to each other, the embryonic trophoblast cells attach to the endometrial epithelial cells. Several families of adhesion molecules are thought to be involved to a greater or lesser degree in human embryo attachment, including the integrins, tastin/trophinin complexes, heparan sulfate proteoglycans, cadherins, lectin/glycan interactions and glycan/glycan interactions. The integrin family is one of the key adhesion factors involved in human implantation. Experimental evidence has shown that hCG upregulates the integrin avb3, and interleukins IL-1a and IL-1b from the embryo are also involved. Human oocytes, early embryos and blastocysts express integrins, and they are also widely expressed by the glandular epithelium. Some are synthesized at a constant rate (a2, a3, a6, b1, b4, b5) whereas others vary during stages of the cell cycle (a1, a9, av, b3, b6). The av family are the integrins most likely to be involved in attachment; they bind fibronectin, osteopontin, vitronectin and other components of the extracellular matrix.

Invasion

Pre-villous trophoblast starts to differentiate between Days 7 and 12, and endometrial transformation (stromal decidualization) is underway by Day 12 post ovulation (Duc-Goiran *et al.*, 1999). Trophoblast cells extensively invade the uterine tissue to establish an interface between fetus and mother, so that nutrients and waste products can be transferred. They also fulfill a mechanical function by stabilizing the placental tissue within the uterus. The embryonic trophectoderm contains cytotrophoblasts, the mononuclear undifferentiated stem cells that are precursors of all trophoblast forms. These differentiate into subsets:

1. Invasive intermediate trophoblasts migrate into the uterine tissue.
2. Villous syncytiotrophoblast makes the majority of the placental hormones, including hCG.
3. Junctional trophoblast cells mediate the attachment of the placenta to the uterus.

Invasive trophoblast cells invade the endometrium and upper layers of the myometrium and remodel the extracellular matrix in order to selectively permeate uterine spiral arteries. The colonized blood vessels are then modified to yield widened, low-resistance channels that can carry an increased maternal blood flow to the placenta. The mother must protect herself from these invasive trophoblasts migrating toward the uterine spiral arteries, and the endometrial stroma transforms itself into a dense cellular matrix known as the decidua, which will form the maternal part of the placenta. During decidualization, the spindle-shaped stromal fibroblasts enlarge and differentiate into plump secretory decidual cells, creating a tough extracellular matrix that is rich in fibronectin and laminin. This transformation occurs under the influence of progesterone.

The reaction is initially localized to cells surrounding the spiral arteries, but subsequently spreads to neighboring cells. The majority of cells of the decidua express leukocyte antigens. The largest single population of white blood cells in the endometrium are large granular lymphocytes (LGLs or NK cells), with smaller numbers of macrophages and T cells also present. The abundance of LGLs increases dramatically between ovulation and implantation, influenced by steroid hormones (King *et al.*, 1998). The decidua forms a physical barrier to invasive cell penetration and also generates a local cytokine milieu that promotes trophoblast attachment.

The first signs of the decidualization reaction can be seen as early as Day 23 of the normal menstrual cycle (10 days after the peak of the luteinizing hormone

surge), when the spiral arteries of the endometrium first become prominent. Over the next few days, the effect of progesterone causes the stromal cells surrounding the spiral arteries to transform and differentiate into predecidual cells. This progressive decidualization of the endometrial stroma prepares the uterine lining for the presence of the invasive trophoblasts, but simultaneously closes the door to implantation. At this stage the embryo first becomes visible to the maternal immune system.

The invasion stage requires a very delicate balancing of conflicting biological needs between the early fetus and the mother, with a complex regulation of adhesion molecule expression, coordinated in time and in space. The invading cells use collagenases, and also express plasminogen activator inhibitor type 1, suggesting that the plasminogen activator system may also be involved. They lose integrins associated with basement membrane interactions (possibly laminin), and gain integrins that can interact with fibronectin and type I collagen. The outer layer of trophoblast cells fuse to form a multinucleated syncytiotrophoblast layer that covers the columns of invading cells. This layer proliferates rapidly, and forms numerous processes, the chorionic villi (the chorion is the layer that surrounds the embryo and extraembryonic membranes). Cyclic AMP and its analogues, and more recently hCG itself, have been shown to direct cytotrophoblast differentiation toward a syncytiotrophoblast phenotype that actively secretes the placental hormones.

At the point where chorionic villi make contact with external extracellular matrix (decidual stromal, ECM), another population of trophoblasts proliferates from the cytotrophoblast layer to form the junctional trophoblast. The junctional trophoblasts make a unique fibronectin, trophouteronectin (TUN), which appears to mediate the attachment of the placenta to the uterus. Transforming growth factor-β (TGF-β) and, more recently, leukemia inhibitory factor (LIF) have been shown to downregulate hCG synthesis and upregulate TUN secretion. These cells also make urokinase-type plasminogen activator and type 1 plasminogen activator inhibitor (PAI-1). Experiments using in-vitro model systems showed that phorbol esters increase trophoblast invasiveness and upregulate PAI-1 in cultured trophoblasts. Uterine prostaglandins (PGF2 and PGE2) are regulated by steroids, and are involved in regulating the formation of the decidua.

The implantation window

The existence of a transient implantation window has been well documented for rodent species. In these species, the window is maternally directed and the receptive state is sustained for less than 24 hours. In the human, the window appears to be approximately 5 days long (Day 6 to Day 10 post ovulation in the normalized 28-day menstrual cycle), and the opening of the receptive phase is not as clearly defined as its termination. In the mouse, hatched blastocysts readily attach and spread in an integrin-dependent process on any surface that includes ligands found in the stroma during pregnancy (fibronectin, collagen, lamininns, vitronectin, thrombospondin, etc.) as well as on artificial substrates like Matrigel. However, humans undergo protrusive penetration of trophoblast through the epithelium and the process is likely to be more strongly regulated by complex and multifactorial interactions between embryo and endometrium.

Summary

Successful implantation requires both a synchronous development and a synchronized interaction between blastocyst and endometrium. Direct signaling from the embryo to the endometrium upregulates molecules such as the integrins, and this signaling promotes blastocyst adhesion. The blastocyst initially derives nourishment from uterine secretions, but in order to continue growing the conceptus must develop its own vascular system, and, as a first step, induces a highly specialized reaction in the uterine stroma that initiates sprouting and growth of capillaries – the primary decidual reaction. The decidualized stromal cells make pericellular fibronectin. There is a dramatic transformation of endometrial stromal cells, and a massive leukocyte infiltration by NK cells and macrophages. Maternal hormones influence the communication between the embryo and the endometrium, via effects on cytokines, adhesion molecules, prostaglandins, metalloproteases and their inhibitors, and angiogenic growth factors.

The molecular mechanisms behind this complex and sophisticated process have been studied using animal models, and knock-out (KO) mouse studies have positively identified genes for receptivity (LIF, HMX3), responses to embryo (Cox2), and decidualization (IL-11R). Other factors identified as having a role include immune response gene (IRG1), progesterone receptor knock-out (PRKO), estrogen receptor knock-out (ERKO), homeobox protein A10 (Hoxa10),

IHH (Indian Hedgehog gene) and immune regulating hormone 1 (IIRH1). Estrogen and progesterone receptors and the signaling pathways that interact with them are clearly important, including the IGF-I and epidermal growth factor (EGF) family and prostaglandins. Research in humans continues, now using microarray technology to look at gene expression in order to identify those factors that determine a receptive endometrium, with the hope of elucidating mechanisms that may enhance successful implantation after ART (Simon *et al*., 2009; Sherwin *et al*., 2007).

Steroid hormones and implantation

Estrogen acts during the proliferative phase of the menstrual cycle to promote the development of the endometrium, and it opens the window of receptivity via several mechanisms:

- Causes loss of surface negative charge, shortening of microvilli and thinning of the mucin coat with changes in its molecular composition
- Stimulates the synthesis of at least 12–14 endometrial polypeptides, as well as estrogen and progesterone receptors
- Acts on luminal epithelial cells to make them responsive or sensitive to a blastocyst signal, promoting trophectoderm attachment to the luminal cells
- Stimulates the release of glandular epithelial secretions that include cytokines, and this activates the implantation process.

Progesterone is secreted by the corpus luteum after ovulation, and stimulates the secretory activity of the uterus:

- Produces intense edema in the stroma
- Increases blood vessel volume threefold
- Stimulates the formation of pinopodes, and primes the decidua.

Steroids regulate the action of **uterine growth factors** which are synthesized at various stages of the menstrual cycle: IGF-I and IGF-II, EGF, HB-EGF, FGF, β-FGF, α-FGF, TGF-β1, PDGF-β.

Early postimplantation embryogenesis

Most of our knowledge about early postimplantation development was gained from studies in chick, mouse and other mammalian species. Although experimental studies in humans are not possible, information has been gained from fixed and stained human embryos after pregnancy loss at different stages of gestation. The Carnegie Collection of Human Development at the National Institute of Child and Human Development in Washington DC has made a collection of several thousand serial microscopic cross-sections, which are available for study (O'Rahilly and Müller, 1987). Human embryos have been classified into 23 developmental stages based on a number of morphological features, known as the Carnegie stages. These stages represent a series of events that occur during development, with overlap between the different stages. From 1994 onwards the Visible Embryo Project (www.visembryo.com) used computer technology to reconstruct accurate 3-D images of human development. A digital image database of serially sectioned human embryos from the Carnegie Collection is available online as The Heirloom Collection, supported by the National Library of Medicine in the USA. Beautiful color 3-D reconstructions and animations can be viewed at: http://virtualhumanembryo.lsuhsc.edu/. Please refer to these images for further details in relation to the brief overview presented here.

7–12 days post ovulation

Once the blastocyst has hatched and trophoblast cells have started the process of implantation, the cells of the inner cell mass start to reorganize into distinct layers, each layer destined for a different developmental fate. Cellular morphology changes, and there is active movement of individual cells and groups of cells, setting up new relationships between them. In the human embryo, the first 14–18 days of development are concerned mainly with the differentiation of various extraembryonic tissues, and after this time separate tissues can begin to be identified.

Ectopic pregnancy

- A hatched blastocyst can sometimes implant in sites outside the uterus, most commonly in the fallopian tube. Other sites include the utero-cervical isthmus (cervical pregnancy) and the utero-tubal junction; more rarely, implantation can take place in the ovary, or in the peritoneal or abdominal cavity.
- Occasionally two embryos will implant at different sites: one in each tube (bilateral tubal pregnancy) or one in the uterus and one at an ectopic site (heterotopic pregnancy).
- An embryo can continue to grow at an ectopic site for several weeks, with signs and symptoms of early pregnancy in the mother. If undetected, ectopic pregnancy can result in a life-threatening crisis due to tubal rupture or erosion into a blood vessel.

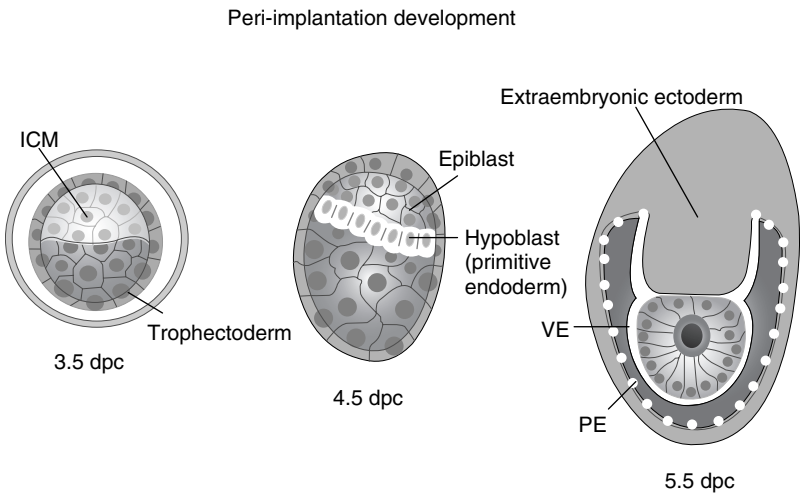

Figure 6.2 Diagram of mouse blastocyst showing epiblast layer. ICM = inner cell mass; VE = visceral endoderm; PE = primitive endoderm; dpc = days post conception. With thanks to Cindy Lu and Elizabeth Robertson.

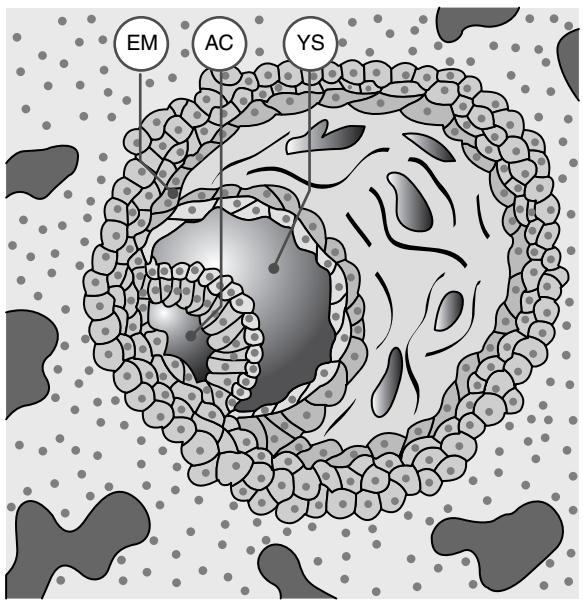

Figure 6.3 Diagrammatic representation of human embryo transverse section on approximately Day 14 post ovulation, indicating extraembryonic mesoblast (EM), amniotic cavity (AC) and yolk sac (YS, also known as umbilical vesicle).

13–15 days post ovulation (second week of development)

Cells of the inner cell mass separate into two different sheets of cells to form a bilaminar embryonic disc (Figure 6.2):

Dorsal germinal layer = epiblast
Ventral germinal layer = hypoblast.

The flatter hypoblast cells lie on top of the epiblast, and will form the yolk sac; the epiblast contains the cells of the future embryo.

The two layers of the embryonic disc divide the blastocyst into two chambers, the amnion and the yolk sac (Figure 6.3); outside the embryo, the extraembryonic spaces (chorionic, amniotic, yolk sac) continue to develop, the endometrium is being converted into the decidua, and early placentation has begun. The amnion fills with fluid that cushions the developing fetus; the yolk sac contributes to the formation of the extraembryonic membranes, chorion and amnion. It is also the site of early blood cell formation, and part of the yolk sac becomes incorporated into the gut later in development. The cells of the trophoblast form another chamber, the extraembryonic coelom around the amnion, yolk sac and developing embryo; this will later become the chorionic sac and placenta.

13–15 days post ovulation (end of second week of development)

Embryonic epiblast cells move along the surface, begin to pile up near the center to form a node, and then move internally to create a furrowed cell mass, the primitive streak (Figure 6.4a). This is a visible feature showing that cells are migrating, and the furrow begins to extend towards the cranial (head) end of the bilaminar disc (Figure 6.4b). Cells near the advancing edge of the streak begin to pull apart slightly to form an open pit, and cells bordering this pit then migrate between the epiblast and hypoblast to create a

new layer, the mesoderm. Cells from the epiblast also displace the hypoblast, creating the endoderm. The cells that remain on the surface will form ectoderm, and this formation of a now trilaminar disc is known as **gastrulation**. During gastrulation, cells from the mesoderm form the notochord, which will define the primitive axis of the embryo and establish craniocaudal orientation and bilateral symmetry of the fully developed body. The three layers of cells form the primary germ cells that will evolve into the specialized cells, tissues and organs of the body:

Endoderm (inner layer): forms the lining of the primitive digestive tract and its associated glandular structures, as well as portions of the liver, pancreas, trachea and lungs.

Mesoderm (middle layer): initially forms a loose aggregate of cells, the mesenchyme; this then organizes into regions that evolve into the vertebral column, skeletal muscle, ribs, skull and the dermis of the skin. Mesenchymal cells also form tubular structures: urogenital system, heart, blood vessels, and the lining of the pericardial, pleural and peritoneal cavities.

Ectoderm (outer layer): develops into skin epidermis, brain and spinal cord. Sensory receptors for vision, hearing and smell, as well as the future autonomic nervous system and adrenal medulla, also develop from ectoderm.

17–19 days (third week of development)

After gastrulation, the mesoderm starts to segment into mesenchymal tissue somites, and the notochord induces rapid growth in the ectoderm. Over the next 2–3 days the ectodermal layer thickens to form a neural plate, which folds to form a neural groove (Figure 6.4c): the nervous system is one of the first organs to develop. During this process of neurolation, the ectoderm will subdivide into neural tissue and epithelial tissue lineages (pigmented cells of the epidermis, adrenal medullary cells, skeletal and connective tissues of the head). Somites continue to form in the mesoderm, the neural groove fuses dorsally to form a tube at the level of the fourth somite, and closes. The cranial end of the neural plate is wider, enclosing the region that will form the brain. The spinal cord will form at the caudal end, which is narrower (the notochord is eventually replaced by the vertebral column).

Genes significant in early development

- Homeobox genes are a highly conserved family of transcription factors that switch on cascades of other genes that are involved in the regulation of embryonic development of virtually all multicellular animals.
- Wnt genes encode short-range secreted proteins that are involved in cell adhesion and cell–cell signaling.
- Cellular adhesion molecules such as the integrins are important for cellular recognition and binding that influence cell migration. Laminin and fibronectin are also involved in cell migration.
- The fate of a migrating cell is apparently determined largely by its final destination. RhoB and Slug proteins, which promote cell migration, are present at the gastrulation stage, and the loss of N-cadherin helps to initiate the migration of neural crest cells.
- Hedgehog genes encode signaling molecules that are involved in patterning processes; this family of genes is named after a *Drosophila* gene whose loss of function produced an embryo covered with pointed denticles, like a hedgehog. Three homologous genes have been found in mammals: Sonic hedgehog, Shh (named after the Sega Genesis video game), Desert hedgehog, Dhh and Indian hedgehog, Ihh.
  - Shh is secreted by the notochord, and has numerous critical roles in development, involved in the patterning of many systems, including the brain and spinal cord, as well as in establishing left–right axis patterning. Differentiation pathways appear to be influenced by different levels of Shh, in combination with other paracrine factors, including Wnt and FGF.
  - The Shh transcription pathway controls cell division in adult stem cells, and has been linked to the formation of some cancerous tumors.

19–23 days (third week)

Rapid cellular growth elongates the embryo and expands the yolk sac. Primordial germ cells can be identified at the root of the allantois. A head fold rises on either side of the primitive streak, and endocardial cells begin to fuse and form two endocardial tubes which will develop into the heart. Pairs of mesodermal somites can be seen on either side of the neural groove (Figure 6.4d), appearing first in the caudal region. The neural folds rise and fuse along the length of the neural tube (Figure 6.5a), along with budding

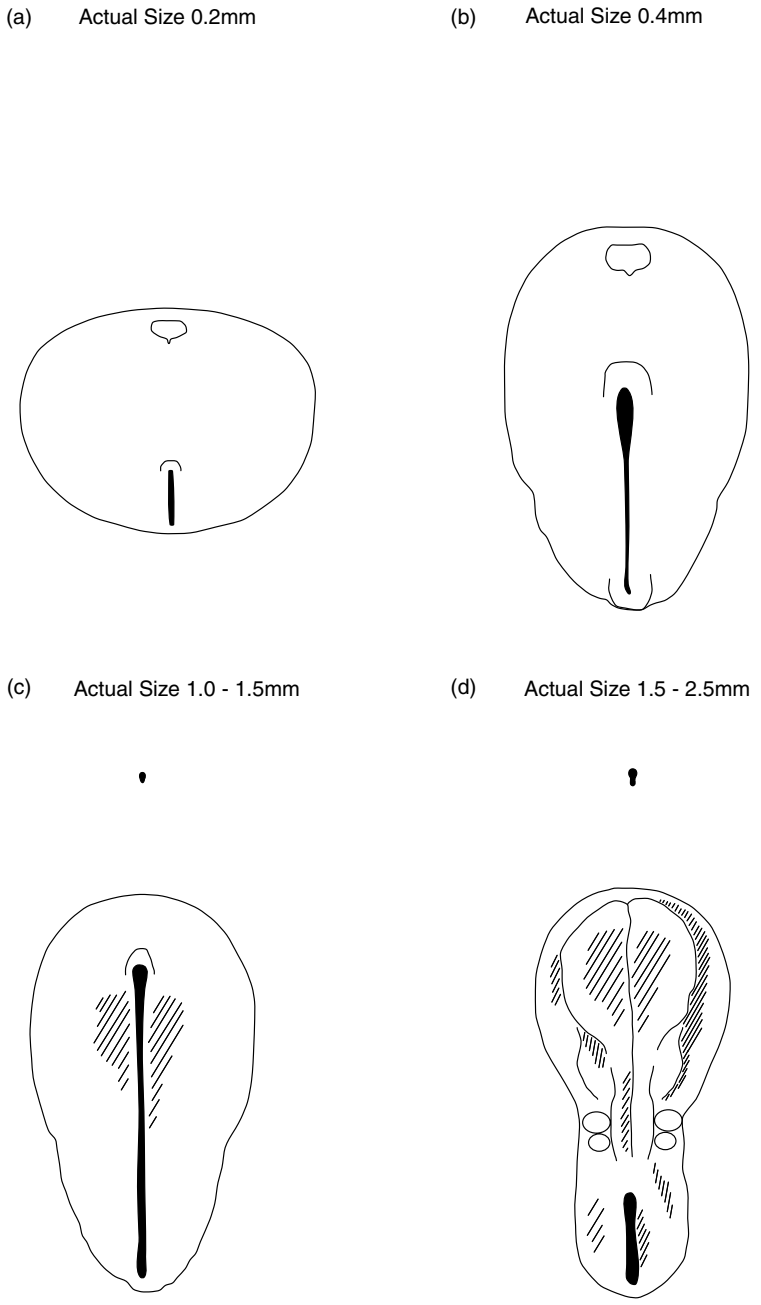

Figure 6.4 Third week of fetal development, dorsal views; dates are approximate, stages overlap. (a) 13 days post ovulation, Carnegie Stage 6; primitive streak and node(bottom) and prechordal plate (top). (b) 16 days post ovulation, Carnegie Stage 7; neurolation. (c) 17–19 days post ovulation, Carnegie Stage 8; primitive pit, notochordal and neurenteric canals. (d) 19–21 days post ovulation, Carnegie Stage 9; appearance of somites. Illustrations by Luke Ebbutt-James, Cambridge, UK.

somites that close the neural tube (like a zipper). The neural tube begins to close in the middle of the embryo (cervical area), and then extends in both cranial and caudal directions. Failure of the neural tube to close correctly at this stage can lead to anomalies such as spina bifida (caudal) or anencephaly (cranial). Masses of cells detach themselves from the side of the neural plate and form the neural crest, precursor cells of numerous differentiated cells of the nervous and glandular systems. The heart tube becomes S-shaped, with the beginning of cardiac muscle contraction. Secondary blood vessels appear in the chorion/placenta, and hematopoietic cells appear in the yolk sac.

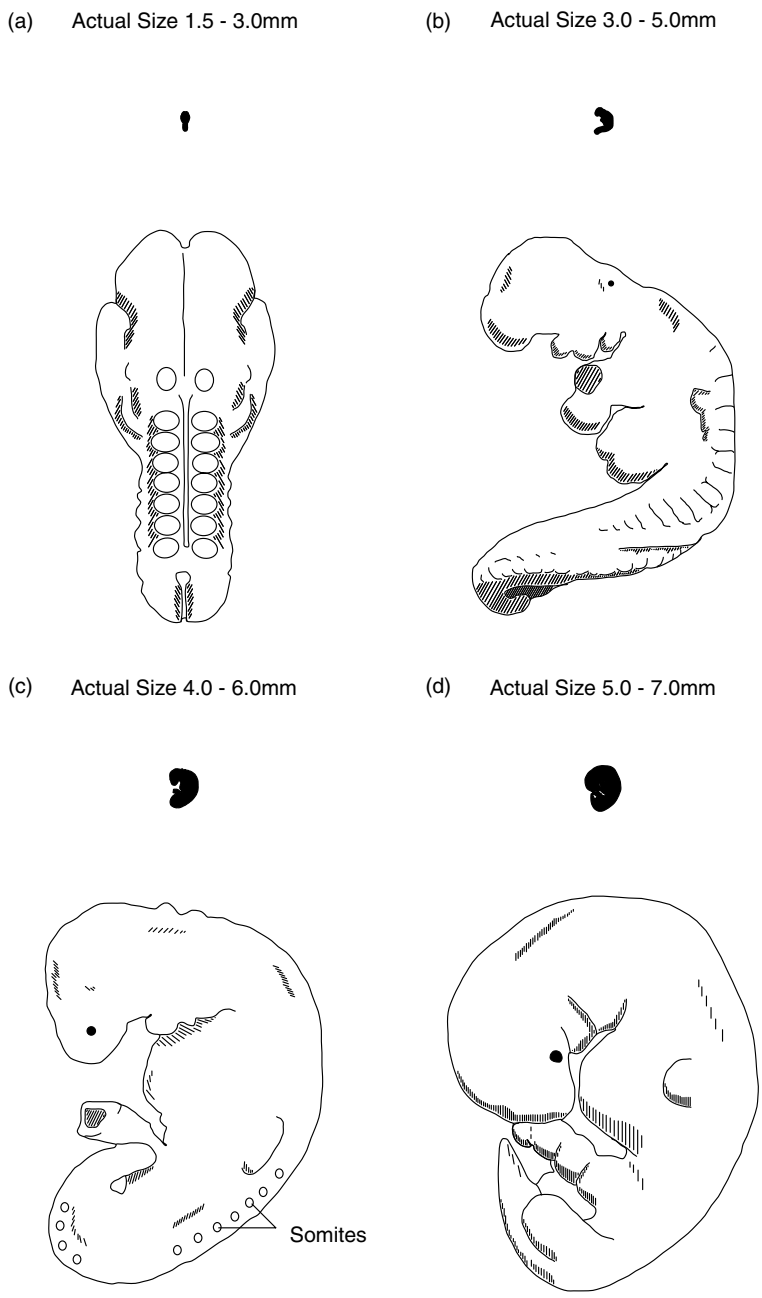

Figure 6.5 Fourth week of fetal development, dorsal views; dates are approximate, stages overlap. (a) 21–23 days post ovulation, Carnegie Stage 10; neural folds/heart folds begin to fuse. (b) 25–27 days post ovulation, Carnegie Stage 12; upper limb buds appear. (c) 26–30 days post ovulation, Carnegie Stage 13; pharyngeal arches and upper limb buds visible. (d) 31–35 days post ovulation, Carnegie Stage 14; esophagus forming, lens vesicle opens to the surface and optic cup develops, upper limb buds elongate and taper. Illustrations by Luke Ebbutt-James, Cambridge, UK.

23–27 days (fourth week)

A primitive S-shaped tubal heart begins to beat, and the developing neural tube curves the embryo into a C-shape (Figure 6.5b). The forebrain is closed when 20 somites are present, eyes and ears begin to form, and pharyngeal arches are present. Valves and septa begin to appear in the heart, and a blood circulatory system continues to develop.

28–35 days (fifth week)

Somites organize themselves into myotomes, the groups of tissues that will develop into the musculoskeletal structure of the body wall, and rudiments of the ribs and limbs begin to appear (Figure 6.5c and d). Sclerotomes give rise to the axial skeleton, myotomes to striated muscle, and dermatomes to subcutaneous tissue and skin. Arches that form the face and neck can

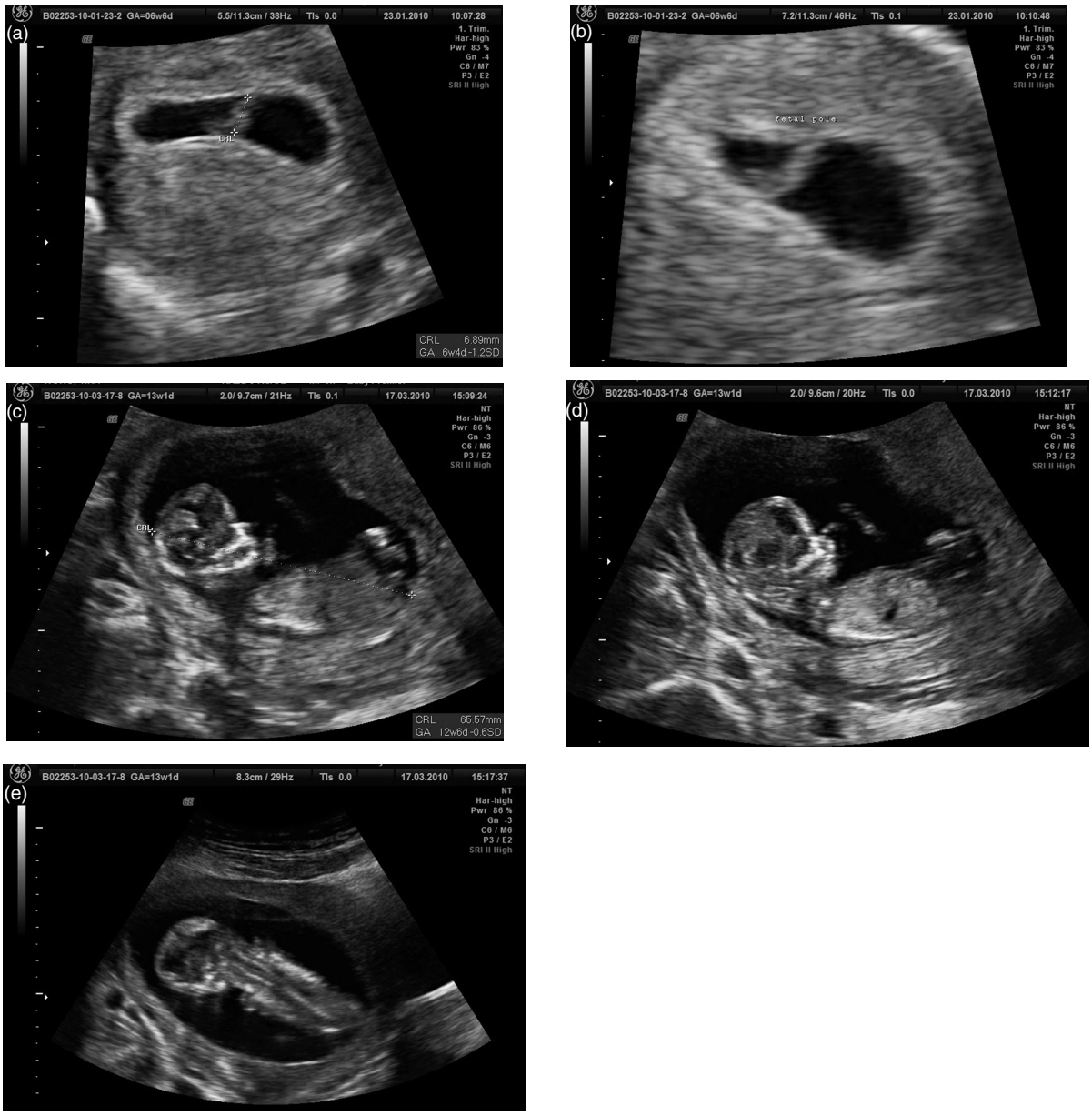

Figure 6.6 Ultrasound scans of early pregnancy. (Images courtesy of Baby Premier, division of Specialist Medical Imaging Ltd.) (a) Six-week scan, showing crown–rump length (crl) = 6.89 mm, = 6 weeks + 4 days. (b) Another view of (a), illustrating fetal pole. (c) Twelve-week scan, crl= 65.57 mm, = 12 weeks+6 days. (d) Thirteen-week scan, clear view of head and body. (e) Another view of (d), showing the vertebral column.

be seen under the enlarging forebrain. The digestive epithelial layer begins to differentiate into the future locations of the liver, lung, stomach and pancreas.

35–42 days (sixth week)

By Day 35 (fifth week of development = 6 weeks from last menstrual period), when clinical pregnancy after IVF can be confirmed by ultrasound visualization of gestational sac and fetal heartbeat (Figure 6.6), the three primary germ layers have expanded and undergone a dramatic process of differentiation and transformation, to form a clearly recognizable fetus. The beating heart has chambers, and all limbs and body systems are under development. Medial thickening of

the coelomic epithelium represents the formation of primordial gonads. The size of the fetus is now approximately 7.0–9.0 mm, and it will continue to grow at a rate of around 1 mm per day during the first trimester.

By the beginning of the second trimester, the basic brain structure is complete, and genitalia begin to show signs of gender characteristics.

Ultrasound confirmation of clinical pregnancy

1. **Gestational sac**: can be seen at around 4 weeks' gestation, but may not be visible until the end of the fifth week. It is characteristic of early pregnancy, but does not correspond to anatomical features of the embryo. Gestational sacs are also found in ectopic pregnancies.
2. **Yolk sac**: visible during the fifth week, and grows to be no larger than 6 mm. Larger yolk sacs usually indicate an abnormal pregnancy; yolk sacs that are misshapen, are "floating" within the gestational sac and contain echogenic (instead of sonolucent) material are ominous findings for the pregnancy.
3. **Fetal heartbeat (FH)**: using transvaginal ultrasound, fetal cardiac activity can sometimes be seen along the edge of the yolk sac before a fetal cell mass is identifiable. In normal pregnancies, the fetal heartbeat may not be seen until the fetal pole is around 4 mm in size. Failure to identify a FH in a fetus >4 mm in size is an ominous sign.
4. **Fetal pole**: the fetus in its somite stage, first visible separation from the yolk sac by transvaginal scan (TVS) just after 6 weeks gestation (Day 35 post ovulation).
5. **Crown–rump length (CRL)**: single most accurate measure of gestational age up to 12 weeks gestation.

Pregnancy failures

1. **Miscarriage**: spontaneous abortion prior to 20 weeks' gestation.
2. **Biochemical pregnancy**: early pregnancy loss, prior to 6 weeks from last menstrual period.
3. **Blighted ovum**: anembryonic gestation. Sac appears normal on TVS, but an embryo never develops; probably due to early embryonic death with continued trophoblast development.
4. **Missed abortion**: nonviable intrauterine pregnancy that has not yet aborted. TVS shows gestational sac with no FH; can be due to a blighted ovum, or early demise of an embryo after detection of FH. The cervical os is closed.
5. **Threatened abortion**: vaginal bleeding and/or abdominal/pelvic pain during early pregnancy; the cervical os is closed, and no tissue has been passed. FH is still present on TVS.
6. **Inevitable abortion**: vaginal bleeding, usually with abdominal pain and cramps. FH is absent, cervical os is open, but no tissue has been passed. Usually progresses to complete abortion.
7. **Incomplete abortion**: Heavy vaginal bleeding, with tissue having been passed, but some remaining in utero.
8. **Complete abortion**: bleeding, abdominal pain, all products of conception have been passed; empty uterus must be confirmed by TVS.
9. **Recurrent abortion**: history of more than three spontaneous abortions.

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Chapter 7

Stem cell biology

Stem cells and stem cell lines

Every cell in an individual has a unique chromosome complement, with 20 000–25 000 genes coded into a DNA sequence of 3 billion base pairs, packed into 23 pairs of chromosomes: a total of 46 chromosomes in each diploid human cell. All of these cells have the same genetic information, copied during mitotic divisions by replicating the DNA in each cell cycle. The pattern of gene activity in each cell (gene expression/transcription) dictates its function and fate, enabling different cells to differentiate and carry out distinct functions.

After an oocyte has been fertilized the one-cell zygote is **totipotent**, with the potential to give rise to a complete organism, including both embryonic and extraembryonic cells. As cell division proceeds, blastomeres lose the potential to give rise to an entire organism, and by the time that a fully expanded blastocyst has formed, three types of morphologically and molecularly distinct cells have emerged: **trophectoderm** cells surround an inner cell mass, which contains **epiblast** progenitor cells and **primitive endoderm** cells. The embryo itself is derived exclusively from epiblast progenitor cells; trophectoderm cells will form fetal components of the placenta, and primitive endoderm will form the yolk sac, which is derived from extraembryonic endoderm. Continued development of the embryo requires the support of both of these extraembryonic cell types.

Epiblast progenitor cells of the blastocyst inner cell mass are considered to be **pluripotent**, as they have the potential to give rise to the three primary germ layers that will form all of the tissues of the fetus: mesoderm, endoderm and ectoderm. Significantly, these progenitor cells do not have the potential to give rise to a whole organism without the supporting extraembryonic cells. Following implantation, the pluripotent embryonic cells become committed to more specialized cells that increasingly lose their potential to contribute to all three germ layers. **Multipotent** cells have been identified in the developing embryo and in the adult, which have the potential to continually give rise to the same cell (**self-renew**) and also have the potential to give rise to other cells with a more specialized function. Hematopoietic stem cells are multipotent cells that replenish all blood cells by dividing to form two types of cell: one daughter cell maintains a stem cell population, and the second daughter cell has the potential to continue to alter its pattern of gene expression and differentiate. The daughter cells that become more specialized with each cell division go through distinct populations of **transit amplifying cells**, proliferative cells that retain their self-renewal property until they reach the end of their production line, and are terminally differentiated. Other types of stem cell, such as neural stem cells are **oligopotent**, giving rise to diverse but restricted populations of specific self-renewing subtypes. **Unipotent** cells, such as spermatogonial stem cells, are self-renewing cells that have the potential to give rise to a single lineage, spermatogonia. Figure 7.1 illustrates a hierarchy of stem cell potential.

Two properties are unique to all types of stem cell:

1. They have the capacity for long-term self-renewal.
2. They have the potential to give rise to cells other than themselves.

A stem cell line is a population of cells that has been grown and maintained in vitro. When maintained under appropriate conditions, these cells continue to grow in tissue culture for very long periods of time.

Mammalian stem cell lines

Following the first derivation and culture of human embryonic stem cell lines in the late 1990s (Thomson *et al.,* 1998), debate and controversy escalated

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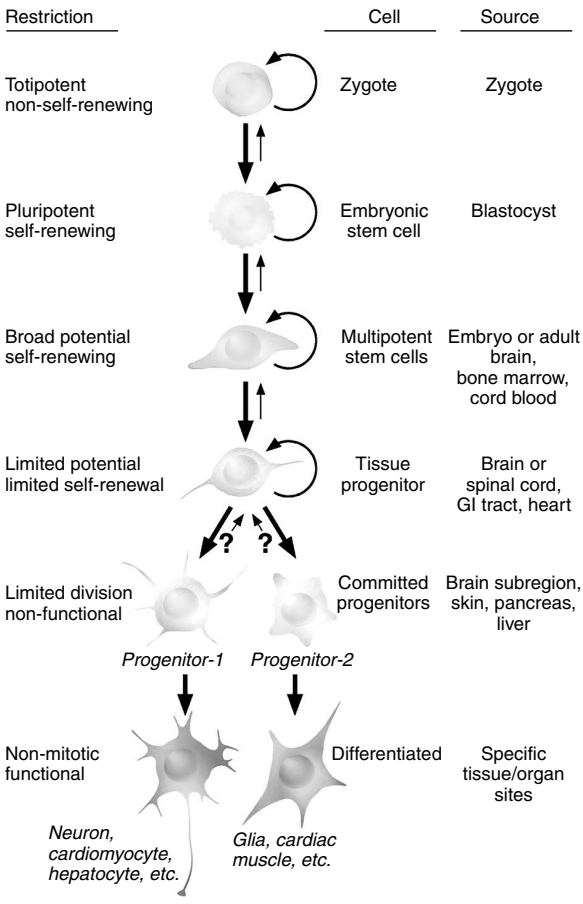


Figure 7.1 Potential pathways for stem cell development (Adapted from: FH Gage, Mammalian neural stem cells (2000) *Science* 1433. With permission from AAAS.)

surrounding the use of surplus human embryos donated for research. Despite the controversial ethico-legal perspectives, in many countries throughout the world, IVF clinics have embryos that are either unsuitable for treatment, or are surplus to the patient's requirements. Given the opportunity for appropriate counseling and informed consent, many patients choose to make a contribution to science by donating surplus embryos for research rather than allowing them to perish (Franklin *et al.*, 2008). There is no doubt that embryos donated for stem cell research represent a very valuable resource for scientific investigation, with the potential to make a significant contribution to our understanding of early developmental processes and the molecular pathology of disease. Stem cell biology has become an integral part of ART; the principles and the science underpinning this new area of developmental and regenerative biology should be viewed within the perspective and frame of reference of the first stages of postimplantation development, as presented in the brief synopsis in Chapter 6.

Human embryonic stem cells (hESCs)

Human embryonic stem cells are derived from the in-vitro expansion of epiblast progenitors within the inner cell mass of a preimplantation blastocyst. hESCs are capable of indefinite self-renewal, and maintain the potential to differentiate into cell types from the three embryonic germ layers (pluripotency). Because they remain pluripotent when maintained in vitro, they provide an ideal resource for investigating and studying the pathways that lead to the establishment of cells that might be relevant to clinical treatment, such as dopamine-producing neurons and insulin-producing cells of the pancreas.

The goal of hESC research is to elucidate the pathways that direct differentiation in vitro, with the aim of providing functional and therapeutically relevant cells. These cells can be used to investigate mechanisms of disease progression, and for research into drugs that might inhibit or reverse pathological processes. hESCs also provide an insight into aspects of early embryonic development that are otherwise inaccessible to research, due to ethical and practical considerations; they can be used as a tool to investigate how cells can be manipulated to regenerate damaged or diseased cells in the human body. Lastly, hESC-differentiated cells may eventually prove to be useful in cell transplantation approaches for the treatment of disease.

Several strategies have been applied to promote the differentiation or selection of therapeutically relevant cell types, including the addition of growth factors, cytokines and other ways of manipulating gene expression. One major hurdle to this approach is that the process of directing the differentiation of hESCs into functionally relevant specialized cells is very inefficient. This inefficiency may be the result of **heterogeneity** within a starting stem cell population, in addition to the lack of information about the developmental events that promote the emergence of specialized cells in vivo. The constraints of in-vitro culture conditions also hinder the ability to mimic in-vivo events.

Tissue stem cells in fetal or adult tissue can supply cells to parts of the body that require a continuous supply of regenerative cells. Tissue stem cells include blood and intestinal stem cells – these are partially committed cells that can function to regenerate their respective adult

tissues as required. They commonly reside in a microenvironment (**niche**) in a **quiescent** state where they are provided with signals and support that promote the maintenance of self-renewal. Stem cells exit the niche as they undergo cellular **commitment**/differentiation.

Multipotent stem cells

- All blood cell types are continuously replaced from a store of hematopoietic stem cells (HSCs) in the bone marrow.
- The lining of the gut (gut epithelium) has intestinal stem cells in the small intestine that produce four different cell lineages (Paneth, goblet, absorptive columnar, enteroendocrine).
- Epidermal stem cells in the skin and hair follicle can regenerate damaged epithelium.
- Skeletal muscle stem cells (satellite cells) are quiescent cells that can also give rise to committed progeny such as myofibers in response to injury or disease.

Oligopotent stem cells

- Neural stem cells are restricted self-renewing subtypes that give rise to three lineages: neurons, oligodendrocytes, astrocytes.

Unipotent stem cells

- Spermatogonial stem cells give rise to spermatogonia.

HSCs in treatment

- Leukemias, lymphomas and other blood disorders have been successfully treated with bone marrow transplants since the 1960s. The donor tissue must be human leukocyte antigen (HLA) matched to that of the recipient, or the cells will be rejected by the recipient's immune system.
- During the past decade, IVF in combination with preimplantation genetic diagnosis (PGD) has been used to select embryos that are HLA matched to a sibling who has a blood disorder. Cord blood isolated from the baby ("savior sibling") at the time of delivery is then prepared to provide a supply of HSCs for transplant to the sibling.
- It had been suggested that HSCs may have "plasticity", i.e., the ability to engraft in other locations and then transdifferentiate to cell types appropriate to their new location. However, so far this concept is unsubstantiated, and there is no evidence that blood-forming stem cells can serve as a significant source of regenerative cells in the repair of non-blood tissues.

Differentiation

Cells can be distinguished from one another by their patterns of gene expression, which includes expression of both protein coding and noncoding RNAs, the secretion of proteins, their response to extracellular signals and the distribution of epigenetic chromatin modification. Changes to any or all of these processes can influence the differentiation of stem cells. For example, hESCs express the transcription factors OCT4, NANOG and SOX2 and require extracellular signals such as fibroblast growth factor (FGF) and Activin/Nodal to maintain self-renewal. Changes in the levels and balance between FGF and Activin/Nodal can influence the maintenance and differentiation of hESCs: in the presence of too much Activin/Nodal they resemble endoderm cells, and too little Activin/Nodal will cause differentiation into ectoderm cells. The mechanisms involved in the maintenance of hESCs is poorly elucidated, but is likely to involve cell signaling pathways that function at different levels, with multiple feedback controls and intercellular gene regulation:

1. Gene expression: transcription factors are proteins that function to promote or repress gene expression at the DNA level. For example, OCT4 is thought to maintain hESCs by binding to genomic regulatory regions to promote the expression of pluripotency-associated genes and repress the expression of genes associated with differentiation.
2. Extracellular signals: small proteins that are produced and secreted (**cytokines**) by a stem cell niche can contribute to the maintenance of self-renewal. These proteins are often required to maintain stem cells in culture, such as the addition of cytokines FGF and epidermal growth factor (EGF) to neural stem cells grown in vitro.
3. Chromatin modifications: DNA is packaged with histones to form nucleosomes. Post-translational modification of histone tails forms a code that can determine states of gene expression that are heritable, modifying gene expression by influencing the access of transcription factors to genomic regulatory regions.
4. Physical context: the presence of extracellular matrix and physical contact with other cells can influence the maintenance and differentiation potential of stem cells.

Potential therapeutic applications for ESCs

1. Platform for drug discovery: hESCs can be differentiated into clinically relevant cells from individuals that harbor disease-associated gene expression. For example, hESCs have been established from individuals with amyotrophic lateral sclerosis and these hESCs were differentiated to motor neurons, the cells that are damaged in these patients. These hES-differentiated cells can be used to screen for small molecules (drugs) that might inhibit the progression of the disease and be effective in patient treatment.
2. Exogenous: transplantation of stem cells that have been differentiated in vitro towards a particular cell lineage, e.g., neuronal stem cells for neurodegenerative diseases that involve death or dysfunction of just one, or a few cell types, such as dopaminergic neurons to treat Parkinson's disease, insulin-producing B-cells to treat diabetes, etc. This approach has at least two significant considerations:
   - in-vitro stem cell differentiation must be completely controlled, as there may be a possibility of malignant transformation
   - transplant rejection unless the cells are immune matched.
3. Autologous: the patient's own stem cells are manipulated in vitro to induce/repress gene expression, and returned to the specific site that requires healing/treatment.
4. Endogenous stem cell renewal: the patient's own stem cells are manipulated to renew themselves in situ; this requires:
   - identification of stem cells in different parts of the body
   - interpretation of how they interact with their niche/microenvironment.

Evolution of stem cell research

The derivation of human ESCs in 1998 ignited an explosion of interest in stem cell biology and its therapeutic potential, and research in the field evolved very rapidly over the subsequent decade. In the words of the French philosopher Auguste Comte (1798–1857) "To understand science, it is necessary to know its history," and this is particularly true of stem cell science, where each significant achievement has been based upon the findings of prior decades of research. Successful derivation of human ESCs was based on research using murine ESCs, and this required information that was gained from previous stem cell models using mouse and human embryonal carcinoma cell lines. Similarly, the recent elucidation of discrete factors that can induce somatic cells into forming pluripotent stem cells (iPSCs) depended on studies in mouse and human embryonic stem cells. There is no doubt that novel trends will continue to emerge, and in order to fully comprehend the potential and the possible directions that lie in the future of stem cell research, it is important to be aware of the milestones that have marked its evolution so far.

Early culture systems

During the early 1960s, Robert Edwards recognized the extraordinary potential of stem cell biology, and in collaboration with Robin Cole isolated stem cells from the inner cell mass (ICM) of rabbit blastocysts. They cultured zona-free rabbit ICM on collagen surfaces, sometimes with HeLa feeder layers, and established cell colonies that showed differentiation to muscle, blood islands, neurons and complex groups of differentiating cells (Figure 7.2). Four immortal cell lines (two epithelioid and two fibroblastic) that survived more than 200 generations of subculture were established and cryopreserved. All cell lines remained diploid for several generations (Cole *et al.*, 1965, 1966; Edwards, 2008).

In further studies with Richard Gardner (1968), ICM cells isolated from mice with marker (coat color) genes were injected directly into the blastocoelic cavity of recipient mice, forming chimeras which showed that the grafted cells had colonized several different tissues; this indicated that the injected ICM cells were multipotent (Gardner, 1968). After human blastocysts first became available through IVF, Edwards and his team attempted to prepare human ESCs in vitro (Fishel *et al.*, 1984), but this early work had to be abandoned due to ethico-legal problems surrounding the use of human embryos for research.

Embryonal carcinoma (EC) and embryonal germ (EG) cell lines

In the early 1970s, stem cell lines that could be propagated in vitro were derived from murine teratocarcinomas (Kahn and Ephrussi, 1970); these cell lines were capable of unlimited self-renewal and multilineage differentiation. Mouse EC cells express antigens and proteins that are similar to cells present in the ICM, which led to the concept that EC cells are an in-vitro counterpart of the pluripotent cells present in the ICM (Martin, 1981); this provided the intellectual framework for working towards derivation of both mouse and human embryonic stem (ES) cells. Human EC

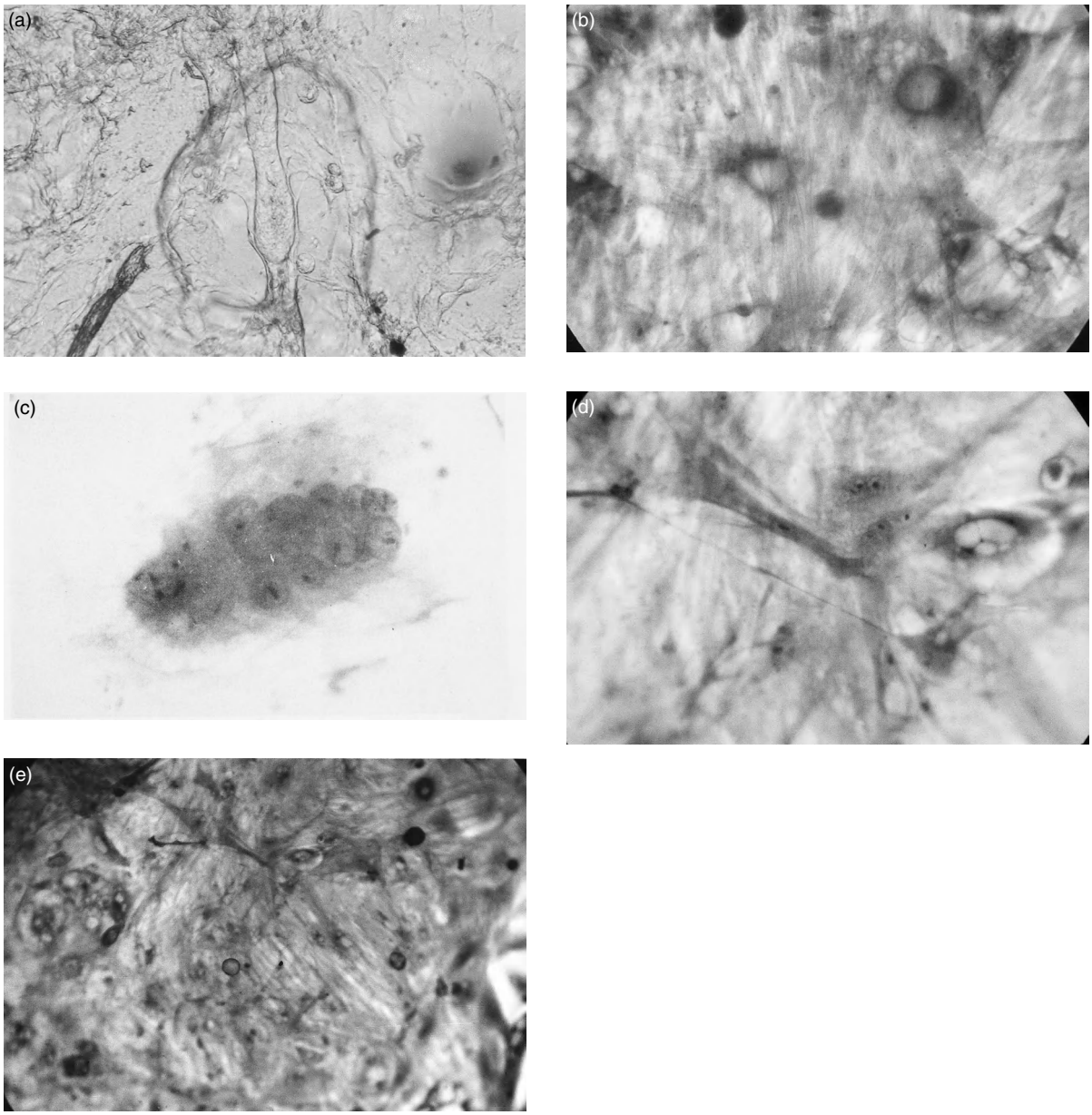


Figure 7.2 Cell colonies derived from intact zona-free rabbit blastocysts after 20 days in culture (Cole *et al.*, 1966). (a) Cell masses derived from a zona-free rabbit blastocyst. (b) Muscle differentiating after continued culture. (c) A single blood island that developed among cell outgrowths from rabbit ICM. (d) A group of neurons in the same outgrowths. (e) A complex group of differentiating cells in which muscle cells are mixed with various types of differentiating cells – a pathologist discerned several cell types in this mass of cells. (Reprinted with permission from: RG Edwards, *Reproductive Biomedicine Online*, 2008)

lines were established in 1977 (Hogan *et al.*, 1977), and these proved to differ from mouse EC lines, both in expression of surface markers, and in their in-vitro properties. Unlike mouse EC lines, the human cells are highly aneuploid, and have a limited ability to differentiate into a wider range of somatic cell types. Nonetheless, these human cell lines provided a useful model in which to study cell differentiation (Andrews *et al.*, 1984b; Thompson *et al.*, 1984; Pera *et al.*, 1989). Both mouse and human EC culture systems enabled

numerous improvements in technique and methodology, as well as initiating studies on factors that are involved in the control of differentiation in vitro.

Stem cell lines derived from mouse testicular teratomas (EG cells) were found to contribute to a variety of tissues in chimeras, including germ cells (Stevens, 1967); this provided a practical way to introduce modifications to the mouse germline (Bradley *et al*., 1984). Pluripotent EG cells were successfully derived directly from primordial germ cells in vitro in 1992 (Matsui *et al*., 1992; Resnick *et al*., 1992).

Mouse embryonic stem cells

Using culture conditions that had been used for mouse EC cells (inactivated fibroblast feeder layers and serum), the first mouse ES cell lines were derived from the ICM of mouse blastocysts (Evans and Kaufman, 1981; Martin, 1981). It was subsequently found that the efficiency of mouse ES cell derivation is strongly influenced by genetic background, and that different culture conditions were required for strains that were initially thought to be nonpermissive. Further experiments revealed that mouse ES cells could be sustained by conditioned medium, harvested from feeder layers, in the absence of the feeder cells themselves. Fractionation of conditioned medium, led to the identification of leukemia inhibitory factor (LIF) as one of the cytokines that sustains mouse ES cells (Smith *et al*., 1988; Williams *et al*., 1988). LIF activates the transcription factor Stat3, which inhibits differentiation and promotes viability. Further investigation of the signaling pathways showed that the proliferative effect of LIF requires a finely tuned balance between positive and negative effectors/factors. If serum is removed from the medium, mouse ES cells can be maintained in an undifferentiated state by adding LIF in combination with bone morphogenetic protein (BMP), a member of the TGF-alpha superfamily (Ying *et al*., 2003).

The subsequent two decades of research using murine ESCs led to numerous advances in culture system techniques and technology, and the identification of several types of in-vitro differentiated cells (including neural tissue) introduced the therapeutic potential and hope of eventual therapies to treat degenerative diseases – neurodegenerative disease in particular. The murine ES model contributed enormously to many different aspects of developmental biology: a large collection of genes, factors, markers, and signaling pathways involved in differentiation have now been identified, providing clues towards achieving directed differentiation of these pluripotent cells in vitro (Yu *et al*., 2007).

Human embryonic stem cells (hESCs)

A considerable delay (17 years) ensued between the derivation of mouse ES cells in 1981, and the first establishment of human ES cell lines in 1998. This was due to at least two factors:

1. Suboptimal media and conditions for human embryo culture meant that human blastocysts were rarely available (especially for research purposes). Media optimized for extended culture was introduced during the mid-1990s, and blastocyst culture then became a more practical reality.
2. Initial culture systems for hESC derivation were based on those that were successful in the mouse, and it eventually became apparent that there are significant species-specific differences between mouse and human ES cells.

Isolation of ICMs from human blastocysts was reported in 1994 (Bongso *et al*., 1994), but they were cultured in conditions that allowed derivation of mouse ES cells, and this resulted only in differentiation of the human cells instead of their derivation into stable pluripotent cell lines. In the mid-1990s, ES cell lines were derived from two nonhuman primates: the rhesus monkey and the common marmoset (Thomson *et al*., 1995, 1996). Using experience gained from these primate models, in 1998 Thompson and his colleagues then reported the isolation of pluripotent stem cell lines derived from human blastocyst ICM cultured on inactivated mouse feeder layers. These cell lines expressed markers for pluripotency, and could be maintained undifferentiated in long-term culture. The cultured cells also maintained the potential to form derivatives from all three germ layers when injected into severe combined immunodeficiency (SCID) mice. Media containing LIF and its related cytokines, required for mouse ESCs, failed to support human or nonhuman primate ES cells (Thomson *et al*., 1998; Dahéron *et al*., 2004; Humphrey *et al*., 2004); the fact that fibroblast feeder layers supported both mouse and human ES cells was apparently a fortunate coincidence.

Reubinoff *et al*. (2000) then reported directed differentiation of hESCs, producing three neural cell lines (astrocytes, dendrocytes, mature neurons) from an early

neural progenitor stem cell in spontaneously differentiating cultures. A huge expansion in research activity followed this initial report, and over the next few years panels of markers associated with pluripotency and with stages of differentiation were identified. Research continues into elucidating pathways of differentiation, and identifying factors that sustain hESCs in culture, as well as active factors that decay with de-differentiation, or change during re-differentiation. The body of published literature surrounding hESCs is now vast; by manipulating culture conditions, spontaneous differentiation to numerous different cell types has been observed, including beating heart cells (Mummery *et al.*, 2002), insulin-secreting cells, hepatocytes, cartilage, etc. Protocols are now available for directing at least partial differentiation of hESCs towards numerous different fates: early endoderm, hepatic cells, pancreatic cells, cardiomyocytes, endothelial cells, osteogenic cells, hematopoietic cells, lymphocytes, myeloid cells, etc (see Sullivan *et al.*, 2007). hESC lines have also been genetically modified, with fluorescent reporter genes introduced into key gene loci that can be traced during in-vitro differentiation in order to identify subsets of cells along developmental pathways (Davis *et al.*, 2008a; Hatzistavrou *et al.*, 2009).

Epiblast stem cells (EpiSCs)

As described in Chapter 6, one of the first stages of postimplantation development is the separation of the ICM into two lineages, the hypoblast and the pluripotent epiblast. Experiments with mouse embryos established that the epiblast in the late blastocyst is functionally and molecularly distinct from blastomeres, and from the ICM (Nichols and Smith, 2009). A strategically important milestone was reached in 2007, with the isolation of pluripotent stem cell lines derived from postimplantation (E5.5 to E6) mouse and rat epiblast (Brons *et al.*, 2007; Tesar *et al.*, 2007). Intriguingly, these EpiSCs differ significantly from mouse ESCs, but have key features in common with human cells. The two factors required for mouse ESC derivation (LIF and/or BMP4) have no effect on epiblast cell isolation, a similar situation to human ESC derivation. Instead, the signaling factors that are important for human ESCs (FGF and Activin/Nodal) are apparently critical for EpiSC derivation. The pattern of gene expression by EpiSCs differs from that of mouse ESCs, but they do retain the ability to proliferate indefinitely, as well as the potential for multilineage differentiation. Mouse ES cells can be induced to become EpiSCs, and the reverse transition has recently been observed (Bao *et al.*, 2009). It is possible that the unique properties that hESCs have in common with EpiSCs could reflect a different origin that had not previously been recognized in hESC, i.e., they may represent a later stage of development (post-implantation epiblast) than mouse ES cells; Activin/Nodal signaling seems to have an evolutionarily conserved role in the maintenance of pluripotency. This also reinforces the significant species-specific differences in embryology and signaling pathways between humans and rodents.

Induced pluripotent cell lines (iPSCs/piPSCs)

The concept of reversing the programming of differentiated tissues to pluripotent states was introduced with the first somatic cell nuclear transfer (SCNT) experiments by John Gurdon during the late 1950s, using *Xenopus* oocytes (Gurdon, 1962). Several decades later, the birth of "Dolly the Sheep" provided the first confirmation in mammals that a differentiated somatic cell could be converted to a totipotent state by inserting its nucleus into an enucleated oocyte.

Somatic cell nuclear transfer: cloning (Figure 7.3)

1. **Therapeutic cloning**: the nucleus of an adult somatic (differentiated) cell is reprogrammed by insertion into an enucleated donor oocyte. The oocyte is then activated by electrofusion to stimulate cleavage, grown in culture to the blastocyst stage, and the ICM of this blastocyst used for stem cell derivation. The resulting stem cell lines are immunologically identical (HLA-matched) to the somatic cell that was reprogrammed, and could theoretically be used for potential therapies without transplant rejection. The technique has recently been successful in nonhuman primates (rhesus macaque), although with very low efficiency: two primate ESC lines were derived from the use of 304 oocytes (Byrne *et al.*, 2007). A report that several patient-specific stem cell lines had been established in Korea subsequently proved to be based on data that was fabricated, and the papers were withdrawn (Hwang *et al.*, 2004, 2005). In view of the extreme difficulty in obtaining donor oocytes, and the inefficiency of the technique so far, it may be that pursuing research with iPS cells is a better strategy than therapeutic cloning.
2. **Reproductive cloning**: SCNT is carried out as for therapeutic cloning, but the resulting embryo is transferred to the uterus. Dolly, the

much-celebrated sheep, was the first example of successful mammalian reproductive cloning, demonstrating that a differentiated adult cell could be reprogrammed to generate an entire organism (Wilmut *et al*., 1997). This success confirmed John Gurdon's remarkable discoveries during the 1950s, when he was able to produce tadpoles after somatic cell nuclear transfer into *Xenopus laevis* eggs (Gurdon, 1962). The technique has been used in a variety of large and small animals, including cows, goats, mice, pigs, cats, rabbits and a gaur. However, reproductive cloning is both expensive and highly inefficient – more than 90% of cloning attempts fail, and imprinting defects have been identified in cloned animals, with abnormalities of immune function, growth and numerous other disorders. Experiments with cloned mice indicate that approximately 4% of their genes function abnormally, due to imprinting defects.

With accumulated information regarding pluripotency in ESC, pinpointing key genes that might be used to reprogram somatic cells became a major research goal. Panels of genes that are enriched in ESC populations, and thought to be involved in maintenance of pluripotency were screened, with the target of identifying factors that have the ability to re-program somatic mouse cells into proliferation. From a panel of 24 target genes, four factors were found that together induced a transformation of mouse fibroblasts into cells that closely resemble mouse ES cells: OCT4, SOX2, c-Myc and Klf4 (Takahashi and Yamanaka, 2006). The experiments were conducted by using retroviruses to insert key pluripotency genes into the fibroblasts; the cells that resulted had properties analogous to ESCs in culture, and also formed teratomas when injected into mice. The same technique was subsequently applied to successfully reprogram human fibroblasts into hES-like cells (Takahashi *et al*., 2007a; Lowry *et al*., 2008). A further independent study using human cells screened a panel of 14 genes that are enriched in hESCs, and succeeded in reprogramming human fibroblasts with the introduction of genes for OCT4, SOX2, NANOG and LIN28 (Yu *et al*., 2007). Human iPS cells satisfy all the original criteria proposed for characterization of hESCs: morphology is similar, they express typical hESC surface antigens and genes, differentiate into multiple lineages in vitro, and when injected into SCID mice they form teratomas containing cells derived from all three primary germ layers. Since this initial report, numerous combinations of somatic cell type

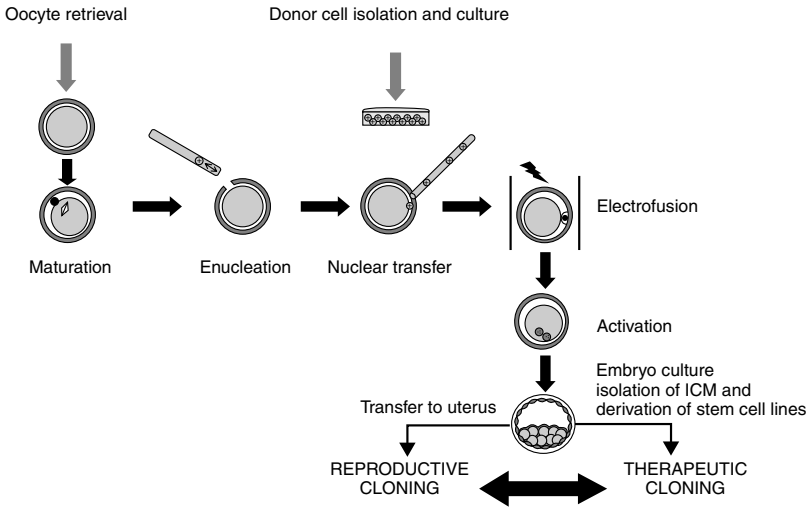


Figure 7.3 Transfer of a diploid somatic cell nucleus into an enucleated metaphase II oocyte followed by activation by electrofusion leads to the creation of an embryo that has the chromosome complement of the somatic cell. A resulting blastocyst stage embryo can be transferred to a recipient uterus and allowed to develop to term into an animal with characteristics of the transferred somatic nucleus (reproductive cloning) or inner cell mass cells may be used to generate stem cell lines that are genetically matched to the donor of the somatic cell (therapeutic cloning).

and cocktails of factors have been studied, and continue to be investigated: some cell types can be reprogrammed more efficiently than others, and different types of cell respond to different expression levels and combinations of factors. The number of factors investigated continues to grow, and the original four now only head a list of related proteins that seem to enhance the efficiency of transformation in some cells (Heng *et al.*, 2010). Efforts to elucidate the mechanisms by which such a limited number of transcription factors can erase and reprogram a differentiated state continue: the DNA binding sites of OCT4, SOX2 and NANOG have been studied, and it seems that these three factors can also activate or repress the expression of many other genes, including transcription factors that are important during early stages of development (Boyer *et al.*, 2005).

Although iPSCs provide an excellent and powerful model for studying the fine details of cell biology and differentiation, the efficiency of reprogramming adult cells is very low (0.1%), and their capacity for diff erentiation is lower, and more variable than that of hESCs (Hua *et al.*, 2010). Selecting the appropriate type of cell, timing, balance of factors, identifying small molecules that might augment efficiency, and the absolute levels of gene expression needed are all aspects that require further research. In terms of therapeutic potential, iPSCs have the major advantage that a patient's own cells can be used for reprogramming, which overcomes the problem of immune matching. However, using viral (retrovirus, lentivirus, adenovirus) or plasmid vectors to introduce genetic material carries the considerable risk of causing genetic mutations at the insertion sites, and alternative methods of delivering the genes are under investigation, including transient transfection and using a "piggyBac" transposon that uses a host factor to carry the genes, and then allows the exogenous genes to be excised (see Müller *et al.*, 2009 and Shao and Wu, 2010 for reviews).

Factors used to induce pluripotency

OCT4
- POU domain transcription factor.
- Expression restricted to pluripotent cells of the embryo.
- Expressed in undifferentiated ES, EC and EG cells.
- Essential for formation of the ICM.
- In the absence of OCT4, all cells in the embryo become trophectoderm.
  - Deletion in ES cells causes differentiation into trophectoderm
  - Overexpression causes differentiation to endoderm/mesoderm

SOX2
- High mobility group (HMG) box transcription factor.
- Expressed in ICM and trophectoderm.
- Required for development of the embryonic lineage.
- Also required for proliferation of extraembryonic ectoderm.
- Interacts specifically with OCT4 to influence expression of target genes.
- SOX2 controls OCT4 expression, and they perpetuate their own expression when they are co-expressed.

c-Myc
- Transcription factor that activates expression of a large number of genes through binding on enhancer box sequences (E-boxes).
- Has a direct role in DNA replication, can drive cell proliferation by upregulating cyclins/downregulating p21.
- Also has roles in cell growth, apoptosis, differentiation, stem cell renewal.
- Proto-oncogene: upregulated in many types of cancers.

Klf4: A member of the Krüppel-like family of transcription factors
- Can act as a transcriptional activator or repressor, depending on promoter context and interaction with other transcription factors.

NANOG
- Homeodomain transcription factor.
- Expression is tightly associated with pluripotency.
  - Essential for early development, and for reprogramming.
  - Required for development of the epiblast.

LIN28: marker of undifferentiated hESCs
- Encodes a cytoplasmic mRNA binding protein that binds to and enhances the translation of IGF2 mRNA·

Protein-induced pluripotent stem cell lines (piPSCs)

In further attempts to overcome the problem of viral vectors, modified versions of the protein products themselves have also been used. Stable iPSCs from human fibroblasts were generated by using a cell-penetrating peptide (CPP) to deliver the four proteins OCT4, SOX2, Klf4, c-Myc (Zhou *et al.*, 2009; Cho *et al.*, 2010). The cells produced by

this DNA-vector free, direct protein transduction method have been called "protein-induced pluripotent stem cells", piPSC. Experiments have been carried out using recombinant proteins, as well as proteins derived from cell fractions. However, so far the generation of piPSC is very slow and inefficient; numerous variables involved in applying this technology remain to be optimized.

hESC culture systems

The first human ESCs were derived using inactivated mouse fibroblasts as feeder layers. A great deal of research has been devoted to both improving the efficiency of derivation/propagation, and towards establishing conditions that are free from xenobiotic hazard in cells, reagents and media supplements (animal cells always carry the risk of pathogen transmission or viral infection, and cells grown in the presence of animal-derived products cannot be used for human therapies). Mouse fibroblast feeder layers can be replaced with human fibroblasts or feeder conditioned medium, substrates such as Matrigel and laminin have been used, and numerous experiments have been carried out using combinations of additional growth factors and cytokines. Basic fibroblast growth factor and members of the TGF-beta superfamily are important in regulating self-renewal, and it has become apparent that maintaining the undifferentiated state involves extensive cross-talk between the intracellular signaling pathways activated by factors such as FGF, TGF-beta and BMP (Rao and Zandstra, 2005). In 2005, Vallier *et al.* reported successful prolonged culture of hESCs in a chemically defined medium (CDM) containing activin A (INHBA)/nodal (NODAL) and FGF2; cells cultured in this medium maintained their fundamental characteristics of pluripotency. Other members of the TGF-beta superfamily (BMP11/GDF11 and myostatin/GDF8) have also been identified that promote self-renewal in feeder-free and serum-free conditions (Hannan *et al.*, 2009). However, to date initial derivation of a stem cell line is still more efficient with the support of a feeder layer.

Derivation and propagation of hESCs requires a significant commitment of time and resources. Establishing a culture of hESCs takes from 3 to 6 weeks, and the cultures need daily attention once established. With the appropriate level of skill and attention, they can be kept in continuous culture for years, with aliquots cryopreserved during subculture. It has become clear that there is probably no standard culture method or medium that is optimal for all lines and all purposes (see under "Books" in Further reading).

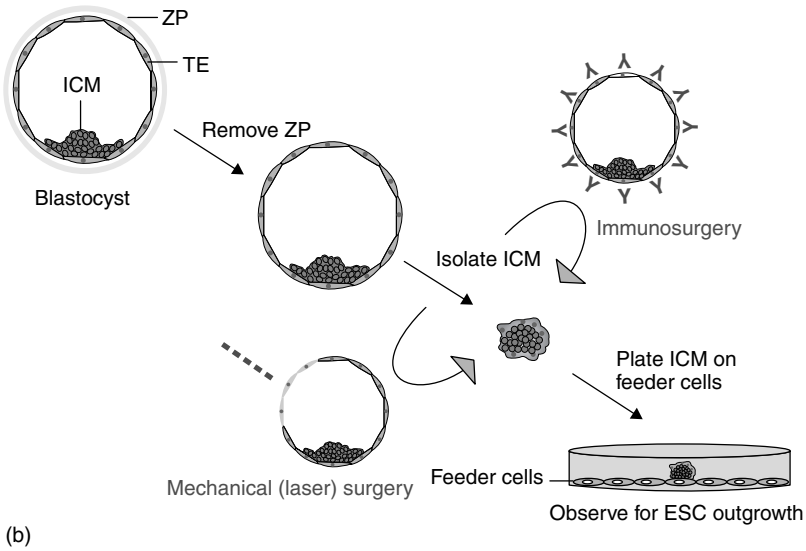
Human ES cells have been derived not only from blastocyst ICM, but also from later stage blastocysts, morulae (Stojkovic *et al.*, 2004), single blastomeres (Klimanskaya *et al.*, 2007), and from parthenogenetic embryos (Mai *et al.*, 2007; Revazova *et al.*, 2007). It is not yet known whether the pluripotent cell lines derived from these sources have any consistent developmental differences, or whether they have an equivalent potential.

Derivation of research stem cell lines from blastocyst ICM (Figures 7.4a and 7.4b)

Protocol based on Sullivan *et al.*, 2007; all animal-based products must be avoided if lines are being derived for potential therapeutic use.

1. Prepare feeder layers: seed mitotically inactivated fibroblast onto gelatinized four-well culture plates at a density of approximately 50 000 cells/cm^3. Mouse embryonic fibroblasts (MEFs) are commonly used, inactivated either with mitomycin C or by gamma-irradiation (human fibroblast cells can also be used, isolated from a variety of sources including newborn foreskin, but for research purposes, derivation on MEF has practical advantages and thus far appears to be more efficient).
2. Remove/dissolve the zona pellucida: different methods include the use of pronase, acid Tyrode's solution, or allowing the blastocyst to hatch completely in culture.
3. Remove the trophectoderm layer, by complement-mediated lysis (immunosurgery), or by manual dissection.
4. Plate the isolated ICM clump onto a well-developed, confluent feeder layer, and incubate in human ES derivation medium, undisturbed, for 48 hours.
5. After several days, outgrowths of cells from the ICM will appear. hESCs grow in flat two-dimensional clumps, and have prominent nucleoli; these can be isolated and subcultured into fresh culture dishes. The explants should not be allowed to become over-confluent and crowd the dish, or they will begin to differentiate.
6. If the subcultured cells continue to proliferate without signs of differentiation, they are a "putative ES line," and require extensive characterization to confirm.
7. Batches of cells can be cryopreserved at intervals during subculture.

(a)



(b)

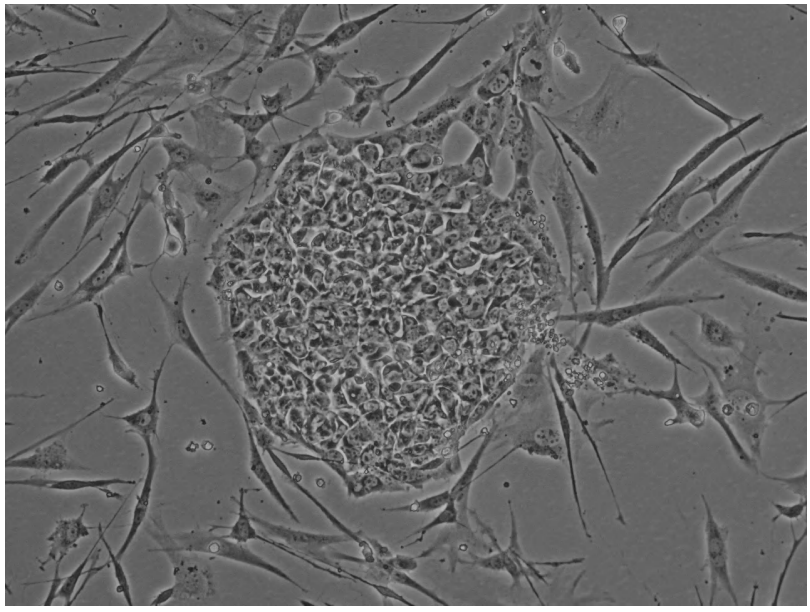


Figure 7.4 (a) Cartoon outlining derivation of a stem cell line from a blastocyst inner cell mass (ICM) (with thanks to Alice Chen). ZP = zona pellucida; TE = trophectoderm; ESC = embryonic stem cell. (b) Phase contrast image of a human stem cell colony, on a background of inactivated mouse fibroblasts (with thanks to Dr. Kathy Niakon).

Characterization of ESC lines

1. The cells should have the ability to be propagated in long-term culture, without visible signs of transformation.
2. Cryopreserved aliquots of cells should maintain the ability to continue propagation after freeze-thawing.
3. Identify cell-surface markers characteristic of ESCs: the glycolipids SSEA3 and SSEA4, and keratan sulfate antigens Tra-1–60 and Tra-1–81.
4. Identify protein markers of pluripotency: Oct4, Nanog, Sox2.
5. Analyze chromosomal karyotype.
6. If feeder layers are removed, they should round up and clump into embryoid bodies containing undirected differentiated cell types from the three germ layers.
7. Injection of the ESCs into an immunosuppressed (SCID) mouse should result in teratoma formation.

More sophisticated molecular biology tools are now being used in order to identify specific transcriptional profiles, combining immunotranscriptional and polysome translation state analyses to identify a large number of genes and cell surface markers (Kolle *et al.*, 2009)

Problems with established human ESCs

- Tendency to become aneuploid after extended passaging.
- Do not like to grow as single cells, making genetic modification (targeting) difficult.
- Difficult to drive along specific differentiation pathways.

ESC markers of pluripotency

A recent report (Hough *et al.*, 2009) suggests that "pluripotency" in hESCs may not be an all-or-nothing state: hESC cultures are in fact heterogeneous. Analyzing transcripts of single hESCs for lineage-specific transcription factors revealed that there is a gradient and a hierarchy of pluripotency gene expression, with many cells co-expressing both pluripotency and lineage specific genes. These authors suggest that only a small fraction of the hESC culture population lies at the top of the developmental hierarchy, and that pluripotent stem cell populations may simultaneously express both stem cell and lineage specific genes. Transcription factor networks that control pluripotency are dependent on upstream extrinsic signaling pathways, and cells along the continuum of differentiation show a progressively decreasing potential for self-renewal, with decreased expression of stem cell surface markers and pluripotency genes. The spatial organization of hESC cultures also influences the fate of individual cells, and engineering the microenvironment (niche) can be used to direct the rate and direction of differentiation by regulating the balance between inducing and inhibiting factors in the signaling pathways (Peerani *et al.*, 2007).

Stem cell banking and registries

Several hundred human embryonic stem cell lines have now been established in countries throughout the world, including several lines carrying gene defects for inherited genetic disease, derived from embryos identified as abnormal in PGD cycles. Research is accelerating via new and sophisticated molecular biology tools for gene sequencing, microarrays to map gene expression in single cells, cytokine and cDNA libraries, etc.

This fast-moving and multidisciplinary research is of critical importance to medical science, and there is a crucial need for collaboration and free exchange of information. Initiatives to facilitate collaboration and establish bench marks and good practice models have been set up (see Andrews *et al.*, 2005; Franklin *et al.*, 2008), and stem cell registries have been developed in order to collect, organize and disseminate information about specific cell lines (see Borstlap *et al.*, 2010 for review of stem cell registries). In May 2004, the world's first Stem Cell Bank was opened in the UK (www.ukstemcellbank.org.uk/), established in order to provide a repository for human stem cell lines of all types, and to supply well-characterized cell lines for use in basic research. The Bank has a catalogue of characterized stem cell lines that have been deposited by research groups in several different countries, and details of lines that are being characterized, or are due for release can also be found on their website. Applications to access the Bank's stem cell lines must be first approved by a UK Medical Research Council (MRC) Stem Cell Steering Committee who will review the research proposal, and the credentials of the research team

In March 2009, in response to a new Executive Order from President Obama, the National Institutes of Health (NIH) in the USA published a registry of cell lines, and guidelines to establish policy and procedures for NIH-funded stem cell research: http://stemcells.nih.gov/research/registry/; http://stemcells.nih.gov/policy/2009guidelines.htm.

Stem cell biology is now arguably the most powerful tool available for the advancement of medical science. The possibility of studying cell-fate transitions, controlling proliferation and directing differentiation by manipulating core sets of transcription factors adds a new dimension to the fields of regenerative medicine, degenerative disease, and the uncontrolled proliferation of cancerous disease. Culture systems and selected populations of stem cells can be used to screen drugs and new approaches to treatment. Understanding the regulation of self-renewal at the molecular level will lead to improved systems for hESC derivation and propagation, and also has the potential to yield further insight into aspects of development that might increase the efficiency of clinical IVF. Human embryonic stem cells undergoing differentiation in culture also give us information about a period that has not previously been accessible for research, the first stages of early postimplantation human development that are described in Chapter 6.

Further reading

Website information

www.hta.gov.uk/

www.regenmd.com/research_links.htm

www.regenmd.com/index.htm

http://stemcells.nih.gov/info/faqs.asp

http://isscr.org/science/faq.htm

www.news.wisc.edu/packages/stemcells/illustration.html

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Chapter 8

The clinical in-vitro fertilization laboratory

Introduction

In the armory of medical technology available for alleviation of disease and quality of life enhancement, there is nothing to match the unique contribution of assisted reproductive technology (ART). There is no other life experience that matches the birth of a baby in significance and importance. The responsibility of nurturing and watching children grow and develop alters the appreciation of life and health, with a resulting long-term impact upon individuals, families and, ultimately, society. Thus, the combination of oocyte and sperm to create an embryo with the potential to develop into a unique individual cannot be regarded lightly, as merely another form of invasive medical technology, but must be treated with the respect and responsibility merited by the most fundamental areas of human life.

Successful assisted reproduction involves the careful coordination of both a medical and a scientific approach for each couple undertaking a treatment cycle, with close collaboration between doctors, scientists, nurses and counselors. Only meticulous attention to detail at every step of each patient's treatment can optimize their chance of delivering a healthy baby. Appropriate patient selection, ovarian stimulation, monitoring and timing of oocyte retrieval should provide the in-vitro fertilization (IVF) laboratory with viable gametes capable of producing healthy embryos. It is the responsibility of the IVF laboratory to ensure a stable, nontoxic, pathogen-free environment with optimum parameters for oocyte fertilization and embryo development. The first part of this book reveals the complexity of variables involved in assuring successful fertilization and embryo development, together with the fascinating and elegant systems of control that have been elucidated at the molecular level. It is essential for the clinical biologist to be aware that the control mechanisms involved in human IVF are complex, and exquisitely sensitive to even apparently minor changes in the environment of gametes and embryos. Temperature and pH are of crucial importance, and many other factors can potentially affect cells at the molecular level. Multiple variables are involved, and the basic science of each step must be carefully controlled, while allowing for individual variation between patients and between treatment cycles. In addition, as technology continues to evolve, the success of new innovations in technique and technology can only be gauged by comparison with a standard of efficient and reproducible established procedures. The IVF laboratory therefore, has a duty and responsibility to ensure that all of the components and elements involved are strictly controlled and regulated via an effective system of quality management (as outlined in Chapter 9).

The assisted conception treatment cycle

- Consultation: history, examination, investigations, counseling, consent(s)
- Drug scheduling regimen: GnRH agonist pituitary downregulation or oral contraceptive pill to schedule withdrawal bleed
- Baseline assessment at start of treatment cycle
- Gonadotropin stimulation
- Follicular phase monitoring, ultrasound/endocrinology
- Induction of ovulation
- Oocyte retrieval (OCR)
- In-vitro fertilization/ICSI
- Embryo transfer
- Supernumerary embryo cryopreservation
- Luteal phase support
- Day 15–18 pregnancy test
- Ultrasound assessment to confirm gestational sac/fetal heartbeat

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Setting up a laboratory: design, equipment and facilities

The design of an IVF laboratory should provide a distraction- and risk-free environment in which uninterrupted concentrated attention can be comfortably and safely dedicated to each manipulation, with sensible and logical planning of workstations that are practical and easy to clean. Priority must be given to minimizing the potential for introducing infection or contamination from any source, and therefore the tissue culture area must provide aseptic facilities for safe manipulation of gametes and embryos, allowing for the highest standards of sterile technique; floors, surfaces and components must be easy to clean on a daily basis. The space should be designated as a restricted access area, with facilities for changing into clean operating theater dress and shoes before entry.

Laboratory space layout

The range of treatment types to be offered, the number of cycles per year, and the manner in which the cycles will be managed all dictate the appropriate layout design and the equipment and supplies required. Four separate areas of work should be equipped according to need, arranged and set up to accommodate the flow of work according to the sequence of procedures in an IVF cycle:

1. **Andrology**: semen assessment and sperm preparation; surgical sperm retrieval
2. **Embryology**: oocyte retrieval, fertilization, embryo culture and transfer
3. **Cryopreservation**: sperm, oocytes, embryos, ovarian and testicular tissue
4. **Micromanipulation**: ICSI, assisted hatching, embryo and polar body biopsy.

Careful consideration should be given to the physical maneuvers involved, ensuring ease and safety of movement between areas to minimize the possibility of accidents. Bench height, adjustable chairs, microscope eye height and efficient use of space and surfaces all contribute to a working environment that minimizes distraction and fatigue. The location of storage areas and equipment such as incubators and centrifuges should be logically planned for efficiency and safety within each working area; the use of mobile laboratory components allows flexibility to meet changing requirements. Many IVF laboratories are now designed with curved joins between walls, floors and ceilings to ensure that no dust settles. For optimal cleanliness on reaching the entrance of the laboratory, two-stage entrance/exits can be incorporated into the changing room areas, so that outdoor clothing is removed at the external section, and scrubs/protective clothing donned at the second stage. Hand washing facilities must be available in the changing area; sinks should be avoided in culture area, as they can act as a source of microbial contamination.

Light exposure during ART procedures

In the course of normal physiology, gametes and embryos exist in a dark environment, and therefore exposure to light is not a "natural" situation. The potential of introducing metabolic stress through light exposure was taken into consideration during the first trials of human IVF in Oldham and Cambridge: dissecting microscopes were fitted with green filters, background lighting in the laboratory was kept low, and during the time of the embryo transfer procedure the lights were extinguished in the operating theater until the embryo transfer catheter had been safely handed over to the physician. Increasingly sophisticated technology in IVF has added the use of more powerful microscopy, with high-intensity light sources. Some spectra of light are known to be associated with generation of reactive oxygen species (ROS), and further data has accumulated about the harmful effects of ROS in IVF. Three groups have recently examined various aspects and effects of light exposure in IVF procedures (Takenaka *et al*., 2007; Ottosen *et al*., 2007; Korhonen *et al*., 2009). These three studies concluded that certain wavelengths of light are potentially harmful in ART, and the extent of the damage is related to the duration of exposure, wavelength and intensity. Wavelengths <300 nm UV are absorbed by plastic; near UV-wavelengths of 300–400 nm are associated with increased apoptosis in mouse blastocysts. Ottosen *et al*. (2007) confirmed that 95% of the radiation exposure was from microscopes, and recommended the use of green filters. They suggest that ambient light was not a significant hazard, but Takenaka *et al*. (2007) found that cool fluorescent light, as commonly used in laboratories, produces a higher level of ROS and apoptosis in mouse and hamster zygotes than does warm fluorescent light or sunlight.

Laboratory equipment

Equipment should be selected based on its suitability for the intended purpose, capacity for the intended

workload, ease of use and maintenance, availability of service and repair contracts to quality standards, and validation of evidence regarding its correct function. All equipment used in clinical treatments must be of highest standard available, validated for the intended use, safety checked and properly calibrated, regularly cleaned and maintained. Temperature and gas levels must be strictly monitored and recorded.

Service contracts should be set up with reliable companies, who must provide calibration certificates for the machines used to service and calibrate equipment in the IVF laboratory. Provision must be made for emergency call-out, with alarms fitted to all vital equipment and back-up machines held in reserve. Essential equipment that require contracts for service and calibration include:

- Incubators
- Air filtration equipment
- Flow cabinets
- Microscopes
- Heated surfaces/microscope stages
- ICSI/Micromanipulation workstation
- Centrifuges
- Refrigerators and freezers
- Embryo/oocyte freezing machines
- Osmometers
- Liquid nitrogen storage dewars
- Electronic witnessing system
- Ultrapure water system.

A camera and monitor system that can display and record images is also recommended as part of the basic laboratory set-up, for teaching, assessment and record keeping.

Incubators

Carefully calibrated and accurately controlled CO_2 incubators are critical to successful IVF. The choice of a humidified or nonhumidified incubator depends upon the type of tissue culture system used: whereas humidity is required for standard four-well "open" culture, the use of an equilibrated humidified overlay of clinical grade mineral oil allows the use of incubators without humidity. Dry incubators carry less risk of fungal contamination, and are easier to clean.

Traditional stand-alone incubators can accommodate culture dishes for many different patients in a secure, well-insulated environment. Recent models deliver a range of gas concentrations, with CO_2 concentrations of 5-6% and variable N_2 levels to reduce oxygen concentrations from ambient 21% to as low as 5% (Meintjes *et al*., 2009). The larger models may incorporate a water-jacket in the door for additional insulation and can be run dry or at 95% relative humidity when water is added to the tray at the bottom of the chamber. The water-jacket not only helps to maintain a consistent temperature under normal circumstances, but also in the event of a power failure. Inner gas-tight split doors are essential in order to minimize recovery time after door opening, and CO_2 levels must be regularly (preferably continuously) monitored using an infrared CO_2 sensor. A single large incubator should not be used to house more than 12 cases at a time.

Smaller models such as the benchtop humidified MINC (COOK IVF) or K-Systems G185 are portable and therefore more flexible, with pre-mixed gas and sealed gas-tight chambers so that the dishes for each patient can be isolated. These mini-incubators are less prone to variation in temperature and pH, exclude the use of laboratory air in the gas mix and do not need constant CO_2 calibration with external monitoring devices. However, it is extremely important to find a reliable source for the gas mixture, both in the accuracy of the percentages of each gas and in their purity.

Large incubators can also be used to equilibrate tubes and bottles of media; this is not possible when only mini-incubators are used, so that HEPES-buffered media might be required for the majority of preparatory procedures. Large incubators also have the advantage of allowing independent probes to be installed, with failure alarms that can operate remotely. Some also have fitted HEPA-VOC filters.

Incubators must be regularly monitored, and readings of the LED display checked and calibrated against independent recordings of temperature and pH monitored by probes placed in a standard "test" culture system. Temperature stability can be monitored with 24-hour thermocouple readings as part of the standard maintenance schedule. There should be a schedule to ensure regular dismantling and cleaning, and a yearly inspection and general servicing by the supplier is recommended. Repeated opening and closing of the incubator affects the stability of the tissue culture environment, and the use of an accessory small benchtop mini-incubator during oocyte retrievals and manipulations helps to minimize disturbance of the larger incubators.

Irrespective of the type used, in all stand-alone incubators the dishes must be removed from the controlled environment for each stage of manipulation,

exposing gametes or embryos to suboptimal temperatures and changes in pH due to reduced CO_2 concentration. This has led to the introduction of integrated workstations that incorporate imaging systems and controlled atmosphere, providing a fully integrated incubator and flow cabinet in one system. These integrated systems reduce the risks inherent in carrying dishes of oocytes or embryos around the laboratory for different procedures. Custom-built isolator units are used in some laboratories, which can provide direct access from the flow cabinet to the incubator so that the culture dish never leaves the controlled environment. In the Ruskinn Ac-tive IVF System, the entire flow cabinet is environmentally controlled and acts as incubator and flow cabinet. These integrated workstations are costly, and evidence of their validation in improving success rates is currently awaited.

Recent advances in incubator technology also incorporate a microscope and imaging system for continuous monitoring of embryo development using time-lapse photography of individual embryos, for example:

EmbryoScope from Unisense FertiliTech A/S (www.fertilitech.com/)

InCu-View Live (www.sanyo-biomedical.com).

Electronic witnessing systems

Systems are available that provide electronic identity checking/witnessing, using either barcode readers or radio frequency identification. IVF Witness (www.ivfwitness.com) and OCTAX Ferti Proof™ (www.mtg-de.com/) utilize RFID (radio frequency identification) technology to read labels attached to all labware, enabling sperm and oocytes to be tracked throughout the IVF process. This technology reduces the chance of human error and helps to ensure that the resulting embryo is transferred to the correct patient.

Automated witnessing systems such as EmbryoMatcher (www.fertqms.com) can also be incorporated into the incubator imaging system, recording bar codes of dishes while monitoring embryo development. The data can be transmitted to a computer for continuous monitoring either on site or remotely.

Electronic witnessing systems have the advantage that they can be programmed to include traceability for all the media and batches of consumables used for a specific case. They also provide information on who performs specific procedures and the time that it takes for completion, which is very useful for audit purposes. However, it is essential that full risk assessments and validation checks are performed before introducing e-witnessing, rather than just accepting that a barcode system will work efficiently.

Ambient air quality

Air cleanliness is classified according to the number and size of particles within a sample of air, measured in particles per cubic foot or cubic meter of air.

Laboratory air quality should be maintained by use of positive air pressure relative to adjacent areas; the airflow pressures in clean areas should be continuously monitored and frequently recorded. Adjacent rooms with different clean area classification should have a pressure differential of 10–15 pascals, with the highest pressure in the most critical areas. High efficiency particulate air (HEPA) filters that remove particles smaller than 0.3 μm and filters to remove volatile organic compounds, which can adversely affect the health of human gametes and embryos, can also be used (Cohen *et al.*, 1997; Cutting *et al.*, 2004). Filters must be regularly inspected and changed to ensure that their efficiency is not reduced.

The environment in a clean room is produced by incorporating parallel streams of HEPA-filtered air (laminar flow) that blow across the room to expel any dust and particles in the airflow by the shortest route, moving at a uniform velocity of 0.3–0.45 meters per second. The air velocity over critical areas must be at a sufficiently high level to sweep particles away from the area and ensure that particles do not thermally migrate from the laminar flow. At least 20 changes of air per hour are usually required for clean rooms classified as Grade B, C and D (see Table 8.1).

The airflow can either be vertical downflow (entering via filters in the roof, exiting through vents in the floor) or horizontal crossflow (entering through filters in one sidewall and exhausted above the floor in the sidewall opposite and/or recirculated via a bank of filters).

The US Federal Standard 209E defines air quality based upon the maximum allowable number of particles 0.5 μm and larger per cubic foot of air: Class 1, 100, 1000, 10 000 and 100 000. The lower the number, the cleaner the air. The ISO classifications are defined as ISO Class 1, 2, 3,4, etc. through to Class 9. The cleanest, ultrapure air is Class 1. Guidelines suggested by

Table 8.1 (a) Comparison of air classification systems

WHO GMP	US 209E	US Customary	ISO/TC (209) ISO 14644	EEC GMP
Grade A	M 3.5	Class 100	ISO 5	Grade A
Grade B	M 3.5	Class 100	ISO 5	Grade B
Grade C	M 5.5	Class 10 000	ISO 7	Grade C
Grade D	M 6.5	Class 100 000	ISO 8	Grade D

Table 8.1(b) Classification of clean areas in terms of airborne particles

	At rest		In operation	
	Maximum permitted number of particles/m^3			
Grade	0.5–5.0 µm	>5 µm	0.5–5.0 µm	>5 µm
A	3 500	0	3 500	0
B	3 500	0	350 000	2 000
C	350 000	2 000	3 500 000	20 000
D	3 500 000	20 000	Not defined	Not defined

"At rest" = equipment installed and operating; "in operation" = installed equipment functioning and specified number of personnel present.

regulatory and legislative authorities for IVF laboratories are based on those for Good Manufacturing Procedures (GMP), which define four grades of clean area (A–D) for aseptic handling and processing of products that are to be used in clinical treatment (Tables 8.1a and 8.1b). Each area has recommendations regarding required facilities, environmental and physical monitoring of viable and nonviable particles, and personnel attire.

- Grade A (equivalent to Class 100 [US Federal Standard 209E], ISO 5 [ISO 14644–1]) is the most stringent, to be used for high risk operations that require complete asepsis, carried out within laminar flow biological safety cabinets (BSC).
- Grade B (equivalent to Class 100, ISO 5) provides the background environment for a Grade A zone, e.g., clean room in which the BSC is housed.
- Grade C (equivalent to Class 10 000, ISO 7) is a clean area for carrying out preparatory stages in manufacture of aseptically prepared products, e.g., preparation of solutions to be filtered.
- Grade D (equivalent to Class 100 000, ISO 8) is a clean area for carrying out less critical stages in manufacture of aseptically prepared products, such as handling of components after washing.

Personnel entering all grades of clean area must maintain high standards of hygiene and cleanliness at all times, and should not enter clean areas in circumstances that might introduce microbiological hazards, i.e., when ill or with open wounds. Changing and washing procedures must be defined and adhered to, with no outdoor clothing introduced into clean areas. Wearing of watches, jewelry and cosmetics is discouraged. Changing rooms for outdoor clothing should lead into a Grade D area (not B or C). Protective clothing for the different areas is defined:

- Grade D: protective clothing and shoes, hair, beard, moustache covered
- Grade C: single or 2-piece suit with high neck, wrists covered, shoes/overshoes, hair beard moustache covered; non-shedding materials
- Grade A and B: headgear, beard and moustache covered, masks, gloves, non-shedding materials, and clothing should retain particles shed by operators.

The Tissue and Cells Directive issued by the European Union (Directive 2003/94/EC) stipulates that where human cells and tissue are exposed to the environment during processing, the air quality should be Grade A with the background environment at least

equivalent to Grade D, unless a less stringent air quality may be justified and documented as achieving the quality and safety required for the type of tissue and cells, process and human application concerned. Since there is no documented evidence of disease transmission in ART treatment that can be attributed to air quality in the laboratory, the European Society for Human Reproduction and Embryology (ESHRE) suggests that less stringent air quality is justified for ART.

The regulatory authority in the UK (Human Fertilisation and Embryology Authority, HFEA) recommends that all work in the IVF laboratory be carried out in Class II flow cabinets delivering Grade C quality air to ensure safe handling of gametes and embryos, with the background environment as close as possible to Grade D (www.hfea.gov.uk).

Cell culture CO_2 incubators are available (Thermo Forma, www.thermo.com) that are equipped with a HEPA filter airflow system that continuously filters the entire chamber volume every 60 seconds, providing Class 100/Grade B air quality within 5 minutes of closing the incubator door. These incubators also incorporate a sterilization cycle (Steri-Cycle™), and can be supplied with an additional ceramic filter that excludes volatile low molecular weight organic and inorganic molecules, collectively known as volatile organic compounds (VOCs). Some air purification systems also incorporate UV filters.

Volatile organic compounds

The importance of ambient air and the possible consequences of chemical air contamination have been reviewed by Cohen *et al.* (1997). Whereas most organisms and species are protected to some extent from hazards in their ambient environment through their immune, digestive and epithelial systems, oocytes and embryos in vitro have no such protection, and their active and passive absorption mechanisms are largely indiscriminate. IVF laboratories set up in buildings within polluted areas, or close to airports or industrial manufacturing sites, may be subject to serious chemical air contamination, which may be reflected by inadequate pregnancy and live birth rates. Large traditional incubators obtain their ambient air directly from the laboratory room; gas mixtures are supplied in gas bottles, which may be contaminated with organic compounds or metallic contaminants. Pressurized rooms using HEPA filtration are used by many IVF laboratories, with standards applied to pharmaceutical clean rooms; however, HEPA filtration cannot effectively retain gaseous low molecular weight organic and inorganic molecules.

The four most common air pollutants are:

1. Volatile organic compounds (VOCs): in urban and dense suburban areas, VOCs are produced by industry, by vehicle and heating exhausts, as well as by a variety of cleaning procedures. Instruments such as microscopes, television monitors or furniture (as a result of manufacturing processes) may also produce VOCs; perfumes, after-shave and other highly scented aerosols are also potential sources, and all theater and laboratory staff should be discouraged from their use.
2. Small inorganic molecules such as N_2O, SO_2, CO.
3. Substances derived from building materials, such as aldehydes from flooring adhesives, substituted benzenes, phenol and *n*-decane released from vinyl floor tiles – flooring adhesives have been found to be particularly aggressive in arresting embryo development. Newly painted surfaces frequently present a hazard, as many paints contain substances that are highly toxic in the IVF laboratory; laboratory renovations and painting should always be planned during a period when treatment cycles are not being performed.
4. Other polluting compounds which may be released by pesticides or by aerosols containing butane or iso-butane as a propellant. Liquids such as floor waxes may contain heavy metals, which have a drastic effect on embryo implantation potential.

Cohen and colleagues conducted a detailed study of chemical air contamination in all areas of their IVF laboratory, which revealed dynamic interactive processes between air-handling systems, spaces, tools, disposable materials and other items unique to their laboratory. Anesthetic gases, refrigerants, cleaning agents, hydrocarbons and aromatic compounds were detected, and some accumulated specifically in incubators. The authors suggest that there may be an interaction between water-soluble and lipid-soluble solid phases such as those in incubators: whereas some contaminants may be absorbed by culture media, this may be counteracted by providing a larger sink such as a humidification pan in the incubator. Mineral oil may act as a sink for other components. Unfiltered outside air may be cleaner than HEPA-filtered laboratory air or air obtained from incubators, due to accumulation of VOCs derived from adjacent spaces or specific laboratory products,

including sterile Petri dishes. Standards for supplies of compressed gases are based upon criteria that are not designed for cultured and unprotected cells, with no perspective of the specific clean air needs of IVF. New incubators can have VOC concentrations more than 100-fold higher than used incubators from the same manufacturer – allowing the emission of gases from new laboratory products is crucial. Systems are now available for installation into existing air conditioning systems that can clean the air and reduce VOCs (http://zandair.com/air-purification-filter-PCOC3.html).

VOCs can be measured in the laboratory using hand-held monitors that use photo ionization monitors (e.g., VOC Meter, Research Instruments, www.researchinstruments.com, Eco sensor C21). These meters are very sensitive, and can be used to screen equipment and consumables to pinpoint sources of VOCs. Active filtration units with activated carbon filters and oxidizing material have now been developed specifically for IVF laboratories, and these can be placed inside cell-culture incubators or in the laboratory spaces themselves (CODA Filters, www.IVFonline.com; Cohen *et al*., 1997; Boone *et al*., 1999). As always, prevention is the best strategy, and efforts should be made to eliminate potential sources such as alcohol disinfectants and anesthetic gases – as well as perfumes/after-shave lotions – from the laboratory.

Biological safety cabinets

A biological safety cabinet or **BSC** is an enclosed workspace that provides protection either to workers, the products being handled, or both. BSCs provide protection from infectious disease agents, by sterilizing the air that is exposed to these agents. The air may be sterilized by UV light, heat or passage through a HEPA filter that removes particles larger than 0.3 μm in diameter. BSCs are designated by class, based on the degree of hazard containment and the type of protection they provide. In order to ensure maximum effectiveness, certain specifications must be met:

(a) Whenever possible, a 30 cm clearance should be provided behind and on each side of the cabinet, to ensure effective air return to the laboratory. This also allows easy access for maintenance.
(b) The cabinet should have 30–35 cm clearance above it, for exhaust filter changes.
(c) The operational integrity of a new BSC should be validated by certification before it is put into service, or after it has been repaired or relocated.
(d) All containers and equipment should be surface decontaminated and removed from the cabinet when the work is completed. The work surface, cabinet sides and back, and interior of the glass should be wiped down (70% ethanol and Fertisafe [www.research-instruments.com] are effective disinfectants) at the end of each day.
(e) The cabinet should be allowed to run for 5 minutes after materials are brought in or removed.

BSC Classes

Class I (Figure 8.1a) – provides personnel and environmental protection, but no product protection. Cabinets have an open front, negative pressure and are ventilated. Nonsterile room air enters and circulates through the cabinet. The environment is protected by filtering exhaust air through a 0.3 μm HEPA filter. The inward airflow protects personnel as long as a minimum velocity of 75 linear feet per minute (lfpm) is maintained through the front opening. This type of cabinet is useful to enclose equipment or procedures that have a potential to generate aerosols (centrifuges, homogenizing tissues, cage dumping), and can be used for work involving microbiological agents of moderate to high risk.

Class II – incorporates both charcoal and HEPA filters to ensure an environment that is close to sterile. It provides product, personnel and environment protection, using a stream of unidirectional air moving at a steady velocity along parallel lines ("laminar flow"). The laminar flow, together with HEPA filtration, captures and removes airborne contaminants and provides a particulate-free work environment. Airflow is drawn around the operator into the front grille of the cabinet, providing personnel protection. A downward flow of HEPA-filtered air minimizes the chance of cross-contamination along the work surface. Exhaust air is HEPA filtered to protect the environment, and may be recirculated back into the laboratory (Type A, Figure 8.1b) or ducted out of the building (Type B).

Class II cabinets provide a microbe-free environment for cell culture, and are recommended for manipulations in an IVF laboratory. They can be modified to accommodate microscopes, centrifuges or other equipment, but the modification should be tested and certified to ensure that the basic systems operate properly after modification. No material should be placed on front or rear grille openings, and laboratory doors should be kept closed during use to ensure adequate

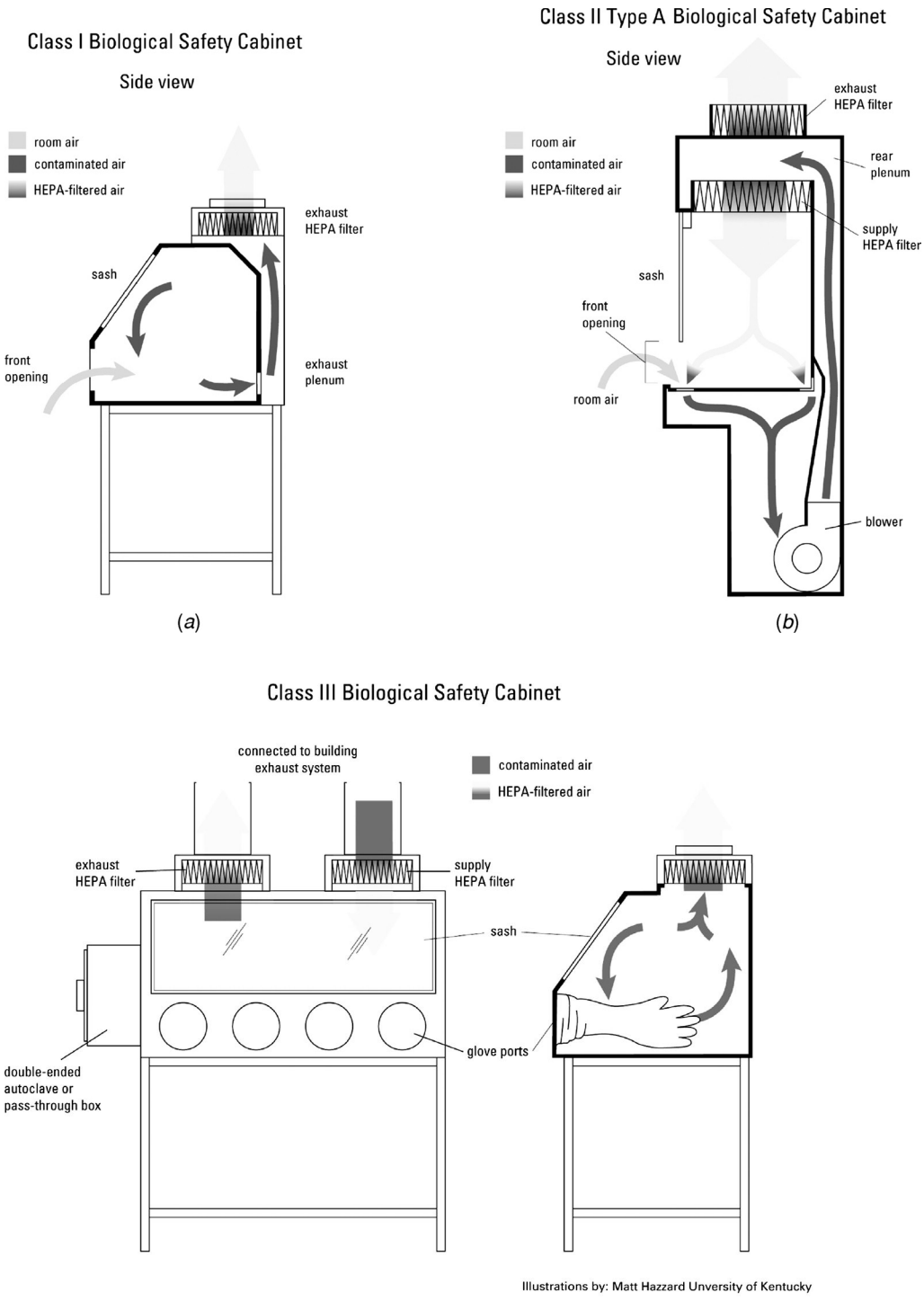

Figure 8.1 (a, b, c) Schematic diagrams of airflow in biological safety cabinets. (Reproduced with permission from Elder, K., Baker, D. J. and Ribes, J.A. 2004. *Infections, Infertility and Assisted Reproduction.* Cambridge: Cambridge University Press.)

airflow within the cabinet. It should be noted that the laminar flow of air can have a significant cooling effect on culture dishes; some laboratories choose to switch off the flow of air at appropriate times when it is safe to do so.

Class III – is used for routine anaerobe work, and is designed for work with high-risk organisms in maximum containment facilities. This cabinet provides maximum protection to the environment and the worker. It is completely enclosed with negative pressure, plus access for passage of materials through a dunk tank or double-door pass through box that can be decontaminated between uses. Air coming into and going out of the cabinet is HEPA filtered, and exhaust air passes through two HEPA filters, or a HEPA filter and an air incinerator before discharge to the outdoors. Infectious material within the cabinet is handled with rubber gloves that are attached and sealed to ports in the cabinet (Figure 8.1c).

Horizontal laminar flow "clean bench" – provides only product protection, and is **not** a BSC. HEPA filtered air is discharged across the work surface towards the user. These can be used for clean activities, but should never be used when handling cell cultures or infectious materials.

Vertical laminar flow "clean bench" – is also **not** a BSC, but is useful in hospital pharmacies for preparation of intravenous drugs. Although they generally have a sash, the air is usually discharged into the room under the sash.

Water quality

Although the majority of media required for an IVF laboratory are now commercially available, if any solutions are to be prepared "in house," a reliable source of ultrapure water is a critical factor. A pure water source is also required for washing and rinsing nondisposable equipment. Weimer *et al*., (1998a) carried out a complete analysis of impurities that can be found in water: this universal solvent provides a medium for most biological and chemical reactions, and is more susceptible to contamination by other substances than any other common solvent. Both surface and ground water are contaminated with a wide range of substances, including fertilizers, pesticides, herbicides, detergents, industrial waste effluent and waste solvents, with seasonal fluctuations in temperature and precipitation affecting the levels of contamination. Four categories of contaminants are present: inorganics (dissolved cationic and anionic species), organics, particles and microorganisms such as bacteria, algae, mold and fungi. Chlorine, chloramines, polyionic substrates, ozone and fluorine may be added to water during treatment processes, and must be removed from water for cell culture media preparation. In water purification, analysis of the feed water source is crucial to determine the proper filtration steps required, and water-processing protocols should be adapted to meet regional requirements. Processing systems include particulate filtration, activated carbon cartridge filtration, reverse osmosis (RO) and electrodeionization (EDI), an ultraviolet oxidation system, followed by a Milli-Q PF Plus purification before final filtration through a 0.22 mm filter to scavenge any trace particles and prevent reverse bacterial contamination from the environment.

IVF laboratory personnel should be familiar with any subtle variations in their water source, as well as the capabilities of their water purification system, and develop protocols to ensure consistently high-quality ultrapure water supplies, following manufacturers' instructions for monitoring, cleaning, filter replacement and maintenance schedules.

Supplies

A basic list of supplies is outlined at in the appendix at the end of this chapter; the exact combination required will depend upon the tissue culture system and techniques of manipulation used. Disposable supplies are used whenever possible and must be guaranteed nontoxic tissue culture grade, in particular the culture vessels, needles, collecting system and catheters for oocyte aspiration and embryo transfer. Disposable glass pipettes are required for gamete and embryo manipulations, and these can be purchased pre-sterilized and packaged. If nonsterile disposable pipettes are purchased, they must be soaked and rinsed with tissue culture grade sterile water and dry heat sterilized before use. In preparing to handle gametes or embryos, examine each pipette and rinse with sterile medium to ensure that it is clean and residue-free.

Important considerations in the selection of supplies include:

- Suitability for intended purpose
- Suitable storage facilities (i.e., storerooms away from excessive heat/direct sunlight)
- Compliance of suppliers to a contract with specified terms and conditions

- Disposable plastics CE marked or mouse embryo tested where possible
- Media with quality certification, mouse embryo tested and proven track record, with validation evidence
- Delivery of perishable items such as media under controlled conditions
- Batch numbers to be recorded for quality assurance purposes
- Health and safety of operators handling potentially infectious bodily fluids.

Routine schedules of cleaning, maintenance, and servicing must be established for each item of equipment, and checklist records maintained for daily, weekly, monthly and annual schedules of cleaning and maintenance of all items used, together with checks for restocking and expiry dates of supplies.

Tissue culture media

Original IVF culture systems were based on simple media developed for organ explant and somatic cell culture, designed to mimic physiological conditions. Analysis of tubal and uterine fluids, together with research into embryo metabolism, then led to the development of complex media. Many controlled studies have shown fertilization and cleavage to be satisfactory in a variety of simple and complex media (comprehensive reviews are published by Bavister, 1995; Edwards and Brody, 1995; Biggers, 2008). Metabolic and nutritional requirements of mammalian embryos are complex, stage specific, and, in many cases, species specific; several decades of research in laboratory and livestock animal systems have shown that, although there are some basic similarities, culture requirements of different species must be considered independently. Understanding metabolic pathways of embryos and their substrate and nutrient preferences has led to major advances in the ability to support embryo development in vitro.

Culture media: physical parameters

- Osmolarity = osmoles of solute particles per liter of solution
  Temperature-dependent (volume changes with temperature)
  Osmolarity of tubal/uterine secretions = 275–305 mOsm/L
- Osmolality = the osmotic concentration of a solution, mOsm/kg of solvent
  Temperature-independent
  One osmole = Avogadro's number of osmotically active particles = 6.02×10^{23}
- Physiological pH range of body fluids = 7.2–7.5. pH is related to an equilibrium between gas phase CO_2 and CO_2/HCO_3 dissolved in the medium, with carbonic acid as an intermediate.
  Henderson–Hasselbach equation:

  CO_2 (gas) $\leftrightarrow$ CO_2 (dissolved) $\leftrightarrow$ $H_2CO_3 + H^+ + HCO_3$

  $pH = pK_a + \log_{10} [H^+]$
  = log of the reciprocal of the molar concentration of hydrogen ions
  pK_a = ionization constant for the acid

  pH is affected by:
  - Temperature (less CO_2 dissolved at higher temperature)
  - Atmospheric pressure (more CO_2 in solution at higher pressure)
  - Presence of other solutes such as amino acids and complex salt mixtures, since pK_a is a function of salt concentration.
- HEPES-buffered medium has been equilibrated to a pH of 7.4 in the presence of bicarbonate; exposure to a CO_2 atmosphere will lower the pH, and therefore culture dishes containing HEPES-buffered medium should be equilibrated at 37°C only, and not in a CO_2 incubator.
- Temperature fluctuations during storage and handling can affect pH, and should therefore be avoided:
  - acid pH destroys glutamine and pyruvate
  - some salts or amino acids may precipitate out of solution, and further affect pH.

Fertilization can be achieved in very simple media such as Earle's, or a TALP-based formulation, but the situation becomes more complex thereafter (see Chapter 5 for details of embryo metabolism). Prior to 1997, single media formulations were used for all stages of IVF. However, research in animal systems during the 1990s led to elucidation of the metabolic biochemistry and molecular mechanisms involved in gamete maturation, activation, fertilization, genomic activation, cleavage, compaction and blastocyst formation. This drew attention to the fact that nutrient and ionic requirements differ during all these different stages. Inappropriate culture conditions expose embryos to cellular stress which could result in retarded cleavage, cleavage arrest, cytoplasmic blebbing, impaired energy production, inadequate genome activation and transcription. Blastocyst formation is followed by an exponential increase in protein synthesis, with

neosynthesis of glycoproteins, histones and new surface antigens.

Although the specific needs of embryos during their preimplantation development have by no means been completely defined, sequential, stage-specific and chemically defined media are used in IVF systems. Media formulations endeavor to mimic the natural in-vivo situation and take into account the significant changes in embryo physiology and metabolism that occur during the preimplantation period. Three different types of protocols are used worldwide:

1. One-step culture using a single medium formulation (nonrenewal monoculture)
2. Single medium formulation, renewed on D2/D3 (renewal monoculture)
3. Two-step culture using two different media formulations (sequential media culture).

Although two-step sequential media have been favored in recent years, doubts have been raised as to whether these more complex protocols have any advantage over one-step protocols (see Biggers and Summers, 2008 for review).

Commercial media is "ready to use," with protein supplements added, and may also contain other components or factors. Different formulations are available for sperm preparation, oocyte washing during retrieval, insemination/fertilization, early cleavage, blastocyst development and freezing/thawing. Media containing HEPES, which maintains a relatively stable pH even in ambient air, can be used for sperm preparation, oocyte harvesting and washing, and during ICSI procedures; however, HEPES-buffered medium will become acidic when placed in a CO_2 atmosphere, and therefore the gametes should be washed in HEPES-free medium before being placed in the culture incubator.

Media used in embryo culture and manipulation (see Table 8.2)

Simple media: balanced salt solutions (BSS)

- Contain combinations of Na, K, Cl, Ca, Pi, Mg, HCO_3, glucose, ± phenol red
- Salt concentrations/balance differ slightly in different formulations:
  Ringer's: contains Na, K, Cl, Ca only
  Earle's, Hank's, Tyrode's etc.: also contain Pi, Mg, HCO_3, glucose.

Complex media

Contain the inorganic salts of BSS, as well as amino acids, fatty acids, vitamins, other substrates (nucleotide bases, cholesterol, glutathione, other macromolecules), antibiotics, and other labile substances to be added before use (pyruvate, lactate, glutamine, methionine, bicarbonate):

- Minimal essential medium (MEM)
- Dulbecco's modified MEM
- Ham's F-10, F-12
- BWW, TC199, KSOM, etc.

Media optimized for preimplantation embryos

- Menezo B2, "complex," designed for bovine embryo culture (1976)
- Human tubal fluid (HTF), "simple," contains pyruvate and lactate (Quinn, 1995)
- P1, Basal IX HTF: no pyruvate or lactate, contains EDTA and glutamine
- HTF-12: no phosphate, low level of glucose + EDTA, taurine, glutathione
- G1/S1, "simple," contains EDTA, lactate/pyruvate, amino acids
- G2/S2, "complex," high level of glucose
- Cook IVF: Sydney IVF Cleavage/Sydney IVF Blastocyst medium
- Cooper Surgical
- Sage
- MediCult EmbryoAssist, BlastAssist
- FertiPro N.V. Ferticult/Ferticult G3
- Irvine Scientific ECM/Multiblast Medium
- Life Global, IVFonline.com

Phosphate buffered saline (PBS) and HEPES buffers: commonly used to stabilize pH solutions used for washing in room atmosphere, e.g., follicle flushing, cryopreservation protocols, sperm preparation.

Rigorous quality control is essential in media preparation, including knowledge of the source of all ingredients, especially the water, which must be tested to make sure that it is endotoxin-free, low in ion content, and guaranteed free of organic molecules and microorganisms. Pharmaceutical grade reagents are required in order to follow Good Manufacturing Practice (GMP). Each batch of culture media prepared must be checked for osmolality (285 ± 2 mOsm/kg) and pH (7.35–7.45), and subjected to quality control procedures with LAL (limulus amebocyte lysate) test for endotoxins and sperm survival or mouse embryo toxicity before use. Culture media can rapidly deteriorate during storage, with a decrease in its ability to support embryo development, and careful attention must be paid to storage conditions and manufacturers' recommended expiry dates. Commercially prepared, pretested high-quality

Table 8.2 International regulations regarding the use of IVF culture media

Australia	Regulated by the Australian Therapeutic Goods Administration (TGA) as a Class III medical device
USA	Regulated by the FDA as a Class II medical device with special conditions under the 510k Pre-market notification scheme
Japan	Classed as a laboratory reagent in Japan, and as such, has no formal regulatory review or approval
China	No official designation of the IVF media as a medical device, but regulatory approval by the Chinese Regulator (SFDA) is required to apply for hospital tenders
France	IVF media products are seen as a "hybrid" product in France (i.e., somewhere between drug and device) and are regulated as a PTA (Produit Therapeutique Annexe) by the French Regulatory Authority (AFSSaPS)
European Union (excl. France)	IVF cell culture media has not been officially declared a medical device in the EU

culture media is available for purchase from a number of suppliers worldwide, so that media preparation for routine use in the laboratory is not necessary, and may not be a cost-effective exercise when time and quality control are taken into account. There is so far no firm scientific evidence that any medium is superior to another in routine IVF, and choice should depend upon considerations such as quality control and testing procedures applied in its manufacture, cost, and, in particular, guaranteed efficient supply delivery in relation to shelf life. After delivery, the medium may be aliquoted in suitable small volumes, such that one aliquot can be used for a single patient's gamete preparation and culture (including sperm preparation). Shelf life is dependent on specific composition and conditions of storage; generally, the more "complex" the media, the shorter its useful lifespan, with an average of approximately 4–6 weeks. All media must be equilibrated for temperature and pH for 4–6 hours before use, ideally by overnight incubation.

Important factors when choosing commercial media

- Information available about composition (not always easy to obtain)
- Information about quality control during production
- Information about endotoxin testing
- Assurance of regular, reliable delivery under controlled conditions
- Acceptable shelf life
- Delivery of batches well before expiry date
- Validation evidence of use in human IVF – i.e., proven track record
- Tested using appropriate bioassay
- Certificates of analysis available
- Serum or serum derivatives should be from virally screened sources

Protein supplement

Protein, or an equivalent macromolecule (e.g., polyvinyl alcohol, hyaluronate), is required in human IVF for:

- Sperm capacitation – involves the removal of sterols from the plasma membrane, and this requires a sterol acceptor molecule (such as albumin) in the medium.
- Handling – a molecule with surfactant properties is needed to facilitate sperm and embryo handling in order to prevent sticking to pipettes and dishes.
- Proteins also act as lipid and peptide carriers, chelators, cell surface protectors, regulators of redox potential, and are a source of fixed nitrogen/amino acids after hydrolysis.

Bovine serum albumin was previously used for human embryo culture, but this is now recognized to carry the risk of disease transmission, including prion disease (Creutzfeldt–Jakob disease, CJD). In domestic animals, media supplemented with whole serum was found to affect embryo development at several different levels (Leese, 1998), and its presence is associated with the development of abnormally large fetuses when used to grow embryos to the blastocyst stage (Thompson *et al.*, 1995). Although the mechanisms involved are unresolved, the findings led to further concerns about the use of whole serum in human IVF, particularly in extended culture systems, and serum substitutes are used instead.

Albumin is the major protein constituent of the embryo's environment; it can be incorporated by the embryo, it binds lipids and may help to bind and

stabilize growth factors. Theoretically it may not be indispensable, but it does effectively replace serum, and has a major role both in maintaining embryo quality and in preventing gametes and embryos from sticking to glass or plastic surfaces, facilitating their manipulation. Commercially prepared media are supplied complete; most contain a serum substitute such as human serum albumin (HSA). Human serum contains approximately 4.5% albumin, i.e., 45 mg/mL. A supplement of 10% human serum albumin (HSA) provides an albumin concentration of 4.5%. Serum Substitute Supplement (SSS) is a 6% protein solution made up of 84% HSA, 16% alpha- and beta-globulins, plus a trace of gamma globulins. Although disease transmission from commercial preparations of HSA were a concern in the past, recombinant preparations are now available and are used routinely in commercial culture media.

Growth factors

Growth factors play a key role in growth and differentiation from the time of morula/blastocyst transition. However, defining their precise role and potential for improving in-vitro preimplantation development is complicated by factors such as gene expression of both the factors and their receptors. There is also the potential of ascribing positive effects to specific factors when the result may in fact be due to a combination of a myriad of other causes. The mammalian blastocyst expresses ligands and receptors for several growth factors, many of which can cross-react, making it difficult to interpret the effects of single factors added to a medium. Insulin, LIF, EGF/TGF-α, TGF-β, PDGG, HB-EGF have all been studied in IVF culture, and, although it is clear that these and other growth factors may influence in-vitro blastocyst development and hatching, further assessment remains an area of research – a comprehensive review was published by Richter in 2008. It has been suggested that the mechanism whereby serum induces abnormalities in domestic animal systems may involve the overexpression of certain growth factors – there is no doubt that complex and delicate regulatory systems are involved.

Culture of embryos in "groups" rather than singly has been found to improve viability and implantation in some systems: it is possible that autocrine/paracrine effects or "trophic" factors exist between embryos. However, observed effects of "group" culture will inevitably be related to the composition of the culture medium and the precise physical conditions used for embryo culture, and in particular the number of embryos in relation to the volume of media used per culture drop.

Follicular flushing media

Ideally, if a patient has responded well to follicular phase stimulation with appropriate monitoring and timing of ovulation induction, the oocyte retrieval will proceed smoothly with efficient recovery of oocytes without the need to flush follicles. If the number of follicles is low or the procedure is difficult for technical reasons, follicles may be flushed with a physiological solution to assist recovery of all the oocytes. Follicles can be flushed with balanced salt solutions such as Earle's (EBSS) or lactated Ringer's solution, and heparin may be added at a concentration of 2 units/mL. HEPES-buffered media can also be used for flushing. Temperature and pH of flushing media must be carefully controlled, and the oocytes recovered from flushing media subsequently washed in culture media before transfer to their final culture droplet or well.

Tissue culture systems

Vessels successfully used for IVF include test-tubes, four-well culture dishes, organ culture dishes and Petri dishes containing microdroplets of culture medium under a layer of clinical grade mineral or silicone oil. Whatever the system employed, it must be capable of rigidly maintaining fixed stable parameters of temperature, pH and osmolarity. Human oocytes are extremely sensitive to transient cooling in vitro, and modest reductions in temperature can cause irreversible disruption of the meiotic spindle, with possible chromosome dispersal. Temperature-induced chromosome disruption may contribute to aneuploidy, and the high rates of preclinical and spontaneous abortion that follow IVF and ICSI. Therefore, it is essential to control temperature fluctuation from the moment of follicle aspiration, and during all oocyte and embryo manipulations, by using heated microscope stages and heating blocks or platforms. Most importantly, the temperature within the media itself must be maintained at the optimal temperature, rather than the temperature of the dish.

An overlay of equilibrated oil as part of the tissue culture system confers specific advantages:

1. The oil acts as a physical barrier, separating droplets of medium from the atmosphere and airborne particles or pathogens.

2. Oil prevents evaporation and delays gas diffusion, thereby keeping pH, temperature and osmolality of the medium stable during gamete manipulations, protecting the embryos from significant fluctuations in their microenvironment (aliquots of medium without an oil overlay begin to show an immediate rise in pH as soon as they are removed from the incubator).
3. Oil prevents evaporation: humidified and pre-equilibrated oil allows the use of nonhumidified incubators, which are easier to clean and maintain.

It has been suggested that oil could enhance embryo development by removing lipid-soluble toxins from the medium; on the other hand, an oil overlay prevents free diffusion of metabolic by-products such as ammonia, and accumulation of ammonia in culture media is toxic to the embryo. The use of an oil overlay also influences oxygen concentration in the medium, with resulting effects on the delicate balance of embryo metabolism; as mentioned previously, it can absorb and concentrate harmful VOCs in the incubator atmosphere. Oil is difficult to sterilize and inappropriate handling can lead it to be a source of fungal contamination.

Two types of oil have been used in culture overlays, from a mineral or a silicone source (see box below). Silicone oil, commonly used as a stationary phase in gas chromatography or as an anti-foaming agent, should not be confused with mineral oil, which is obtained from fractionated distillation in the petrol industry.

Types of oil used in culture overlays

1. Paraffin oil – also known as white mineral oil or liquid paraffin
   - Merck Index: "Liquid Petrolatum" (Petrolatum = Vaseline)
   - Is derived from petroleum, and exists in many different forms with differing melting points and viscosities
   - Available in "light" or "heavy" forms
   - Light – density = 0.83–0.89
   - Batch to batch variations are common, since they are a mixture of hydrocarbons with fluctuating composition
2. Silicone oil (dimethyl-polysiloxane) (Erbach *et al.*, 1995)
   - Polymer, available in many viscosities, from 10 to 600 000 centistokes
   - Composition should be more stable than that of paraffin
   - Problems have been encountered with Zn^{2+} contamination, and toxicity after degradation as a result of exposure to sunlight (Provo and Herr, 1998)

Oil preparation

Commercial companies now supply washed, sterilized oil that is ready for use in overlays. If obtained from other sources, mineral or silicone oil should be sterile as supplied, and does not require sterilization or filtration. High temperature for sterilization may be detrimental to the oil itself, and the procedure may also "leach" potential toxins from the container. Provo and Herr (1998) reported that exposure of mineral oil to direct sunlight for a period of 4 hours resulted in a highly embryotoxic overlay, and they recommend that washed oil should be shielded from light and treated as a photoreactive compound. Contaminants have been reported in certain types of oil. Washing procedures remove water-soluble toxins, but non-water-soluble toxins may also be present which will not be removed by washing. Therefore it is prudent not only to wash, but to test every batch of oil before use with, at the very least, a sperm survival test as a quality control procedure. Erbach *et al.* (1995) suggested that zinc might be a contaminant in silicone oil, and found that washing the oil with EDTA removed a toxicity factor that may have been due to the presence of zinc. Some mineral oil products may also contain preservatives such as alpha-tocopherol. Oil can be carefully washed in sterile disposable tissue culture flasks (without vigorous shaking) with either Milli-Q water, sterile saline solution or a simple culture medium without protein or lipid-soluble components, in a ratio of 5:1 (oil:aq). The oil can be further "equilibrated" by bubbling 5% CO_2 through the mixture before allowing the phases to separate and settle. Washed oil can be stored either at room temperature or at 4°C in equilibrium with the aqueous layer, or separated before storage, but should be prepared at least 2 days prior to its use.

Oil overlays must be further equilibrated in the CO_2 incubator for several hours (or overnight) before introducing media/gametes/embryos. Conaghan (2008) established experimentally that even relatively small volumes of medium (50 μL) with an oil overlay require a minimum of 8 hours equilibration in order to establish a stable pH under normal culture conditions. However, once equilibrated, the oil acts as an effective buffer, maintaining pH for up to 10 minutes

after removing dishes from the incubator, whereas media without an oil overlay show a dramatic rise in pH as soon as the dishes are removed from the CO_2 atmosphere.

Co-culture systems

Co-culture systems played an important role in the evolution of modern culture systems, using a substrate layer of feeder cells in order to support the growth of human embryos and regulate their metabolic turnover processes. A variety of different types of homologous and autologous cells were used as feeder layers in the past, including tubal epithelial cells, explants of endometrial tissue, granulosa cells, as well as the animal cells used in commercial vaccine production, African green monkey kidney (Vero). For the same developmental stage, embryos co-cultured to the blastocyst stage were found to have higher numbers of cells and a fully cohesive inner cell mass when compared with embryos cultured in simple media. It was postulated that improved development occurs as a result of four different possible mechanisms:

1. "Metabolic locks": co-culture cell layers can provide a supply of small molecular weight metabolites which simpler culture media lack. This supply may assist continued cell metabolism required for genome activation, and divert the potential for abnormal metabolic processes which may lead to cleavage arrest.
2. The feeder cell layer may supply growth factors essential for development.
3. Toxic compounds resulting from cell metabolism can be removed: heavy metal ions may be chelated by glycine produced by feeder cells, and ammonium and urea may be recycled through feeder cell metabolic cycles.
4. Feeder cells can synthesize reducing agents which prevent the formation of free radicals.

The use of feeder cells during the late 1980s played an important role in research into embryo metabolism and preimplantation development, and the observations and data obtained were instrumental in helping to develop more appropriate stage-specific culture media and systems. However, maintaining a safe and effective co-culture system is difficult and labor intensive, and their use carries the risk of potential disease transmission, or even retrovirus-induced cell transformation in the feeder layer. Although accumulated experimental data suggest that the ability of human embryos to develop in vitro during the early cleavage stages is more a measure of their adaptability and survival capabilities than of the suitability of the medium, the use of stage-specific media, with composition that is based upon research into early embryo metabolism (see Chapter 5), has now largely made the use of co-cultures redundant, as well as introducing an unacceptable risk in clinical practice.

Emerging technologies

Research continues into technologies that will improve the outcome of in-vitro embryo culture, with the goal of minimizing environmental stress imposed upon the embryo during laboratory manipulations. The active integrated workstation is one approach, aiming to eliminate fluctuations in the physical parameters of the environment. However, carrying out the manipulations in these systems is more difficult for the operator, and involves additional training, experience and a learning curve. An alternative approach is to mimic dynamic in-vivo conditions by exposing them to continuous movement: "embryo rocking" using a dynamic microfunnel culture system (Heo *et al.*, 2010), tilting platforms (Matsuura *et al.*, 2010) or with microfluidic technology, the use of chambers or devices that enable continuous fluid movement in a micro- or nano-environment. The gametes/embryos can be perfused with media that gradually changes in composition, providing different chemical substrates for different stages during development. This could have the effect of removing metabolic by-products such as ammonia, or simply disrupting any micro-gradients that may form in small culture droplets. Although microfluidic platforms for ART procedures have theoretical advantages, and a number of different devices are under trial, there are practical considerations yet to be addressed before they can be successfully implemented (for review, see Swain *et al.*, 2008, 2009).

Appendix

Equipment and supplies for embryology

CO_2 incubator
Dissecting microscope
Inverted microscope
Heated surfaces for microscope and manipulation areas
Heating block for test-tubes

Laminar flow cabinet
Oven for heat-sterilizing
Small autoclave
Water bath
Pipette 10–1000 mL Eppendorf
Refrigerator
Supply of medical grade CO_2
Supply of 5% CO_2 in air (or special gas mixtures)
Wash bottle + Millex filter for gas
Rubber tubing
Pipette canisters
Clinical grade mineral or paraffin oil
Culture media
Glassware for media preparation
Osmometer (for media preparation)
Weighing balance
Tissue culture plastics: (Nunc, Corning, Sterilin)
Flasks for media and oil: 50 mL, 175 mL
Culture dishes: 60, 35 mm
OCR (oocyte retrieval) needles
Test-tubes for OCR: 17 mL disposable
Transfer catheters and stylets: embryo, GIFT, IUI
Syringes
Needles
Disposable pipettes: 1, 5, 10, 25 mL
"Pipetus" pipetting device
Eppendorf tips, small and large
Millipore filters: 0.22, 0.8 mm
Glass Pasteur pipettes (Volac)
Pipette bulbs
Test-tube racks
Rubbish bags
Tissues
Tape for labeling
7X detergent (Flow)
70% ethanol
Sterile gloves, latex and non-latex
Oil: Boots, Squibb, Sigma, Medicult
Supply of purified water: Milli-Q system or Analar
Glassware for making culture media: beakers, flasks, measuring cylinder

Further reading

ASRM Guidelines

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ESHRE Guidelines

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Website information

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COSHH – www.hse.gov.uk/coshh

Department of Health – www.dh.gov.uk

The Human Fertilisation and Embryology Authority – www.hfea.gov.uk

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Chapter 9

Quality management in the IVF laboratory

There is no doubt that success in ART is crucially dependent on carefully controlled conditions in every aspect of the IVF laboratory routine (Figure 9.1).

From the beginning of the twenty-first century onwards, laboratories that offer human ART treatment or are involved in the handling of human gametes and/or tissue became subject to increasing demands and precepts set down by regulatory and legislative authorities. The regulations differ from country to country, and some directives (e.g., the European Tissue Directive 2004/23/EC-2006/86/EC) are subject to interpretation according to legislation or guidelines set down by national authorities in individual countries. In many countries, it has become necessary to obtain accreditation and/or certification by a national or international body that will carry out in-depth assessments and inspections to ensure that all aspects of facilities and treatment meet a required standard.

ISO9001:2000

The International Organisation for Standardisation (ISO) (www.iso.org) is a network of the national standards institutes of 159 countries, consisting of one member per country; a Central Secretariat in Geneva, Switzerland, coordinates the system. The ISO is an independent body, not associated with the government of any particular country. Some of the 159 member institutes may be a part of their country's government structure, but others have been set up by national partnerships of industry associations, and therefore are in the private business domain. ISO therefore tries to reach a consensus on issues that concern both business requirements, and the broader needs of society. Their standard for quality management is published as ISO9001:2000.

In order to obtain ISO certification, a unit with an established quality management system (QMS) is assessed by an external auditor to evaluate the effectiveness of the QMS. If all the requirements of the ISO standard are met, a certificate is issued stating that the QMS conforms to the standards laid down in ISO9001:2000. Many countries now require that IVF clinics and laboratories show evidence such as this, that an effective QMS is in place. ART laboratories in the USA should conform to the American Society for Reproductive Medicine guidelines (ASRM, 2008) and must undergo certification and accreditation by an appropriate agency, such as the College of American Pathologists (CAP) or Joint Commission International (JCI). In the UK, all IVF clinics must apply for a license from the Human Fertilisation and Embryology Authority (HFEA) and ensure that all of their procedures are compliant with the HFEA Code of Practice. The HFEA monitors all UK data, and carries out annual audits and inspections.

The final impact of legislative, regulatory, accreditation and certification requirements is that every ART laboratory should have an effective total quality management (TQM) system. TQM is a system that can monitor all procedures and components of the laboratory; this must include not only pregnancy and implantation rates, but also a systematic check and survey of all laboratory material, supplies, equipment and instruments, procedures, protocols and staff performance.

Irrespective of the numerous fine details involved in TQM, the first, and ultimate, test of quality control must rest with pregnancy and live birth rates per treatment cycle. An ongoing record of the results of fertilization, cleavage and embryo development provide the best short-term evidence of good quality control (QC). Daily records in the form of a laboratory logbook or electronic database are essential, summarizing details of patients and outcome of laboratory procedures: age, cause of infertility, stimulation protocol, number

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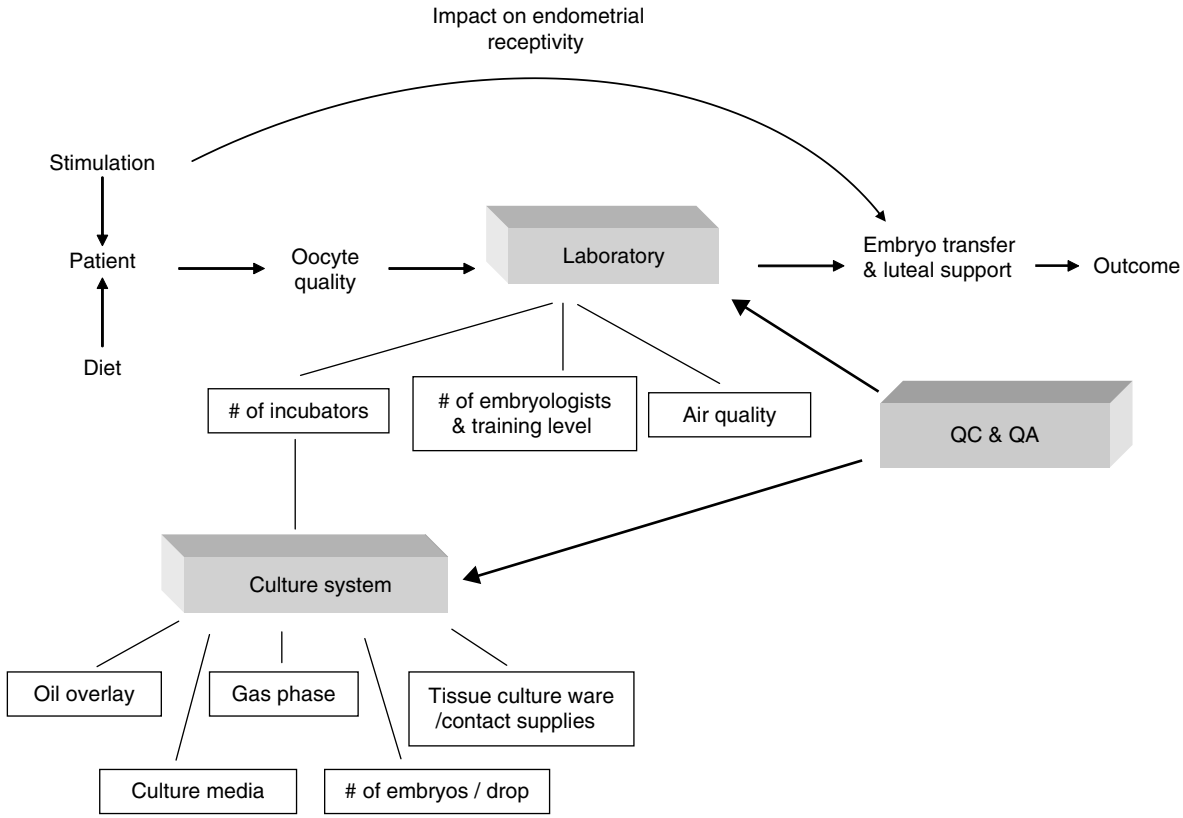

Figure 9.1 Critical elements of an ART treatment cycle (with thanks to David Gardner, Melbourne, Australia).

of oocytes retrieved, semen analysis, sperm preparation details, insemination time, fertilization, cleavage, embryo transfer and cryopreservation. Details of media and oil batches and all consumables that come into contact with gametes and embryos must also be recorded for reference, and the introduction of any new methods or materials must be documented.

Definitions used in quality management

- Quality: fitness for purpose.
- Quality management system: a system that encompasses quality control, quality assurance and quality improvement by providing defined sets of procedures for the management of each component.
- Quality policy: a statement defining the purpose of the organization and its commitment to a defined quality objective.
- Quality control: inspection of a system to ensure that a product or service is delivered under optimal conditions.
- Quality assurance: monitoring the effectiveness of QC and indicating preventive and corrective action taken when errors are detected.
- Quality Audit: review and checking of the quality management system to ensure its correct operation.
- External Quality Assurance: testing a product or service against an external standard.

A QMS in the IVF laboratory aims to achieve specific goals:

1. Identify all of the processes to be included in the QMS:
   - provision and management of resources
   - ART processes
   - evaluation and continual improvement
   - monitoring of key performance indicators (KPIs)
2. Make available the resources and information necessary to operate and support these processes
3. Implement any actions necessary to ensure that the processes are effective and subject to continual improvement.

A QMS ensures continuous assessment and improvement of all component parts of the patient

treatment cycle. KPIs to be monitored include rates of fertilization, cleavage, survival of injected oocytes, pregnancy, implantation and live births; patient satisfaction with the quality of the service should also be monitored. Graphic analysis of these parameters can reveal problems early so that corrective action can be taken promptly to minimize the extent of any problem that might arise (Kastrop, 2003).

Basic elements of a quality management system

A formal QMS should include:

- Scope: a list of all treatments provided and links to the forms and documents used
- Normative reference values: definition of a successful outcome
- Terms and definitions: those used in IVF
- Management responsibility: organization chart showing lines of responsibility and accountability of all staff
- Resource management: provision of sufficient resources, including staff, to deliver the service
- Product realization: treatment plans, procedures, purchasing, traceability, witnessing
- Measurement, analysis, improvement: monitoring of KPIs, reporting of adverse incidents, corrective and preventive action to improve service.

Implementing QMS in the IVF laboratory

The first key requirement for implementing a formal QMS is appropriately educated and trained personnel, with training records that are regularly updated. Other key requirements include:

1. Complete list/index of standard operating procedures (SOPs)
2. Housekeeping procedures: cleaning and decontamination
3. Correct operation, calibration and maintenance of all instruments with manual and logbook records
4. Proper procedure, policy and safety manuals
5. Consistent and correct execution of appropriate techniques and methods
6. Comprehensive documentation, record-keeping and reporting of results
7. System for specimen collection and handling, including verification of patient identity and chain of custody
8. Safety procedures including appropriate handling and storage of materials
9. Infection control measures
10. Documentation of suppliers and dates of receipt and expiry of consumables
11. System of performance appraisal, correction of deficiencies and implementation of advances and improvements
12. Quality materials, tested with bioassays when appropriate
13. Quality assurance program.

Laboratory equipment

Equipment failure or suboptimal operation in an IVF laboratory can seriously jeopardize the prognosis for patients undergoing treatment, and therefore service contracts should be set up with reliable companies. As part of the service, the companies should be able to provide calibration certificates for the tools that they use in servicing and calibrating the equipment. Alarms should be fitted to all vital equipment, and provision made for emergency call-out. Back-up equipment should be held in reserve.

Electrical appliances must be tested for safety before first use (e.g., by portable appliance testing) and then regularly tested by a trained operator. They should be designated as a potential source of fire, and must not be used if faulty; any faults must be reported and repaired immediately. Contracts for service and calibration should be held for:

- Incubators
- Incubator alarms
- Flow cabinets
- Microscopes
- Micromanipulator stations
- Heated surfaces/microscope stages
- Centrifuges
- Refrigerators and freezers
- Embryo/oocyte freezing machines
- Osmometers
- Liquid nitrogen storage dewars
- Low-level nitrogen alarms in the dewars
- Air filtration equipment
- Electronic witnessing system.

Key items of equipment in daily use also require systems of continuous monitoring to ensure optimal performance. Computer-controlled data acquisition

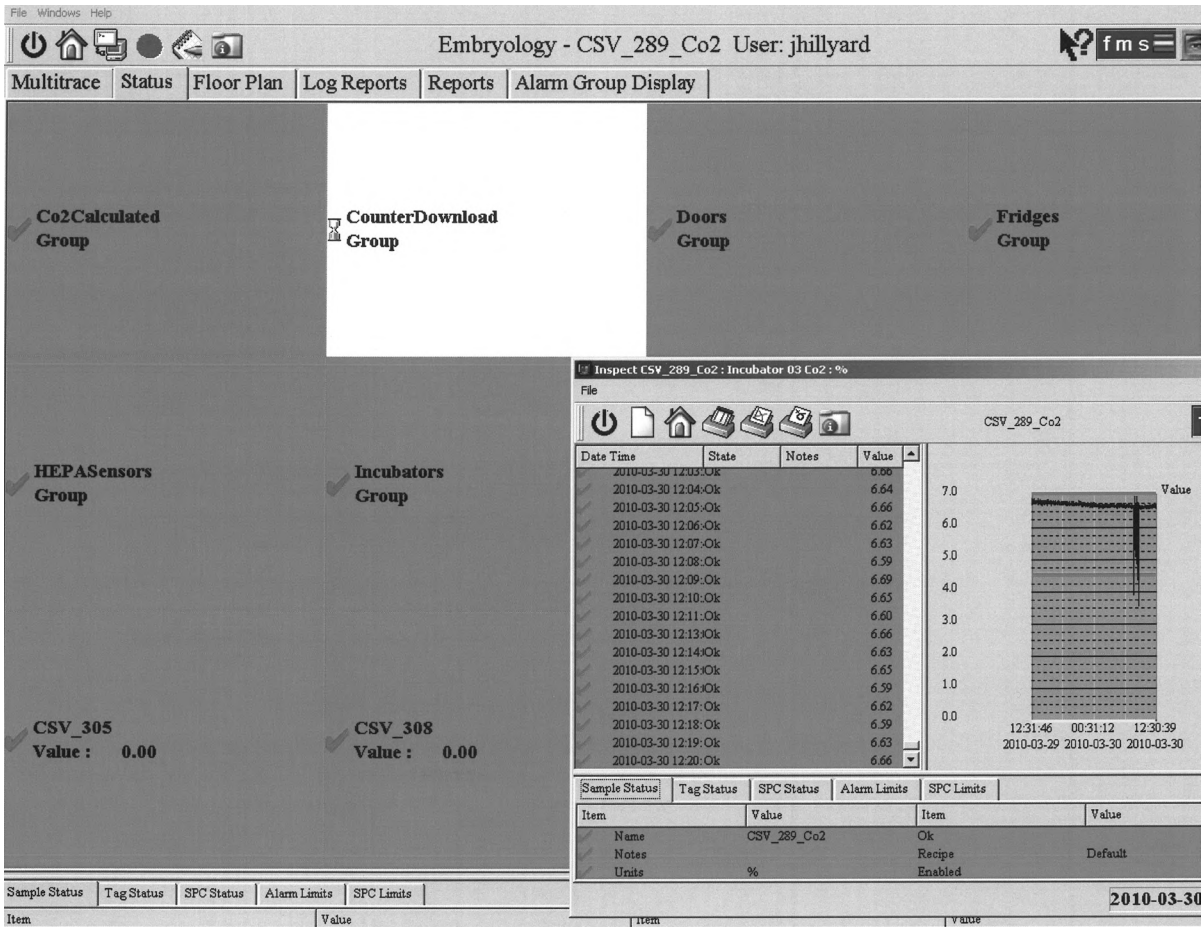

Figure 9.2 Data from FMS in the IVF laboratory at Bourn Hall Clinic. The background shows the different monitoring modules that can be viewed: CO_2 sensors, HEPA sensors, incubator temperature, refrigerators, freezers. Insets: 24-hour records from a single incubator showing (a) CO_2 concentration and (b) temperature. Note dips during peak laboratory activity times, 8–10.30 am. See color plate section.

and monitoring systems are now available, with continuous monitoring and logging software systems that support multiple instrument inputs (Figure 9.2, Facilities Monitoring Systems, www.fmonsys.com/). This system can continuously check and record airborne particle counts, as well as data from key equipment such as incubators, freezers, refrigerators and liquid nitrogen storage vessels. The data is monitored in real-time, and the computerized system can display historical data acquisition and analysis, trendlines, correlation studies and various levels of alarm notification. Variation outside set parameters is covered by an alarm system that is monitored 24 hours a day.

Temperature and pH are known to be critical parameters that must be carefully controlled, and their measurement in an IVF culture system requires special attention:

Temperature

The temperature of incubators, water baths, heating blocks, heated surfaces and microscope stages should not rely on a digital display from the equipment, but must be independently recorded and controlled, with individual fine tuning for each. Always note that the temperature of the incubator, bath or surface will differ from that in the culture media within a drop, tube or dish; 37°C should be the temperature to aim for within the media, not for the incubator or heating device. Setpoints should be defined for each dish on each separate device, using a calibrated thermometer with type K thermocouple.

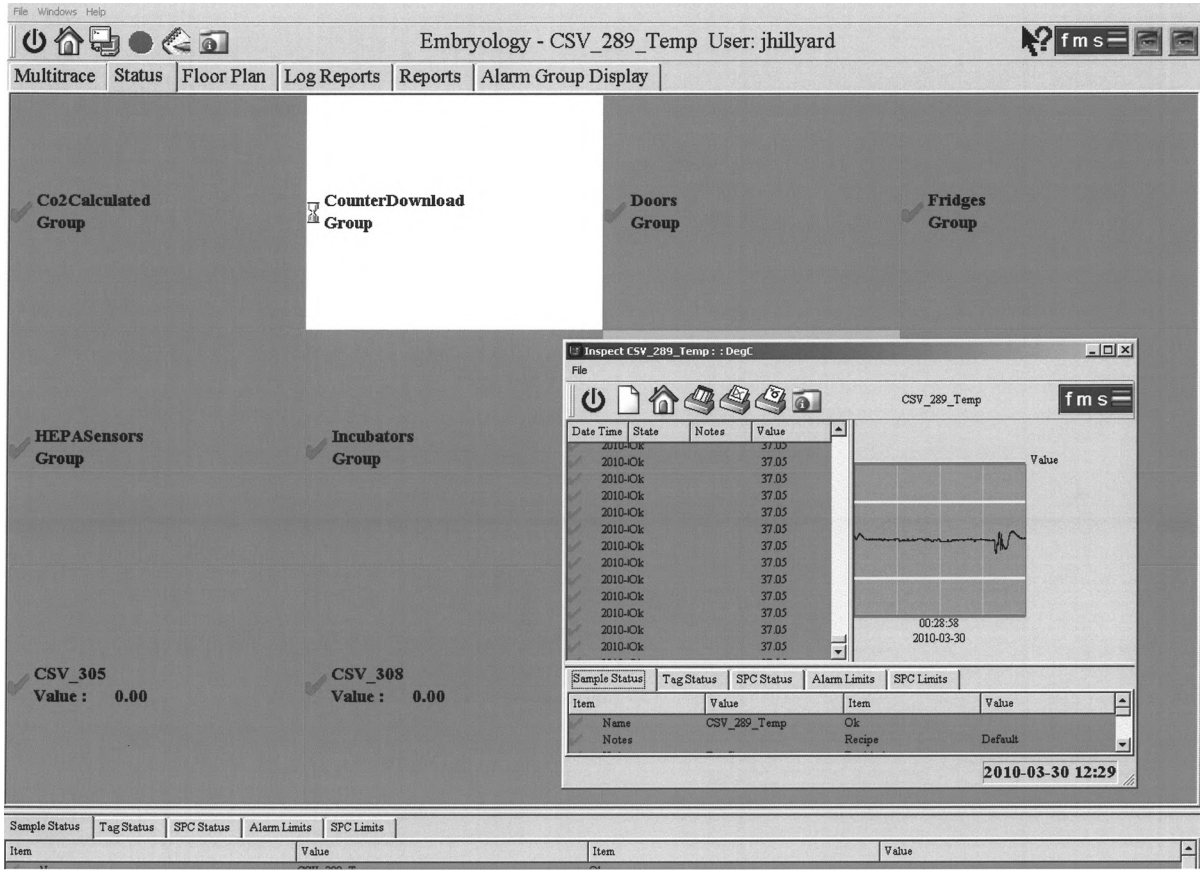

Figure 9.2 (*cont.*)

pH

An "optimal" pH for in-vitro culture has not been precisely defined or characterized, and according to the manufacturers' information, the range of acceptable pH differs with media type, since the hydrogen ion concentration is determined by the overall composition, including concentrations of amino acids and protein supplement. Different media companies advise that incubators are run at different CO_2 concentrations in order to optimize the pH for their media.

Media	pH range
P1	7.1–7.3
Quinn's Advantage	7.1–7.3
Basal XI	7.2–7.35
Global	7.25–7.3
G1.3	7.30 ± 0.10
IVF 500	7.35 ± 0.10
HTF	7.40 ± 0.10
Medicult	None specified; "pH tested"

pH is a number that reflects dynamic culture conditions – it changes rapidly if acid/base concentrations change. Although it is a critical parameter, in practice it is not easy to monitor effectively, especially in microdrop culture under oil. A standard glass probe pH meter is fragile, requires a volume of at least 1 mL that needs to be equilibrated (cannot be used for microdrops), is not standardized, and therefore readings taken do not represent actual culture conditions. A number of alternative devices are available for use in IVF culture systems, but none provide an ideal solution to the problem of pH monitoring, and the different devices can yield different results when used to test a variety of different media and solutions. Therefore, whatever device is used, careful validation and calibration is essential; it is unwise

to rely on only one method. Although pH measurement is important, and is useful in monitoring manipulation and handling procedures, a change in pH reflecting CO_2 concentration takes time, and depends upon the volume and the culture system being used. Please refer to Pool (2004) for a comprehensive review of the science behind culture media pH, and its importance in human IVF.

Devices used to monitor pH in culture media

1. ISFET (ion sensitive field effect transistor) probes:
   - Can be used in small volumes outside the incubator, is simple and fast.
   - Requires frequent calibration and cleaning (sensitive to protein deposits), expensive.
2. RI pH meter:
   - Can be used for measurements inside the incubator, but not for microdrop culture.
   - Slow, drifts over time, and calibration is difficult/time consuming.
3. MTG pH meter: pH Online™, "fluorescent decay time" pH meter:
   - Allows continuous pH measurement inside the incubator; can be used to monitor pH in up to 10 incubators simultaneously.
   - Requires disposable four-well Nunc dish, with one well fitted with a pH reactive fluorochrome spot.
   - Simple to use, but expensive and slow.
4. Blood gas analyzer:
   - Accurate, but not suitable for microdrops.
   - Method of choice for initial media pH testing.
   - Sampling errors can be a problem.
5. Beckmann pH meter:
   - Can be used to measure pH in 5 mL aliquots of media after overnight incubation in test-tubes with loose-fitting caps (see Pool, 2004).
   - Electrode selection, cleaning and regular replacement is important.
   - Must be correctly calibrated before each use.

Incubator CO_2

In contrast to pH measurements, a properly calibrated device can measure CO_2 and provide fast and reproducible results, as well as detect rapid changes in gas concentration: very important in case of incubator malfunction. The "standard" Fyrite kit for measuring CO_2 is inaccurate, and Fyrite is toxic – these kits should not be used in the IVF laboratory. Infrared gas analyzers (Hereaus/Thermo/Bacharach, K-Systems, Vaisala) are available that can provide accurate measurements of gas concentrations. The devices must be calibrated with a reference gas, and should be used at least once per week to measure actual incubator CO_2 with reference to the digital display. Incubator gases can also be monitored with independent probes as part of a facilities monitoring system.

Equipment monitoring for QMS

1. Incubators
   - Independent readings of temperature and gases; calibrated thermometer inside the incubator as standard.
   - Monitor CO_2 supply cylinders regularly; ensure that autochangers are functional.
   - For humidified incubators, check water levels daily.
   - Each incubator should have a 24-hour surveillance system, with alarms accessible to staff when at home or at work.
   - A back-up secondary power source should be installed, and a contingency plan made available to all laboratory staff with back-up arrangements clearly outlined in writing.
2. Heated surfaces and water baths
   - Record the temperature of water baths daily, using a calibrated thermometer in a tube of water.
   - Use a thermocouple to record the temperature of heated surfaces of flow cabinets and the temperature of media drops in culture dishes (the temperature of the surface usually differs from the temperature of media in the culture dish).
   - Define acceptable limits of maxima and minima, and record action taken when these are exceeded.
3. Refrigerators and freezers
   - Record temperatures daily, using calibrated thermometers.
   - Define acceptable limits of maximum and minimum temperatures, and record any action taken when these are exceeded.
4. Liquid nitrogen dewars
   - Should always have an alarm to indicate low nitrogen levels (or increasing temperature, or both).
   - Top up nitrogen levels at least once per week (be careful not to overfill, as liquid nitrogen pouring over the top can damage the seal at the neck).
   - Monitor weekly nitrogen usage by recording

the liquid N_2 level before top-up, and plot the readings on a graph in order to detect any gradual increase in usage, indicating a slow vacuum leak.

Records of all readings should be kept and can be plotted on a graph, with acceptable limits and any corrective action noted on the records. A graph provides a useful means of quickly monitoring and analyzing results visually:

1. Dispersion: increased frequency of both high and low numbers
2. Trend: progressive drift (in one direction) from a mean
3. Shift: abrupt change from the mean.

Bioassays

Guidelines issued by ESHRE (2008), the HFEA (2008), ASRM (2008) and the Association of Clinical Embryologists (ACE) in the UK, state that all tissue culture media prepared in the laboratory should undergo quality control testing with an appropriate bioassay system. A range of bioassays to detect toxicity and suboptimal culture conditions have been tried, including:

1. Human or hamster sperm survival (Critchlow *et al*., 1989; Nijs *et al*., 2009)
2. Continued culture of multipronucleate embryos
3. Somatic cell lines: LAL test for endotoxins (Blechová and Pivodová, 2001), HeLa – test for cytotoxins (Painter, 1978)
4. Culture of mouse embryos from either the one-cell or two-cell stage.

The validity of a mouse embryo bioassay has been questioned as a reliable assay for extrapolation to clinical IVF: it assumes that the requirements of human and mouse embryos are the same, and we know that this is a false assumption. The mouse embryo cannot regulate its endogenous metabolic pool before the late two-cell stage; this is not the case for human or bovine embryos. Mouse embryos will develop from the two-cell stage onwards in a wide variety of cell culture media.

Although none of the systems currently available can guarantee the detection of subtle levels of toxicity, they can be helpful in providing baseline data for comparative purposes, and for identifying specific problems. Any bioassay done routinely and frequently with baseline data for comparing deviations from the norm will be helpful in minimizing the random introduction of contaminants into the system, and is a useful investment of time and resources in an IVF laboratory.

New batches of media, oil, material or supplies used in the culture system, if not pretested, should be tested before use. The physico-chemical limits of culture media testing are crucial: osmolarity must be within the limits of 275-305 mOsm, with a total variation of no more than 30 mOsm. pH must be within the limits of 7.2–7.5, with a maximal variation of 0.4 units of pH. Larger variations in either parameter indicate poor technique/technology and inadequate controls during manufacture, leading to poor reproducibility.

Tissue culture plastics have on occasion been found to be subject to variation in quality: well–well variations have been observed even within a single four-well plate, and rinsing plates with media before use may be a useful precaution. Studies have shown that oil can interact with different plastic supports, and this can affect embryo development. Manufacturers of plasticware used for tissue culture may change the chemical formulation of their products without notification, and such changes in manufacture of syringes, filters and culture dishes may sometimes be embryotoxic. It is also important to store plasticware at an ambient room temperature, away from direct sunlight, as this can affect the properties of the plastic.

Embryos are very sensitive quality control indicators: in routine IVF culture a normal fertilization rate in the order of >70% and cleavage rate of >95% is expected. The cleavage rate is important, as a block at the two-pronucleate stage indicates a serious problem. At least 65% of inseminated oocytes should result in cleaved embryos on Day 2. The appearance of the blastomeres and the presence of fragmentation are also good indicators: blastomeres in the early human embryo should be bright and clear, without granules in the cytoplasm. During cell division, the nucleocytoplasmic ratio is important, and this can be affected by culture medium osmolarity; in the presence of low osmolarity, the size of the embryo increases relative to the volume of the cytoplasm, and cytoplasmic blebs are formed to compensate; in order to achieve an adequate nuclear/cytoplasmic ratio for entry into mitosis. However, in forming blebs, the embryo loses not only cytoplasm but also mRNA and proteins, which are necessary for further development.

Any trend or shift in data should be investigated, with consideration of patient age and activity level: batches of materials (plasticware and media), air quality, temperature/CO_2 levels in incubators and

flow cabinets, water quality, oil and gloves should all be investigated.

Useful routine QC procedures

Sperm survival test

The test should be performed in protein-free media, since protein may act as a buffer for potential toxicity. Select a normal sample of washed prepared spermatozoa and assess for count, motility and progression. Divide the selected sample into four aliquots: add test material to two aliquots, and equivalent control material (in current use) to the remaining two aliquots. Incubate one control and one test sample at 37°C, and one of each at room temperature. Assess each sample for count, motility and progression after 24 and 48 hours (a computer-aided system can be used if available). Test and control samples should show equivalent survival. If there is any doubt, repeat the test.

Culture of surplus oocytes

Surplus oocytes from patients who have large numbers of oocytes retrieved, may be used to test new culture material. Culture at least six embryos in the control media, and a maximum of four in test media.

Multipronucleate embryo culture

Oocytes that show abnormal fertilization on Day 1 after insemination can be used for testing new batches of material. Observe, score and assess each embryo daily until Day 6 after insemination.

Culture of surplus embryos

Surplus embryos after embryo transfer that are not suitable for freezing have also been used for testing new culture material, but this practice is not allowed under an HFEA treatment license in the UK: "An embryo may not be kept under a treatment license if it is no longer to be used in the treatment of a woman, or if any information that may be obtained from keeping it will not be of specific use in the treatment or diagnosis of the individuals whose gametes were used to produce it. If there is some specific value for a couple in continuing to culture spare embryos from a particular treatment cycle, this should be done with the knowledge and agreement of the couple concerned" (HFEA Code of Practice, Ch(94)09). The HFEA Act as amended in 2009 does now allow surplus embryos to be used for training purposes, provided that written patient consent is obtained.

Key points in the use of bioassays

- Choice of bioassay
- Selection of materials to test
- Frequency of testing
- Establish a set of standards, routine and schedule
- Establish acceptable performance range
- Document all results
- Review results regularly
- Do not use anything that fails the bioassay
- Write a standard operating procedure for any corrective action required.

Risk assessments and standard operating procedures

The standard IVF unit has many areas of risk which should be assessed:

- Transport and storage of liquid nitrogen and compressed gases (e.g., transport of liquid nitrogen in elevators requires the dewar to be transported on its own, with "hazard" signs on all floors in between, to prevent the elevator from being used by anyone else)
- Fire
- Infection (bacterial, viral) in theater, scan rooms, laboratories, treatment rooms, consultation rooms, waiting area
- Staff health and safety in all clinical areas and during all clinical activities
- Patient health and safety during all procedures including diagnostic
- Patient confidentiality
- Equipment: use, maintenance, assessment, emergency cover
- Regulatory restrictions for:
  - confidentiality of patients
  - security and confidentiality of current and archived patient records
  - storage and confidentiality of data
  - audit
- IT support
- Security of laboratory stores
- Witnessing (a risk assessment is needed when introducing electronic witnessing systems to ensure they are as effective).

Standard operating procedures (SOPs) must be listed on a regularly updated document, and be available to all laboratory personnel. Each SOP should exist

in only one version, with version number, date and author, and must be updated at least annually, or when any changes are made. They should refer to all associated documentation including patient consent, be part of a document control system, and be archived and no longer available when new version created.

The SOPs should describe in detail:

- The procedures used
- The expected end product
- All equipment and reagents required for each procedure.

They should also include:

- Information on health and safety and infection control
- SOPs for:
  - housekeeping, cleaning and decontamination
  - patient identification, chain of custody and witnessing of all procedures
  - storage and audit of cryopreserved material
- Validation evidence of the procedure described.

Key points of a QMS

- Review and update all processes regularly.
- Ensure traceability by recording batch numbers and expiry dates of all consumables, including plastics and culture media.
- Monitor and service equipment frequently, keeping detailed records.
- Monitor KPIs to check laboratory performance.

Basic housekeeping procedures in the IVF laboratory

Daily cleaning routine

During the course of procedures any spillage should be immediately cleaned with damp tissue and the use of an appropriate disinfectant (e.g., Trigene wipes). No detergent or alcohol should be used whilst oocytes/embryos are being handled. Should it be necessary to use either of the above, allow residual traces to evaporate for a at least 20 minutes before removing oocytes/embryos from incubators.

At the end of each day:

1. Heat seal, double bag, and dispose of all waste from procedures
2. Remove all pipette holders for washing and sterilizing before reuse
3. Reseal and resterilize pipette canisters
4. Clean flow hoods, work benches, and all equipment by washing with a solution of distilled water and 7X laboratory detergent (Flow Laboratories), followed by wiping with 70% denatured alcohol or specific detergent sprays developed for IVF laboratories such as Oosafe (www.parallabls.com), or Fertisafe
5. Prepare each workstation for the following day's work, with clean rubbish bags, pipette holder and Pasteur pipettes.

Washing procedures

If the laboratory has a system for preparation of ultrapure water, particular attention must be paid to instructions for maintenance and chemical cleaning. Water purity is essential for washing procedures, and the system should be periodically checked for organic contamination and endotoxins.

Pipettes

Pre-washed and sterilized pipettes are available, but the following procedure can be used if necessary:

1. Soak new pipettes overnight in fresh Analar or Milli-Q water, ensuring that they are completely covered
2. Drain the pipettes and rinse with fresh water
3. Drain again and dry at 100°C for 1–2 hours
4. Place in a clean pipette canister (tips forward), and dry heat sterilize for 3 hours at 180°C
5. After cooling, record date and use within 1 month of sterilization.

Nondisposable items (glassware, etc.): handle with nonpowdered gloves, rinsed in purified water

1. Soak in distilled water containing 3–5% 7X (Flow Laboratories)
2. Sonicate small items for 5–10 minutes in an ultrasonic cleaning bath
3. Rinse eight times with distilled water, then twice with Analar or Milli-Q water
4. Dry, seal in aluminum foil or double wrap in autoclave bags as appropriate
5. Autoclave, or dry heat sterilize at 180°C for 3 hours

6. Record date of sterilization, and store in a clean, dust-free area.

Incubator maintenance

- Schedule for cleaning should be based on:
  External environment (climate: humid or dry?)
  How often the incubator is used
  Type of incubator
  Where it is housed (clean room, hospital room?)
- Follow manufacturer's instructions for the particular incubator regarding cleaning agents (hydrogen peroxide can normally be used, followed by rinsing with distilled water)
- Fungal contamination in a humidified incubator can be avoided by placing a piece of autoclaved copper in the water pan.

Microbiological testing and contamination in the laboratory

The risk of introducing infection into the laboratory can be minimized by screening patients for infectious agents where indicated by medical history and physical examination. The risk of infectious agent transmission in ART procedures varies in different populations and geographical regions; a risk assessment should be carried out according to the prevalence of disease in the specific patient population, bearing in mind the possibility of "silent" infection prior to detectable seroconversion. National and international guidelines in many countries now recommend that patients attending for ART procedures should undergo routine testing for HIV 1 and 2, hepatitis B and C at least annually; in some cases, viral screening for both partners prior to each cycle is mandatory. Human T-cell lymphotropic virus (HTLV I and II) has a low prevalence in Western countries, but HTLV I is principally endemic in Japan, Central Africa, the Caribbean and Malaysia, and HTLV II is prevalent in Central America and the southern USA. Screening for these viruses prior to blood or organ donation is now mandatory in some countries; guidelines for HTLV I and II patient screening prior to ART procedures should be adapted to local regulations and epidemiology. Routine screening for genital infections, i.e., syphilis, gonorrhea, chlamydia, cytomegalovirus, herpes simplex, human papilloma virus and vaginal infections, should be assessed within the context of patient population, prevalence of disease and full medical history/physical examination of both partners (e.g., malaria, sickle cell disease, *Trypanosoma cruzi*). For syphilis testing, a validated testing algorithm must be applied to exclude the presence of active infection with *Treponema pallidum*.

Effective handling, cleaning and maintenance schedules, together with strict adherence to aseptic technique should make routine microbiological testing unnecessary, but it may be required in order to identify a source of contamination in a culture system. It is required as part of some of the ISO 9000 series quality management protocols and is recommended by the UK Department of Health for laboratories that offer tissue banking facilities, including ovarian and testicular tissue.

Culture systems should be under constant vigilance to detect early signs of possible microbial contamination, in order to avert serious subsequent problems. Any turbidity or drastic color change in media is a clear reflection of contamination, and an inseminated culture dish that shows all sperm dead or immotile should prompt further investigation for possible microbial contamination. A practical and simple method that can be used for checking bacterial and fungal contamination in the incubator or culture medium is to leave an aliquot of culture medium without an oil overlay in the incubator for 5 days. Organisms contaminating the medium or the incubator that are a hazard under IVF culture conditions will multiply in this optimal growth environment of nutrients, temperature, pH and humidity. The aliquot can then be checked for contamination by looking for turbidity and change in color (if medium with a pH indicator is used), and stained for microscopic observation of bacteria or fungi. This test can be used as an ongoing procedure for sterility testing of the incubator as well as the culture medium.

Methods used for microbiological testing of the environment include air sampling, settle plates, contact plates and glove print tests. Although air sampling by either settle plates or Anderson air filtration systems are rarely indicated in an ART laboratory, except in evaluating an episode of contamination or outbreak, specific air quality testing is now a requirement under the European Tissue Directive. Table 9.1 shows average values used for guidance in defining acceptable limits for viable particle detection (microbiological contamination) in monitoring areas of different air quality.

Routine culture of bacteria or fungal spores is expected in most environments, and does not reflect the environment in the sterile hood where procedures are performed. Settle plates are noninhibitory culture

Table 9.1 Average values for limits of microbial detection in areas of defined air quality

Grade	Air sample (CFU/m³)	Settle plates (90 mm diameter) (CFU/4 hours)	Contact plates (55 mm diameter) (CFU/plate)	Glove print (5 fingers) (CFU/glove)
A	<3	<3	<3	<3
B	10	5	5	5
C	100	50	25	–
D	200	100	50	–

CFU, colony forming units.

media plates that are left out on a work surface so that bacteria and mold spores can settle out of the air onto the plate. This method represents an unconcentrated air volume assessment, and the procedure used should specify the length of time of exposure for the plate (1 hour vs. 12 or 24 hours). Air filters such as the Anderson air filter apparatus actively collect (suck in) air through a filter. The filter is then placed on a culture medium to allow bacterial and fungal growth. Using this technique, the number of cubic feet of air to be processed must be specified; it is used mainly in clinical transplant areas (e.g., bone marrow transplant). Isolation of spores is expected and usually does not correlate with patient disease.

Fungal contamination in the laboratory

The ability of fungi to form spores that can survive in a wide range of physical extremes makes them a continuous source of potential contamination, and a laboratory that is not kept strictly and rigorously clean at all times provides an ideal environment for them to thrive. Fungal spores may be introduced from the environment, or from central heating/air conditioning systems, and can grow in incubators, water baths, sinks, refrigerators and on walls and surfaces that escape regular thorough cleaning. They thrive in a moist environment where there is a substrate, such as air filters, heat exchangers, humidifiers, water pumps, cooling units, wet carpet, ceiling tiles, condensation on windows – even indoor plants. In most cases, a thorough routine of regular cleaning together with comprehensive visual inspection should eliminate fungal contamination in the laboratory.

Contamination in an incubator is usually detected when the same fungus grows in multiple culture dishes. In this case, the incubator must be decontaminated according to the manufacturer's instructions: each manufacturer has specifications about safe and appropriate decontamination procedures. The use of quaternary ammonium compounds, chlorine and alcohol solutions must be dictated by the reactivity of these compounds with the components of the incubator. Following decontamination, the incubators must be wiped down with sterile water to remove any residual cleaning solution that might volatize and contaminate the cultures. Full decontamination procedures should be carried out during laboratory "down" periods, when no embryos are being cultured. Decontamination should include all surfaces, the water pan and the fan blades (which are very efficient at dispersing spores). Occasionally decontamination may fail – some institutions have been forced to replace the incubators. Many incubators now incorporate an in-built sterilizing cycle to assist in regular decontamination (Thermo, Forma, Steri-Cycle™).

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Books

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Keel BA, May JV, De Jonge, CJ (eds.) (2000) *Handbook of the Assisted Reproduction Laboratory*. CRC Press, Boca Raton, FL.

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Useful websites

Association of Clinical Embryologists – www.embryologists.org.uk

College of American Pathologists, Accreditation and Laboratory Improvement – www.cap.org/apps/cap.portal?_nfpb=true&_pageLabel=accreditation

COSHH – www.hse.gov.uk/coshh

Health and Safety – www.hse.gov.uk

Human Fertilisation and Embryology Authority – www.hfea.gov.uk

International Organisation for Standardisation (ISO) – www.iso.org

Joint Commission International (JCI) – www.jointcommissioninternational.org/

Department of Health – www.dh.gov.uk

ASRM Guidelines

The Practice Committee of the American Society for Reproductive Medicine and the Practice Committee of the Society for Assisted Reproductive Technology (2008) Revised guidelines for human embryology and andrology laboratories. *Fertility and Sterility* 90: S45–59.

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Tomlinson M (2005) Managing risks associated with cryopreservation. *Human Reproduction* 20: 1433–1438.

Chapter

10 Sperm and ART

Introduction

At least 50% of couples referred for infertility investigation and treatment are found to have a contributing male factor. Male factor infertility can represent a variety of defects, which result in abnormal sperm number, morphology or function. Detailed analysis of sperm assessment and function are important for accurate diagnosis, and are described in detail in numerous textbooks of practical andrology and semen analysis. A comprehensive review of semen analysis is beyond the scope of this book, and only details relevant to assisted conception treatment will be described here.

The World Health Organization (WHO) laboratory manual (2010) describes standard conditions for the collection of semen samples, their delivery and the standardization of laboratory assessment procedures. This manual represents a major revision over the previous (WHO, 1999) edition and includes new "Reference Values" to allow decisions to be made about patient management, and proposes that the 5th centile of a population of men, whose partners conceived within 12 months of stopping contraceptive use, potentially defines "normal" from "abnormal." Therefore the lower reference limits (5th centile and 95% confidence intervals) are 15×10^6/mL (12–16) for concentration, 32% (31–34) for good forward progressive movement within 60 minutes of ejaculation and 4% (3–4) for normal morphology (see WHO manual 2010; Appendix A1.1). The introduction of external quality control and quality assurance schemes in semen assessment have highlighted the fact that accurate analysis of seminal fluid is notoriously difficult to standardize, with many technical variables, and the quality of semen analysis in different laboratories can be highly variable (Matson, 1995). This implies that diagnosis and treatment modality chosen for a patient could differ according to the laboratory carrying out the assessment. Without good semen analysis data, patients may be offered inappropriate treatments or no treatment at all; it is essential that an assisted reproductive service should ensure that laboratory personnel are adequately and correctly trained in basic semen assessment techniques according to WHO guidelines. Even the most confident of laboratories should have a discipline of monitored standards. The routine application of intracytoplasmic sperm injection (ICSI) provides effective treatment for even the most severe cases of male infertility which were previously felt to be beyond hope, and the fact that fertilization can be achieved from semen with "hopeless" sperm parameters has forced a review of standard semen analysis and sperm function testing. This chapter will address only the basic principles required in the practical features of sperm preparation procedures for assisted conception techniques.

Semen assessment

Sample collection and handling

(a) *Record information* – prior to sample production the patient should be asked to confirm their personal details and if necessary provide suitable identification. They should be asked when they last ejaculated, and for a history of recent illness, medication taken, smoking and alcohol consumption; this information should be noted on the final report form. Once the sample has been produced the patient should sign a consent form agreeing to the use of that sample for analysis or treatment.

(b) *Provide adequate instructions* – the patient should be given written instructions about the process involved, including precise details about the location and time that their sample will be

In-Vitro Fertilization: Third Edition, ed. Kay Elder and Brian Dale. Published by Cambridge University Press. (©) K. Elder and B. Dale 2011.

required. They should be informed of the need to abstain from sexual intercourse or masturbation for between 3 and 5 days before their sample is to be produced. Care should be taken over language difficulties and for patients with special needs.

(c) *Methods of sample production* – the sample should ideally be produced by masturbation after the required period of abstinence. However, it is acknowledged that this is not possible for all patients. A small number of men can only produce a sample at intercourse and in these cases they should be provided with a silastic (nonspermicidal) condom. No other condoms are permissible. Samples produced by coitus interruptus without a condom should not be accepted for analysis.

(d) *Location of production* – whenever possible all samples for analysis or treatment should be produced on site. It is unacceptable to ask a patient to produce a sample in a lavatory, and there should be a special room set aside for this purpose. Where it is not possible to produce a sample on site, a sample produced at home should be brought to the laboratory within one hour.

(e) *Specimen container* – samples should be collected into a pre-weighed wide-necked plastic or glass container. All specimen containers should be cytotoxically tested as some plastics are detrimental to sperm motility (see Box 2.1 of WHO, 2010, for details). Samples previously assessed as having high viscosity benefit from collection into pots containing 1 mL of medium. Prior to production the patient should be asked to pass urine and then rinse his hands and penis.

(f) *Treatment of samples post-production* – once produced, the patient and a member of staff should check that the sample container is identified with the patient's name or identification number, and the time of sample production. The samples should be placed in an incubator at 37°C for up to one hour to allow liquefaction.

Macroscopic evaluation

Once the process of liquefaction has occurred (usually within 30–60 minutes of ejaculation), the sample should be examined macroscopically, with evaluation of:

- *Appearance and consistency* – semen should be a grayish opalescent liquid with a neutral odor. Any unpleasant smell or discoloration (e.g., contains blood), or the presence of mucus or jelly should be reported.
- *Liquefaction and viscosity* – although semen is ejaculated as a coagulum, it should liquefy within 30 minutes. If a sample fails to liquefy or is highly viscous after liquefaction this should be noted.
- *Volume* – measured by weighing the container in which the specimen was ejaculated and subtracting the full weight from the empty weight. The volume can be inferred from the weight assuming the density of semen to be 1 g/mL. The use of volumetric methods to measure semen volume is no longer recommended.
- *pH* – the most convenient way to measure the pH of a sample is to use pH paper.

Macroscopic anomalies can provide important information about the patient and should not be ignored. For example, a low pH can indicate infection of the genital tract, and a low volume could suggest a retrograde ejaculation, a leakage from the sample container, or that the patient failed to collect the entire sample he produced.

Sperm motility

Since sperm motility decreases with increasing exposure to seminal plasma, this should be the first assessment carried out. There are three important aspects to correctly estimating sperm motility:

- *Observation chamber* – a variety of types are available, but this should have a minimum depth of 20 μm to allow the sperm to move freely. A number of companies produce disposable chambers designed for motility observation (e.g., Microcell), but an alternative strategy is to place a 10 μL drop of semen on a glass microscope slide and cover it with a 22 mm diameter coverslip. NB: the depth of a Makler chamber (10 μm) makes it unsuitable for accurate motility measurements. However, when the purpose of assessment is the selection of an appropriate method of preparing the sample for assisted conception procedures, the Makler chamber does allow simultaneous judgment of approximate motile and immotile concentrations, and a quick assessment of type of sperm motility.
- *Temperature* – the microscope slide should be maintained at 37°C on a heated stage during motility assessment for correct identification of motility grade.

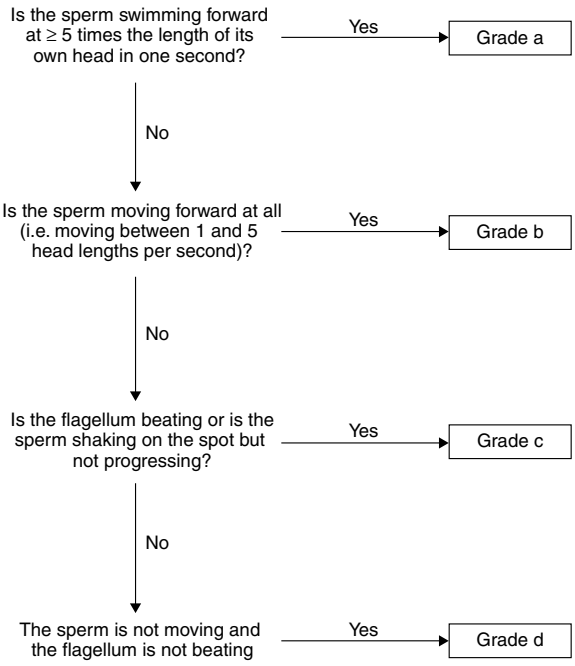

Figure 10.1 Flow chart for grading sperm motility.

- *Microscope* – observe the sample at ×200 or ×400 magnification using a phase contrast objective.
- *Grading system* – approximately 200 sperm should be examined and each sperm classified as belonging to one of four motility grades. Figure 10.1 outlines the difference between the motility grades, with a flow chart explaining how to classify them. A percentage for the number of sperm belonging to each category should be calculated.

Aggregated or agglutinated spermatozoa

- A high number of aggregated or agglutinated spermatozoa can make accurate motility assessment impossible. A motility count should then be performed only on the free-swimming portion, with this noted in the report.

Less than 50% of the spermatozoa are motile

- If less than 50% of the spermatozoa are motile, a vitality test, such as a hypo-osmotic swelling (HOS) test, is recommended in order to determine if the nonmotile sperm are dead or alive.

Sperm concentration

Methods used to determine sperm concentration have long been a subject of debate. Andrologists tend to agree that using a hemocytometer is the most appropriate, as it provides the most reproducible result with the lowest coefficient of variation when used properly. Since it normally relies upon the use of fixatives to kill spermatozoa before they are placed on the counting chamber, its use is often thought to be in conflict with the principles of trying to reduce chemical contaminants in the IVF laboratory. Many embryologists, however, use water instead of fixative in which to dilute the spermatozoa and the osmotic shock is sufficient to immobilize sperm sufficiently for a count to be undertaken. Others prefer to use a Makler chamber or disposable chambers such as the Microcell. If an alternative chamber is chosen, then it is important that its accuracy is regularly checked using a thorough internal quality control system. This should preferably be checked against a hemocytometer as the gold standard. Whatever chamber is chosen, it is important to pay special consideration to samples in which no spermatozoa are observed. These samples should be centrifuged at >3000 *g* for 15 minutes; a sample can be classified as truly azoospermic only if no sperm are observed in the pellet after centrifugation.

Before performing the count, note the presence of agglutination and type if present (H-H, T-T, H-T), and any debris and cells other than spermatozoa, such as red or white blood cells.

Examine the counting grid and count the number of *motile* sperm in 20 squares. If the count appears on initial observation to be less than 10 million/mL, all 100 squares should be counted. Count the *total* number of sperm in the same group of squares, and calculate motility:

Sperm density in millions/mL = the number of sperm in 10 squares of the grid.

The sperm concentration should be reported in millions per mL.

Sperm antibody detection

Antibodies directed against sperm can be detected by two methods, the mixed antiglobulin reaction (MAR) test and the immunobead test. They differ slightly in their approach and methodology, but their interpretation is similar, in that they rely upon the identification of motile spermatozoa with adherent latex spheres or beads. Kits (SpermMar, FertiPro N.V., Belgium; Microm, UK) are available for antisperm antibody screening in semen samples; the MAR test will nonspecifically detect IgG, IgA or IgM antibodies,

and specific latex particle immunobeads (BioRad) can be used to distinguish between the different categories of antibody. Although the results of the two tests do not always agree, it is generally considered that a test is clinically significant only if >50% of sperm have antibodies directed against them. In cases where the test cannot be performed due to an insufficient number of motile sperm, the sample can be tested indirectly by using the sperm of a donor (known to have no sperm antibodies in his semen) as part of the test: the donor sperm acts as a reagent in the assay. The percentage of spermatozoa with adherent particles should be recorded on the report form after evaluating 200 sperm.

Sperm morphology estimation

Sperm morphology assessment is one of the most controversial measures in semen analysis. This is due partly to several changes in reporting the dimensions of normal spermatozoa in successive editions of the WHO manual, but is also due to the technical difficulty of making accurate morphology measurements without the aid of a computerized system. All morphology measurements should be made using fixed smears stained by the Papanicolaou method or the Diff-Quick or Shorr stains (prestained slides are also available: Testsimplets, Waldeck GmbH, www.waldeck-online.com). Stained slides should be examined by bright-field optics using an oil-immersion objective at ×1000 magnification. At least 200 spermatozoa should be examined and an eyepiece graticule can be used to measure individual spermatozoa if necessary. The normal head has an oval shape with a length:width ratio of 1.50:1.75. A well-defined acrosomal region should cover 40–70% of the head area. No neck, midpiece or tail defects should be evident, and cytoplasmic droplets should constitute no more than one-third the size of a normal sperm head. Figure 10.2 illustrates some examples of typical sperm abnormalities. Count the number of sperm that display:

1. Abnormal heads
2. Tail abnormality
3. Midpiece abnormalities
4. Immature forms.

All borderline forms are classified as abnormal. Calculate the percentage of each abnormal form, and add together the percentages to yield the total percentage of abnormal forms in the sample.

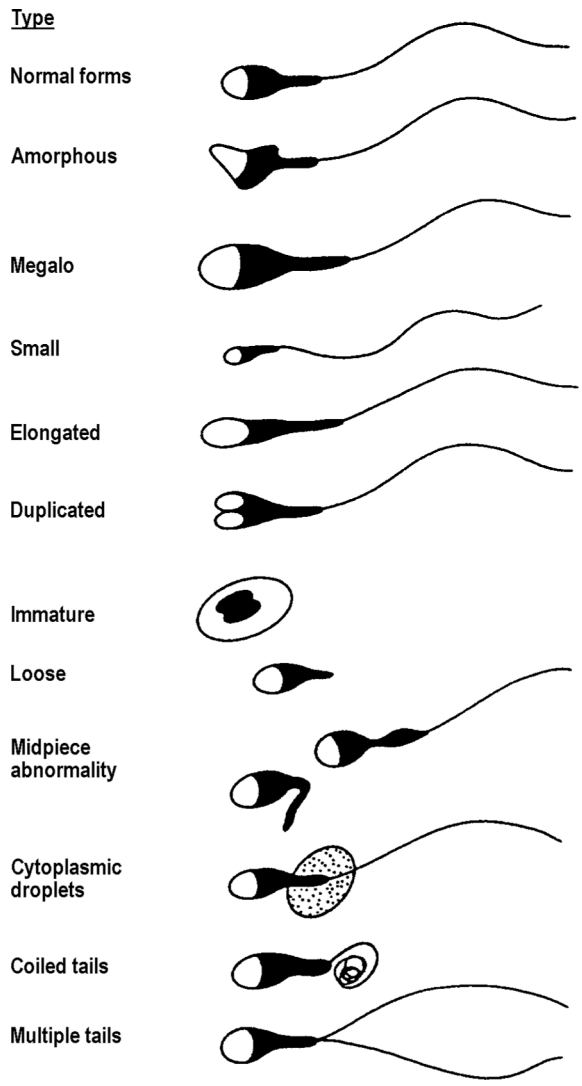

Figure 10.2 Common abnormalities found in human sperm morphology.

A normal, fertile semen sample contains a very high proportion of morphologically abnormal forms, and the significance of abnormal sperm morphology is not entirely understood. Although sperm of abnormal morphology evidently have reduced fertilizing potential, the true anomalies present in abnormal sperm cells have been only partially characterized; a correlation has been found with specific deficiencies such as poor zona pellucida binding and penetration, poor response to agonists that modulate intracellular calcium concentrations, and with biochemical markers such as reactive oxygen species production and enhanced creatine phosphokinase activity. The significance of sperm morphology is

discussed further in Chapter 13, under intracytoplasmic morphologically-selected sperm injection (IMSI).

Other cells in semen

Other (non-sperm) cells can sometimes be observed during the semen analysis, either in the wet (motility) preparation, or in the stained morphology slide. These include epithelial cells from the urethra, erythrocytes, germ cells and leukocytes. Whilst epithelial cells and erythrocytes are easily identifiable from their morphology, germ cells and leukocytes can easily be confused. Therefore specific stains are needed to discriminate between the two cell types and to correctly enumerate their concentration on the semen analysis report. Leukocytes can be identified using a peroxidase-based stain, or with the use of specific monoclonal antibodies. The concentration of any non-sperm cell can be calculated relative to the sperm concentration using the equation $c = n \times s/100$, where n is the number of a given cell type in the same field as 100 sperm, and s is the sperm concentration in millions per mL.

Internal and external quality control procedures

A final but important part of the semen analysis is the application of internal and external quality control procedures to the semenology laboratory. Several studies have shown that samples analyzed in different laboratories can give rise to radically different results, in some cases leading to an inappropriate diagnosis for the patient. Many techniques have been outlined that can be used to monitor the performance of a laboratory, and these are described in more detail in the 5th edition of the WHO manual. Any laboratory involved in making diagnoses should have such protocols in place and should be members of an external quality assessment scheme for andrology.

Sperm kinematics

In the early 1980s several studies used time lapse photography to analyze detailed movement characteristics of sperm in time and space (sperm kinematics). The motion of spermatozoa can be described in a number of different ways (see Mortimer, 1994):

VSL = straight line velocity
VCL = curvilinear velocity
VAP = average path velocity
ALH = amplitude of lateral head displacement.

These early data led to the suggestion that specific patterns of sperm motility behavior were advantageous. For example, only spermatozoa with a high degree of lateral head displacement are able to penetrate cervical mucus. The development of computerized systems allowed such measurements to be made more rapidly as well as allowing the analysis of more sophisticated behavior patterns, such as sperm hyperactivation. Although the measurement of sperm hyperactivation has been controversial, it has been linked with IVF success. However, this technology is not used routinely as the prognostic value is poor and the high cost of the machines precludes their use in all but the most specialized laboratories (see Tomlinson *et al*., 2010 for review).

DNA fragmentation

As detailed in Chapter 3, spermatogenesis is a complex and dynamic process of proliferation and differentiation, involving mitosis, meiosis, changes in cytoplasmic architecture, replacement of histones with transition proteins and the final addition of protamines, leading to a highly packaged chromatin. It is not surprising therefore that ejaculated spermatozoa have a variety of abnormalities at the nuclear, cytoskeletal and organelle levels. There is now evidence to suggest that sperm DNA integrity may be useful in predicting male fertility potential. The first manuscript describing in-situ detection of sperm DNA fragmentation was published 25 years ago, and a surge in published reports about sperm DNA fragmentation then appeared between 2005 and 2010. These studies provide strong evidence that semen samples in which more than a third of the DNA is fragmented have a reduced chance of resulting in clinical pregnancy (Sakkas and Alvarez, 2010). Sperm DNA fragmentation may result from aberrant chromatin packaging during spermatogenesis, apoptosis before ejaculation, excessive production of reactive oxygen species in the ejaculate, exposure to environmental or industrial toxins, genetics, oxidative stress, smoking, etc. Current standard sperm preparation techniques depend on a sedimentation or migration approach to separate spermatozoa based on their motility or density with molecular events being overlooked. Thus, the use of sperm with DNA damage during IVF may be one of the reasons for suboptimal pregnancy and low live birth rates.

Sperm chromatin assays

Sperm condensation quality and sperm morphology studies suggest that the quality of chromatin packaging in human sperm, as assessed by its binding capacity for specific dyes and fluorochromes, can be used as an adjunct to the assessment of morphology. Sperm of poor morphology may possess loosely packaged chromatin, and this may contribute to a failure in sperm decondensation during fertilization.

Damaged chromatin will take up the following dyes:

1. Chromomycin (CMA3) staining

- Fix prepared semen samples or semen smears in 3:1 v/v of methanol/glacial acetic acid, at 4°C for 5 minutes.
- Treat each slide for 20 minutes with 100 mL CMA3 solution: 0.25 mg/mL in McIlvane's buffer, pH 7.0, containing 10 mM $MgCl_2$.
- Evaluate the slides using fluorescent microscopy.

2. Aniline blue staining (AAB)

- Fix the samples in 3% buffered glutaraldehyde for 30 minutes.
- Stain the slides with 5% aqueous aniline blue and mix with 4% acetic acid (pH 3.5) for 7 minutes.
- Three classes of head staining can be noted:
  unstained (gray/white)
  partially stained
  entire sperm head dark blue intensity.

Detection of DNA fragmentation by the TUNEL assay

Kits for DNA fragmentation analyses are now available, for example the in situ cell death detection kit by Roche Diagnostics, Mannheim, Germany.

- Wash a semen aliquot containing $1–2 \times 10^6$ spermatozoa with phosphate-buffered saline (PBS) by centrifugation at 500 *g* at room temperature for 5 minutes.
- Remove the seminal plasma and wash the pellet twice in PBS with 1% bovine serum albumin (BSA).
- Suspend the pellet in 100 µL of PBS/BSA 1% and fix it in 100 µL of 4% paraformaldehyde in PBS (pH 7.4) for 1 hour at room temperature, with agitation.
- Wash the cells again in PBS/1% BSA, spot a 10 µL aliquot onto a demarcated area on a clean microscope slide and allow this to air dry.
- Rinse the slides twice in PBS, and then permeabilize using 0.1% Triton X100 in 0.1 % sodium citrate for 2 min on ice.
- Wash again twice with PBS, add terminal deoxyribonucleotidyl transferase (TdT)-mediated dUTP nick-end label in order to allow DNA elongation, and incubate the slides in a humidified chamber at 37°C for 60 min.
- Rinse slides twice in PBS and counterstain with 1 mg/mL 4,6 diamidoino-2-phenylindole (DAPI) to visualize the nucleus.
- Include negative (omitting TdT from the reaction mixture) and positive (using only DNAse I, 1 mg/mL for 30 min at room temperature) controls in each sample tested.
- Evaluate a total of 500 sperm per sample using fluorescence microscopy for fluorescein isothiocyanate (FITC). Count the number of sperm per field stained with DAPI (blue); the number of cells with green FITC fluorescence (TUNEL positive) is expressed as a percentage of the total sample.

Preparation of sperm for in-vitro fertilization

At the time of oocyte retrieval or intrauterine insemination (IUI), the laboratory should already be familiar with the male partner's semen profile, and can refer to features that might influence the choice of sperm preparation method used. Semen is a nonsterile body fluid that can transmit infection, and viral screening tests should be confirmed as negative before the sample is handled for preparation in the laboratory. Aseptic technique should be maintained throughout.

The choice of sperm preparation method or combination of methods depends upon the assessment of:

- motile count
- ratio between motile:immotile counts
- volume
- presence of antibodies, agglutination, pus cells or debris.

Ejaculated semen is a viscous liquid composed of a mixture of testicular and epididymal secretions containing spermatozoa, mixed with prostatic secretions produced at the time of ejaculation. This seminal plasma contains substances that inhibit capacitation and prevent fertilization. The purpose of sperm preparation is to concentrate the motile spermatozoa in

a fraction that is free of seminal plasma and debris. Early IVF practice involved preparing sperm by simple washing and centrifugation, but this method also concentrates cells, debris and immotile sperm, which can jeopardize fertilization. Aitken and Clarkson (1987) demonstrated that leukocytes and dead sperm in semen can generate reactive oxygen species (ROS), and these can initiate lipid peroxidation in human sperm membranes. Peroxidation of sperm membrane unsaturated fatty acids leads to a loss of membrane fluidity, which inhibits sperm fusion events during the process of fertilization. When preparing sperm for assisted conception it is advantageous to separate motile sperm from leukocytes and dead sperm as effectively and efficiently as possible. However, if intracytoplasmic sperm injection (ICSI) is the treatment of choice, sperm fusion events are of course bypassed, and direct high-speed centrifugation of these suboptimal sperm samples does not appear to jeopardize fertilization by ICSI.

Sperm samples that show moderate to high counts ($>35 \times 10^6$ motile sperm/mL) with good forward progression and motility can be prepared using a basic overlay and swim-up technique. Discontinuous buoyant density gradient centrifugation is the method of choice for samples that show:

1. Low motility
2. Poor forward progression
3. Large amounts of debris and/or high numbers of cells
4. Antisperm antibodies.

At the end of each preparation procedure, adjust the pH of the resulting samples by gassing gently with 5% CO_2, and store the samples at room temperature until final preparation for insemination.

Standard swim-up or layering

1. Pipette 2 mL of HEPES-buffered culture medium into a round-bottomed disposable test-tube.
2. Gently pipette approximately 1.5 mL neat semen underneath the medium (being very careful not to disturb the interface formed between the semen and the medium).
3. Tightly cap the tube and allow it to stand at room temperature for up to 2 hours (the ejaculate can also be divided into several tubes for layering if necessary).
4. Harvest the resulting top and middle clouded layers into a conical test-tube and spin at 200 *g* for 5 minutes.
5. Remove the supernatant and resuspend the pellet in 2 mL medium. Centrifuge again, discard the supernatant, and resuspend the pellet in 1 mL medium.
6. Assess this sample for count and motility, gas the surface gently with 5% CO_2 in air, and store at room temperature prior to dilution for the insemination procedure.

Alternatively, 2 mL of medium can be gently layered over the semen sample in its pot, which provides a larger interface surface area. After 10–45 minutes, suspend an aliquot of this layer in 1 mL of medium, and process as above. The time allowed for swim-up should be adjusted according to the quality of the initial sample: the percentage of abnormal sperm that will appear in the medium increases with time, and continues to do so after normal motile density has reached its optimum level.

Pellet and swim-up

This method is used when the semen has been collected into medium or medium + albumin. It is also useful for viscous samples (once the viscosity has been decreased, by vigorous pipetting or syringing) and when the total volume of semen is very low. This method is not recommended when motility is very poor or when there is a large degree of cellular contamination and debris (the sperm will be concentrated with this prior to the swim-up).

1. Mix semen and medium and centrifuge once. *Note*: In some cases (i.e., oligo/asthenospermia) much more semen will need to be prepared, and the volume of medium used should be increased accordingly. As a general rule be careful not to take far more of the semen than is required.
2. Carefully remove all the supernatant and then very gently pipette about 0.75 mL of medium over the pellet, taking care not to disturb it.
3. Allow the sperm to swim up into the medium. If the sample has poor motility it sometimes helps to lay the centrifuge tube on its side.
   - 10 minutes is sufficient for very motile sperm (activity 3–4)
   - 1 hour plus may be required for poorly motile sperm.

- In general do not leave for too long, as some cells and debris will become resuspended.

4. Carefully remove supernatant from pellet and place in a clean centrifuge tube.
5. Centrifuge again, resuspend in medium, assess count and motility, and gas with CO_2 before storing at room temperature.

This method has the disadvantage of exposing motile sperm to peroxidative damage during centrifugation with defective sperm and white cells. Aitken (1990) has shown that unselected sperm exhibit higher levels of ROS production in response to centrifugation than the functionally competent sperm selected prior to centrifugation by the layering method. Sperm that are selected prior to centrifugation produce much lower levels of ROS and their functional capacity is not impaired.

Discontinuous buoyant density gradient centrifugation

Buoyant density gradient "kits" for sperm preparation are commercially available. These are based upon either coated silica particles, a mixture of Ficoll and iodixanol, or highly purified arabinogalactan. Individual experiences comparing the use of these products have reported no significant differences between them. Buoyant density gradients apparently protect the sperm from the trauma of centrifugation, and a high proportion of functional sperm can be recovered from the gradients. Discontinuous two- or three-step gradients are simple to prepare and highly effective in preparing motile sperm fractions from suboptimal semen samples. A single layer of 90–100% density can also be used for simple filtration by layering the sample on top of the column and allowing the sperm to migrate through the density medium, where they can be harvested from the bottom of the test-tube.

Methods

Manufacturers' instructions should be followed for the different commercial preparations, but "recipes" can be adapted according to each individual semen sample, in particular with respect to volumes, speed of centrifugation, and length of centrifugation. In general, a longer centrifugation *time* increases the recovery of both motile and immotile sperm; normal immotile sperm are only decelerated by the particles, and after long spinning they will reach the bottom of the gradient. Higher centrifugation *speeds* increase the recovery of motile sperm, and also of lower density particles; therefore, if the gradients are spun at a higher speed, a shorter time should be used. Debris, round cells, and abnormal forms with amorphous heads and cytoplasmic droplets never reach the bottom of the tube because of their low density. Gradients with larger volumes result in improved filtration, but decreased yield. The three layers of a mini-gradient improve filtration, and the smaller volumes improve recovery of sperm from severely oligospermic samples. Large amounts of debris can disrupt gradients and prevent adequate filtration. There is a limit to the number of cells that can be loaded onto any gradient, and samples with high density or a large amount of debris should be distributed in smaller volumes over several gradients. Severely asthenozoospermic samples, with a normal sperm density but poor motility can also be distributed over a series of mini-gradients.

The temperature of the prepared gradients also affects the "merging of gradients," which is improved at 37°C compared to room temperature.

Isotonic gradient solution

10 × concentrated EBS (or other media concentrate):	10 mL
5% human serum albumin:	19 mL
Sodium pyruvate:	113 mg
Sodium lactate 60%:	0·37 mL
1 M HEPES:	12 mL
Gentamicin sulfate, 5 mg/mL:	12 mL

Mix, and filter this solution through a 0.22 mm Millipore filter

Add 90 mL density gradient preparation media
Store at + 4°C for up to 1 week.

Two-step gradient, 80/40

- Can be used for all samples which contain> 4×10^6 motile sperm/mL.
- Should be used for all specimens with known or suspected antisperm antibodies:
  80%: 8 mL isotonic + 2 mL culture medium
  40%: 4 mL isotonic + 6 mL culture medium.

1. Gradients: pipette 2.0–2.5 mL of 80% into the bottom of a conical centrifuge tube, and gently overlay with an equal volume of 40%.
2. Layer up to 2 mL of sample on top of the 40% layer.
3. Centrifuge at 600 *g* for 20 minutes.

Cells, debris and immotile/abnormal sperm accumulate at the interfaces, and the pellet should contain functionally normal sperm. Recovery of a good pellet is influenced by the amount of debris and immotile sperm, which impede the travel of the normal motile sperm.

4. Carefully recover the pellet at the bottom of the 80% layer, resuspend in 1 mL of medium, and assess (even if there is no visible pellet, a sufficient number of sperm can usually be recovered by aspirating the bottom portion of the 80% layer).
5. If the sample looks sufficiently clean, centrifuge for 5 minutes at 200 *g*, resuspend the pellet in fresh medium, and assess the final preparation.
6. If there is a high percentage of immotile sperm, centrifuge at 200 *g* for 5 minutes, remove the supernatant, carefully layer 1 mL fresh medium over the pellet, and allow the motile sperm to swim up for 15–30 minutes. Collect the supernatant and assess the final preparation.

If at least 10^6 motile sperm/mL have been recovered, spin at 200 *g* for 5 minutes and resuspend in fresh medium. This will be the final preparation to be diluted before insemination, therefore the volume of medium added will depend upon the calculated assessment.

Mini-gradient (95/70/50)

95%	9.5 mL gradient solution + 0.5 mL culture medium
70%	7.0 mL + 3.0 mL
50%	5.0 mL + 5.0 mL

1. Gradients: make layers with 0.3 mL of each solution: 95, then 70, then 50.
2. Dilute the semen 1:1 with culture medium, and centrifuge at 200 *g* for 10 minutes.
3. Resuspend the pellet in 0.3 mL culture medium, and layer over mini-gradient (resuspend in a larger volume if it is to be distributed over several gradients).
4. Centrifuge at 600 *g* for 20–30 minutes.
5. Recover the pellet(s), resuspend in 0.5 mL culture medium, and assess count and motility. Proceed exactly as for two-step gradient preparation: either centrifuge at 200 *g* for 5 minutes and resuspend the pellet, or layer over the pellet for a further preparation by swim-up. The concentration of the final preparation should be adjusted to a sperm density of approximately 10^6 motile sperm per mL if possible.

If a sample is being prepared for ICSI, note that residual polyvinylpyrrolidone (PVP)-coated particles in the preparation will interact with PVP used for sperm immobilization, resulting in a gelatinous mass from which the sperm cannot be aspirated. Careful washing of the preparation to remove all traces of gradient preparation is essential when handling samples for ICSI (one wash is usually sufficient).

Sedimentation method or layering under paraffin oil

This method is useful for samples with very low counts and poor motility. It is very effective in removing debris, but requires several hours of preparation time.

1. Mix the semen with a large volume of medium, pipetting thoroughly to break down viscosity etc., and wash the sample by dilution and centrifugation twice.
2. Alternatively: process the entire sample (undiluted) on an appropriate discontinuous buoyant density gradient.
3. Resuspend the pellet in a reduced volume of medium so that the final motile sperm concentration is not too dilute.
4. Layer this final suspension under paraffin oil (making one large droplet) in a small Petri dish. Place in a desiccator and gas with 5% CO_2.
5. Leave at room temperature for 3–24 hours. The duration of sedimentation depends upon the amount of cells, debris and motile spermatozoa; a longer period is usually more effective in reducing cells and debris, but may also reduce the number of freely motile sperm in the upper part of the droplet.
6. Carefully aspirate motile sperm by pipetting the upper part of the droplet. Aspiration can be made more efficient by using a fine drawn pipette and also by positioning the droplet under the stereomicroscope, to ensure that as little debris as possible is collected.

High-speed centrifugation and washing

Cryptozoospermic (or nearly cryptozoospermic) samples which must be prepared for ICSI can be either centrifuged directly (without dilution) at 1800 *g*

for 5 minutes, or diluted with medium and then centrifuged at 1800 *g* for 5 minutes.

1. Wash the pellet with a small volume of medium (0.5 mL approximately) and centrifuge at 200 *g* for 5 minutes.
2. Recover this pellet in a minimal volume of medium (20–50 mL), and overlay with mineral oil. Single sperm for microinjection can then be retrieved from this droplet using the micromanipulator.

It is important to bear in mind that every semen specimen has different characteristics and parameters, and it is illogical to apply an identical preparation technique to each specimen. All preparation methods are adaptable in some way: layering can be carried out in test-tubes, but a wider vessel increases the area exposed to culture medium and decreases the depth of the specimen, increasing the potential return of motile sperm from oligoasthenospermic samples. Centrifugation times for buoyant density gradients may be adjusted according to the quality of the specimen to give optimum results. It is important to tailor preparation techniques to fit the parameters of the semen specimen, rather than to have fixed recipes. A trial preparation prior to oocyte retrieval may be advisable in choosing the suitable technique for particular patients.

Chemical enhancement of sperm motility prior to insemination

Pentoxifylline is a methylxanthine-derived phosphodiesterase inhibitor which is known to elevate spermatozoal intracellular levels of cAMP in vitro. It has been postulated that the resulting increase in intracellular adenosine triphosphate (ATP) enhances sperm motility in samples that are assessed as having poor progressive motility, with an increase in fertilization and pregnancy rates for suboptimal semen samples. 2-Deoxyadenosine has also been used to achieve a similar effect. The protocol involves a 30-minute preincubation of prepared sperm with the stimulant; the resulting sperm suspension is then washed to remove the stimulant, and the preparation is used immediately for insemination.

Stock solutions

1 mM PF: dissolve 22 mg pentoxifylline in 10 mL medium.

3 mM 2-DA: dissolve 8 mg 2-deoxyadenosine in 10 mL medium.

Gas with 5% CO_2 to adjust pH.

Store at 4°C for a maximum period of one month.

Procedure

1. Gas and warm PF or 2-DA solutions, and also an additional 10 mL medium.
2. 35–40 minutes prior to insemination time, add an equal volume of PF or 2-DA solutions to the sperm preparation suspension.
3. Incubate at 37°C for 30 minutes.
4. Centrifuge, 5 minutes at 200 *g*, and resuspend pellet in warm medium.
5. Analyze the sperm suspension for count and motility, dilute to appropriate concentration for insemination, and inseminate oocytes immediately.

Sperm preparation for ICSI/IMSI

A combination of sperm preparation methods can be used; extremely oligospermic/asthenozoospermic samples cannot be prepared by buoyant density centrifugation or swim-up techniques.

1. Centrifuge the whole sample, 1800 *g*, 5 minutes, wash with medium, and resuspend the pellet in a small volume of medium.
2. Apply this sample directly to the injection dish (without PVP/SpermSlow), or add an aliquot of the suspension to a drop of HEPES-buffered medium.
3. If possible, use the injection pipette to select a moving sperm with apparently normal morphology from this drop and transfer it into the drop to be used for sperm immobilization.
4. If there is debris attached to the sperm, clean it by pipetting the sperm back and forth with the injection pipette.
5. If the sperm still has some movement in the immobilization drop, immobilize it by crushing the tail and proceed with the injection as described in Chapter 11.
6. It may sometimes be helpful to connect the sperm droplet to another small medium droplet by means of a bridge of medium, and allow motile sperm to swim out into the clean droplet. Overloading a PVP/SpermSlow droplet with very poor sperm can seriously hamper the selection procedure, due

to the presence of excessive amounts of debris. Using a bridge of medium allows motile sperm to swim into the second, clean droplet.

Even if the results of semen analysis show no motile sperm, it may be possible to see occasional slight tail movement in a medium drop without PVP. If absolutely no motile sperm are found, immotile sperm may be used. The fertilization rate with immotile sperm is generally lower than that with motile sperm, and oocytes with a single pronucleus are seen more often in these cases, possibly indicating incomplete oocyte activation. Previous assessment with a vital stain may be helpful before deciding upon ICSI treatment. However, embryologists may be left with the dilemma of making a decision regarding injection in situations where there are only immotile sperm, the vitality stain/HOS test indicates the sperm tested are not alive and there is no donor back-up.

Hypo-osmotic swelling test (HOS)

The HOS test assesses the osmoregulatory ability of the sperm, and therefore the functional integrity of its membranes. It can be used to discriminate viable from nonviable sperm cells in a sample which has zero or little apparent motility. The test is based upon the ability of live spermatozoa to withstand the moderate stress of a hypo-osmotic environment – they react to this stress by swelling of the tail. Dead spermatozoa whose plasma membranes are no longer intact do not show tail swelling. HOS test diluent is a solution of 150 mOsm/kg osmolarity, and can be made by dissolving 7.35 g sodium citrate and 13.51 g fructose in 1000 mL of reagent-grade water (alternatively, sperm preparation medium can be diluted 1:1 with reagent-grade water). Mix an aliquot of the sample with approximately 10 times the volume of diluent, incubate at 37°C for 30 minutes, remix and transfer one drop of the mixture to a clean microscope slide. Cover with a coverslip, and examine using phase-contrast microscopy at a magnification of ×400–500 for the presence of swollen (coiled) tails. Osmotically incompetent and dead spermatozoa swell so much that the plasma membrane bursts, allowing the tail to straighten out again.

100% abnormal heads

If the semen analysis shows 100% head anomalies, it may still be possible to find the occasional normal form in the sample. In cases where no normal forms are found, the fertilization and implantation rates may be lower; however, debate continues about this subject, and individual judgment should be applied to each case, with careful assessment of several different semen samples.

Fertilization and pregnancy have now been demonstrated using samples from men with globozoospermia, a 100% head anomaly where all sperm lack an acrosomal cap; in these cases the oocytes need to be artificially activated post-ICSI for fertilization to occur. However, there is evidence to suggest that such defects which are genetically determined have a high probability of being transmitted to the offspring, and debate continues as to whether it is ethically advisable to offer treatment to these men.

Sticky sperm

Sperm that have a tendency to stick to the injection pipette make the injection procedure more difficult. If the sperm is caught in the pipette, try to release it by repeatedly aspirating and blowing with the injection system.

Excessive amounts of debris

Large amounts of debris in the sperm preparation may block the injection pipette, or become attached to the outside of the pipette. A blocked pipette may be cleared by blowing a small amount of the air already in the pipette through it. Another useful technique is to insert the injection pipette into the lumen of the holding pipette, and then apply negative suction to the holding pipette and simultaneous positive pressure to the injection pipette. Debris attached to the outside of the injection pipette can be cleaned by rubbing the pipette against the holding pipette, against the oocyte, or against the oil at the edge of the medium drop. It may be necessary (and preferable) to change the pipette if it cannot be quickly cleared.

Sperm preparation after retrograde ejaculation and electroejaculation

When treating patients with ejaculatory dysfunction, with or without the aid of electroejaculation, both antegrade and retrograde ejaculation (into the bladder) are commonly found. When retrograde ejaculation is anticipated, the patient should first empty his bladder, and then drink an alkaline drink (e.g., bicarbonate of soda), and empty his bladder again 30 minutes

later before producing a sample that can then be collected from a urine sample by centrifugation. In cases of spinal cord injury, the bladder is emptied via a catheter and approximately 20 mL of culture medium then instilled. After electroejaculation, the bladder is again emptied, and the entire sample centrifuged. The resulting pellet(s) are resuspended in medium and processed on appropriate density gradients. As with all abnormal semen samples, a flexible approach is required in order to obtain a suitable sample for insemination; ICSI is recommended as the treatment of choice.

Surgical sperm retrieval

Until the mid-1990s, virtually all patients with obstructive or nonobstructive azoospermia (see Appendix for list of pathologies) had untreatable male sterility; this situation was completely reversed by the ability to combine ICSI with surgical techniques to recover samples from the epididymis and directly from the testis.

1. Epididymal sperm can be obtained by open microscopic surgery (MESA) or by percutaneous puncture (PESA), using a 21-gauge "butterfly" or equivalent needle to aspirate fluid. If large numbers of sperm are found, they can be processed by buoyant density gradient centrifugation. Samples with fewer sperm can be washed and centrifuged with IVF medium a number of times, and the concentrated sample is then added to microdroplets in the injection dish. Motile spermatozoa "swim out" to the periphery of the droplets, where they can be collected and transferred to clean drops of medium for injection later.
2. Testicular sperm is obtained by open biopsy (testicular sperm extraction, TESE) or by percutaneous needle biopsy (testicular fine needle aspiration, TEFNA), and samples can be processed in a variety of ways:
   - Crush the biopsy sample between two microscope slides, and expose sperm by shredding the tissue either with glass slides, by needle dissection, by dissection using microscissors, or by maceration using a microgrinder (available from Hunter Scientific). Concentrate the debris by centrifugation and examine under high-power microscopy to look for spermatozoa. Large quantities of debris are invariably present, and it may be difficult to find sperm (especially with cases of focal spermatogenesis). Further processing steps will depend upon the quality of the sample: it may be loaded onto a small single-step buoyant density gradient, or sperm simply harvested "by hand" under the microscope. Use a large needle, assisted hatching pipette or biopsy pipette to collect and pool live sperm in a clean drop of medium.
   - Tubules in the biopsy sample can be carefully unraveled under the dissecting microscope, using fine watchmakers' forceps. Cut the tubules into small lengths of 1–2 cm, and "milk" the contents by squeezing from the middle to an open end (analogous to squeezing a tube of toothpaste). The cells can be picked out of the dish and examined under the ICSI microscope, or placed into a centrifuge tube of clean medium for further preparation. An alternative approach is to slit the segments of tubule rather than "milking" to release the cells. Fresh testicular sperm are often immotile and combined with Sertoli cells, but free-swimming sperm are usually seen after further incubation. In cases of obstructive azoospermia, pregnancies have been achieved from testicular sperm incubated up to 3 days after the biopsy procedure, but the proportion of motile sperm in a testicular biopsy sample is usually highest after 24 hours' incubation. Incubation at 32°C instead of 37°C may also be of benefit (Van den Berg, 1998). If fresh sperm is to be used, the biopsy procedure should be carried out the day before the planned oocyte retrieval. In cryopreserved samples, a higher proportion of frozen testicular sperm have been found to retain their motility on thawing if they have been incubated for 24–48 hours before freezing; however, in cases of nonobstructive azoospermia, incubation for longer than 24 hours is not recommended. When biopsied sperm are processed in advance, any motile sperm found using an injection needle can be stored in empty zonae before freezing (see Chapter 12); this has the advantage that the sperm are then readily available at the time of ICSI, which can save considerable time.

Spermatid identification

In some cases of severe testicular dysfunction, no spermatozoa can be found either in the ejaculate or in testicular tissue, but precursor cells (round, elongating or elongated spermatids) may be identified. Although there was initial enthusiasm in the late 1990s with the technique of round spermatid nucleus injection (ROSNI), this was short-lived with the prospect of unresolved genetic concerns and poor activating capacity of the immature sperm cells. Spermatid injection is forbidden by the HFEA in the UK, and also by regulatory authorities in some other countries. Males with meiotic arrest of spermatogenesis are counseled towards the use of donor sperm.

Using Hoffman Modulation Contrast systems, four categories of spermatids can be observed and identified according to their shape, amount of cytoplasm, and size of tail: round, elongating, elongated and mature spermatids just prior to their release from Sertoli cells. However, in practice it can be difficult to confidently identify immature sperm cells in a wet preparation. Round spermatids must be distinguished from other round cells such as spermatogonia, spermatocytes, polymorphonuclear leukocytes, lymphocytes and erythrocytes. Their diameter (6.5–8 μm) is similar to that of erythrocytes (7.2 μm) and small lymphocytes. Round spermatids may be observed at three different phases: Golgi, cap and acrosome phase (where the nucleus moves towards a peripheral position). When the cell is rotated, a centrally located smooth (uncondensed) nucleus can be seen, and a developing acrosomal structure may be observed as a bright spot or small protrusion on one side of the cell, adjacent to the spermatid nucleus. Sertoli cell nuclei are very flat and transparent, with a prominent central or adjacent nucleus, whereas the ROS is a three-dimensional round cell (Figure 10.3a). Phase 3 is a transition between round and elongated forms – elongated spermatids have an elongated nucleus at one side of the cell, and a larger cytoplasmic region on the other side, surrounding the developing tail (Figure 10.3b).

Pathology of azoospermia

The Johnsen score is an assessment of the degree of spermatogenesis found in a biopsy: a number of tubules are assessed, and each one is given a score for the most advanced stage of spermatogenesis seen:

1 = no cells present in the tubule
2 = Sertoli cells
3 = spermatogonia

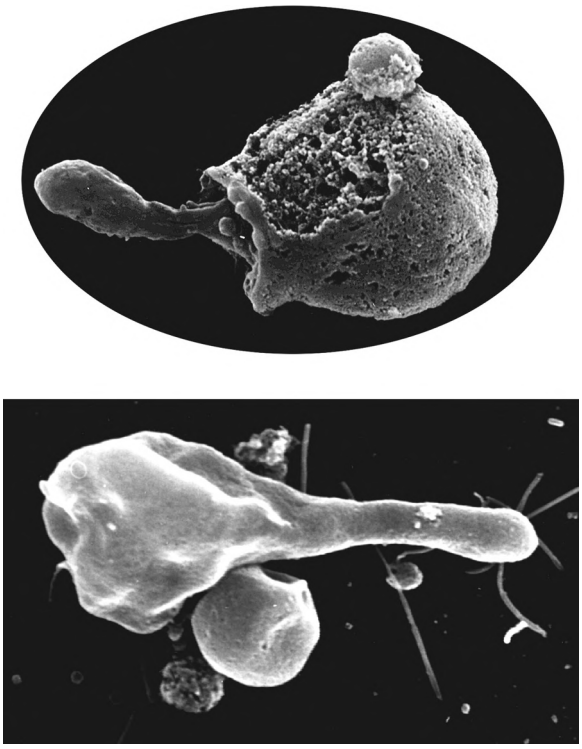

Figure 10.3 (a and b) Scanning electron micrographs of early spermatid detected in azoospermic ejaculate. (Courtesy of Professor B. Bartoov, Israel.)

4–5 = spermatocytes
6–7 = spermatids
8–10 = spermatozoa.

Mean Johnsen score (MJS) = average of all the tubules assessed, i.e.:

MJS = 2 is the Sertoli cell only syndrome
MJS = 8–10 is normal spermatogenesis
MJS between 2 and 8 represents varying degrees of subnormal spermatogenesis, but a qualitative description is required.

There is a correlation between testicular size and MJS.

Pathologies

A. Pretesticular: deficient gonadotropin drive – low FSH
B. Androgen resistance: familial pseudohermaphroditism
C. Testicular failure: no spermatogenesis – raised FSH

D. Post-testicular duct obstruction: functional sperm usually present, size of testes is normal, FSH is not raised.

Pretesticular

These are pathologies that result in secondary testicular failure (hypogonadotropic hypogonadism) due to decreased gonadotropin release (low serum FSH). Testicular biopsy may show a prepubertal appearance, with precursors of Sertoli cells, prespermatogenic cells and absence of Leydig cells.

1. Congenital
   - partial or complete Kallman's syndrome, GnRH deficiency associated with agenesis of the first cranial nerve and thus anosmia
   - low FSH and LH, small but potentially normal testes.
2. Acquired: space-occupying lesions
   - pituitary tumors
   - craniopharyngioma
   - trauma, meningitis, sarcoidosis
   - Cushing's syndrome (adrenal hypoplasia)
   - congenital adrenal hyperplasia
   - hemochromatosis.

Androgen resistance

Familial incomplete male pseudohermaphroditism, type 1: partial or complete defects in amount or function of the androgen receptor. Patients fall into a wide spectrum of disorders, probably due to variable manifestations of a single gene defect. Cryptorchidism is common, and the testes remain small in size. The testes demonstrate normal Leydig cells and tubules containing both germ cells and Sertoli cells, but there is usually no maturation beyond the primary spermatocyte. Plasma testosterone and LH are high, suggesting that there is a defect in the feedback control of testosterone on the hypothalamus. There are four (phenotypically) separate clinical disorders:

1. Rosewater's syndrome (mildest form)
2. Reinfenstein's syndrome
3. Gilbert–Dreyfus syndrome
4. Lub's syndrome (most severe) – phenotypic females with partial Wolffian duct development and masculine skeletal development.

Testicular failure

This can be congenital or acquired, and testicular biopsy can show a wide variation in appearance, e.g.: Sclerosing tubular degeneration is seen in Klinefelter's syndrome. Disorganization with extensive hyalinization and tubular atrophy is seen after orchitis.

1. Congenital
   - Klinefelter's syndrome (XXY)
   - autosomal abnormalities
   - torsion (maturation arrest)
   - cryptorchidism, anorchia
   - sickle cell disease
   - myotonic muscular dystrophy
   - Noonan's syndrome (male Turner's).
2. Acquired
   - mumps orchitis
   - epididymo-orchitis
   - testicular trauma
   - inguinal/scrotal surgery
   - radiotherapy.

Post-testicular: obstructive causes of azoospermia

Testicular biopsy shows well-preserved normal spermatogenesis, and there may be sloughing of superficial layers of the seminiferous epithelium. The upper epididymis is the most common site of genital tract obstruction (two-thirds of lesions), and multifocal sites of obstruction may be present. Obstructive lesions can be caused by specific or nonspecific infection, and edema and/or hematoma as a result of trauma can lead to epididymal or vasal obstruction.

1. Congenital:
   - Congenital absence of the vas deferens (CAVD – female partner should be screened for cystic fibrosis mutations)
   - Cystic fibrosis
   - Young's syndrome
   - Zinner's syndrome: congenital absence of the vas deferens, corpus and cauda epididymis, seminal vesicle, ampulla and ejaculatory duct – may be bilateral or unilateral and can be associated with ipsilateral renal agenesis – due to failure of the Wolffian (mesonephric) duct.
2. Acquired
   - TB
   - Gonococcal or chlamydial infection
   - surgical trauma
   - smallpox
   - bilharziasis

- filariasis
- vasectomy.

Other causes of spermatogenic failure or disorder

These may be associated with defective testosterone synthesis, decreased metabolic clearance rates, increased binding of testosterone to plasma proteins, increased plasma estradiol and low, normal or moderately elevated serum FSH levels.

1. Systemic illness
   fevers, burns, head trauma
   chronic renal failure
   thyrotoxicosis, diabetes
   male anorexia nervosa
   surgery, general anesthesia.
2. Drugs/industrial toxins
   (a) Therapeutic: sulfasalazine, nitrofurantoin, cimetidine, niridazole, colchicine, spironolactone, testosterone injections, cytotoxic agents
   (b) Occupational: carbon disulfide (rayon), lead, dibromochloropropane, radiation
   (c) Recreational abuse: alcohol, opiates, anabolic steroids.
3. Absent spermatogenesis: germinal aplasia or hypoplasia, Sertoli cell only (del Castillo) syndrome. Only Sertoli cells are present in the tubular epithelium, none of the spermatogenic elements remain. In germinal cell hypoplasia, there is a generalized reduction in the numbers of germ cells of all stages. The numbers of more mature cells are greatly reduced, and the germinal epithelium has a loose, poorly populated appearance. There are two forms:
   (a) serum FSH is grossly elevated, Sertoli cells show severe abnormalities on electron microscopy – no inhibin production
   (b) serum FSH is normal – normal inhibin production.
   Testis size is often not markedly reduced, and this may lead to diagnostic difficulties. These patients are frequently misdiagnosed as having an obstructive lesion, and biopsy is the only means of making a correct diagnosis.
4. Leydig cell failure leads to low testosterone levels, and raised serum FSH and LH. In this situation the testis is atrophied, with gross reduction in size.
5. Immotile sperm – Kartagener's immotile cilia syndrome. Normal numbers of sperm are present in the semen, but they are all immotile. Transmission electron microscopy shows that the central filaments of the tails are absent, and this anomaly may be present in cilia throughout the body, resulting in chronic sinusitis and bronchiectasis.
6. Retrograde ejaculation: diabetes, multiple sclerosis, sympathectomy, prostatectomy, funnel bladder neck.
7. Ejaculatory failure: spinal cord injury, multiple sclerosis, diabetes, abdominal aortic surgery, abdominoperineal resection, psychomimetic/antihypertensive drugs, hypogonadism.

Chromosomal anomalies

Klinefelter's syndrome

- Bilateral testicular atrophy, signs of hypogonadism with a greater span than height, often with gynecomastia.
- FSH and LH are extremely high, often with low testosterone.
- Diagnosis can be made on clinical and biochemical grounds, and confirmed by buccal smear or karyotype (XXY).
- Affects 1 in 400 live-born males, and is found in around 7% of infertile men.
- Testicular histology: obvious and gross spermatogenic failure with disappearance of all the spermatogenic elements in all the tubules. Marked hyperplasia of the Leydig cells.

46XX Klinefelter's

Patients are phenotypically male, with same clinical and endocrinological features as the XXY patient. H-Y antigen has been demonstrated: despite apparent absence of Y chromosome, there is expression of some Y genes.

46XX (Noonan's syndrome)

Male equivalent of Turner's (XO): normal male phenotype, but are usually cryptorchid and show varying degrees of hypoandrogenization.

There is testicular atrophy, raised FSH and LH, and reduced testosterone.

Robertsonian translocation

A form of chromosomal aberration which involves the fusion of long arms of acrocentric chromosomes at the centromere. Breaks occur at the extreme ends of the short arms of two nonhomologous acrocentric chromosomes; these small segments are lost, and the larger segments fuse at their centromeric region, producing a new, large submetacentric or metacentric chromosome.

"Balanced translocations" may produce only minor deficiencies, but translocation heterozygotes have reduced frequencies of crossing over and are usually subfertile through the production of abnormal gametes.

Appendix

Sperm preparation: equipment and materials

Semen sterile collection pot 60 mL
Microscope (phase is useful)
Counting chamber (Makler, Sefi Medical Instruments, POB 7295, Haifa 31070 Israel, or Horwell Haemocytometer)
Centrifuge with swing-out rotor (Mistral 1000, MSE)
Centrifuge tubes (15 mL, Corning)
Microscope slides
Coverslips
Disposable test-tubes: 4 mL, 10 mL
Culture media
Buoyant density media:
- Pure Sperm (Scandinavian IVF AB)
- IxtaPrep (MediCult)
- Sil-Select (MICROM)
- Isolate (Irvine Scientific)

Glass Pasteur pipettes
Disposable pipettes: 1, 5, 10 mL
Spirit burner + methanol or gas Bunsen burner
Plastic ampules or straws for sperm freezing
Sperm cryopreservation media (Chapter 12)
Supply of liquid nitrogen and storage dewars

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Chapter 11

Oocyte retrieval and embryo culture

Preparation for each case

Every individual treatment cycle involves a number of different stages and manipulations in the laboratory, and each case must be assessed and prepared for in advance; the afternoon prior to the procedure (the day after hCG administration) is a convenient time to make the preparations. The laboratory staff should ensure that all appropriate consent forms have been signed by both partners, including consent for special procedures and storage of cryopreserved embryos. Details of any previous assisted conception treatment should be studied, including response to stimulation, number and quality of oocytes, timing of insemination, fertilization rate, embryo quality and embryo transfer procedure, and judgements regarding whether any parameters at any stage could be altered or improved in the present cycle should be made. The risk of introducing any infection into the laboratory via gametes and samples must be absolutely minimized: screening tests such as human immunodeficiency virus (HIV 1 and 2: Anti-HIV 1, 2) and hepatitis B (HbsAg/Anti-HBc) and C (Anti-HCV-Ab) should be confirmed, as well as any other tests indicated by the patients' history (e.g., HTLV-I antibody, RhD, malaria, *Trypanosoma cruzi*). If donor gametes are to be used, additional tests for the donor are required: chlamydia, cytomegalovirus and a validated testing algorithm to exclude the presence of active infection with *Treponema pallidum* for syphilis testing.

Laboratory preparation: checklist for each case

- Results of viral screening tests for both partners
- Consent forms signed by both partners
- Specific details or instructions regarding insemination, cryopreservation, number of embryos for transfer, etc.
- Results of semen assessments; note any special features or precautions for semen collection or preparation
- Previous history, current response to stimulation; note any special features of ultrasound scans, endocrine assays, or IVF laboratory results
- Current cycle history: number of follicles, endocrine parameters, potential ovarian hyperstimulation syndrome (OHSS)

Laboratory case notes, media, culture vessels and tubes for sperm preparation, with clear and adequate labeling throughout should be prepared. All labeling should have a minimum of the patient's full name and a unique identifier, for example patient number. When donor sperm is used, the donor code should uniquely identify that specific donor. Tissue culture dishes or plates must be equilibrated in the CO_2 incubator overnight. The choice of culture system used is a matter of individual preference and previous experience; microdroplets under oil, four-well dishes and organ culture dishes are amongst those most commonly used.

Microdroplets under oil

- Pour previously equilibrated mineral oil into 60 mm Petri dishes that are clearly marked with each patient's full name.
- Using either a Pasteur pipette or adjustable pipettor and sterile tips, carefully place eight or nine droplets of medium around the edge of the dish. One or two droplets may be placed centrally, to be used as wash drops.
- Examine the follicular growth records to assess approximately how many drops/dishes should be prepared; each drop may contain one or two oocytes. Droplet size can range from 50 to 250 µL per droplet.

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Four-well plates

This system may also be used in combination with an overlay of equilibrated mineral oil.

- Prepare labeled and numbered plates containing 0.5–1 mL of tissue culture medium; each well is normally used to incubate up to three oocytes.
- Equilibrate overnight – if used without an oil overlay, the incubator must be humidified.
- Small Petri dishes with approximately 2 mL of HEPES-containing medium may also be prepared, to be used for washing oocytes immediately after identification in the follicular aspirates (media containing HEPES should not be equilibrated in a CO_2 atmosphere).

Organ culture (center-well) dishes can also be used for group culture: place three 250 µL drops in the center well, and three to four oocytes in each drop.

Laboratory supplies for labeling and preparation

- Pots for semen collection
- Test-tubes for sperm preparation: conical tubes, small and large round-bottomed tubes
- Aliquots of media for each patient
- Culture vessels for overnight equilibration
- Paperwork for recording case details and results

Oocyte retrieval (OCR) and identification

A Class II biological safety cabinet is recommended for handling of follicular aspirates to avoid risk of infection, but be aware that the laminar flow of air can have a dramatic cooling effect on the samples.

Prior to the follicle aspiration procedure:

1. Ensure: heating blocks, stages and trays are warmed to a temperature that will maintain the medium in the dishes at 37°C; media to be used for flushing/rinsing must also be warmed and equilibrated to correct pH.
2. Prewarm collection test-tubes and 60 mm Petri dishes for scanning aspirates.
3. Prepare a sterile Pasteur pipette plus holder, a fine-drawn blunt Pasteur pipette as a probe for manipulations, and 1 mL syringes with attached needles for dissection.
4. Check patient ID, confirm names and unique identifiers on dishes and laboratory case notes with medical notes.

If follicular aspirates cannot be examined immediately, they should be collected into test-tubes that are completely filled with fluid, tightly sealed, and rigorously maintained at 37°C until they reach the laboratory. Aliquot the contents of each test-tube into two or three Petri dishes, forming a thin layer of fluid that can be quickly, carefully and easily scanned for the presence of an oocyte, using a stereo dissecting microscope with transmitted illumination base and heated stage. Low-power magnification (×6–12) can be used for scanning the fluid, and oocyte identification verified using higher magnification (×25–50). Always work quickly and carefully, with rigid attention to sterile technique, maintaining correct temperature and pH at all times.

Oocyte identification

The oocyte usually appears within varying quantities of cumulus cells and, if very mature, may be pale and difficult to see (immature oocytes are dark and also difficult to see). Granulosa cells are clearer and more "fluffy," present in amorphous, often iridescent clumps. Blood clots, especially from the collection needle, should be carefully dissected with 23-gauge needles to check for the presence of cumulus cells.

The presence of blood clots within the cumulus–oocyte complex (COC) may be a reflection of poor follicular development, with an effect on the competence of the corresponding oocyte (Ebner, 2008). When a COC is identified, assess its stage of maturity by noting the volume, density and condition of the surrounding cumulus cells and the expansion of coronal cells. It is unlikely that the oocyte itself can be seen, since it will most commonly be surrounded by cumulus cells. However, when an oocyte can be observed with minimal cumulus cells, the presence of a single polar body indicates that it has reached the stage of metaphase II. The appearance of the COC can be used to classify the oocyte according to the following scheme (Figure 11.1):

1. *Germinal vesicle*: the oocyte is very immature. There is no expansion of the surrounding cells, which are tightly packed around the oocyte. A large nucleus (the germinal vesicle) is still present and may occasionally be seen with the help of an inverted microscope. Maturation occasionally takes place in vitro from this stage, and germinal vesicles are preincubated for 24 hours before insemination (Figure 11.1e).
2. *Metaphase I*: the oocyte is surrounded by a tightly apposed layer of corona cells; tightly

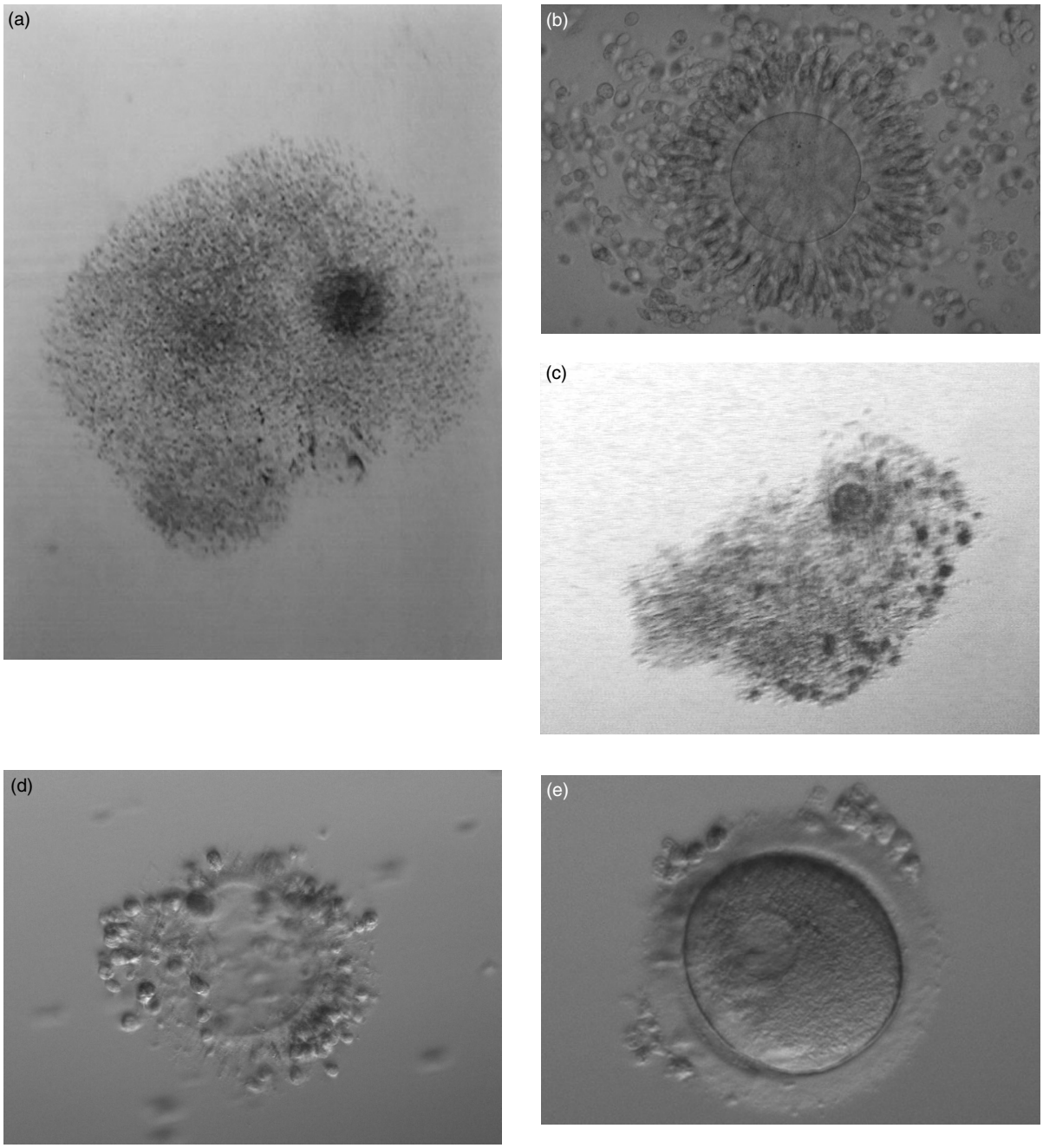


Figure 11.1 Images after oocyte retrieval, before and after hyaluronidase denudation.
Cumulus–oocyte complexes, without denudation: (a) Cumulus–oocyte complex visualized under dissecting microscope, ×25 magnification. See color plate section; (b) phase-contrast image of mature metaphase II cumulus–oocyte complex, first polar body visible. See color plate section; (c) cumulus–oocyte complex with clumped refractile areas indicating signs of luteinization. See color plate section; (d) empty zona pellucida.
Phase contrast images after denudation: (e) Germinal vesicle; (f) metaphase I oocyte, polar body not extruded; (g) preovulatory metaphase II oocyte, polar body extruded; (h) postmature oocyte, showing granularity in the perivitelline space; (i) dysmorphic metaphase II oocyte showing a large necrotic first polar body. (Images (h) and (i) courtesy of Thomas Ebner, Austria.)

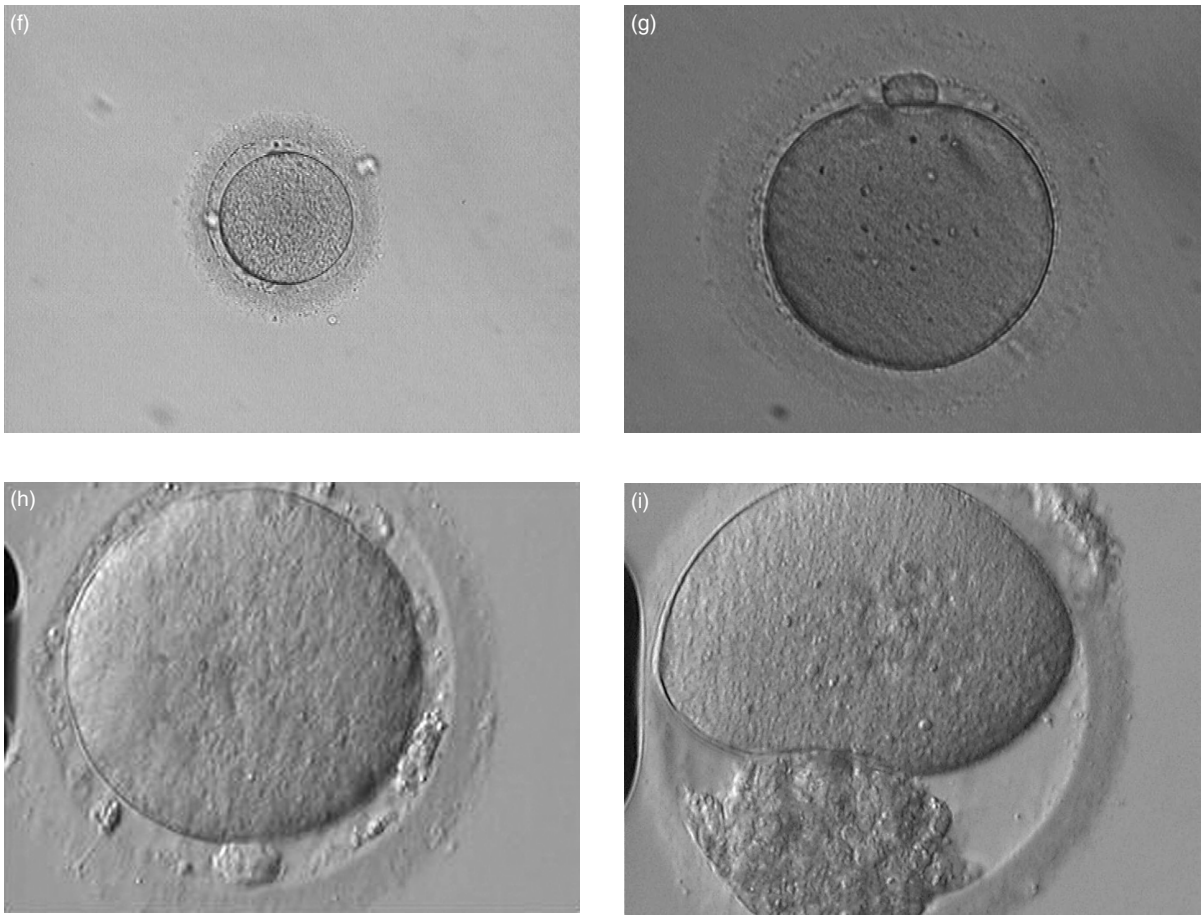


Figure 11.1 (*cont.*)

packed cumulus with little extracellular matrix may surround this with a maximum size of approximately five oocyte diameters. If the oocyte can be seen, it no longer shows a germinal vesicle. The absence of a polar body indicates that the oocyte is in metaphase I, and these immature oocytes can be preincubated for 6–24 hours before insemination (Figure 11.1f).

3. *Metaphase II*
   (a) *Preovulatory* (harvested from Graafian follicles): this is the optimal level of maturity, appropriate for successful fertilization. Coronal cells are still apposed to the oocyte, but are fully radiating; one polar body has been extruded. The cumulus has expanded into a fluffy viscous mass that can be easily stretched, with abundant extracellular matrix (Figure 11.1a and b).
   (b) *Mature*: the oocyte can often be seen clearly as a pale orb; little coronal material is present and is dissociated from the oocyte. The cumulus is very profuse but is still cellular. The latest events of this stage involve a condensation of cumulus into small black (refractile) drops, as if a tight corona is reforming around the oocyte. The perivitelline space often shows granularity (Figure 11.1h).
   (c) *Luteinized*: the oocyte is very pale and often is difficult to find. The cumulus has broken down and becomes a gelatinous mass around the oocyte. These oocytes have a low probability of fertilization, and are usually inseminated with little delay (Figure 11.1).
   (d) *Atretic*: the oocyte is very dark, and can be difficult to identify. Granulosa cells are fragmented, and have a lace-like appearance.

Gross morphological assessment of oocyte maturity is highly subjective, and open to inaccuracies. In preparation for intracytoplasmic sperm injection (ICSI), the oocytes are completely denuded of surrounding cells using hyaluronidase, allowing accurate assessment of nuclear maturity and cytoplasm; this process has made it apparent that gross COC morphology does not necessarily correlate with nuclear maturity, and there is considerable conflict in the data available regarding the association between oocyte morphology and treatment outcome (see Ebner, 2006, for review). A number of dysmorphic features can be identified in denuded oocytes, including areas of necrosis, organelle clustering, vacuolation or accumulating aggregates of smooth endoplasmic reticulum (sER). Anomalies of the zona pellucida and nonspherical oocytes can also be seen. In practice, a wide variety of unusual and surprising dysmorphisms are often observed – please refer to the image database at www.ivf.net for an interesting collection of photographs and histories.

Some features of dysmorphism may be associated with the endocrine environment during ovarian stimulation, in particular the structure of the zona pellucida and/or oolemma (Ebner, 2002, 2006). Although aberrations in the morphology of oocytes are not necessarily of any consequence to fertilization or early cleavage after ICSI, it is possible that embryos generated from dysmorphic oocytes have a reduced potential for implantation and further development. Repeated appearance of some dysmorphic features such as sER aggregation, central granulation or vacuoles in an individual patient's oocyte cohort may indicate an underlying intrinsic problem in the process of oocyte development within the ovary.

Dysmorphic oocyte features (Figure 11.2)

- Irregular shape
- Areas of necrosis in the cytoplasm
- Cytoplasmic granularity
- Organelle clustering
- Aggregates of sER
- Vacuoles/Vesicles
- Anomalies of the zona pellucida

Van Blerkom and Henry (1992) reported aneuploidy in 50% of oocytes with cytoplasmic dysmorphism; it is not clear whether oocyte aneuploidy is a fundamental developmental phenomenon, or a patient-specific response to induced ovarian stimulation. In 1996, the same group related the oxygen content of human follicular fluid to oocyte quality and subsequent implantation potential. They propose that low oxygen tension associated with poor blood flow to follicles lowers the pH and produces anomalies in chromosomal organization and microtubule assembly, which might cause segregation disorders. Measurement of blood flow to individual follicles by power color Doppler ultrasound (Gregory and Leese, 1996) confirmed the observations of Van Blerkom *et al.* (1997) in correlating follicular blood flow with implantation; the incidence of triploid zygotes was also found to be significantly higher when oocytes were derived from follicles with poor vascularity. Follicular vascularity may also influence free cortisol levels in follicular fluid by promoting its diffusion across the follicle boundary.

Insemination

Oocytes are routinely inseminated with a concentration of 100 000 progressively motile sperm per milliliter. If the prepared sperm show suboptimal parameters of motility or morphology, the insemination concentration may be accordingly increased. Some reports have suggested that the use of a high insemination concentration of up to 300 000 progressively motile sperm per milliliter may be a useful prelude before deciding upon ICSI treatment for male factor patients. Traditionally, inseminated oocytes were incubated overnight in the presence of the prepared sperm sample; however, sperm binding to the zona pellucida normally takes place within 1–3 hours of insemination, and fertilization occurs very rapidly thereafter. A few hours of sperm–oocyte contact, yields the same time course of events that is observed after overnight incubation, and oocytes can be washed free of excess sperm after 3 hours incubation (Gianaroli *et al.*, 1996; Ménézo and Barak, 2000).

For a culture system of microdroplets under oil, each oocyte is transferred into a drop containing motile sperm at a concentration of approximately 100 000 sperm/mL. In a four-well system, a measured volume of prepared sperm is added to each well, to a final concentration of approximately 100 000 progressively motile sperm per well.

Insemination

For microdroplets under oil, the oil overlays for insemination dishes must be prepared earlier, so that there is at least 4–6 hours of equilibration time.

1. Prepare a dilution of prepared sperm, containing 100 000 motile sperm/mL

- Assess a drop of the dilution on a glass slide, under ×10 magnification – at least 20 motile sperm should be visible in the field.
- Equilibrate the suspension at 37°C for 30 minutes, 5% CO_2.
- Place droplets of the sperm suspension under the previously prepared and equilibrated oil overlays.
- Examine each oocyte before transfer to the insemination drop, and dissect the cumulus to remove bubbles, large clumps of granulosa cells or blood clots if necessary.

2. If oocytes are in pre-measured culture droplets, e.g., 240 µL, add 10 µL of a prepared sperm suspension that has been adjusted to 2.5 ×10^6/mL.
   - Final concentration = approximately 100 000 sperm/mL, or 25 000 sperm per oocyte.
3. Prepare labeled 35 mm Petri dishes containing equilibrated oil, to be used for culture of the zygotes after scoring for fertilization the following day·

Four-well dishes and organ culture dishes

- Add 0.5–1.0 mL of prepared sperm suspension to each well or drop.
- Total: approximately 100 000 motile sperm per well/drop.

Scoring of fertilization on Day 1

Dissecting fertilized oocytes

Inseminated oocytes are dissected 17–20 hours following insemination in order to assess fertilization. Oocytes at this time are normally covered with a layer of dispersed coronal and cumulus cells, which must be carefully removed so that the cell cytoplasm can be examined for the presence of two pronuclei and two polar bodies, indicating normal fertilization. The choice of dissection procedure is a matter of

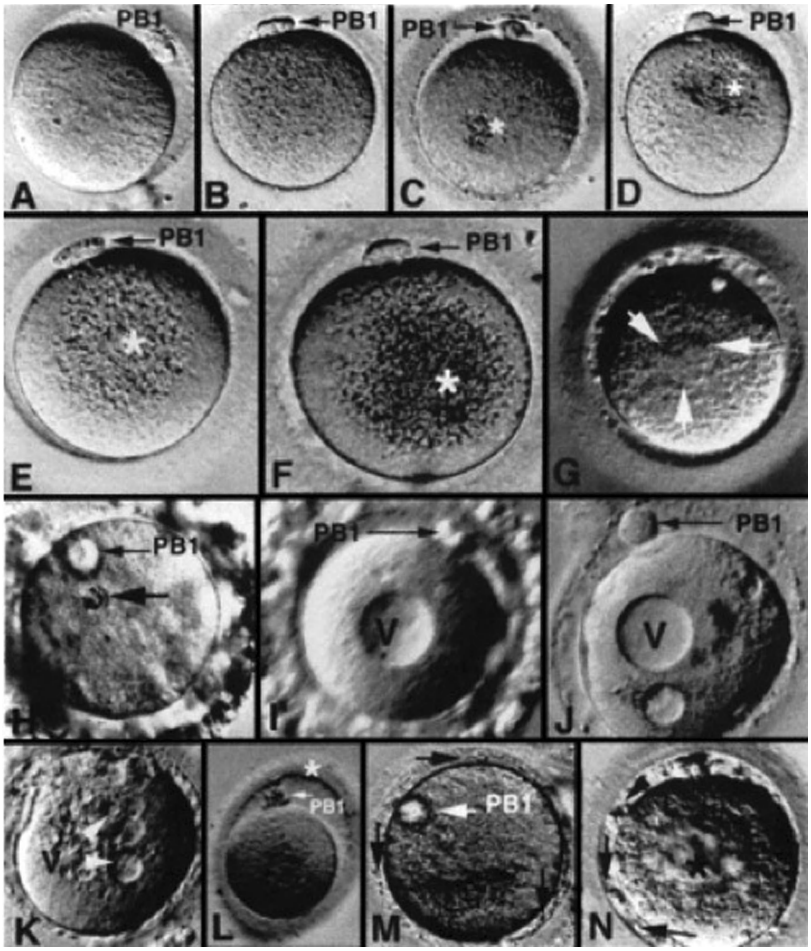


Figure 11.2 Normal and dysmorphic oocytes (A, B). Normal appearing oocytes with no visually outstanding features (C–F). Varying degrees of organelle clusters (*) (central granularity) observed from mild to very severe. (G, N) Aggregation (arrows) of smooth endoplasmic reticulum as a flat, clear disc in the middle of the cytoplasm of the oocyte. (H) A dark "horse shoe shaped" (large arrow) cytoplasmic inclusion. (I, J, K) Varying degrees (mild to severe) of fluid-filled vacuoles within the cytoplasm. (L) Organelle cluster with fragmented polar body (arrow) and increased perivitelline debris (*) and space. (K–M) Combination of cytoplasmic dysmorphisms and extracytoplasmic phenotypes. PB1 = first polar body. (From: Meriano *et al.*, 2001 *Human Reproduction* 16(10): 2118–23, with permission from Oxford University Press).

individual preference, and sometimes a combination of methods may be necessary for particular cases. Whatever the method used, it must be carried out carefully, delicately and speedily, taking care not to expose the fertilized oocytes to changes in temperature and pH. Scoring for pronuclei should be carried out within the appropriate time span, before pronuclei merge during syngamy: cleaved embryos with abnormal fertilization are indistinguishable from those with two pronuclei.

Dissection techniques

1. *Narrow-gauge pipetting*: narrow-gauge pipettes can be made (see below), but commercial hand-held pipetting devices are simpler and more convenient. "Flexipet" and "Stripper" are hand-held pipetting devices for cumulus/corona removal, with sterile disposable polycarbonate capillaries of specified inner diameters ranging from 135 up to 175 or 600 μm. Variations that incorporate a capillary that attaches to a tiny pressure "bulb" inserted into a hollow metal tube are also available, e.g., Swemed.
   - Use the microscope at ×25 magnification, and choose a tip with a diameter slightly larger than the oocyte (a tip that is too small will damage the oocyte, therefore take care in selecting the appropriate diameter).
   - Aspirate approximately 2 cm of clean culture medium into the tip, providing a protective buffer. This allows easy flushing of the oocyte, and prevents it from sticking to the inside surface of the tip.
   - Place the tip over the oocyte and gently aspirate it into the shaft.
   - If the oocyte does not easily enter, change to a larger diameter pipette (however, if the diameter is too large, it will be ineffective for cumulus removal).
   - Gently aspirate and expel the oocyte through the pipette, retaining the initial buffer volume, until sufficient cumulus and corona is removed to allow clear visualization of the cell cytoplasm and pronuclei.
2. *Needle dissection*: use two 26-gauge needles attached to 1 mL syringes, microscope at ×25 magnification. Use one needle as a guide, anchoring a piece of cellular debris if possible; slide the other needle down the first one, "shaving" cells from around the zona pellucida, with a scissors-like action.
3. *Rolling*: use one 23-gauge needle attached to a syringe, and a fine glass probe. With the microscope at ×12 magnification, use the needle to score lines in each droplet on the base of the plastic dish. Adjust the magnification to ×25, and push the oocyte gently over the scratches with a fire polished glass probe until the adhering cells are teased away. This technique may be helpful to remove adherent sticky blood clots. However, it should not be used with dishes that are coated with a specific non-embryotoxic layer.

Great care must be taken with any technique to avoid damaging the zona pellucida or the oocyte either by puncture or overdistortion. Breaks or cracks in the zona can sometimes be seen, and a small portion of the oocyte may extrude through the crack (this may have occurred during dissection or during the aspiration process). Occasionally the zona is very fragile, fracturing or distorting at the slightest touch; it is probably best not to continue the dissection in these cases.

Making narrow-gauge pipettes

The preparation of finely drawn pipettes with an inner diameter slightly larger than the circumference of an oocyte is an acquired skill which requires practice and patience.

- Hold both ends of the pipette, and roll an area approximately 2.5 cm below the tapered section of the pipette over a gentle flame (Bunsen or spirit burner).
- As the glass begins to melt, quickly pull the pipette in both directions to separate.
- Before the glass has a chance to cool, carefully and quickly break the pipette at an appropriate position.
- The tip must have a clean break, without rough or uneven edges; these will damage the oocyte during dissection.
- Examine the tip of each pipette to ensure that it is of accurate diameter, with smooth clean edges.

Pronuclear scoring

An inverted microscope is recommended for accurate scoring of fertilization; although the pronuclei can be seen with dissecting microscopes, it can often be difficult to distinguish normal pronuclei from vacuoles or other irregularities in the cytoplasm. Normally fertilized oocytes should have two pronuclei, two polar

bodies, regular shape with intact zona pellucida, and a clear healthy cytoplasm. A variety of different features may be observed: the cytoplasm of normally fertilized oocytes is usually slightly granular, whereas the cytoplasm of unfertilized oocytes tends to be completely clear and featureless. The cytoplasm can vary from slightly granular and healthy-looking, to brown or dark and degenerate. The shape of the oocyte may also vary, from perfectly spherical to irregular (see Figure 11.2). A clear halo of peripheral cytoplasm 5–10 mm thick is an indication of good activation and re-initiation of meiosis. The pattern and alignment of nucleoli may also be significant (Scott and Smith 1998; Tesarik and Greco, 1999).

Approximately 5% of fertilized oocytes in human IVF routinely show abnormal fertilization, with three or more pronuclei visible; this is attributed to polyspermy, or non-extrusion of the second polar body. Fluorescent in-situ hybridization (FISH) analysis indicates that 80–90% of these zygotes are mosaic after cleavage. Single pronucleate zygotes obtained after conventional IVF analyzed by FISH to determine their ploidy, reveal that a proportion of these zygotes are diploid (Levron *et al.*, 1995). It seems that during the course of their interaction, it is possible for human gamete nuclei to associate together and form diploid, single pronucleate zygotes. These findings may indicate a variation of human pronuclear interaction during syngamy, and the authors suggest that single pronucleate zygotes which develop with normal cleavage may be selected for transfer in cases where no other suitable embryos are available.

Details of morphology and fertilization should be recorded for each zygote, for reference when choosing embryos for transfer. Remove zygotes with normal fertilization at the time of scoring from the insemination drops or wells, transfer into new dishes or plates containing pre-equilibrated culture medium, and return them to the incubator for a further 24 hours of culture. Those with abnormal fertilization such as multipronucleate zygotes should be discarded, so that there is no possibility of their being selected for embryo transfer; after cleavage these are indistinguishable from normally fertilized oocytes.

Although the presence of two pronuclei confirms fertilization, their absence does not necessarily indicate fertilization failure, and may instead represent either parthenogenetic activation, or a delay in timing of one or more of the events involved in fertilization (Figure 11.3). Numerous studies have accumulated evidence to demonstrate that up to 40% of oocytes with no sign of fertilization 17–27 hours after insemination may have the appearance of morphologically normal embryos on the following day, with morphology and cleavage rate similar to that of zygotes with obvious pronuclei on Day 1. However, around a third of these zygotes may subsequently arrest on Day 2 (Plachot *et al.*, 1993). Cytogenetic analysis of these embryos reveals a higher incidence of chromosomal anomalies and a high rate of haploidy, confirming parthenogenetic activation (Plachot *et al.*, 1988, 1993).

Delayed fertilization with the appearance of pronuclei on Day 2 may also be observed, and these embryos also tend to have an impaired developmental potential. Delayed fertilization can be attributed to morphological or endocrine oocyte defects in some cases, and to sperm defects in others. No obvious association with either oocyte or sperm defects can be found in a number of cases (Oehninger *et al.*, 1989).

Reinsemination

Reinsemination of oocytes that fail to demonstrate clear pronuclei at the time of scoring for fertilization is a practice that has been widely questioned scientifically. Fertilization or cleavage may subsequently be observed on day 2, but this may be as a consequence of the initial insemination, and the delay in fertilization may be attributed either to functional disorders of the sperm, or maturation delay of the oocyte. These embryos generally have a poor prognosis for implantation.

"Rescue ICSI" is another option, whereby unfertilized oocytes are microinjected with a single sperm from the original sample. This practice is banned in some countries such as the UK, since it cannot be certain if a sperm has already entered the oocyte and fertilization is delayed. Others reserve rescue ICSI only for cases where there is complete failure to fertilize following conventional IVF. A recent report indicates that a better pregnancy rate can be achieved following rescue ICSI if the fertilized oocytes are frozen and transferred in a subsequent frozen-thawed cycle, to ensure better synchronization of the embryo and uterus for implantation (Sermondade *et al.*, 2010).

Selection of pronucleate embryos for cryopreservation

Legislation in some countries forbids embryo freezing, but allows cryopreservation at the zygote stage, before

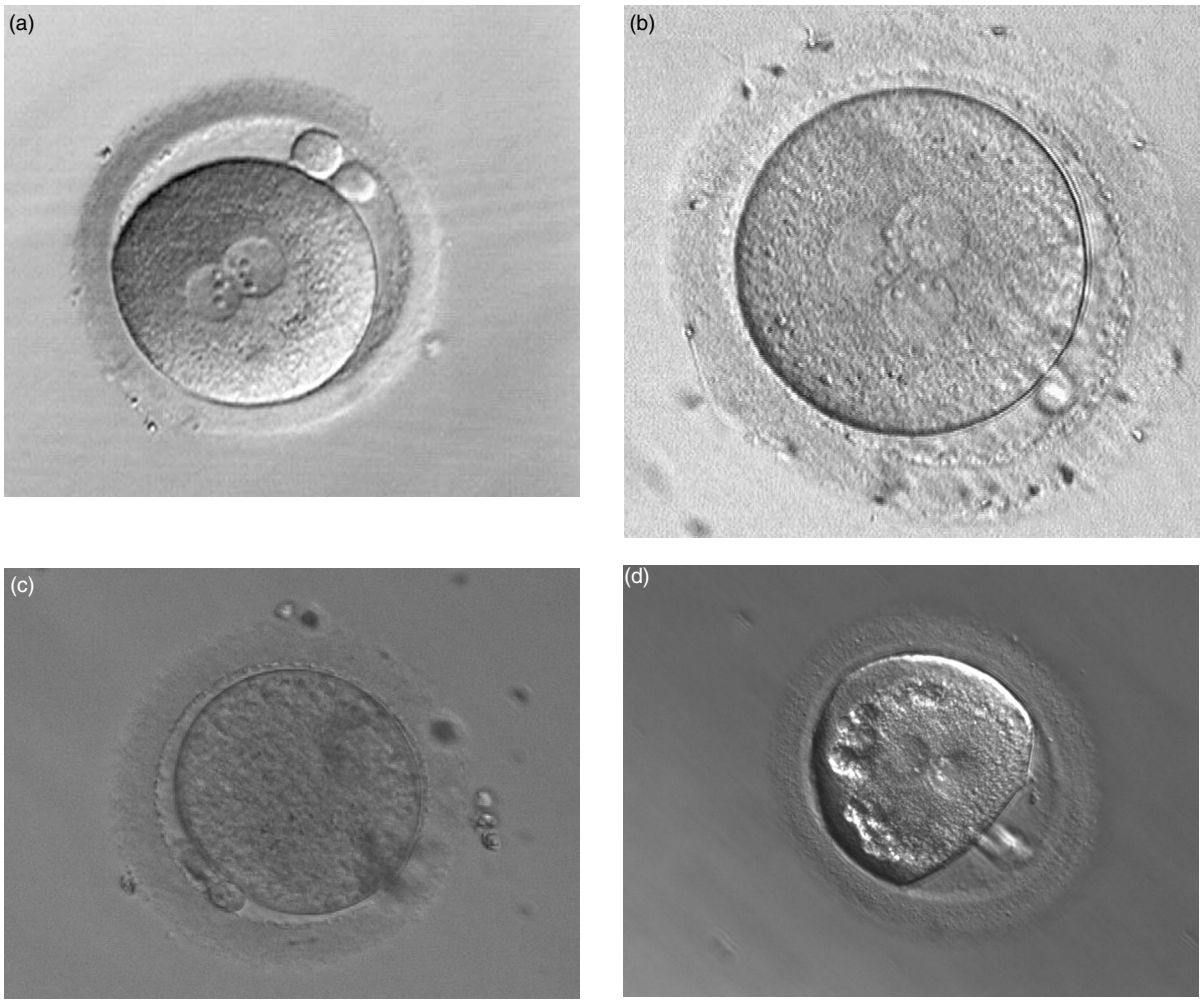


Figure 11.3 Phase contrast micrographs of fertilized human oocytes. (a) Normal fertilization: two pronuclei, one polar body. See color plate section. (b) Abnormal fertilization: three pronuclei. See color plate section. (c) Abnormal fertilization, no pronuclei, two polar bodies. (d) Zygote showing two pronuclei, numerous vacuoles, and irregular perivitelline space, illustrating that severely dysmorphic oocytes are capable of fertilization (with thanks to Marc van den Bergh).

syngamy. In countries that allow both zygote and embryo freezing, a patient who has a large cohort of oocytes with two clearly visible pronuclei on Day 1 may have a selected number kept in culture for transfer on Day 2 or 3, and the remainder considered for pronucleate stage cryopreservation. The decision as to number of embryos to be frozen at the pronucleate stage should take into consideration the patient's previous history regarding cleavage and quality of embryos. Zygotes to be frozen should have a regular outline, distinct zona and clearly visible pronuclei. The cryopreservation procedure must be initiated while the pronuclei are still visible, before the onset of syngamy.

Selection of embryos for transfer

Historically, embryo transfer was carried out 2 days (approximately 48–54 hours) after oocyte retrieval, but transfer has been carried out from as early as one hour post-ICSI (AOT, activated oocyte transfer, Dale *et al.*, 1999) to 5 days later, at the blastocyst stage. Trials of zygote transfer on Day1 also achieved acceptable pregnancy rates (Scott and Smith, 1998; Tesarik and Greco, 1999; Tesarik *et al.*, 2000); it seems that the specific timing of transfer may not be crucial for the human implantation process. On Day 2, cleaved embryos may contain from two to six blastomeres. Embryo transfer

one day later, on Day 3, or on Day 5 at the blastocyst stage is advocated as a means of selecting better quality embryos, by the elimination of those that arrest at earlier cleavage stages in vitro.

Sequence of events observed by time-lapse cinematography (Mio and Maeda, 2008)

Day 0 = day of OCR, insemination approximately 4–5 hours post OCR.

Hours post insemination:	Observation:
1.5 ± 0.2	Sperm penetrated
2.0 ± 0.2	Sperm incorporated
2.5 ± 1.2	Second polar body extruded
2.5 ± 0.5	Cone appearance (interaction between sperm midpiece and oocyte plasma membrane)
3.7 ± 0.7	Appearance of cytoplasmic flare (may represent sperm aster)
4.5 ± 0.8	Cone disappears
5.5 ± 0.5	Cytoplasmic flare disappears
6.6 ± 0.3	Male pronucleus formed
6.8 ± 0.3	Female pronucleus formed
8.8 ± 0.7	Both pronuclei abutted
9.0 ± 0.5	Halo appears
19.2 ± 1.1	Halo disappears
24.8 ± 1.0	Syngamy
27.3 ± 1.0	First cleavage commenced
37.2 ± 1.2	Second cleavage commenced

Activation of the zygote genome begins at this stage, with massive increase in transcription and translation; this cell cycle requires a full 24 hours.

By 96 hours post insemination, on Day 4: compacting morula stage (around 32 cells).

Day 5: differentiation to blastocyst stage. Cell number may vary considerably, from 50 to 100–120 cells for early expanding blastocysts.

Hatching may start as early as the morning of Day 5, but is usually observed on Day 6/7.

Two major problems continue to hinder the effectiveness of ART treatment: low implantation rates and a high incidence of multiple pregnancies. Poor endometrial receptivity and adverse uterine contractions can both contribute to early embryo loss, but the low efficiency of assisted conception is widely attributed to genetic defects in the embryo. More than 40% of ART-derived embryos are known to harbor chromosomal abnormalities. Errors in meiotic and mitotic segregation of chromosomes in the oocyte and during the cleavage of early embryos can lead to different patterns of aneuploidy, including polyploidy and chaotic mosaics, which account for around one-third of aneuploidies involving more than one chromosome per cell. However, despite the fact that grossly abnormal chromosome complements are lethal, in most cases the morphology of embryos that are genetically normal does not differ markedly from those with aneuploid, polyploid or mosaic chromosomal complements. Consequently, genetically abnormal embryos after IVF or ICSI may be graded as suitable for transfer using subjective selection criteria. Developing a reliable diagnostic test that can be used to identify embryos with the greatest developmental competence continues to be a major priority in human ART, in the hope of eventually selecting a single embryo that is likely to result in a healthy live birth following transfer.

In selecting embryos for transfer, the limitations of evaluating embryos based on morphological criteria alone are well recognized: correlations between gross morphology and implantation are weak and inaccurate, unless the embryos are clearly degenerating/fragmented. Objective criteria for evaluating embryos are available in laboratories with research facilities, but may be out of reach for a routine clinical IVF laboratory without access to specialized equipment and facilities. Objective measurements of human embryo viability that have been applied include:

- High-resolution videocinematography
- Computer-assisted morphometric analysis
- Blastomere or polar body biopsy for cytogenetic analysis
- Culture of cumulus cells
- Oxygen levels in follicular fluid/perifollicular vascularization
- Distribution of mitochondria and ATP levels in blastomeres
- Metabolic assessment of culture media (amino acid profiling, metabolomics)
- Gene expression/expression of mRNA in cumulus cells and/or embryos.

High-resolution videocinematography

As early as 1989, Cohen *et al.* carried out a classic experiment that aimed to clearly define morphological criteria that might be used for embryo assessment, using a detailed analysis of videotaped images. Immediately before embryo transfer, embryos were recorded on VHS for 30–90 seconds, at several focal points, using Nomarski

optics and an overall magnification of ×1400. The recordings were subsequently analyzed by observers who were unaware of the outcome of the IVF procedure, and they objectively assessed a total of 11 different parameters:

Cell organelles visible	Cellular extrusions
Blastomeres all intact	Cytoplasmic vacuoles
Identical blastomere size	Blastomeres contracted
Smooth membranes	% variation in zona thickness
Dark blastomeres	% extracellular fragments
Cell–cell adherence	

Nine parameters were judged (+) or (–), and variation in zona thickness and percentage of extracellular fragments were given a numerical value. Analysis of these criteria showed no clear correlation with any intracellular features of morphology, but that the most important predictor of fresh embryo implantation was the percentage of variation in thickness of the zona pellucida. Embryos with a thick, even zona had a poor prognosis for implantation; those whose zona had thin patches also had "swollen," more refractile blastomeres, and had few or no fragments. This observation was one of the parameters that led the group to introduce the use of assisted hatching (see Chapter 13). In analyzing frozen-thawed embryos, the best predictor of implantation was cell–cell adherence. The proportion of thawed embryos with more than one abnormality (77%) was higher than that of fresh embryos (38%) despite similar implantation rates (18% versus 15%).

More than twenty years have elapsed since these observations were published, and the quest to identify specific morphological markers of embryo implantation potential still continues – now with the help of more sophisticated technology to measure both properties of the zona and detailed embryo morphology.

Zona pellucida birefringence

Polarized microscopy allows three layers to be distinguished in the zona, with the innermost layer showing the greatest birefringence (i.e., a higher level of light retardance). Several studies have investigated a possible correlation between this zona property and implantation potential (Montag *et al*., 2008; Madaschi *et al*., 2009); there is no doubt that properties of the zona may be important in assessing oocyte/embryo potential, particularly in response to exogenous FSH stimulation, but further studies are required in order to establish a clear correlation.

Computer-assisted morphometric analysis

High-resolution digital images of embryos can be assessed in detail with the help of computer-assisted multilevel analysis, which provides a three-dimensional picture of embryo morphology. The FertiMorph multilevel system from IH-Medical, Denmark is equipped with a computer-controlled motorized stepper mounted on the microscope, and this system will automatically focus through different focal planes in the embryo to produce a sequence of digital images. Automatic calculations of morphometric information from the image sequences describe features and measurements of each embryo, including size of nucleus and blastomeres and their spatial positions within the embryo, as well as features of the zona pellucida; all of the information is stored in a database. To date, clinical results following the use of this system are limited. Preliminary results indicated that implantation was affected by the number and size of blastomeres on Day 3, and prediction of embryo implantation was superior to that of traditional manual scoring systems (Paternot *et al*., 2009). Additional information about morphology and embryo development is also accumulating from the use of modern systems that allow time-lapse photography in combination with culture systems.

Aneuploidy screening

Cytogenetic analysis of a biopsied polar body or blastomere can be used to screen embryos in order to detect those with an abnormal chromosome composition, a strategy known as aneuploidy screening or preimplantation genetic screening (PGS). The techniques employed for biopsy and diagnosis are described in Chapters 13 and 14, as well as the associated pros and cons. PGS has been a subject of considerable debate (Kuliev and Verlinsky, 2008; Mastenbroek *et al*., 2008; Sermondade and Mandelbaum, 2009), and it is now discouraged in guidelines issued by the European Society for Human Reproduction and Embryology (ESHRE) and the American Society for Reproductive Medicine (ASRM).

Follicular indicators of embryo health

Each assisted conception cycle generates a number of waste products: luteinized granulosa cells, follicular

fluids harvested from follicles at the time of oocyte retrieval and cumulus cells that can be removed and separated from the oocytes. These products have all been assayed to provide indices of embryo developmental competence. Biomarkers quantified in serum and follicular fluid include cytokines, C-reactive protein and leptin (Wunder *et al*., 2005), inhibin B (Chang *et al*., 2002) and reactive oxygen species (Das *et al*., 2006). Cumulus cell gene expression profiles have also been linked to the implantation potential of oocytes and embryos (McKenzie *et al*., 2004). While many of these parameters are indicative of follicular differentiated status at the time of oocyte harvest, follicular fluid is a highly concentrated cellular exudate, which is accumulated over an extended period. Consequently, to date neither follicular fluid nor granulosa cell assays at the time of oocyte collection have provided a consistent measure for assessing the implantation potential of individual embryos. The degree of follicular vascularization and its relationship to mitochondrial segregation in embryonic blastomeres has also been promoted as a determinant of embryo developmental competence (Van Blerkom *et al*., 2000).

Secreted factors

Assessment of products secreted by the embryo, the embryonic "secretome," may be a better indicator of embryo development in vitro and in vivo than measurements of the follicular environment. For example, secretion of factors that regulate gamete transport and/or prepare the female tract for implantation has been used to predict embryo health. In this context, measurement of the amount of soluble human leukocyte antigen-G (HLA-G) into embryo culture media has been directly related to embryo quality and viability (Sher *et al*., 2004). mRNAs for HLA-G can be detected in human blastocysts, but the cellular origins and biology of soluble HLA-G are not clear (Sargent, 2005). The suitability of HLA-G as a predictor of embryo developmental potential has also been questioned, as the amount of HLA-G measured in embryo culture media appears to exceed the total protein content of the embryo itself (Ménézo *et al*., 2006).

Embryo metabolism

Advances in technology have facilitated noninvasive measurement of amino acid uptake/output into the spent culture medium of individual embryos; it is now possible to quantify the products of embryo metabolism, and use these measurements as a basis for identifying healthy embryos. During early development in vivo and in vitro, each preimplantation embryo will utilize numerous substrates from its immediate environment: oxygen for respiration, sugars, energy sources such as glucose and proteins/amino acids. The embryo also secretes many waste products of metabolism into its immediate environment. The turnover of these substrates and products can be measured either in the embryos themselves or in their culture environment (Gardner, 2007).The rapid development of technologies to measure embryo metabolism has created a new analytical science for embryo selection, the science of "embryo metabolomics." Metabolomics is defined as "the systematic analysis of the inventory of metabolites – as small molecule biomarkers – that represent the functional phenotype at the cellular level" (Posillico *et al*., 2007). The field of embryo metabolomics is rapidly expanding and includes the noninvasive measurement of amino acid turnover in spent embryo culture media using established technologies such as high performance liquid chromatography (HPLC, as detailed above), as well as methods such as gas chromatography and mass spectrometry, nuclear magnetic resonance spectroscopy and Raman near infrared spectroscopy. Quantifying embryo metabolism has also been extended to include measurement of embryo oxygen consumption by respirometry. Details of the advantages and disadvantages associated with each of these different methods are reviewed by Posillico *et al*. (2007).

The use of amino acid turnover to predict the developmental competence of individual embryos is based on the premise that metabolism is intrinsic to early embryo health and that the embryonic metabolome is immediately perturbed when embryos are stressed (Houghton and Leese, 2004; Lane and Gardner, 2005). Different methods can be used to quantify the metabolome (for review see Hollywood *et al*., 2006); to date the methodologies that best suit the measurement of amino acid turnover by individual embryos are HPLC and mass spectrometry.

Amino acid profiling has been extensively tested as a valid clinical diagnostic test for embryo selection in animal species, including mice, cows and pigs, as well as humans (Houghton *et al*., 2002). Collectively the data on amino acid metabolism across these species indicates that:

(i) the net rates of depletion or appearance of amino acids by individual embryos vary between

amino acids and the stage of preimplantation development

(ii) there is no difference between the turnover of essential and non-essential amino acids as defined by Eagle in 1959

(iii) the turnover of amino acids is moderated by the concentrations of the amino acids in the embryo culture media.

Net depletion of glutamine and arginine and the net appearance of alanine have been found to be common features between pig, cow and human embryos. The association of metabolic profiles of certain amino acids (particularly asparagine, glycine and leucine) with embryo viability is based on a variety of complex interactions and may involve energy production, mitochondrial function, regulation of pH and osmolarity. Nevertheless, on the basis of the turnover of three to five key amino acids, it is possible to discriminate with some confidence between morphologically similar cleavage stage human embryos which are metabolically "quiet" (Leese, 2002), but have the capacity to undergo zygotic genome activation and blastocyst development, and embryos which are metabolically active but are destined to undergo cleavage arrest (Houghton *et al.*, 2002). Interestingly, amino acid turnover by early cleavage embryos appears to be linked to embryo genetic health (Picton *et al.*, 2010). In this context inadequate energy production has been postulated as a cause of aneuploidy induction, due to errors during the energy-dependent processes of chromosome alignment, segregation and polar body formation (Bielanska *et al.*, 2002a; Ziebe *et al.*, 2003).

Preliminary trials of in-vitro amino acid profiling suggested that this strategy can be used to identify cleavage stage embryos with high implantation potential (Brison *et al.*, 2004). Metabolic profiles of developmentally competent, frozen-thawed human embryos were also consistent with those of fresh embryos (Brison *et al.*, 2004), and metabolic profiles could be used to identify frozen-thawed embryos with the potential to develop to the blastocyst stage in vitro.

Although initial results have been encouraging, the relevance of embryo metabolomics to the selection of the best embryo for transfer has been the subject of considerable debate, reviewed in a collection of papers published as the proceedings of a meeting on this specific topic (Sturmey *et al.*, 2008).

Gene expression studies

The activity of individual genes in an embryo change continuously in response to fluctuating intrinsic requirements or environmental conditions. Embryonic gene expression has been assessed at different stages of preimplantation development, using real-time PCR analysis of cDNA fragments as a measure of mRNA transcripts (Adjaye *et al.*, 1998). More recently, in-vitro transcription techniques have been developed that allow oocyte and embryo mRNA to be amplified to a level that can be analyzed by microarrays (Wells, 2007). Although the technology is far from routine clinical application, as a research tool it is hoped that data accumulated over time from gene expression studies may eventually lead to the identification of markers for embryo viability and implantation potential.

Embryo grading

Divergent national strategies define the maximum number of embryos that can be transferred in any one cycle, and there is now a trend towards elective single embryo transfer (eSET) for selected patient populations, in order to decrease the incidence of multiple births associated with ART. In the UK, the HFEA introduced a "Multiple Births, Single Embryo Transfer Policy" in 2009, setting a maximum multiple birth rate for each UK clinic of 20%. In routine clinical practice, embryo selection for transfer continues to be based primarily on morphological assessment, often with multiple observations over the course of the embryo's development; for cleavage stage embryos, assessment criteria include:

- rate of division judged by the number of blastomeres
- size, shape, symmetry and cytoplasmic appearance of the blastomeres
- presence of anucleate cytoplasmic fragments
- appearance of the zona pellucida.

Criteria are frequently combined to produce composite scoring systems which may incorporate pronuclear scoring of zygotes. Online Quality Control schemes are available, where participants can compare their scoring criteria for embryo assessments with those of others (www.fertaid.com, www.embryologists.org.uk/).

Although morphological assessment is recognized to be highly subjective, arbitrary and unsatisfactory, it is quick, noninvasive, easy to carry out in routine practice, and does help to eliminate those embryos with the

poorest prognosis. Evaluation of blastomere shape, size and number will reflect synchronous cleavage of the blastomeres, and embryos with asynchrony in either the timing of cleavage, or the process of blastomere division will be given lower scores. Unfortunately embryo cleavage in vitro rarely follows the postulated theoretical timing of early development, and computer-assisted morphometric analyses confirm that large variations in blastomere size and fragmentation are frequently observed; large variations in blastomere size have been linked to increased chromosomal errors (Hnida and Ziebe, 2007).

Zygote scoring

Schemes for identifying healthy viable embryos at the zygote stage were proposed by Scott *et al.* (2000) and Tesarik and Greco (1999). Criteria suggested as predictive of optimal implantation potential include:

- close alignment of nucleoli in a row
- adequate separation of pronuclei
- heterogeneous cytoplasm with a clear "halo"
- cleavage within 24–26 hours.

The scoring systems were reviewed by James (2007), and are compared in Table 11.1. The timing of assessment is critical, as pronuclear development is a dynamic process and zygote scoring should therefore be used with caution and only in conjunction with other methods of evaluation.

Early cleavage

Timing of the first cell division of the embryo has been investigated as a predictor of developmental competence, with the suggestion that "early cleavage" is associated with higher pregnancy rates. However, there is only a certain window where true early cleavage can be seen, and this extra assessment may be difficult to fit into the normal routine of an IVF laboratory. Once the embryos have undergone the second division to the four-cell stage, those that might have cleaved a few hours earlier will have similar morphology to those that did not, and it is not possible to differentiate between them.

Multinucleation

Multinucleated blastomeres can sometimes be observed at early cleavage stages, most easily on Day 2 (Figure 11.4). Karyotyping and FISH analysis confirms that their presence may be more common in arrested embryos, and may occur more readily in some patients.

Multinucleated blastomeres have larger volumes than mononucleated blastomeres within the same embryo, and these mononucleated blastomeres are also smaller than mononucleated blastomeres in embryos with no multinucleation. Transfer of embryos with multinucleated blastomeres may be associated with decreased implantation, pregnancy and birth rates and should be excluded from transfer when possible (Meriano *et al.*, 2004; Hnida and Ziebe, 2007).

Cumulative scoring systems

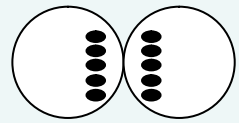
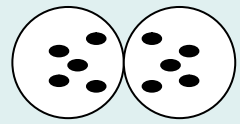
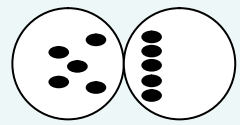
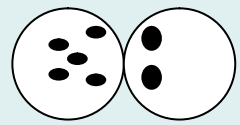
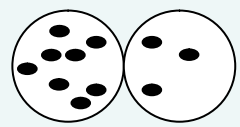
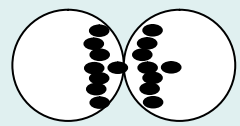
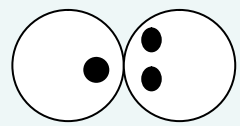
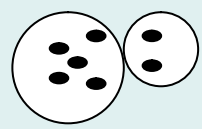
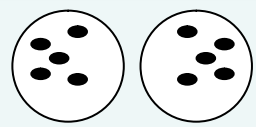
Multi-day scoring systems can enhance embryo selection by combining both developmental rate and morphological assessment (Skiadas and Racowsky, 2007).

These should provide a more accurate picture of developmental progression than can be obtained from a single observation. However, the ultimate combination of morphological features required for optimal evaluation of developmental competence has yet to be resolved. The optimal timing of embryo transfer will be more accurately determined when agreement on this is reached.

Fragments

Fragmentation in the human embryo is very common, affecting up to 75% of all embryos developed in vitro (Alikani, 2007); it is not clear whether this is an effect of culture conditions and follicular stimulation, or a characteristic of human development (Figure 11.5). Extensive fragmentation is known to be associated with implantation failure, but the relationship between the degree of fragmentation and the developmental potential of the embryo is far from clear. Alikani *et al.* (1999) found that when embryos with more than 15% fragmentation were cultured to blastocyst stage, they formed fewer morulas, fewer cavities and fewer blastocysts compared to those embryos with less than 15% fragmentation. When fragmentation was greater than 35%, all processes were compromised. Retrospective analysis of embryo transfer data revealed that nearly 90% of embryos selected for transfer were developed from embryos with less than 15% fragmentation observed on Day 3.

Table 11.1 Comparison of pronuclear morphology scoring systems, with a representative illustration of pronuclei in each scoring group

Scott et al., 2000 Series 1	Scott et al., 2000 Series 2	Scott, 2003	Tesarik and Greco, 1999	Tesarik et al., 2000	Pronuclear morphology
Grade 1	Z1	Z1	Pattern 0	Pattern 0	
Grade 3	Z2	Z2	Pattern 0	Pattern 0	
Grade 2	Z3	Z3–2	Pattern 2	Non-pattern 0	
Grade 4	Z3	Z3–1	Pattern 5	Non-pattern 0	
Grade 4	Z3	Z3–4	Pattern 1	Non-pattern 0	
Grade 4	Z3	Z3–4	Pattern 3	Non-pattern 0	
Grade 5	Z3	Z3–1	Pattern 4	Non-pattern 0	
Grade 5	Z4	Z4–2	Pattern 4	Non-pattern 0	
Grade 5	Z4	Z4–1	NA	Non-pattern 0	

Alikani and Cohen (1995) used an analysis of patterns of cell fragmentation in the human embryo as a means of determining the relationship between cell fragmentation and implantation potential, with the conclusion that not only the degree, but also the pattern of embryo fragmentation determine its implantation potential. Five distinct patterns of fragmentation which can be seen by Day 3 were identified:

Type I: <5% of the volume of the perivitelline space (PVS) occupied by fragments.

Type II: small, localized fragments associated with one or two cells.

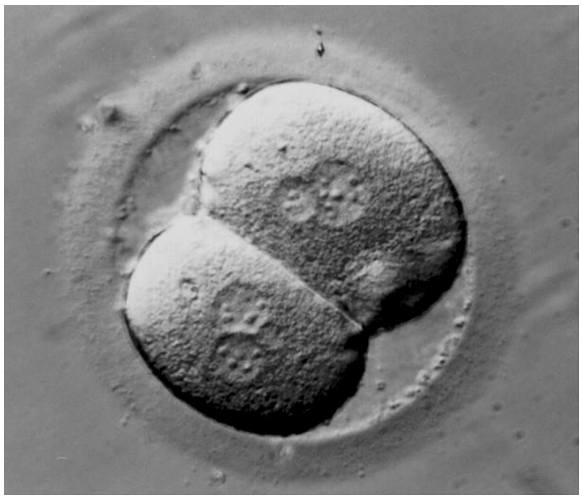
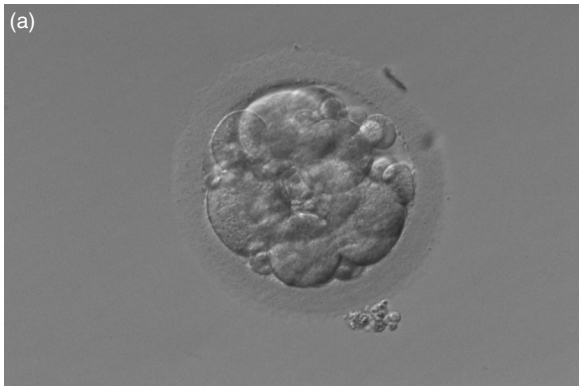


Figure 11.4 A two-cell embryo showing two nuclei in each. (Reprinted with permission, J Meriano 2004, *Reproductive Biomedicine Online*.)



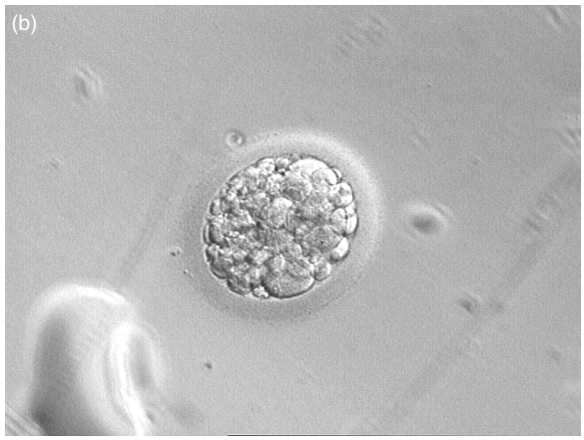


Figure 11.5 (a) Day 3 human embryo with type 3 fragments. (b) Day 3 embryo with type 4 fragmentation.

Type III: small, scattered fragments associated with multiple cells.

Type IV: large, scattered fragments associated with several unevenly sized cells and scattered throughout the PVS.

Type V: fragments throughout the PVS, appearing degenerate such that cell boundaries are invisible, associated with contracted and granular cytoplasm.

Some embryos have no distinct pattern (NDP) of fragmentation.

No definite cause of fragmentation has been identified, although speculations include high spermatozoal numbers and consequently high levels of free radicals, temperature or pH shock, and stimulation protocols. Apoptosis has been suggested as a possible cause, and a progressive shortening of telomere length, which induces apoptosis, has been linked with fragmentation (Keefe *et al.*, 2005); however, this study was not definitive. Mitotically inactive cells do not exhibit fragmentation (Liu *et al.*, 2002), and it has been suggested that aberrant cytokinesis in the presence of spindle and cytoskeletal abnormalities may be associated with fragmentation.

Observed via the scanning electron microscope, the surface of fragments is made up of irregularly shaped blebs and protrusions, very different to the regular surface of blastomeres, which is organized into short, regular microvilli (Figure 11.6).

Interestingly, programmed cell death in somatic cells also starts with surface blebbing, and is caused, in part, by a calcium-induced disorganization of the cytoskeleton. We can speculate that similar mechanisms operate within human embryos, but there is so far no scientific evidence that this is the case.

There does appear to be an element of programming in this partial embryonic autodestruction, as embryos from certain patients, irrespective of the types of procedure applied in successive IVF attempts, are always prone to fragmentation. Surprisingly, fragmented embryos, repaired or not, do implant and often come to term. Time-lapse photography technology has clearly demonstrated that an individual embryo can radically change its morphological appearance in a short period of time: fragments that are apparent at a particular moment in time can be subsequently absorbed with no evidence of their prior existence (Hamberger *et al.*, 1998; Hashimoto *et al.*, 2009; Mio and Maeda, 2008). This demonstrates the highly regulative nature of the human embryo, as

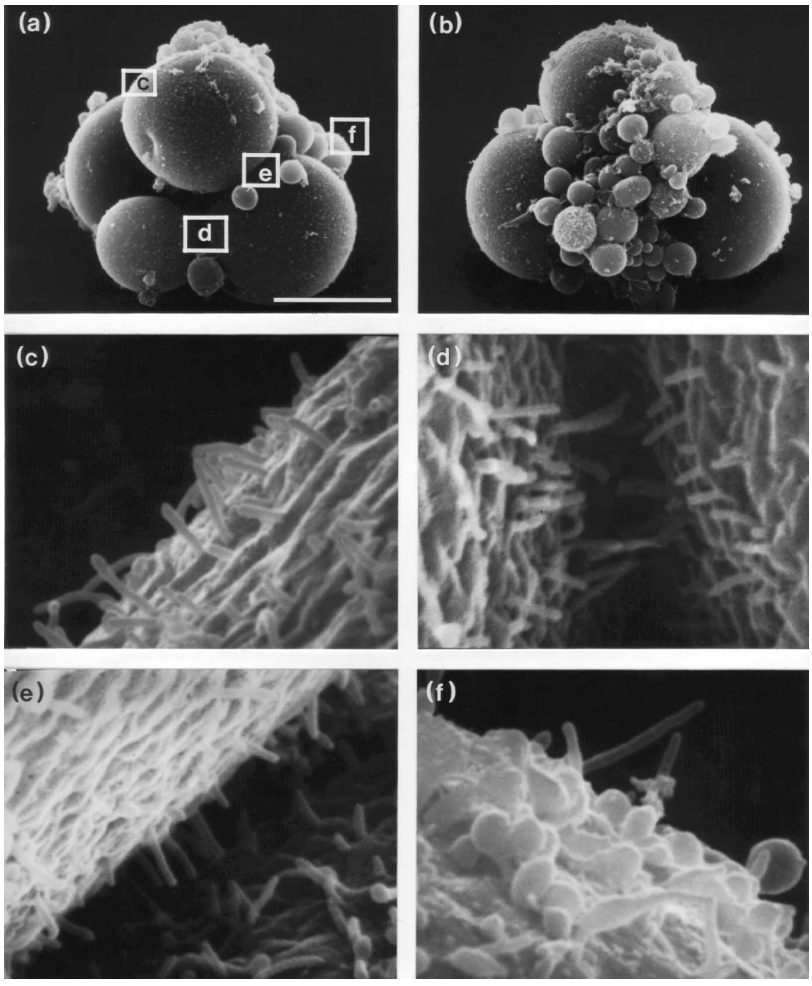


Figure 11.6 Scanning electron micrographs. (a, b) Two views of a human four-cell embryo showing 20% fragmentation. (c–e) Magnification of corresponding areas showing regular short microvilli of vital blastomeres and intercellular areas. (f) Magnification of the surface of a cytoplasmic fragment showing irregular blebs and protrusions. (With permission from Dale *et al.*, 1995.)

it can apparently lose over half of its cellular mass and still recover, and also confirms the general consensus that the mature oocyte contains much more material than it needs for development. The reasons why part, and only part, of an early embryo should become disorganized and degenerate are a mystery. Different degrees of fragmentation argue against the idea that the embryo is purposely casting off excess cytoplasm, somewhat analogous to the situation in annelids and marsupials that shed cytoplasmic lobes rich in yolk, and favors the idea of partial degeneration. Perhaps it involves cell polarization, where organelles gather to one side of the cell. It is certain that pH, calcium and transcellular currents trigger cell polarization, which may in certain cases lead to an abnormal polarization, and therefore to fragmentation. Describing fragmentation as a degenerative process may not be justified, but more research is needed to elucidate whether the implantation of embryos with extensive fragmentation at the cleavage stages has any long-term effects.

Embryo grading: cleavage stages

After more than 30 years, and the birth of close to 10 million babies, the basic selection criteria in a routine IVF laboratory without research facilities continue to be based on assessment of morphology (Figure 11.7). Preimplantation genetic screening by FISH analysis of biopsied blastomeres has shown a surprising discrepancy between gross morphology and genetic normality of embryos. Even the most "beautiful" Grade 1 embryos may have numerical chromosomal anomalies, whilst those judged to be of "poorer" quality, with uneven blastomeres and fragments, may have a normal chromosome complement. The image database on www.ivf.net has

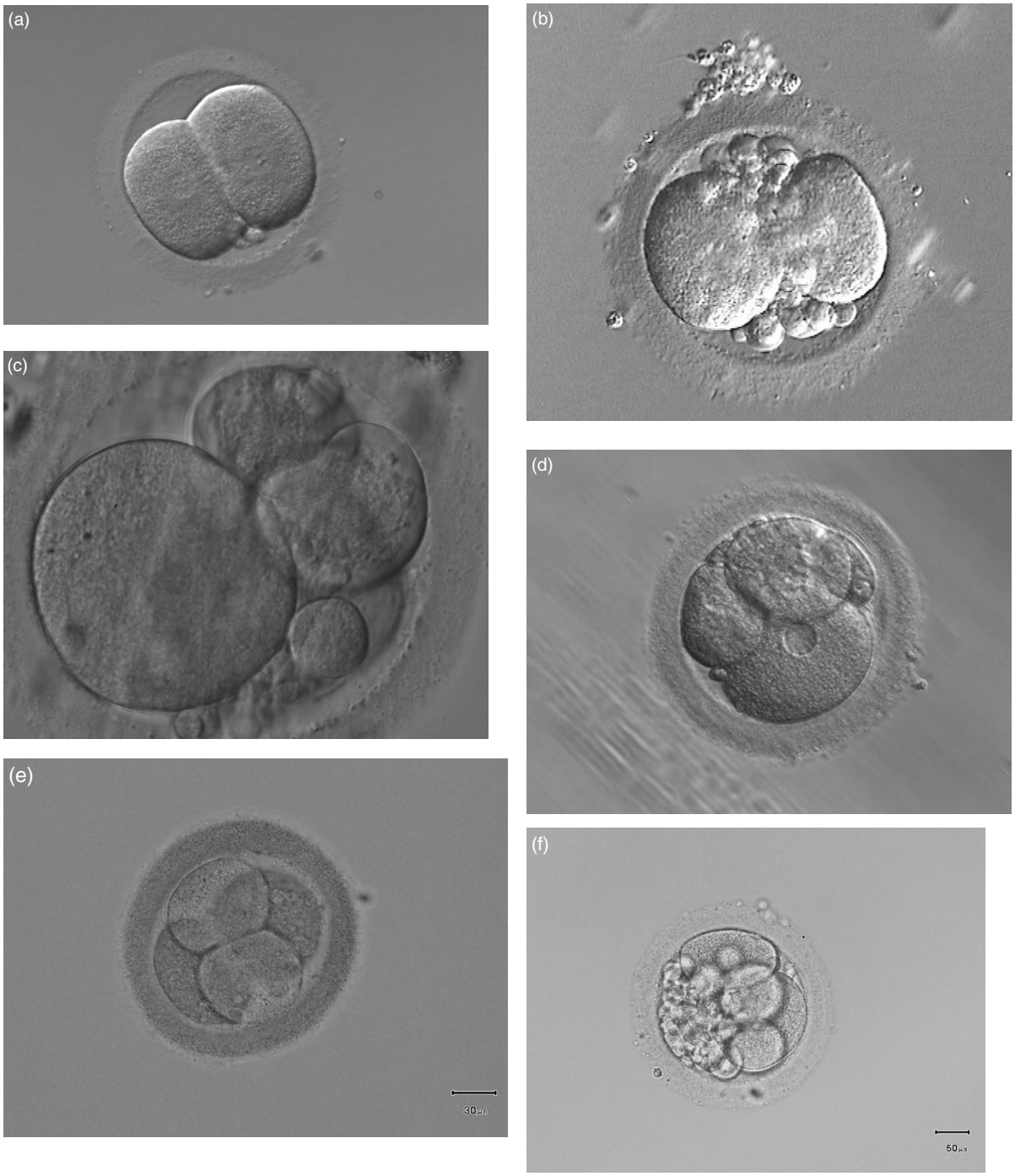
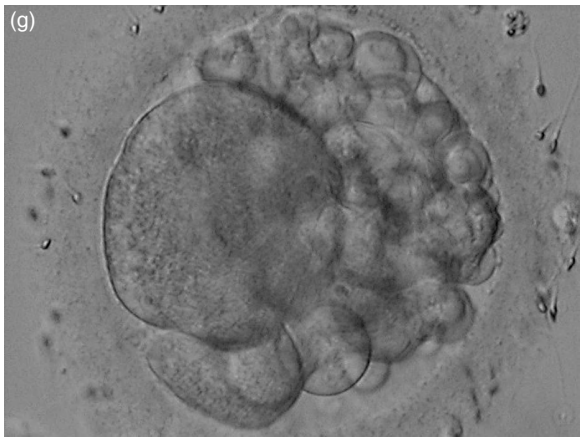
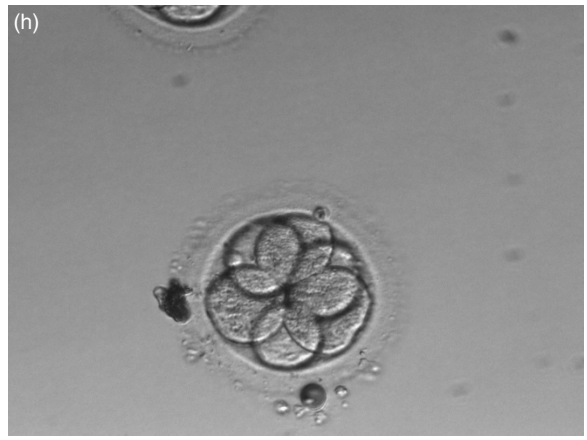


Figure 11.7 Morphological variations in cleavage stage human embryos. (a) Two-cell embryo on Day 2, no fragments. (b) Day 2 embryo, Grade 3. (c) Day 2 embryo, uneven blastomeres with one large dominant blastomere. (d) Day 2 embryo, one blastomere shows large vacuole, Grade 2/3. (e) Four-cell embryo on Day 2, Grade 1. See color plate section. (f) Grade 3 embryo on Day 2. See color plate section. (g) Grade 4 embryo on Day 2. (h) Day 3 six-cell embryo, Grade 1. (i) Day 3 eight-cell embryo, Grade 1. Images (c) and (g) kindly supplied by Thomas Ebner, Austria; (g) by Marc van den Bergh, Switzerland; (e) and (f) by Oleksii Barash, Ukraine; (h) by Gemma Fabozzi, Naples. Despite the extreme difference in their morphology, the single transfer of embryos (e) and (f) both led to healthy live births (photos and history courtesy of Oleksii Barash, Ukraine).





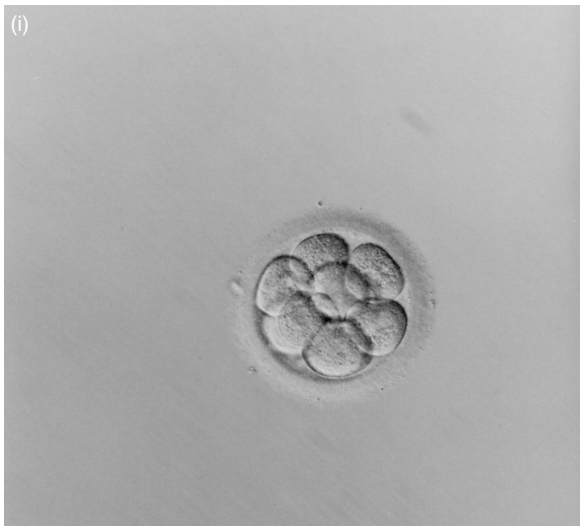


Figure 11.7 (*cont.*)

a valuable collection of photographs showing single embryos that led to a live birth after transfer, which clearly confirm that our subjective assessment of embryo morphology often fails to predict its implantation potential in reality.

Grade 1/A

- Even, regular spherical blastomeres
- Moderate refractility (i.e., not very dark)
- Intact zona
- No, or very few, fragments (less than 10%)

Allowance should be made for the appearance of blastomeres that are in division or that have divided asynchronously with their sisters, e.g., three- five- six- or seven-cell embryos, which may be uneven. As always, individual judgment is important, and this is a highly subjective assessment.

Grade 2/B

- Uneven or irregular shaped blastomeres
- Mild variation in refractility
- No more than 10% fragmentation of blastomeres

Grade 3/C

- Fragmentation of no more than 50% of blastomeres

- Remaining blastomeres must be at least in reasonable (Grade 2) condition
- Refractility associated with cell viability
- Intact zona pellucida

Grade 4/D

- More than 50% of the blastomeres are fragmented
- Gross variation in refractility.
- Remaining blastomeres appear viable

Grade 5

- Zygotes with two pronuclei on Day 2 (delayed fertilization or reinsemination on Day 1)

Grade 6

- Nonviable: fragmented, lysed, contracted or dark blastomeres
- No viable cells

It is important to bear in mind that the time during which the assessment and judgment is made represents only a tiny instant of a rapidly evolving process of development. Embryos can be judged quite differently at two different periods in time, as may be seen if a comparison is made between assessments made in the morning, and later in the day immediately before transfer. Individual judgment should be exercised in determining which embryos are selected. In general, those embryos at later stages and of higher grades are preferred, but the choice is often not clear cut. The Grade 2 category covers a wide range of morphological states but, provided the blastomeres are not grossly abnormal, a later stage Grade 2 embryo may be selected in preference to an earlier stage Grade 1 embryo. Attention should also be paid to the appearance of the zona pellucida and to the pattern of fragmentation. Embryos of Grade 3 or 4 are transferred only where no better embryos are available. If only pronucleate embryos are available on Day 2, they should be cultured further and transferred only if cleavage occurs.

Blastocyst transfer

The transfer of blastocysts on Day 5 or 6 may have the advantage of allowing better synchrony between the embryo and endometrium, as well as eliminating embryos that cannot develop after activation of the zygote genome due to genetic or metabolic defects. Following the development of new generation sequential embryo culture media, single blastocyst transfer has been promoted as a means of improving the success of IVF, while at the same time reducing multiple birth rates (Gardner *et al.*, 1998). However, blastocyst generation in vitro is costly both in terms of time and laboratory resources, and there is continuing debate as to whether sequential, two-step culture confers significant benefit over one-step culture systems (Biggers *et al.*, 2005). An important prerequisite for blastocyst culture is an optimal IVF laboratory culture environment; there is no advantage in extended culture unless satisfactory implantation rates are already obtained after culture to Day 2 or 3. Published literature indicates that a policy of careful embryo evaluation at cleavage stages can lead to success rates after Day 3 transfer that broadly match the success rates achieved with blastocyst transfer. One of the hesitations of culturing embryos to the blastocyst stage was the poor survival rate for supernumerary blastocysts after slow freezing. However, the increasing use of vitrification for blastocyst freezing with associated high survival rate post-thaw (see Chapter 12) has made single blastocyst transfer a more viable option, and this also facilitates a policy for elective single embryo transfer (eSET).

Concern has also been expressed about the safety of prolonged culture to the blastocyst stage and the risk of potential aberrant epigenetic programming during extended in-vitro development (Huntriss and Picton, 2008; see Chapter 15).

Potential benefits of blastocyst transfer (Gardner *et al.*, 2007)

- True embryo viability can be assessed, post embryonic genome activation.
- Embryos with limited developmental potential are eliminated.
- Embryonic stage is synchronized with the uterus, reducing cellular stress on the embryo.
- Exposure of the embryo to a hyperstimulated uterine environment is minimized.
- Possibility of uterine contractions is reduced, minimizing the chance of embryo expulsion.
- Cleavage stage embryo biopsy can be carried out, and embryos transferred at blastocyst stage when results are available.
- High implantation rate; reduces the need to transfer multiple embryos.

- Reduction in multiple gestation rate as only one or two embryos need be transferred.

The ability to identify healthy viable blastocysts is an important factor in the success of blastocyst transfer, and a grading system has been devised that takes into consideration the degree of expansion, hatching status, the development of the inner cell mass (ICM) and the development of the trophectoderm (Gardner and Schoolcraft, 1999). Careful assessment of all morphological parameters available will optimize the chance of achieving satisfactory implantation rates after blastocyst transfer.

Blastocysts are initially graded numerically, from 1 to 6 (this can be performed under a dissecting microscope):

1. Early blastocyst: the blastocoele occupies less than half the volume of the embryo.
2. Blastocyst: the blastocoele occupies half the volume of the embryo or more.
3. Full blastocyst: the blastocoele completely fills the embryo, but the zona has not thinned.
4. Expanded blastocyst: the volume of the blastocoele is larger than that of the embryo, and the zona is thinning.
5. Hatching blastocyst: the trophectoderm has started to herniate through the zona.
6. Hatched blastocyst: the blastocyst has completely escaped from the zona.

The morphology of the ICM and trophectoderm are then assessed under an inverted microscope:

ICM	Trophectoderm
A. Tightly packed, many cells,	A. Many cells forming a cohesive epithelium
B. Loosely grouped, several cells	B. Few cells forming a loose epithelium
C. Very few cells	C. Very few large cells

Examples of stages of human blastocyst development are illustrated in Figure 11.8; Figure 11.9 illustrates the limitations of morphological assessment.

Blastocyst transfer has also been used as a strategy for the treatment of patients who carry chromosomal translocations; chromosome translocations cause a delay in the cell cycle, and abnormal or slowly developing embryos are eliminated during in-vitro culture. Several normal pregnancies have been successfully established after transfer of healthy blastocysts in a group of patients carrying translocations (Ménézo *et al.*, 2001).

Remaining embryos

After embryo transfer, remaining embryos of Grades 1 or 2 which show less than 20% fragmentation at the time of assessment may be cryopreserved on Day 2 or Day 3. Embryos of suboptimal morphology at this time can be further cultured until Day 6, with daily assessment. Those that develop to blastocysts on Days 5 or 6 can also be cryopreserved.

Embryo transfer procedure

Materials

1. Pre-equilibrated, warmed culture medium
2. 1 mL disposable syringe
3. Embryo transfer catheter
4. Sterile disposable gloves (nonpowdered)
5. Clean Petri dish
6. Sterile Pasteur pipette and glass probe
7. Dissecting microscope with warm stage

Although it may seem obvious that correct identification of patient and embryos is vital, errors in communication do happen and can lead to a disastrous mistake, especially should there be patients with similar names undergoing treatment at the same time. Electronic identity/witnessing systems are now available to confirm identities of doctor, nurse, embryologist and patient, but if such a system is not available, a routine discipline of identification should be followed to ensure that there is no possibility of mistaken identity:

1. Ensure that medical notes always accompany a patient who is being prepared for embryo transfer.
2. Name and medical numbers on medical notes and patient identity bracelet should be checked by two people, i.e., the clinician in charge of the procedure and the assisting nurse.
3. The doctor should also check name and number verbally with the patient, and doctor, nurse and

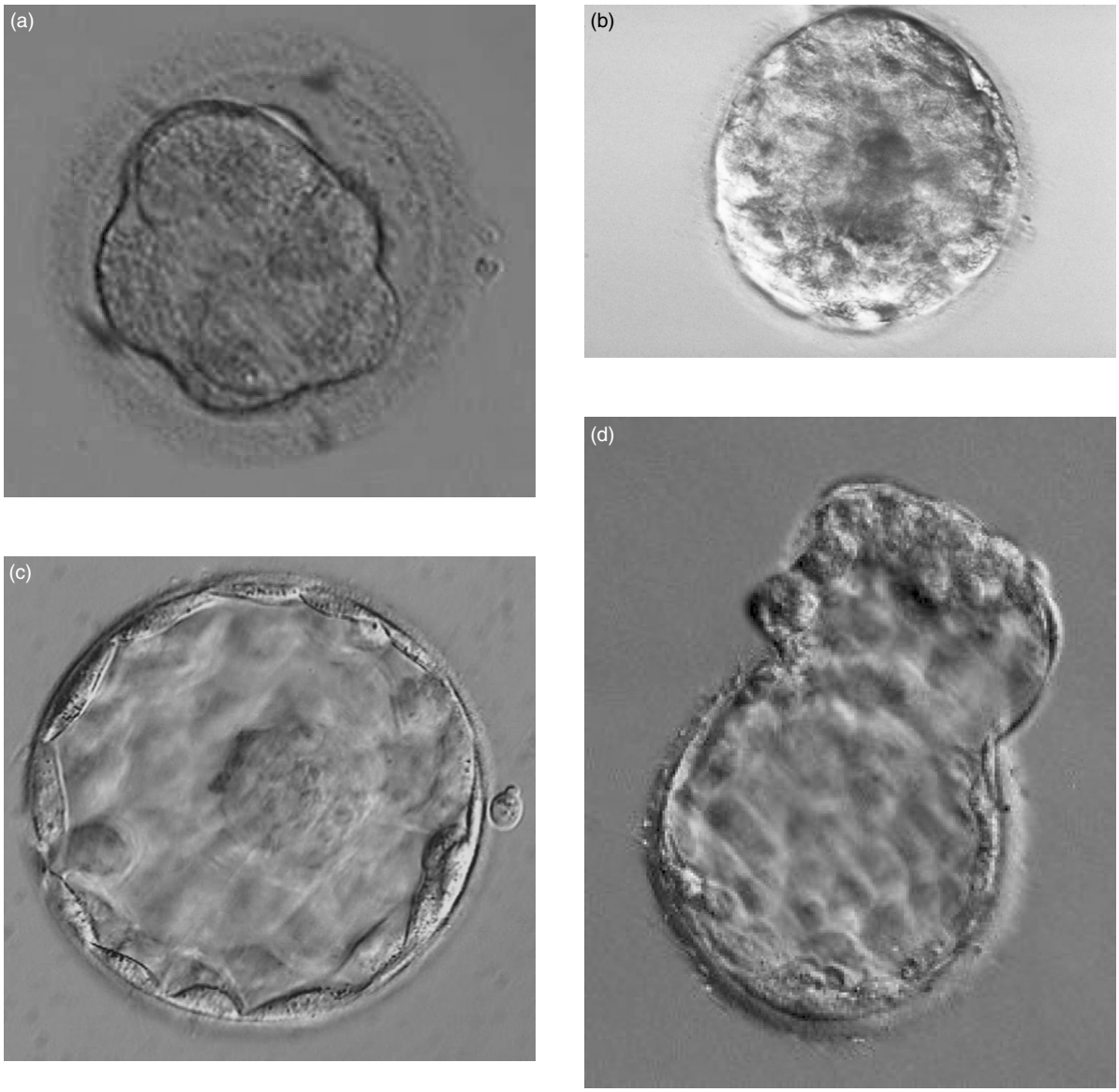


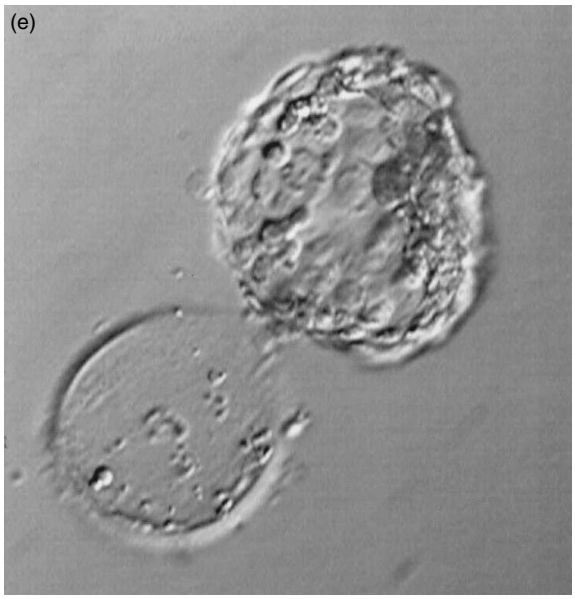
Figure 11.8 Stages in blastocyst development. (a) Compacting morula (see color plate section, between pp. 00 and 00, for color version of this figure). (b) Early stages of cavitation, See color plate section. (c) Fully expanded top-grade blastocyst with well-developed ICM and trophectoderm, thinned zona, hatching process initiated. See color plate section. (d) Top-grade blastocyst in the process of hatching. (e) Hatching blastocyst about to leave the zona. See color plate section. (f) Fully hatched blastocyst and empty zona.

patient may sign an appropriate form confirming that the details are correct.

4. The duty embryologist should check the same details with the embryology records, and also sign the same form in the presence of the doctor.

Preparation of embryos for transfer

The rate of multiple gestation resulting from IVF/ET is unacceptably high, and legislation in the UK and a few other European countries now prohibits the transfer of more than two embryos in a treatment cycle for



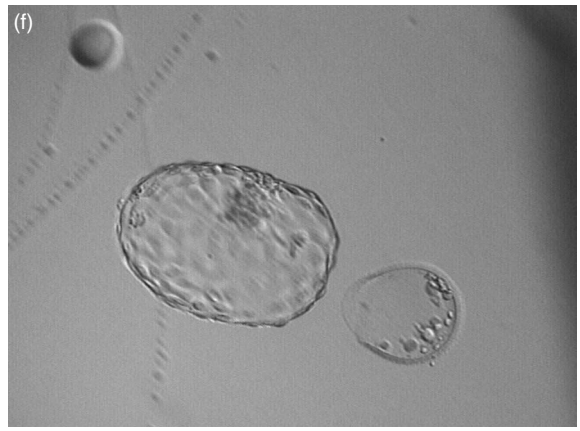
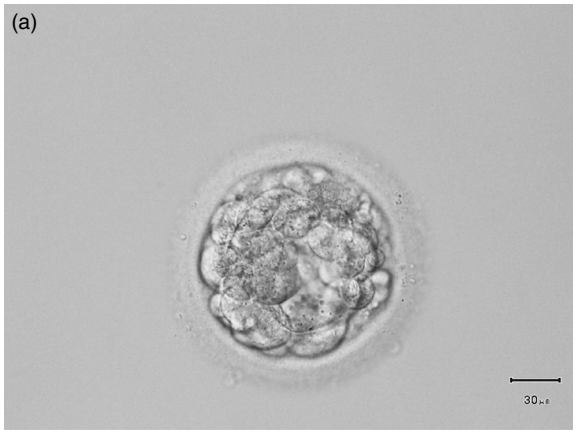


Figure 11.8 (*cont.*)



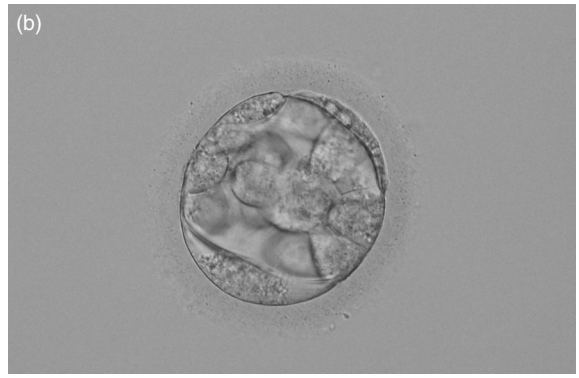


Figure 11.9 Limitations of morphological blastocyst assessment. Although each of the embryos pictured here would be graded as lesser quality compared with the "classic" optimal grade blastocysts seen in Figure 11.8, each of these embryos resulted in a healthy live birth after single embryo transfer on Day 5. Image (a) is a thawed embryo (two blastocysts were thawed on Day 5 [slow-freeze protocol], the one pictured here survived and was transferred on the same day). (With thanks to Oleksii Barash, Clinic of Reproductive Medicine "Nadiya," Ukraine.) See color plate section.

certain patient groups. Elective single embryo transfer (eSET) is recommended for selected patients with a good prognosis, i.e., young age, tubal infertility only, first attempt or previous history of pregnancy and/or delivery.

1. Prepare a droplet (or well) of fresh medium for the selected embryos.
2. When the embryos for transfer have been identified and scored, and their details recorded (number, developmental stage and grade), place them together in the pre-equilibrated droplet or well. No more than two embryos should be selected. If medium with a higher density is used (i.e., with 50–75% serum), this transfer should be carried out

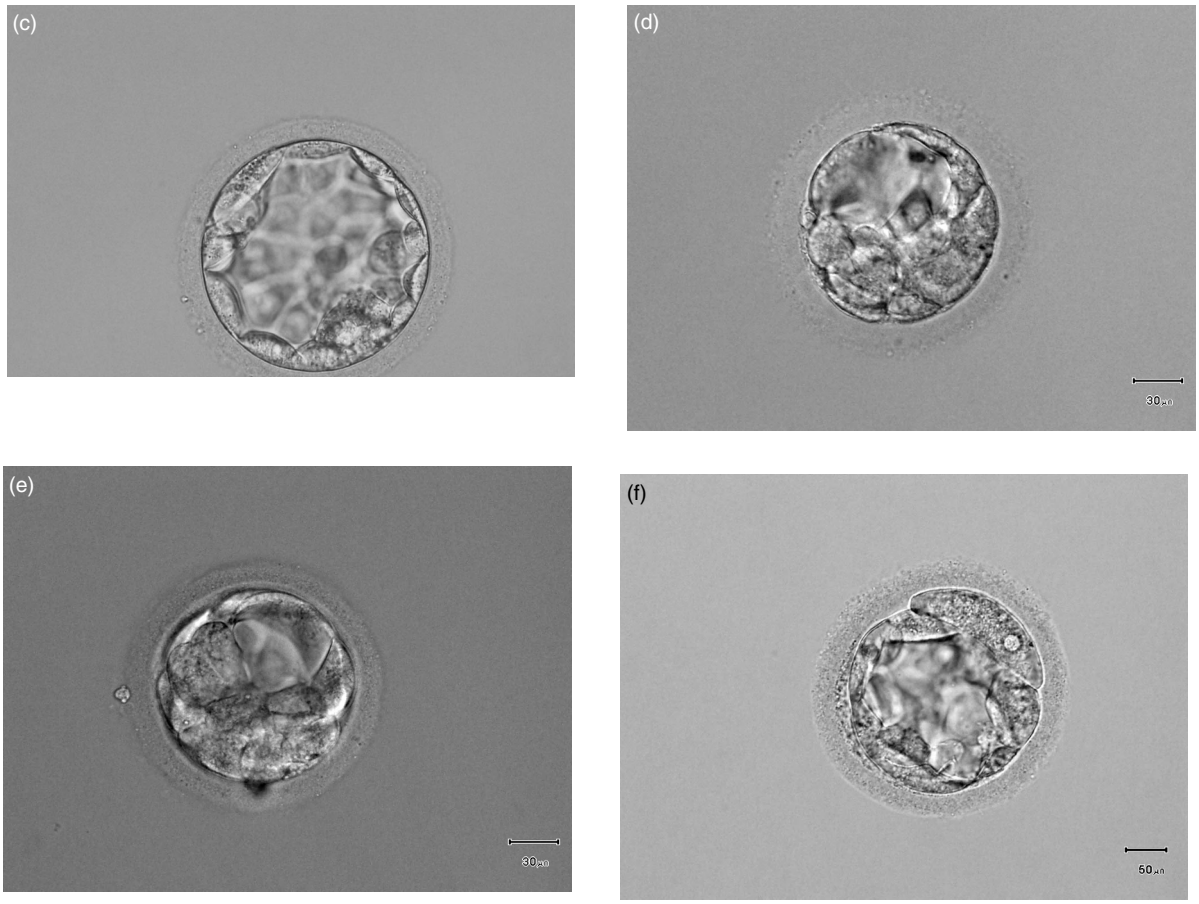


Figure 11.9 (*cont.*)

carefully, as the embryos will "float" in the higher density medium and must be allowed to settle before aspiration into the embryo transfer catheter.

3. After gently pushing the embryos together, leave them under low power on the heated stage of the microscope, in focus.
4. Turn off the microscope light.
5. Wash your hands with a surgical scrub preparation, and don sterile gloves.
6. Fill a 1 mL sterile syringe with warm medium, and eject any air bubbles.
7. Check that the catheter to be used moves freely through its outer sheath, attach it to the syringe and eject the medium from the syringe through the catheter, discarding the medium. Syringes designed specifically for embryo transfer are available, and these allow better control than a standard 1 mL syringe.
8. Draw up warm medium through the catheter into the syringe, and then push the piston down to the 0.1 mL mark, ejecting excess medium and again discarding it.
9. Pour some clean warm medium into the warm Petri dish on the microscope stage (for rinsing the catheter tip).
10. Place the end of the catheter into the drop or well, away from the embryos, and inject a small amount of medium to break the boundary of surface tension that may appear at the end of the catheter. Aspirate the embryos into the catheter, so that the volume to be transferred is 15–20 μL.

11. If the embryos have been loaded from a droplet under oil, rinse the tip of the catheter in the Petri dish containing clean warm medium.

Hand the catheter and syringe to the clinician for transfer to the patient. When the catheter is returned after the procedure, carefully inspect it, rotating under the microscope. It is especially important to ensure that no embryo is buried in any mucus present; note and record the presence of mucus and/or blood. Loosen the Lueur fitting, and allow the fluid in the catheter to drain into the clean Petri dish while continuing to observe through the microscope. Inform the doctor and patient as soon as you have confirmed that no embryos have been returned. If any embryos have been returned, they should be reloaded into a clean catheter, and the transfer procedure repeated. If difficulties arise during the transfer procedure causing delay, return the embryos to the culture drop in the interim, until the physician is confident that they can be safely transferred to the uterus of the patient.

There is no doubt that the technique of embryo transfer, although apparently a simple and straightforward procedure, is absolutely critical in safe delivery of the embryos to the site of their potential implantation. Studies repeatedly show that pregnancy rates can vary in the hands of different operators, and with the use of different embryo transfer catheters. In a study of embryo transfer procedures under ultrasound-guided control, Woolcott and Stanger (1998) observed guiding cannula and transfer catheter placement in relation to the endometrial surface and uterine fundus during embryo transfer. Their results indicated that tactile assessment of embryo transfer catheter was unreliable, in that the cannula and the catheter could be seen to abut the fundal endometrium, and indent or embed in the endometrium in a significant number of cases. Endometrial movement due to sub-endometrial myometrial contraction was obvious in 36% of cases, and this movement was associated with a reduced pregnancy rate. Their studies highlight the fact that "blind" embryo transfer procedures may often lead to an unsatisfactory outcome, and they recommend the use of ultrasound guidance as a routine during embryo transfer.

In-vitro maturation

In-vitro maturation (IVM) of human oocytes from antral follicles is an emerging new technology which has several advantages over traditional controlled ovarian hyperstimulation (COH) for IVF, particularly in reducing the risk of OHSS for patients with polycystic ovaries (PCO), as well as maintaining lower costs of treatment. Although IVM is still in a developmental stage for human clinical IVF, several hundred children have been born following the use of this technique, and consequently it may be considered as an alternative treatment strategy for selected patient groups. IVM technology has been successfully used in farm animals for decades, and collection of immature oocytes continues to be routine. Pincus and Enzmann (1935) were the first to show that immature rabbit oocytes removed from their natural ovarian environment were capable of undergoing spontaneous maturation and fertilization in vitro. Robert Edwards made similar observations in human oocytes in 1965. Lucinda Veeck and colleagues matured human oocytes in the laboratory that had been retrieved from COH cycles in 1983, and these subsequently fertilized and resulted in live births. However, such "rescued" oocytes are often suboptimal, showing a high incidence of aneuploidy as well as other defects. Current technology aims to retrieve immature oocytes from unstimulated or slightly stimulated small antral follicles, and culture these cumulus-enclosed oocytes in a specifically designed medium. Cha *et al.* (1991) reported the first human live births after retrieval of immature oocytes from antral follicles.

The phrase "cytoplasmic maturity" was coined by Delage in 1901, to point out that it was not necessarily synchronous with nuclear maturity. This is certainly true for human oocytes, where a metaphase II nucleus does not guarantee developmental competence. Whereas nuclear maturation is relatively visible, cytoplasmic maturity is much more difficult to assess microscopically. The aim of IVM is to retrieve and rescue immature oocytes before they are adversely affected by the endocrine and paracrine influences of the growing dominant follicle. Studies in the bovine have shown that oocytes retrieved in later phases of follicular development have more mitochondrial RNA (mRNA) transcripts compared with oocytes from less developed follicles, correlated with good embryonic developmental potential. Data from animals suggest that mRNA instability and the absence or abundance of certain transcripts such as LH and FSH receptors, connexin 43, and cyclooxygenase-2 in the cumulus of oocytes from small antral follicles after resumption of meiosis in vitro are predictors of

oocyte quality. The presence of other key components that have been described in rodents, such as growth differentiation factor-9 (GDF-9) and bone morphogenetic protein-15 (BMP-15), mediate important biochemical changes in oocytes required for normal postmeiotic events.

Although protocols are by no means standard, it seems that an initial priming of the ovaries with low doses of FSH from Days 2–6 improves success, followed by administration of hCG and co-culture of the oocytes with granulosa/cumulus cells in a medium that is supplemented with FSH and LH or hCG. There are several considerations to be taken into account while handling immature oocytes: since the volume of follicular fluid retrieved in IVM procedures is small and often contains blood, it is advisable to use heparin (2–5 IU/mL) in HEPES-buffered medium to minimize blood clotting. As in traditional IVF technology, attention should be given to pH and temperature settings. Oocytes from small antral follicles differ morphologically from mature oocytes and are often found enclosed within a compacted mass of surrounding granulosa cells. The degree of expansion of the cumulus cells may be influenced by the size of the follicle, as well as by the exposure to gonadotropins and/or hCG in vivo.

Immediately after retrieval, cumulus-enclosed immature oocytes are placed in IVM medium for 24–48 hours. Numerous IVM media formulations are commercially available, composed of standard IVF medium with the addition of recombinant FSH and hCG. Patient-inactivated serum or follicular fluid is often added to the maturation media as an exogenous protein source. Growth factors, lipids, glycoproteins, steroid hormones, cytokines and other factors in the serum or follicular fluid may be instrumental in the regulation of oocyte maturation and account for its preference over synthetic serum substitute or human albumin.

ICSI is the preferred method of insemination in IVM, since oocytes are frequently denuded of granulosa cells for evaluation of maturational status and there are fears of zona pellucida hardening during the IVM process. Studies comparing ICSI versus IVF for the insemination of IVM oocytes have shown that ICSI results in higher rates of fertilization than IVF; however, the developmental potential of the fertilized oocytes was similar irrespective of the insemination method. Child *et al.* (2002) compared the outcome of IVM cycles with IVF in women of comparable age with PCO and polycystic ovary syndrome. The absolute number of oocytes collected, metaphase II oocytes, fertilized oocytes and cleaved embryos was significantly higher in the IVF group. The implantation rate was higher in the IVF group (17.1%) than in the IVM group (9.5%), but there were 12 (11.2%) cases of OHSS in the IVF group, compared with none in the IVM group.

Although results are promising, IVM in the human requires further basic research before being applied routinely to the IVF laboratory. The complexities of oocyte maturation, in particular the unknowns in cytoplasmic maturity (see Chapter 4) and the inherent problems in methylation that may be induced by suboptimal culture conditions (Ménézo *et al.*, 2010) warrant caution.

Transport IVF and transport ICSI

The facilities of a central expert IVF laboratory can be used to offer treatment in hospitals that do not have the necessary laboratory space and personnel. Carefully selected patients undergo ovarian stimulation, monitoring and oocyte retrieval under the care and management of a gynecologist who has a close liaison with an IVF laboratory team in a location that can be reached ideally within 2 hours of the hospital or clinic where the oocyte retrieval procedure takes place. It is an advantage to select patients with simple, uncomplicated infertility and good ovarian response, and close communication and coordination between the patient, physician and IVF laboratory team is essential.

Selection criteria for transport IVF/ICSI

1. Women 35 years of age or less
2. Tubal damage as the sole cause of subfertility

Exclusion criteria

1. Women over 35 years of age
2. Patients with LH:FSH ratio higher than 3:1
3. Patients with laparoscopically proven moderate or severe endometriosis
4. Patients requesting oocyte donation or donor insemination
5. Three previously unsuccessful IVF treatment cycles

A GnRH-agonist/FSH long protocol is used for superovulation so that a simplified monitoring

regimen can be used (ultrasound assessment and optional serum estradiol levels) and to allow scheduled admission of patients into the stimulation phase. This protocol also allows latitude in the administration of hCG, so that the timing of oocyte retrieval can be scheduled in a routine operating list. Ovulation is induced by hCG administration 36 hours prior to the planned follicular aspiration.

The couple under treatment must visit the central unit before hCG is given, both to receive detailed information and consent forms, and to familiarize themselves with the journey and the facilities. The husband will return to the central unit on the morning of oocyte retrieval to produce a semen sample and to collect a prewarmed portable incubator. The portable incubator, plugged into a car cigarette lighter, is then used to transport the follicular aspirates which have been collected in the peripheral hospital or clinic. Follicular aspirates are collected under ultrasound-guided control into sterile test-tubes (without flushing). It is essential that each test-tube is *filled completely* and tightly capped in order to prevent pH fluctuations. A heated test-tube rack during aspiration must be used to prevent temperature fluctuations in the aspirates. At the end of the oocyte retrieval procedure, the partner transports the follicular aspirates, together with the treatment records, to the central laboratory for oocyte identification and subsequent insemination and culture.

The embryo transfer procedure is carried out 48 hours later at the central unit. Patient follow-up is carried out by the physician at the peripheral unit. Provided that the instructions and inclusion criteria are strictly adhered to, a highly motivated, well-coordinated team working in close liaison can achieve success rates comparable to those obtained in the specialist center, and IVF treatment can thus be offered to couples to whom it might otherwise be unavailable. Transport ICSI can also be successfully offered on a similar basis (see Chapter 13).

Conclusion

In view of the complexity of the elegant biochemistry and physiology involved in the development of a competent oocyte that will fertilize and develop successfully in vitro, as well as the delicate balance that must be required within each contributing component and compartment, it seems miraculous indeed that the application of essentially ill-defined strategies has led to the successful birth of so many children. Although success rates have improved significantly over the past two decades, there continues to be a wastage of embryos that fail to implant. A great deal more research is required to identify and define factors involved in the development of competent oocytes and viable embryos, and new data from the application of current research using metabolomic and proteomic strategies are awaited.

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Chapter

12 Cryopreservation of gametes and embryos

Principles of cryobiology

The first live births following frozen-thawed embryo transfer were reported in 1984 and 1985 by groups in Australia, the Netherlands and the UK. Since that time, the original protocols have been modified and simplified such that cryopreservation as an adjunct to a routine IVF program may lead to successful survival of up to 80% of the embryos frozen, with subsequent pregnancy and live birth rates that contribute significantly to cumulative conception rates after fresh embryo transfer. Although the routine protocols required are simple and easily undertaken, an understanding of the basic principles of cryobiology involved is essential to ensure that the methodology is correctly and successfully applied, in order to minimize cell damage during the processes of freezing and thawing. There are two major classes of physical stresses that cells are exposed to during freezing:

1. Direct effects of reduced temperature
2. Physical changes associated with ice formation.

Direct effects of reduced temperatures

Cold shock injury (damage to cell structure and function arising from a sudden reduction in temperature) is species-specific and well documented for spermatozoa (cattle, pig), oocytes and embryos (pig). This phenomenon is associated with modifications in membrane permeability and changes in the cytoskeletal structure (reviewed by Watson and Morris, 1987), but is not evident following conventional cooling of human sperm and embryos. However, when freezing oocytes, ovarian tissue and testicular tissue, the possibility of sublethal injuries does exist, particularly those associated with breakdown of mitotic spindles.

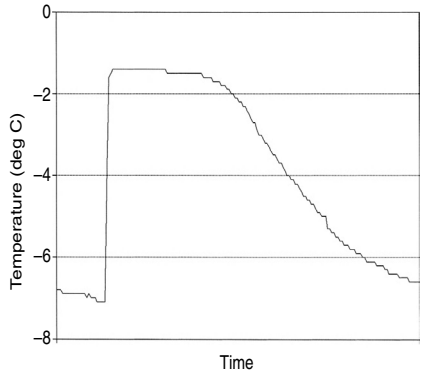

Figure 12.1 Temperature changes during the freezing of an aqueous solution of glycerol (5% w/v).

Physical changes associated with ice formation

The temperature changes observed during the freezing of an aqueous solution are illustrated in Figure 12.1. Water and aqueous solutions have a strong tendency to cool below their melting point before nucleation of ice occurs: this phenomenon is referred to as supercooling, or more correctly undercooling. For example, whilst 0°C is the melting point of ice, the temperature of water may be reduced significantly below 0°C before ice formation occurs, and in carefully controlled conditions water may be cooled to approximately –40°C before ice nucleation becomes inevitable (homogeneous nucleation temperature). Following ice nucleation and initial crystal growth the temperature rises to its melting point and remains relatively constant at that temperature during the subsequent phase change to ice ("latent heat plateau"), when the temperature then changes more rapidly to the environment temperature.

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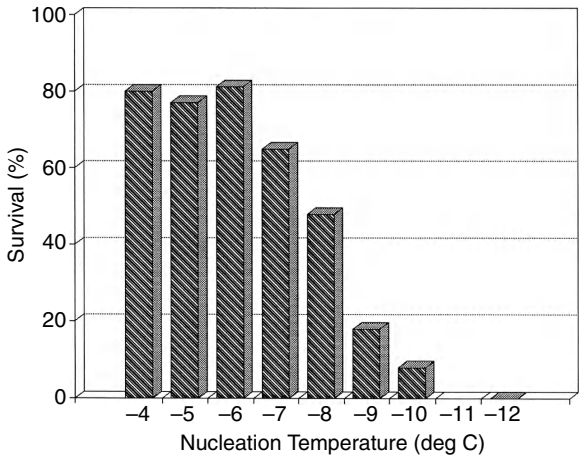


Figure 12.2 The survival of mouse cell embryos after seeding at various subzero temperatures. Redrawn from Whittingham (1977).

The tendency of a system to supercool is related to a number of factors including temperature, rate of cooling, volume, exclusion of atmospheric ice nuclei and purity of particulates. In cryopreservation of cells and tissues in IVF systems, there is thus a strong tendency for supercooling to occur. To avoid the damaging effects of supercooling on cells and in particular embryos (see below) ice formation is initiated in a controlled manner. This is commonly referred to as "seeding" – although, strictly speaking, this term refers to the introduction of a crystal to an undercooled solution. "Nucleation" is the initiation of ice other than by seeding, and this is the process generally practiced in IVF.

Supercooling and cell survival

Controlled ice formation during freezing is recognized to be a key factor in determining the viability of embryos following freezing and thawing (see Whittingham, 1977). In a carefully controlled series of experiments, samples which were nucleated below –9°C had a low viability, whilst nucleation at higher subzero temperatures of –5°C to –7.5°C resulted in much higher viability (Figure 12.2).

An analysis of the spontaneous nucleation behavior of straws (Figure 12.3) clearly demonstrates that if nucleation is not controlled, a poor recovery of embryos would be expected.

The physical basis of this injury is clear from examination of the thermal histories of supercooled straws (Figure 12.4). The differences between laboratories that achieve good results and those that are less successful can often be attributed to the practical step of ice nucleation, or "seeding." Straws can be frozen horizontally or vertically – this has no effect on viability or ease of ice nucleation. Embryos sink in the cryoprotective additive and will always be found at the wall of the straw when frozen horizontally, or at the bottom of the column of liquid when frozen vertically. Following thermal equilibration ("holding") at the nucleation temperature (–7°C), ice formation is initiated by touching the outside of the straw or ampule with a liquid nitrogen cooled spatula, forceps, cotton bud etc., at the level of the meniscus (seeding at both ends of a horizontal straw has also been advocated). This causes a local cold spot on the vessel wall, which leads to ice nucleation. Immediately following ice nucleation the temperature will rise rapidly (cf. Figure 12.1) and the ice front will propagate through the sample. Following ice formation the temperature returns at a rate of 2.5°C/min to –7°C. Cellular dehydration then occurs during subsequent slow cooling.

By contrast, in a straw supercooled to –15°C, spontaneous ice formation again results in a temperature rise followed by a rapid rate of cooling at 10°C/min to –5°C. The combination of a rapid rate of cooling and a large reduction in temperature does not allow the cell to dehydrate, and lethal intracellular ice formation is then inevitable (Table 12.1). This has been observed by direct cryomicroscopy.

Ice crystallization in an aqueous solution effectively removes some water from solution. The remaining aqueous phase becomes more concentrated, and a two-phase system of ice and concentrated solution coexists. As the temperature is reduced, more ice forms and the residual non-frozen phase becomes increasingly concentrated. For example, in glycerol and water a two-phase system occurs at all temperatures to –45°C. At –45°C the non-frozen phase solidifies, with a glycerol concentration of 64% w/v; this is the eutectic temperature. In dilute aqueous solutions such as culture media, there is a dramatic increase in ionic composition following ice formation and by –10°C the salt concentration reaches *c.* 3 molal – not surprisingly, this is lethal to cells. Cryoprotective additives reduce cellular damage during freezing and thawing by simply increasing the volume of the residual unfrozen phase. This reduces the ionic composition of the solution at any subzero temperature. It must also be noted that all

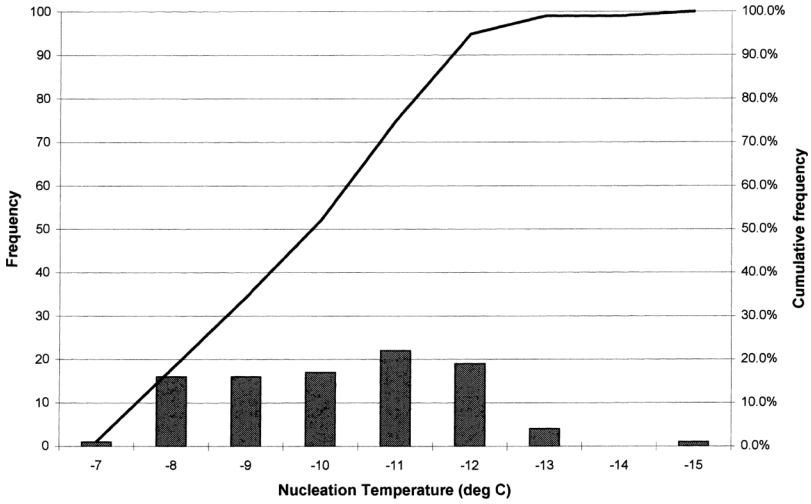


Figure 12.3 The measured nucleation temperatures within 0.25 mL straws cooling at 0.3°C/min.

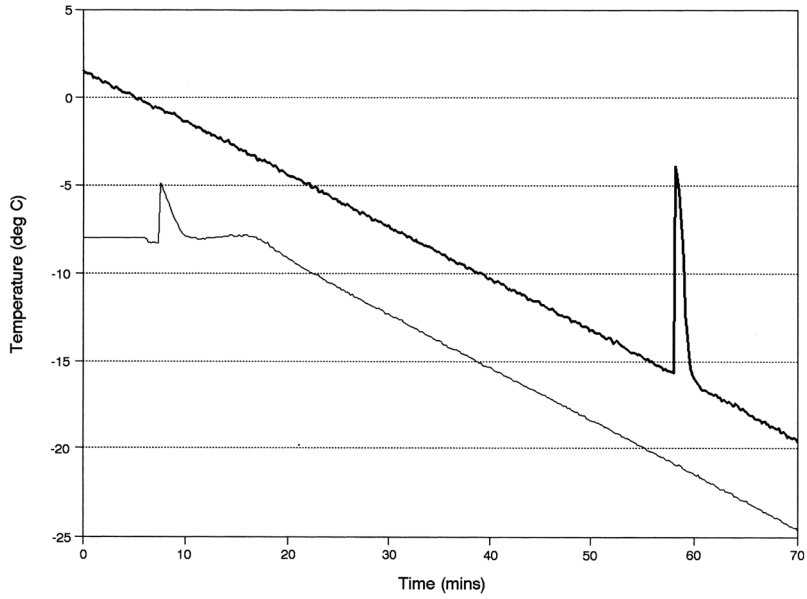


Figure 12.4 Measured temperatures within straws. During conventional cryopreservation the straws are held at a temperature of –7°C and nucleated, the resultant rise in temperature following ice nucleation is small. In the absence of induced nucleation the straws may reach very low temperatures before spontaneous nucleation occurs. A large rise in temperature to the melting point of the suspending medium then occurs followed by a rapid reduction in temperature; this will inevitably result in intracellular ice formation within embryos or oocytes.

other physical parameters of the solution change following the formation of ice, including gas content, viscosity and pH.

Cryoprotectants

Cryoprotectant additives (CPAs) are thought to protect cells by stabilizing intracellular proteins, reducing or eliminating lethal intracellular ice formation, and by moderating the impact of concentrated intra- and extracellular electrolytes. The first successful use of a cryoprotectant was in 1949, when Polge *et al.* used glycerol to freeze semen; glycerol is still commonly used as a cryoprotectant today. All cryoprotectants are completely miscible with water, easily permeate cell membranes, and some (but not all) are nontoxic even at high concentrations. All are hyperosmotic, and they can be divided into two groups:

1. Permeating: glycerol, ethylene glycol (EG), 1,2-propanediol (PROH) and dimethylsulfoxide (DMSO) penetrate the cell membrane, although more slowly than water. Inside the cell, they stabilize intracellular proteins, reduce the

Table 12.1 Factors associated with cooling and cryopreservation that contribute to cellular injury and death in biological systems (reproduced with permission from Shaw *et al.* 2000)

System	Type/cause of damage
All	Intracellular ice formation, extracellular ice formation, apoptosis, toxicity, calcium imbalance, free radicals, ATP levels, general metabolism, fertilization failure, cleavage failure, intracellular pH, parthenogenetic activation, cleavage
Membrane	Rupture, leakage, fusion, microvilli, phase transition
Chromosomes	Loss/gain, polyspermy, polygny (failure to extrude polar body), tetraploidy.
DNA	Apoptosis, fusion, rearrangements
Cytoskeleton	Microtubules dissolve, actin
Proteins/enzymes	Dehydration, loss of function
Ultrastructure	Microvilli, mitochondria, vesicles, cortical granules, zona pellucida
Zona pellucida	Hardening, fracture
Lipids	Free radicals?

temperature at which intracellular ice forms, and minimize osmotic damage due to electrolyte concentration effects. Glycerol penetrates tissue less readily than DMSO, PROH and EG. These newer cryoprotectants have higher water solubility, rapid tissue penetration and induce less osmotic damage at high concentrations.

2. Non-permeating: a variety of sugars, polymers and amphipathic compounds have been used as non-permeating cryoprotectants. Raffinose and lactose decrease the percentage of unfrozen water and/or decrease salt concentrations. Glycine, proline and trehalose are amphipathic compounds that interact with membrane lipids and proteins to alter phase transitions and hydration status.

Cryoprotectants should always be used in combination with non-permeating osmolytes such as sucrose or mannitol. These act as osmotic buffers to protect against cell swelling during the addition/removal of cryoprotectants. Egg yolk has been added to cryopreservation medium used to preserve animal sperm, since the low-density lipoprotein fraction of egg yolk is thought to protect against cold shock. However, this is no longer recommended for freezing human cells, as it introduces batch-to-batch variation and the possibility of bacterial contamination.

The pioneering studies on mammalian embryo cryopreservation used either glycerol or DMSO as cryoprotectants, but these have been superseded by the protocol of Lasalle *et al.* (1985), which uses PROH. PROH is considered to have a higher permeability to human embryos than either glycerol or DMSO, and is less toxic than DMSO. The real toxicity of cryoprotectants in cells is largely unknown; Stanic *et al.* (2000) reported that most of the decrease in sperm motility during cryopreservation was due to exposure to cryoprotectants rather than to the freezing process.

Factors that affect cellular response to freezing

Surface area

In general, the larger the cell, the slower it must be cooled to survive freezing. For example, a human oocyte has a diameter of 120 μm, a volume of 9.05×10^5 μm^3 and a surface area of 4.5×10^4 μm^2; a human sperm cell has a volume of 28 μm and a surface area of 120 μm^2. Thus, the flattened paddle-shaped sperm cell has a surface area to volume ratio of 4.3, whereas the ratio in the spherical oocyte is only 0.05. Consequently, when exposed to CPAs, spermatozoa reach osmotic equilibrium much faster than oocytes, and optimum

cooling rates for spermatozoa are much higher than those for oocytes and embryos.

Cell volume changes

At temperatures near 0°C, abrupt changes in volume can immediately damage cells and also make them more susceptible to stress during subsequent cooling or thawing procedures; therefore extreme fluctuations in cell volume must be avoided during CPA equilibration. The duration of exposure to these potentially toxic chemicals should also be minimized: CPA toxicity can be reduced by lowering the temperature of exposure, but this would require a longer exposure time.

Cooling to subzero temperature

When large cells such as oocytes are frozen in molar concentrations of CPAs, their survival is strongly dependent on cooling rate, and the specific optimum cooling rate depends on both the type and the concentration of CPA. Cell survival is equally dependent on warming rate, and the optimum warming rate depends on both the CPA and its concentration, as well as on the cooling rate that preceded it. Cryobiology studies have shown that different types of cell have different optimal cooling rates, even when frozen in the same solution. This is especially relevant to the cryopreservation of tissues such as ovarian cortex that are made up of many different types of cell, each with its own characteristic size, shape and permeability properties. Cooling and warming conditions that are optimal for one cell type may be harmful to others.

Osmotic events

As mentioned above, when aqueous solutions are frozen, water is removed in the form of ice, causing the cells to become increasingly concentrated as the temperature falls. The reverse occurs during thawing. Cells in suspension are not punctured by ice crystals (see Figure 12.5), nor are they mechanically damaged by ice. Cells partition into the unfrozen fraction and are exposed to increasing hypertonic solutions. Varying amounts of water may be removed osmotically from the cell, dependent on the rate of cooling. At "slow" rates of cooling, cells may remain essentially in equilibrium with the external solution. As the rate of cooling is increased, there is less time for water to move from the cell, which becomes increasingly supercooled until eventually intracellular ice is formed. An optimum rate of cooling results from the balance of these two phenomena. At rates of cooling slower than the optimum, cell death is due to long periods of exposure to hypertonic conditions. At rates of cooling faster than the optimum, cell death is associated with intracellular ice formation, which is inevitably lethal. The actual value of the optimum rate is determined by a number of biophysical factors, including cell volume and surface area, permeability to water, Arrhenius activation energy, and type and concentration of cryoprotective additives. As the cells are frozen, they must respond osmotically to large changes in extracellular fluid concentrations: the efflux of water during slow freezing (<2°C/min) causes oocytes to undergo osmotic dehydration, with resulting contraction.

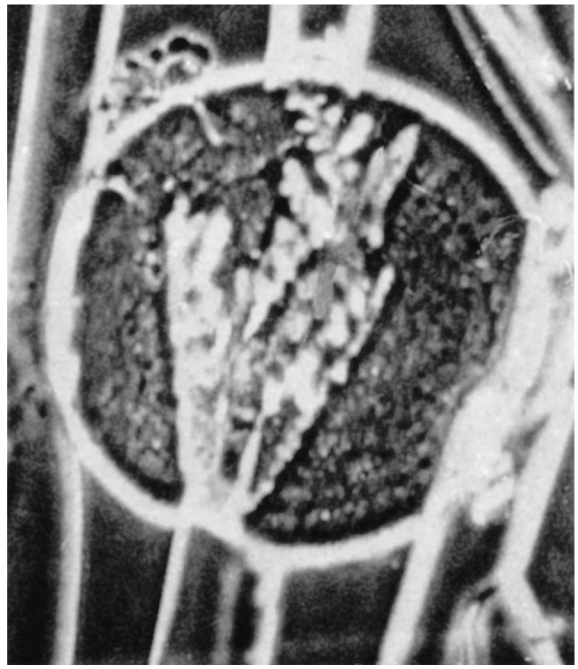


Figure 12.5 Light microscopy of a human oocyte following ice nucleation in the extracellular medium. The oocyte is not punctured by ice crystals.

Removal of cryoprotectant

Although cells in suspension can tolerate exposure to very high concentrations of CPAs, whether or not they survive the freeze-thaw process depends on how the CPA solution is removed. When frozen cells are warmed rapidly, the melting process is equivalent to rapid dilution of the CPA that was concentrated during the freezing process. As the extracellular milieu

begins to melt, the rapid influx of water into the cells can cause osmotic shock at subzero temperatures. Sensitivity to osmotic shock is therefore a function of the cell's permeability to water and solutes. This shock can be reduced by using a non-permeating substance such as sucrose as an osmotic buffer. Tissues are even more sensitive to osmotic effects than are cell suspensions, because cells located in the interior of a piece of tissue can respond osmotically only when the neighboring cells have also responded.

Cryopreservation protocols

Two basic types of cryopreservation are used for cells and tissues: slow freezing and vitrification.

Equilibrium freezing (slow freezing)

The procedures used for slow freezing of oocytes, embryos and ovarian cortex are generally quite similar. The cells or tissues are equilibrated in an aqueous solution containing an optimal concentration (1.0–1.5 M) of cryoprotectant and sucrose (0.1 M) and frozen in straws or ampules. Following CPA exposure the temperature is slowly lowered, and ice crystal growth is initiated in the solution ("seeding"). The ampules or straws are seeded at –6.5 to –7°C, cooled slowly at 0.3–0.5°C /min to approximately –40°C, then quickly cooled to –150°C before final transfer into liquid nitrogen for storage. Embryos and spermatozoa do not deteriorate when stored even for decades in liquid nitrogen.

Non-equilibrium protocols: vitrification/ultra-rapid freezing

Vitrification combines the use of concentrated cryoprotectant solutions with ultra-rapid cooling in order to avoid the formation of ice. Samples reach low temperatures in a state that has the molecular structure of a viscous liquid without crystals, forming an amorphous glassy solid. Rapid cooling is often carried out by direct contact with liquid nitrogen, and therefore programmable freezing machines are not needed. There is an inverse correlation between the cryoprotectant concentrations and cooling rates required, and successful vitrification is based on the use of very high concentrations of cryoprotectant or cryoprotectant mixtures, with an extremely high cooling rate. However, CPAs are toxic, and high CPA concentrations increase the risk of osmotic shock to the cells, due to a rapid change in ionic conditions. In order to use vitrification in ART programs, experiments have been directed towards finding a practical means of achieving very high cooling and warming rates, with minimal potential toxicity:

- A balance must be achieved between the lowest level of least hazardous CPA and maximal cooling rate.
- Adequate dehydration and permeation of cells is essential, and therefore exposure time is important.
- In order to avoid the formation of both intracellular and extracellular ice, the initial cooling rate must exceed the critical cooling rate (CCR) of the solution; in human ART, experimental evidence suggests that the cooling rate should be >20 000°C/min, and preferably >50 000°C/min.

The cooling rate is affected by several parameters, and different methods have been employed in order to find an effective and practical solution, by varying CP solutions, combinations, exposure times, and temperature. The addition of sugars (sucrose, trehalose, fructose, sorbitol saccharose, raffinose) reduces the concentration of CP required; the permeability of mixtures is higher than that of individual components, and different combinations of CP have also been tried. Vitrification solutions are developed with the lowest possible concentration of CP compatible with achieving glass formation. Reducing volume to a minimum reduces potential toxicity and osmotic damage, and methods have been devised to reduce the volume of CP down to between 0.1 µL and 2 µL. Reducing the drop size, or increasing the number of embryos per drop risks diluting the CP with medium carried over from the culture drop, and this could allow the sample to freeze, with lethal results Two different types of carrier have been used:

1. "Open" systems: the sample comes into direct contact with liquid nitrogen, and a cooling rate of 23 000°C/min can be achieved.
   Electron microscope grid
   Open pulled straw
   Cryoloop
   Cryoleaf™ (McGill)
2. "Closed" systems: the carrier, but not the sample itself, is in direct contact with liquid nitrogen; the cooling rate is slower, around 12 000°C/min.
   Cryotip

CryoLock
Cryo BioSystem HSV straw

The thawing rate must also be rapid, in order to prevent devitrification and ice crystal formation during the transition state. The samples are kept in air (room temperature) for 1–3 seconds; for open systems, the sample is then immersed in dilution medium at 37°C, and for closed systems, the carrier is immersed in a 37°C water bath before transferring the sample to the dilution medium. The CP is diluted in several steps, in order to counterbalance osmotic effects as the CP leaves the cell.

Comparison of slow freezing vs. vitrification

Slow freezing

- Low concentrations of permeating + non-permeating cryoprotectants (1.5/0.1–0.3), PROH or DMSO/sucrose.
- Requires controlled-rate freezing machine, 2°C/min, then 0.3°C/min.
- Nucleation (seeding) and transfer to liquid nitrogen is critical.
- Well-established protocols and techniques.

Vitrification

- Avoids formation of ice that can damage membranes.
- Rapid method, simple equipment.
- No specialized controlled rate freezer required.
- Application into clinical practice has been slow, due to concerns about toxicity of high CP concentrations (up to 6 M).
- Use of very low volumes reduces toxicity risk.
- Requires critical process control: zero tolerance to any changes/fluctuations.
- Samples must be handled and moved very rapidly.
- Avoid accidental warming: stored samples are very fragile, and could be susceptible to mini-devitrification cycles during routine dewar use in a busy IVF laboratory.
- "Open" systems: direct contact with liquid nitrogen.

Numerous reports have now been published that confirm successful use of vitrification in human ART cryostorage, and several authors recommend vitrification instead of slow freezing for oocytes and blastocyst stage embryos in particular. However, questions remain about the long-term stability of the "glassy state" of the vitrified cells, which are prone to fracture; this may be a hazard under normal working conditions in the IVF laboratory with routine access to storage tanks. Questions have also been raised about the safety of "open" vitrification, in the perspective of the discussion below.

Storage of cryopreserved samples

During the early 1990s the transmission of hepatitis B virus between frozen bone marrow samples in a liquid nitrogen storage tank was demonstrated. This incident raised the possibility of pathogen transmission between samples in ART laboratories, and led to further consideration of potential sources of contamination and means of avoiding the transmission of infection.

Potential sources of contamination include:

1. *Within the freezing apparatus*. Vapor phase controlled rate freezers spray nonsterile liquid nitrogen directly onto the samples. This may be further compounded by liquid condensation that may accumulate within ducting between freezing runs. Ideally, a freezing apparatus should have the capability of being sterilized between freezing runs, but this is not a practical option.
2. *During storage*. Straws may be contaminated on the outside, or seals and plugs may leak. Particulates may then transfer via the liquid nitrogen within the storage vessel.
3. *From liquid nitrogen*. Generally, liquid nitrogen has a very low microbial count when it is manufactured. However, contamination may occur during storage and distribution. Any part of the distribution chain that periodically warms up, in particular transfer dewars or dry shippers, may become heavily contaminated. The microbial quality of the liquid nitrogen when delivered from the manufacturer varies widely with geographical region and more extreme reports of microbial contamination may reflect local industrial practices.

The HFEA in the UK prepared a consultation document with guidelines for safe storage of human gametes in liquid nitrogen (HFEA, 1998); basic recommendations include patient screening for hepatitis B, hepatitis C and HIV, careful hygiene throughout, double containment of storage straws and the use of sealed ampules. The risks of cross-contamination during the quarantine period need to be assessed and procedures put in place to minimize these risks. However, the literature now available on animal models and human IVF has been reviewed by Pomeroy *et al.* (2009), and this

review suggests that in practice, the risk of cross-contamination in IVF working conditions is negligible.

Embryo cryopreservation

Following fresh embryo transfer in a stimulated IVF cycle, supernumerary embryos are available for cryopreservation in a large number of cycles. In a routine IVF practice, more than half of stimulated IVF cycles may yield surplus embryos suitable for cryopreservation (although this is now subject to legislative control in particular countries of the world). In addition to enhancing the clinical benefits and cumulative conception rate possible for a couple following a single cycle of ovarian stimulation and IVF, a successful cryopreservation program offers other benefits including the possibility of avoiding fresh embryo transfer in stimulated cycles with a potential for ovarian hyperstimulation syndrome, or in which factors that may jeopardize implantation are apparent (e.g., bleeding, unfavorable endometrium, polyps or an extremely difficult embryo transfer).

Consent to storage after cryopreservation

A unit that offers embryo cryopreservation must also be aware of logistic, legal, moral and ethical problems that can arise, and ensure that all patients are fully informed and counseled. Both partners must sign comprehensive consent forms indicating how long the embryos are to be stored, and define legal ownership in case of divorce or separation, death of one of the partners, or loss of contact between the Unit and the couple. Cryopreserved samples cannot in practice be maintained in storage indefinitely, and there must be a clear clinic policy to ensure that records are correctly maintained, with regular audits of the storage banks. Clinic administration may mandate that all couples with cryopreserved embryos in storage must be contacted annually, and asked to return a signed form indicating whether they wish to continue storage. In the UK, options for couples include:

1. Continue storage.
2. Return for frozen embryo transfer.
3. Donate their embryos for research projects approved by appropriate ethics committees/Internal Review Boards and the HFEA.
4. Donate their frozen embryos for transfer to another infertile couple.
5. Have the embryos thawed and disposed of.

Selection of embryos for freezing

Using PROH as cryoprotectant, embryos can be frozen at either the pronucleate or early cleavage stages. Careful selection of viable embryos will optimize their potential for surviving freeze-thawing.

Pronucleate

The cell should have an intact zona pellucida, and healthy cytoplasm with two distinct pronuclei clearly visible. Accurate timing of zygote freezing is essential to avoid periods of the cell cycle that are highly sensitive to cooling. For example, during the period when pronuclei start to migrate before syngamy, with DNA synthesis and formation of the mitotic spindle, the microtubular system is highly vulnerable to temperature fluctuation, leading to possible scattering of the chromosomes. Zygotes processed for freezing at this stage will no longer survive cryopreservation. The timing of pronucleate freezing is crucial, and the process must be initiated while the pronuclei are still distinctly apparent, no later than 20–22 hours after insemination.

Cleavage

Two- to eight-cell embryos should be of good quality, grade 1 or 2, with less than 20% cytoplasmic fragments. Uneven blastomeres and a high degree of fragmentation jeopardize survival potential; embryos with damage after thawing may still be viable and result in pregnancies, but their prognosis for implantation is reduced.

Embryo cryopreservation: method

Details of each patient and the associated embryos must be carefully recorded on appropriate data sheets. Meticulous and complete record keeping is crucial, and must include the patient's date of birth, medical number, date of oocyte retrieval (OCR), date of cryopreservation, number and type of embryos frozen, number of straws or ampules used, together with clear and accurate identification of storage vessel and location within the storage vessel. The data sheets should also confirm that both partners have signed consent forms. Both ampules and straws have been successfully used for embryo storage, and each has advantages and disadvantages. The choice between them is a matter of individual preference, as well as availability of storage space and laboratory time to prepare and sterilize ampules. When straws are used, they must be handled with care to avoid external contamination, and to avoid inadvertent temperature fluctuations during seeding or transfer to the storage dewar. The measured temperature excursions within straws can be very dramatic (see

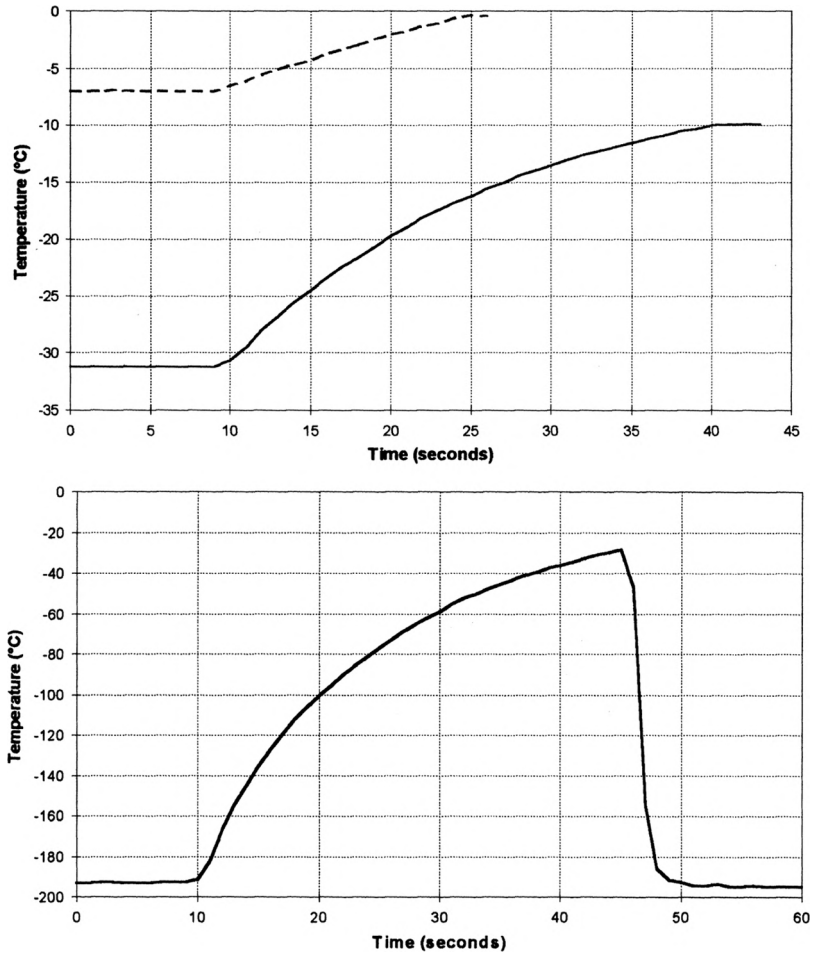


Figure 12.6 Measured temperatures within straws following removal from a controlled rate freezer or from a liquid nitrogen vessel at various points during the freezing cycle. Prior to nucleation the temperature rise within 5 seconds is sufficient to prevent ice nucleation. At –30°C the sample temperature may rise very quickly and if transfer to liquid nitrogen is carried out at this point of the freezing program, care must be taken to ensure that the increase in temperature is minimized. Following liquid nitrogen immersion the temperature of straws may rise by 130°C within 20 seconds.

Figure 12.6). It is likely that in straws frozen horizontally the embryos will be adjacent to the wall, where they will be exposed to the highest thermal gradient; great care must be taken in handling cryopreserved material. Plastic cryovials are not recommended for embryo freezing.

Ready-to-use media for freezing and thawing embryos are available from the majority of companies who supply culture media. Individual methods and protocols vary slightly with the different preparations, and manufacturers' instructions should be followed for each.

- Care must be taken to ensure that no air bubbles are trapped within the freezing medium after the sample has been loaded, into either ampules or straws. Air bubbles can sometimes be seen in both vessels on thawing, and these present a hazard to the fragile dehydrated embryo. Warming solutions to 37°C before starting the procedure may effectively act as a "degassing" mechanism.
- It is common practice to cool human embryos within the controlled rate freezing apparatus down to below –100°C after the slow cooling to –30°C, before transfer to liquid nitrogen. In veterinary IVF cryopreservation, straws are often transferred to liquid nitrogen directly from –30°C. This procedure would give equally good results for human embryos and is indeed used by some laboratories with no reduction in viability. However, it is essential that the transfer is carried out rapidly (within 5 seconds) because the temperature of the straws may rise very rapidly when they are removed from the controlled rate device (see Figure 12.6). Cooling to temperatures below –100°C within the freezing machine carries less risk, but does consume considerably more liquid nitrogen.

Sample protocols

Sample protocol for embryo slow freezing

1. Equilibrate selected and washed embryos in 1,2-propanediol (1.5 M) at room temperature, to allow uptake of the CP into the cells. This is usually done in two steps, the second step incorporating 0.1 M sucrose.
2. Load equilibrated embryos into straws or ampules.
3. Cool the samples at a rate of 2°C/min to –7°C, and "hold" at this temperature to allow thermal equilibration before ice nucleation (seeding).
4. Following seeding, with initiation and growth of ice crystals, cool the samples at a slow rate, –0.3°C/min, down to –30°C.
5. Cool the samples rapidly to liquid nitrogen temperatures, then plunge and store in liquid nitrogen.

Sample protocol for embryo thawing after slow freezing

1. Samples are thawed in two stages: hold straws in air for 40 seconds, and then transfer to a 30°C water bath for a further minute.
2. Remove cryoprotectant by dilution through solutions containing 0.2 M sucrose, and then wash three times in culture medium.

The thawing protocol is carried out at room temperature, and the embryos placed in equilibrated culture medium at room temperature before being allowed to warm gradually to 37°C in the incubator. Pronucleate embryos may be cultured overnight to confirm continued development, and cleavage stage embryos are incubated for a minimum of 1 hour before transfer.

Use of glass ampules

Tissue-culture washed borosilicate glass ampules with a fine-drawn neck can be used for embryo cryostorage.

- Fill the ampule with approximately 0.4 mL of the sucrose/PROH solution using a needle and syringe.
- Carefully transfer the embryos using a fine-drawn Pasteur pipette.
- Using a high-intensity flame, carefully heat-seal the neck of the ampule. It is important to ensure (under the microscope) that the seal is complete, without leaks: leakage of liquid nitrogen into the ampule during freezing will cause it to explode immediately upon thawing. It is often impossible to detect whether the glass neck is completely sealed, and the possibility of explosion can be avoided by opening the ampule under liquid nitrogen before thawing.

Ice nucleation: practical points

1. Because straws have a large surface area, small diameter and a thin wall, very rapid warming occurs when they are removed from a cold environment. Measured temperature excursions that occur at different points of the cryopreservation procedure are illustrated in Figure 12.6. If straws are removed from the controlled rate freezing apparatus for excessive lengths of time during the nucleation procedure, they can warm to a temperature that is too high for ice nucleation to occur. Ice nucleation may occur because of the local cooling induced by the nucleating tool, but it is possible that the bulk temperature of the fluid may not allow ice crystal growth to propagate through the sample. In some laboratories, it is common practice to check that ice propagation has occurred throughout the sample, usually 1 minute after the seeding procedure. If straws are removed from the controlled rate cooling equipment, this in itself may cause melting of the nucleated ice.
2. The thermal control of the freezing apparatus may not be sufficiently accurate or stable at the nucleation temperature. The temperature achieved may allow nucleation to occur because of the thermal mass of the nucleating tool, but may not be sufficiently low to allow subsequent ice propagation. Any thermal fluctuations within the freezing apparatus may also lead to the melting of ice.
3. Within straws, nucleation of ice at temperatures very close to the melting point results in a very slow propagation of ice through the sample. In some cases, the ice propagation can actually become blocked, and embryos are then effectively supercooled. In this case the embryos would not be expected to survive further cooling.

Blastocyst freezing

The first reports of successful human blastocyst cryopreservation were published in 1985 (Cohen *et al.*, 1985; Fehilly *et al.*, 1985), but blastocyst freezing became routine in IVF only after media for effective extended culture became available during the 1990s. Using Vero cell co-culture to enhance extended culture, Ménézo *et al.* (1992) explored the use of a combination of glycerol and sucrose as cryoprotectants to

freeze surplus expanded blastocysts, and the protocols were later modified to obtain satisfactory freeze-thaw rates. Inconsistent success rates were reported initially, but this may have been partly due to lack of experience with selection criteria for freezing, and also a need to understand the subtleties of cryopreservation and the impact that even the slightest variation might have on consistency. Extended culture to blastocyst stage is now routine in many IVF laboratories, and the companies that supply blastocyst media also offer blastocyst cryopreservation media and protocols. Glycerol is the cryoprotectant of choice for slow freezing of blastocysts, and extra sucrose dilutions in the thaw have led to a substantial overall improvement.

Using strict criteria to select potentially viable blastocysts is crucial to success:

- Growth rate: expanded blastocyst stage on Day 5/Day 6.
- Overall cell number >60 cells (depending on day of development).
- Relative cell allocation to trophectoderm/inner cell mass.
- Original quality of early stage embryo: pronucleus formation and orientation, blastomere regularity, mono-nucleation, fragmentation, appropriate cleavage stage for time of development.

Blastocyst vitrification

Although it is currently too early to reach conclusions about pregnancy rates, blastocyst vitrification has recently become an increasing trend, with reports of very favorable survival, implantation and clinical pregnancy rates (Hong *et al*., 2009). Initial trials with human blastocysts were reported by Lane *et al*. in 1999, and commercial kits for blastocyst vitrification are available – as always, the ultimate success of the protocol will be related to the operator's experience and careful attention to detail. In common with all aspects of human ART, careful research into the consequences of such new therapies continues to be essential. In large expanded blastocysts, collapsing the blastocoelic cavity with an ICSI needle immediately before processing increases survival rates after both slow freezing and vitrification (Kader *et al*., 2009).

Assisted hatching and cryopreservation

Freeze-thawing is known to cause hardening of the zona pellucida, and the application of assisted hatching, particularly at the blastocyst stage, has been suggested as beneficial to implantation after freeze-thawing (Tucker, 1991). In some cases, zona fracture can be a routine result of some cryopreservation protocols (Van den Abbeel *et al*., 2000). Embryos with existing holes in the zona following PGD procedures can successfully survive and implant (Magli *et al*., 2006).

Clinical aspects of frozen embryo transfer

Freeze-thawed embryos must be transferred to a uterus that is optimally receptive for implantation, in a postovulatory secretory phase. Patients with regular ovulatory cycles and an adequate luteal phase may have their embryos transferred in a natural cycle, monitored by ultrasound and blood or urine luteinizing hormone (LH) levels in order to pinpoint ovulation. Older patients or those with irregular cycles may have their embryos transferred in an artificial cycle: hormone replacement therapy with exogenous steroids is administered after creating an artificial menopause by downregulation with a GnRH agonist.

Transfer in a natural menstrual cycle

1. Patient selection: regular cycles, 28 ± 3 days, previously assay luteal phase progesterone to confirm ovulation. An ovulation "kit" such as Clearplan can also be used in a previous cycle to confirm that the patient has regular ovulatory cycles.
2. Cycle monitoring from Day 10 until ovulation is confirmed, by ultrasound scan and plasma LH. Ultrasound scan should also confirm appropriate endometrial development; the cycle should be canceled if the endometrial thickness is <8 mm at the time of the LH surge.
3. Timing of the embryo transfer:
   - Pronucleate embryos: thaw on Day 1 after ovulation (3 days after the LH surge: LH + 3), culture overnight
   - Cleavage stage embryos: thaw and transfer on Day 2 or 3 after ovulation (LH + 4/5)
   - Blastocysts: thaw and transfer on Day 4 or 5 after ovulation (LH + 6/7).

Patients with irregular cycles may be induced to ovulate using clomifene citrate or gonadotropins, and

embryo transfer timed in relation to the endogenous LH surge or following administration of human chorionic gonadotropin (hCG). Although it is possible to estimate embryo transfer time using an ovulation "kit" to detect the LH surge, this is far less accurate, and does present a risk of inappropriate timing.

Schedule for frozen embryo transfer in a hormone replacement cycle

1. Patient selection: oligomenorrhea/irregular cycles, or age >38 years.
2. Downregulate with GnRH analogue (buserelin or nafarelin) for at least 14 days; continue downregulation until the time of embryo transfer.
3. Administer estradiol valerate:

Days 1–5	2 mg
Days 6–9	4 mg
Days 10–13	6 mg
Days 14 onwards	4 mg

4. Progesterone from Day 15–16, choice between:
   - Gestone 50 mg intramuscular or
   - Cyclogest pessaries 200 mg twice daily or
   - Utrogestan pessaries 100 mg three times daily or
   - 8% Crinone gel per vaginam, once daily.

   Double the dose from Day 17 onwards (100 mg Gestone, 400 mg twice daily Cyclogest, 200 mg three times daily Utrogestan, 8% Crinone gel PV, twice daily).
5. Embryo transfer
   (a) Pronucleate: thaw on Day 16 of the artificial cycle, culture overnight before transfer on Day 17 or 18.
   (b) Cleavage stage embryos: thaw and replace on Day 17 or 18.
   (c) Blastocysts: thaw and replace Day 19 or 20.
6. If pregnancy is established, continue hormone replacement (HRT) therapy with 8 mg estradiol valerate and the higher dose of progesterone supplement daily until Day 77 after embryo transfer. Gradually withdraw the drugs with monitoring of blood P4 (progesterone) levels.

   This protocol is also successfully used for the treatment of agonadal women who require ovum or embryo donation. In combination with prior gonadotropin releasing hormone (GnRH) pituitary suppression, the artificial cycle can be timed to a prescheduled program according to the patient's (or clinic's) convenience.

Semen cryopreservation

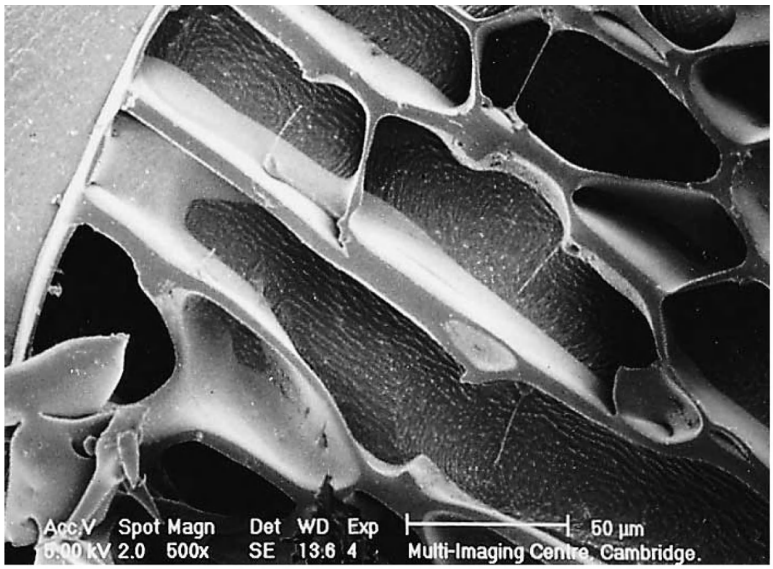
Cryopreserved semen has long been used successfully for artificial insemination (AI), intrauterine insemination (IUI) and IVF. Although freeze-thawing does produce damage to the cells with loss of up to 50% of pre-freeze motility, since large numbers of cells are available, successful fertilization can be achieved even with low cryosurvival rates. There is, however, a noticeable difference in sperm cryosurvival rates between normal semen and semen with abnormal parameters such as low count and motility; samples from men who require sperm cryopreservation prior to chemotherapy treatment for malignant disease frequently show very poor cryosurvival rates. The routine introduction of ICSI into IVF practice has surmounted this problem, so that successful fertilization using ICSI is possible even with extremely poor cryosurvival of suboptimal samples.

Effects of cryropreservation on sperm

Sperm membranes have an unusual lipid composition, with relative proportions of phospholipids, glycolipids and sterols that differ from those of other cell membranes. Reduction in temperature alters the membrane lipid organization and modifies the kinetics or intra-membrane proteins, leading to lowered permeability and loss of fluidity. This loss of fluidity is associated with lower sperm survival on thawing. Figure 12.7 illustrates the ultrastructure of human sperm following freezing with 10% glycerol as cryoprotectant. Frozen/thawed sperm behave in a similar way to capacitated sperm, which may lead to a shortened lifespan within the female tract; therefore the timing of insemination is important when using frozen sperm samples.

Semen can be successfully cryopreserved using either glycerol alone at a concentration of 10–15%, or a complex cryoprotective medium such as Human Sperm Preservation Medium (HPSM, Sperm Freeze™, FertiPro, Belgium) or Test Yolk Buffer (TYB medium, Irvine Scientific, Santa Ana, CA), Quinn's Sperm Freeze Medium (SAGE), Sperm Freezing Medium (Medicult). Adding the cryoprotectant gradually, dropwise, helps to minimize potential damage due to volume changes within the cell. Cooling and freezing can be carried out by using a programmed cell freezer, or by simply suspending the prepared specimens in liquid nitrogen vapor for a period of 30 minutes.

(a)



(b)

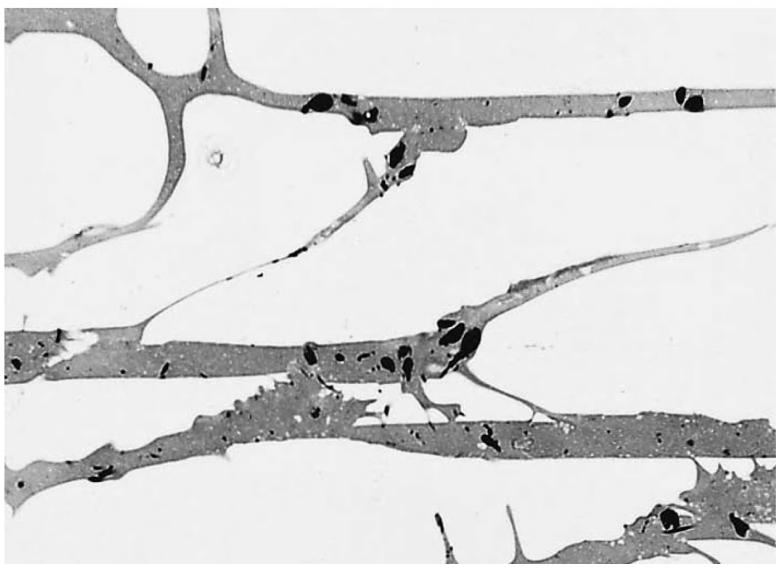


Figure 12.7 Ultrastructure of human sperm following freezing in a 0.25 mL straw; cells were suspended in glycerol (10%). (a) Freeze fracture followed by etching reveals the structure of ice crystals, cells are entrapped within the freeze concentrated material and few cell structures are evident. (b) Freeze substitution followed by sectioning shows cells entrapped within the freeze concentrated matrix.

Method

Samples should be prepared and frozen within 1–2 hours of ejaculation.

1. Allow the sample to liquefy, and perform semen analysis according to standard laboratory technique; label two plastic conical tubes and an appropriate number of 0.5 mL freezing straws or ampules for each specimen. Record all details on appropriate record sheets.
2. Add small aliquots of cryoprotectant medium (CPM) to the semen at room temperature over a period of 2 minutes, to a ratio of 1:1. If the ejaculated volume is greater than 5 mL, divide the sample into two aliquots before mixing with CPM.
3. Aliquot the diluted sample into straws or ampules, labeling aliquots for assessment of post-thaw count and motility.
4. Dilute specimens with CPM according to count.

60 million/mL	dilute 1:1 semen:CPM
20–60 million/mL	dilute 2:1 semen:CPM
20 million/mL	dilute 4:1 semen:CPM

5. Reassess the number of motile sperm/mL, which should ideally be 10 million or above.
6. Aliquot into prelabeled straws.

Manual freezing

1. Place the ampules or straws (in goblets) on a metal cane.
2. Refrigerate at 4°C for 15 minutes.
3. Place into liquid nitrogen vapor for 25 minutes.
4. Plunge into liquid nitrogen for storage, and record storage details.

The sample must be carefully washed or prepared by density gradient centrifugation to remove all traces of cryoprotectant medium before it is used for insemination by intrauterine insemination (IUI) or IVF.

Methods to improve sperm survival

Sperm survival and pregnancy rates are lower when the frozen samples used are from infertile men compared with samples from fertile donors, and there is evidence to suggest that sperm preparation in order to remove immotile and damaged sperm prior to freezing may help to select a population of sperm with a better chance of survival. The use of stimulants such as pentoxifylline may also improve survival after thawing (see Chapter 10).

Cryopreservation of testicular and epididymal sperm

Whereas the relatively poor survival rates (50%) obtained after freeze-thawing semen samples have not in the past presented a major problem due to the abundance of cells in the original specimens, it is not always possible to obtain an ejaculated semen sample; the current use of suboptimal ejaculate, epididymal and testicular samples in combination with ICSI demands a different approach in order to recover as many sperm cells as possible from each sample. Sample cryopreservation in cases of epididymal and testicular aspiration or biopsy has considerable advantages both to the patient and to the clinical and laboratory staff, in that sperm and oocyte retrieval procedures may be carried out on separate occasions; this strategy is now a successful routine in the majority of ART programs offering this form of treatment. Generally epididymal and testicular samples are cryopreserved using protocols developed for ejaculated sperm: this may not be optimal. Many changes to the membranes of sperm occur during maturation, and it is likely that the water permeability of testicular sperm, a major factor in determining the cellular response to freezing, is very different from that of ejaculated sperm.

In cases where prolonged washing and searching yield only very few sperm, sperm can be frozen individually or in small groups by injecting them into empty zona pellucida "shells," using a crude freezing solution with 8% glycerol in phosphate-buffered saline supplemented with 3% human serum albumin. Samples are recovered after washing the zonae through droplets, and more than 70% of sperm survive using this procedure with resulting successful pregnancy rates (Walmsley *et al*., 1999).

The criteria for sperm freezing have now changed, in that even the most inadequate samples can be frozen/thawed for successful ICSI. It is no longer necessary to do testicular sperm aspiration/percutaneous epididymal sperm aspiration on the same day as the oocyte retrieval – numerous groups report success with frozen/thawed testicular and epididymal samples. All biopsy samples can be successfully frozen. The use of cryopreservation buffers without egg yolk is recommended for testicular and epididymal samples.

Testicular biopsy samples

Freezing whole biopsy samples without prior processing is not recommended, as cryoprotectant solution will not equilibrate evenly throughout the tissue. Pieces of macerated or minced tissue can, however, be frozen with some success. It has also been reported that a higher proportion of testicular sperm retain their motility on thawing if they have been incubated 24–48 hours before freezing, and Van den Berg (1998) suggests that incubation at 32°C may be beneficial. If there is doubt about sperm viability after thawing, a simple hypo-osmotic swelling test will identify viable sperm before injection.

Cryopreservation of semen for cancer patients

Patients who are to be treated with combined chemotherapy for various types of cancer, such as Hodgkin's disease and testicular tumors, are frequently young or even adolescent. Recent progress in oncology has given these patients a greatly improved prognosis for

successful recovery, and cryopreservation of spermatozoa before initiating treatment can preserve fertility for the majority of patients.

All cancer treatment regimens are toxic to spermatogenesis, and the majority of patients will be azoospermic after 7–8 weeks of treatment. In some cases spermatogenesis is restored after some years, but in others there is minimal recovery even after a decade. Animal studies have indicated that spermatogenesis may be protected from the adverse effects of chemotherapy by inhibiting pituitary control of spermatogenesis with GnRH agonists, androgens or male contraceptive regimens. Similar protective regimens in humans are currently ineffective, and the strategy remains experimental.

Currently, there are no pretreatment parameters that can predict a patient's prognosis for recovery of fertility; the possibility of erectile dysfunction after treatment should also be borne in mind. Patients should be given general advice about the need for contraception when recovery is unpredictable, and advised to seek medical help early if fertility is required. Informed consent forms should be signed after discussion and counseling. Ideally, three sperm samples are collected before chemotherapy is initiated; animal studies suggest that chemotherapy may have a mutagenic effect on late-stage germinal cells, but in the absence of a known clinical significance in humans, sample collection after the start of treatment is preferable to no storage at all. Patients should be informed of the potential risks and receive appropriate counseling in such cases. Spermatogenesis is often already impaired due to the effects of the disease: many demonstrate hypothalamic dysfunction, and in severe cases pituitary gonadotropin secretion is altered. The tremendous stress caused by cancer reduces fertility potential by the action of stress hormones in the brain, leading to altered catecholamine secretion, rise in prolactin and corticotropin-releasing factor, which in turn suppress the release of GnRH. However, in the light of ICSI treatment success rates, semen samples should be frozen regardless of their quality. Prior to sample collection for storage, patients should be screened for hepatitis B and C and HIV.

Patients are naturally concerned that their cancer treatment might cause an increased risk of congenital malformation in a subsequent pregnancy: the results of studies to date are reassuring, although insufficient data have been accumulated for each cancer or treatment regimen. There are now several published reports of successful treatment for couples using sperm stored prior to treatment; type of treatment depends upon the quality of the sample, but pregnancies and live births are reported after AI, IUI, IVF and ICSI.

In the future, autotransplantation of cryopreserved testicular tissue may become an alternative option for young men who are not yet producing sperm, or who are unable to produce an ejaculate. Research has shown that gonocytes from immature mice injected into the tubules of sterilized hosts restore spermatogenesis and produce fertile spermatozoa – hopefully, this strategy may one day provide another option for cancer patients, especially for children.

Oocyte cryopreservation

Prior to 1997, the options for preserving a young woman's fertility after treatment for malignant disease were very limited: a full IVF treatment cycle with cryopreservation of embryos prior to the initiation of chemotherapy, or oocyte or embryo donation following recovery from the malignant disease. The first option is available only to women with partners to provide a semen sample for fertilization of the harvested oocytes. However, the success of frozen embryo cryopreservation in a competent IVF program is such that these patients maintain a very good chance of achieving a pregnancy after transfer of frozen-thawed embryos following recovery from their disease. On the other hand, this strategy also raises the risk of creating embryos with a higher than average chance of being orphaned. Many of the legal and ethical problems created by the cryopreservation and storage of embryos might be overcome by preserving oocytes, especially for young women about to undergo treatment for malignant disease that will result in loss of ovarian function. Oocyte cryopreservation may also be indicated in patients with a known family history of premature ovarian failure, and may be advantageous in various clinical scenarios, such as in ovarian hyperstimulation syndrome, unexpected lack of sperm following oocyte retrieval, egg donation programs and in order to extend the duration of natural fertility.

Human oocytes are particularly susceptible to freeze-thaw damage due to their size and complexity. They must not only survive thawing, but also preserve their potential for fertilization and development. The first pregnancies with human oocyte freezing were reported in the 1980s (Chen, 1986; Al Hasani *et al*., 1987), but the procedure was abandoned for approximately 10 years due to low survival and fertilization rates, thought to be

due primarily to hardening of the zona pellucida and to spindle damage causing aneuploidy. Since 1997, focus has intensified on modifying protocols to increase survival rates, in particular to avoid activation/premature release of cortical granules, zona hardening and the detection/avoidance of spindle damage and aneuploidy. In 2009, Noyes *et al.* reported the birth of more than 900 babies after oocyte cryopreservation, with no apparent increased incidence of congenital anomalies. Attempts to monitor alterations in the permeability of the plasma membrane, assess warming and rehydration protocols and use ICSI to improve fertilization rates have resulted in significant clinical progress.

Several intrinsic difficulties are associated with human oocyte freezing, due mainly to their high volume:surface area ratio and low membrane permeability. Intracellular ice formation causes critical damage to the cytoskeleton, which is also sensitive to osmotic stress. Disruption of the meiotic spindle can cause chromosome defects and aneuploidy. Lowering the temperature, or the cryoprotectant agents themselves may cause an increase in intracellular Ca^{2+} leading to changes in the intracellular signaling mechanisms and oocyte activation. Finally, since the zona hardens after freezing it is necessary to employ ICSI for fertilization of the thawed oocyte.

Freezing can result in parthenogenetic activation, leading to premature release of cortical granules (CGs). It is also important to consider the cytoplasmic maturity of the oocyte at freezing and the potentially toxic effects of cryoprotectants.

Several modifications have been made in order to improve the effectiveness of oocyte freezing:

1. Complete removal of the cumulus and coronal mass increases survival rates.
2. Altering sucrose concentrations from 0.1 to 0.2, 0.3 or 0.5 mol/L increases oocyte dehydration and survival.
3. Choline has been used as a substitute for sodium (Stachecki *et al.*, 2006; Boldt *et al.*, 2006) on the basis that cryodamage to the Na^+/K^+ pump might lead to high intracellular concentrations of Na^+ with a resulting efflux of protons. Choline does not cross the plasma membrane, is less toxic than high sucrose and does not affect osmotic pressure of the cell.

Not surprisingly, damage caused by oocyte freezing appears to be protocol-dependent (Rienzi *et al.*, 2004). Using a Polscope to observe the meiotic spindle following freeze-thaw procedures, these authors observed that the spindle disintegrates during freeze-thawing, and oocytes must reconstruct their spindles after thawing. Other authors using confocal or electron microscopy have shown that elevated sucrose concentrations may prevent spindle damage (Cottichio *et al.*, 2006; Nottola *et al.*, 2008). The timing of freezing after oocyte retrieval also seems to be important, with lower pregnancy rates reported from oocytes that were frozen more than 2 hours after OCR (Parmegiani *et al.*, 2009). Germinal vesicle stage oocytes show better survival (Sereni *et al.*, 2000), probably because these immature oocytes lack a spindle apparatus; however, the oocytes need to be matured in vitro post thaw, and this currently has limited success.

The thawing process is equally fraught with difficulties. Osmotic stress caused by rehydration must be minimized in order to prevent degeneration, and reassembly of the spindle post-thaw takes at least 3–4 hours.

Vitrification of human oocytes has many potential advantages over slow freezing. The first live birth was reported by Kuleshova *et al.* in 1999, and numerous studies with favorable results were published between 2005 and 2009. The main concerns with vitrification are the toxicity of high concentrations of cryoprotectant, and extreme osmotic changes. Huang *et al.* (2007) showed less damage to the spindle and chromosomes after vitrification compared to slow freezing. Cobo *et al.* (2008) compared sibling fresh oocytes with vitrified donor oocytes using the Cryotop method, reporting very high survival rates after warming (97%), and fertilization, blastocyst development and pregnancy rates for recipients that were equivalent to those obtained with the use of fresh donor oocytes.

Unfortunately, the published data on the subject of oocyte preservation are not homogeneous and are thus difficult to analyze. Different studies used sources of oocytes from different populations (pre-chemotherapy, egg donation, IVF), and there is also immense variability in protocols and procedures. In addition, results are reported in different ways, referring to success ranging from live birth per oocyte to simple freeze-thaw survival per oocyte. However, oocyte preservation is an emerging technology with encouraging results: to date, more than a thousand births have been reported after transfer of embryos generated from both slow-freeze and vitrified oocytes. Follow-up will continue to be important, and patients

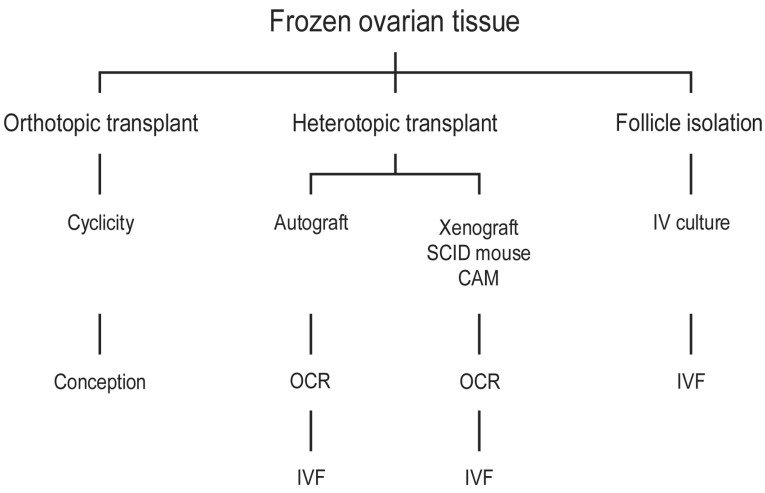


Figure 12.8 Options for ovarian tissue preservation.

should be offered realistic information and appropriate counseling until further research improves the outcome and confirms the safety of both slow freezing and vitrification techniques.

Ovarian tissue cryopreservation

Cryopreservation and banking of ovarian tissue is another attractive strategy for fertility conservation, indicated primarily for young women who will suffer anticipated loss of ovarian function due to premature ovarian failure, cancer or other diseases. Since follicle number diminishes with age, this option is open only to patients who are less than 30 years old and have had no previous chemo- or radiotherapy. The uterus must be functional and the patient should have a high probability of long-term survival after treatment. The type of malignancy is also important, as any risk of ovarian metastasis must be avoided. As discussed in the previous section, cryopreservation of mature metaphase II oocytes is also an option for women wishing to preserve their fertility. However, there are several potential advantages of freezing ovarian tissue rather than oocytes:

1. Small pieces of tissue contain very large numbers of primordial follicles and can be stored for children as well as for young adults.
2. Laparoscopic ovarian biopsy/oophorectomy can be carried out rapidly before chemotherapy, any time during the menstrual cycle, thereby avoiding delays in initiating therapy.
3. Germline cells are removed from cytotoxic harm and the entire tissue can be returned to the patient by grafting.
4. Storing cortical tissue theoretically preserves natural cell–cell interactions and intra-ovarian signals, and grafting can potentially restore both steroidogenic and gametogenic function – both important factors for the quality of life of the patient.

There are several options for freezing ovarian tissues (Figure 12.8), which depend on permeability properties, optimal cooling rates, susceptibility to cryo-injury and potential options after thawing.

Fragments or thin slices of ovarian cortex

Although storage of slices of ovarian cortex is an attractive alternative to mature oocyte freezing, there are a number of technical problems associated with the cryopreservation of ovarian tissues compared to isolated oocytes. Tissues respond very differently to ice formation than do cell suspensions. Cells in tissues are usually closely packed, and they also have interacting connections with each other and with basement membranes. Tissues have a three-dimensional structure and are traversed by fine capillaries or other blood vessels. Changes in extracellular ice surrounding the tissue during the freezing process, and recrystallization during warming of the tissue are both hazardous. In the hands of experienced cryobiologists morphological assessments of cryopreserved human ovarian cortex at the light microscope (Gook *et al.*, 1999) and electron microscope (Picton *et al.*, 2000; Kim *et al.*, 2001) have confirmed that cellular damage in the tissue can be minimal. However, the choice of an inappropriate CPA together with poor laboratory practice can lead to extensive cellular damage which

will compromise tissue viability on thawing (Picton *et al*., 2000). The problems of achieving adequate permeation of tissue fragments with CPA can be overcome either by preparation of thin strips of tissue <1 mm thick which provides maximal surface area for solute penetration (Newton *et al*., 1998), or by dissociation of the tissue into follicles or isolated cells before freezing (Cox *et al*., 1996). Most procedures stipulate that ovarian cortex slices are thawed rapidly by being swirled in a water bath at ~20°C or 37°C, and the CPA is progressively diluted from the tissue by repeated rinses with fresh medium.

Hemi-ovaries or whole ovaries

There are obvious advantages in conserving the whole ovary, in that the entire primordial population is retained and the cells and oocytes remain in their natural environment. There is also the potential for restoration of endocrine function and the menstrual cycle, preventing menopausal symptoms and improving quality of life. The main challenge with whole ovary preservation is that permeation is difficult and requires perfusion via the ovarian artery or vascular pedicle. The tissue is denser, with a complex structure of different cell types, a high cell density, a low surface area/volume ratio and decreased efficiency of heat transfer. Vitrification is particularly difficult since large amounts of cryoprotectant are required and different parts of the ovary will vitrify at different times. The cortex and primordial follicles are sustained by very small capillaries which are easily damaged, and this can lead to ischemic follicle loss and death of cortical tissue. The vessels can also fracture during warming, and pedicle lacerations can lead to possible thrombosis. Finally, revascularization is a time-consuming surgical procedure.

Dissected intact isolated primordial follicles and denuded primordial oocytes from isolated primordial follicles

While it may be possible to store ovarian tissue for young cancer patients, where there is any risk of reintroducing malignant cells in the tissue graft (Shaw and Trounson, 1997), a far safer strategy is to culture the follicles to maturity in vitro (Picton *et al*., 2000). Following fertilization by IVF or ICSI, embryos that are free from contamination could be transferred back to the patient. Freezing isolated primordial follicles has several advantages over freezing metaphase II oocytes, including their availability, size, lack of accessory cells, nuclear status (prophase I with intact nuclear membrane), absence of zona pellucida, CGs and low metabolic rate. The disadvantage of the technique is that prolonged in-vitro maturation is required, and follicle culture is difficult and unreliable – the growth from primordial to antral follicle takes approximately 70–90 days. Human follicle culture is still an emerging technology, but recent data are encouraging, and it may eventually be possible to grow primordial follicles to antral stages after cryopreservation (Picton *et al*., 2000). Nonetheless, a considerable amount of research is still needed to confirm that this strategy is safe, and that it does not induce epigenetic alterations in the female gametes, a possibility that has already been confirmed in a murine model system (Eppig *et al*., 2009).

Techniques for cryopreservation of ovarian tissue

Slow freezing of tissue has many intrinsic problems, but experimental slow freezing of human ovarian tissue slices have resulted in high rates of follicle survival after thawing (Nugent *et al*., 1998; Hovatta *et al*., 2004).

Tissue vitrification involves difficult challenges:

- Delivery of high intracellular concentrations of cryoprotectant in order to achieve high levels of dehydration.
- Contact time with solutions must be controlled so that rapid cooling and high heat transfer rates can be achieved whilst avoiding toxicity.

The outcome of the procedure depends on the sample size, the logistics of cooling and storage, direct contact with the liquid nitrogen and specialized containers. Protocols are either "open" (direct contact with liquid nitrogen) or "closed" (container in contact with liquid nitrogen. "Open" protocols are advantageous for rapid cooling:

- Direct cover vitrification (DCV, Chen *et al*., 2006)
- Solid surface vitrification (SSV, Huang *et al*., 2008)
- "Carrier-less" (Li *et al*., 2007) – drop tissue into shallow container of liquid nitrogen
- Copper grids, 42 mm holes (Isachenko *et al*., 2007)
- Cryotissue – metal strip full of holes (Kagawa *et al*., 2008).

Since tissue is involved, the danger of contamination using the open methods is much greater than in the case of oocytes.

Several closed devices offer an alternative: Cryotube, Cryobag, Straw, Crovial, Cryotip, Cryopette and the Ohio-Cryo (Kader *et al*., 2008).

Success of ovarian tissue preservation

There are varying degrees of success with all the above techniques. Intact or denuded follicles survive the thaw, but do not grow well after isolation, whereas tissue fragments or slices may show signs of injury/necrosis after culture. Tissues may be xenotransplanted in a SCID mouse or in chorioallantoic membrane (CAM) in the chick embryo. Alternatively tissue may be orthotopically or heterotopically autotransplanted; orthotopic sites include the pelvic peritoneum, the ovary or the ovarian fossa, and heterotopic sites include the rectus abdominis muscle, the abdominal wall, the breast or the forearm.

In whole ovaries, ultrastructural and apoptotic analyses have been used to assess viability after thaw/recovery. To date there has been success with cryo/transplant in rodents, rabbits, sheep and marmoset monkeys, and successful restoration of fertility in sheep after orthotopic transplantation of cortical strips to the ovarian pedicle. These grafts continue to function for up to 2 years. Cryopreservation experiments that used whole ovaries led to tissue survival in rats, rabbits, dogs, sheep and humans (Martinez-Madrid *et al*., 2004). Whole ovary vitrification and transplantation has been successfully performed in infantile rats, mice, rabbits, pigs and sheep.

Current status of ovarian transplantation (2010)

- Short-term restoration of natural fertility is possible.
- Graft follicle content is influenced by patient age and previous treatment and possibly by graft location.
- Lipid peroxidation occurs in murine and human ovarian grafts; vitamin E can significantly reduce lipid peroxidation and increase follicle survival.
- Gonadotropin environment probably has no effect on graft survival although low gonadotropins may be detrimental.
- Follicle survival has been demonstrated in orthotopic and heterotopic human autografts.
- Live births after autotransplantation are now being reported in clinical practice.

Autotransplantation in the human

Donnez *et al*. (2004) reported the first live birth after orthotopic transplantation of human cryopreserved ovarian tissue; they detected live follicles from 16 to 26 weeks after grafting, and some of the grafts still functioned after 3 years. Overall results to date are very encouraging, with 17 pregnancies, and 10 live births reported up to February 2010. A patient who had ovarian cortical tissue cryopreserved prior to treatment for Ewing's sarcoma in 2004 had six cortical strips transplanted after recovery from the disease, and has since delivered two healthy daughters: the first in 2007, and the second in 2008 (Andersen *et al*., 2008).

Considering the experimental status of this new technique in ART, it is paramount to test and optimize freeze-thawing results before offering a service to patients. Post-thaw tissue integrity, follicle counts and viability stains should be carried out. The EU Tissue Banking Directive regulations ask for centralized specialized cryobanks with rigid quality control to prevent transmission of disease. Clinical, psychological and ethical considerations must be considered since transplantation can only be performed as an experimental procedure and there is a potential risk of re-seeding tumor cells. On a more sober note, consent forms must be adequate and discussed with the patient in the event of death. In conclusion, cryopreservation of ovarian tissue is in its infancy, but is already successful as a tool for fertility preservation.

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Chapter

13 Micromanipulation techniques

Introduction

Biologists and physiologists began to micromanipulate cells during the last century, using a variety of manipulator systems to dissect or record from cells. The earliest attempt to inject sperm was recorded in 1914, when G.I. Kite injected starfish oocytes with sperm, but with inconclusive results (Lillie, 1914). Experiments in which sperm were injected into eggs around the mid-1960s were primarily designed to investigate the early events of fertilization, i.e., the role of membrane fusion, activation of the oocyte and the formation of the pronuclei. Two series of early experiments by independent groups demonstrated major species differences. Hiramoto showed in the 1960s that microinjection of spermatozoa into unfertilized sea urchin oocytes did not induce activation of the oocyte or condensation of the sperm nucleus, whereas others demonstrated the opposite in frog oocytes. Ryuzo Yanagimachi and his group later demonstrated that isolated hamster nuclei could develop into pronuclei after microinjection into homologous eggs, and a similar result was obtained when freeze-dried human spermatozoa were injected into a hamster egg. These experiments indicated that membrane fusion events can be bypassed during the activation of mammalian oocytes, without compromising the initiation of development. The experiments not only provided information on the mechanism of fertilization, but also led to a new technique in clinical embryology.

The first clinical application of microsurgical techniques was partial zona dissection (PZD) developed by Jacques Cohen and colleagues to aid fertilization in human oocytes This mechanical technique involves breaching a slit in the zona pellucida with a sharp glass micropipette and subsequently placing the dissected oocyte into a suspension of spermatozoa, on the assumption that sperm entry is facilitated by the slit. In the same year, S.C. Ng and colleagues in Singapore reported the first pregnancy from subzonal sperm injection (SUZI), where several spermatozoa are inserted into the perivitelline space. In 1990, the same group reported activation of human oocytes following intracytoplasmic injection (ICSI) of human spermatozoa, and in 1992 Palermo and colleagues in Brussels reported the first live birth from this technique of ICSI. Since the time of these pioneering reports, many healthy ICSI babies have been born worldwide. The technique of assisted hatching was also developed during the 1990s, using micromanipulation to cut a slit in the zona pellucida or dissolve a hole in the zona with an acid solution (zona drilling, ZD). Assisted hatching was proposed as a means of facilitating embryo implantation in selected cases. Figure 13.1 shows a diagrammatic representation of these micromanipulation procedures.

ICSI – intracytoplasmic sperm injection

Genetic implications

Prior to 1992, the majority of cases of severe male infertility were virtually untreatable, and failure of fertilization was observed in up to 30% of IVF treatments for male infertility. The introduction of micromanipulation techniques such as ZD, PZD and SUZI raised the hopes of a better prognosis for these cases, but did not overall provide a substantial improvement in success rates. The introduction and successful application of ICSI by GianPiero Palermo and colleagues at The Free University in Brussels, Belgium, produced a dramatic improvement in the treatment of severe male infertility by assisted reproductive technology.

The establishment of ICSI as a routine technique was quickly followed by the introduction of techniques

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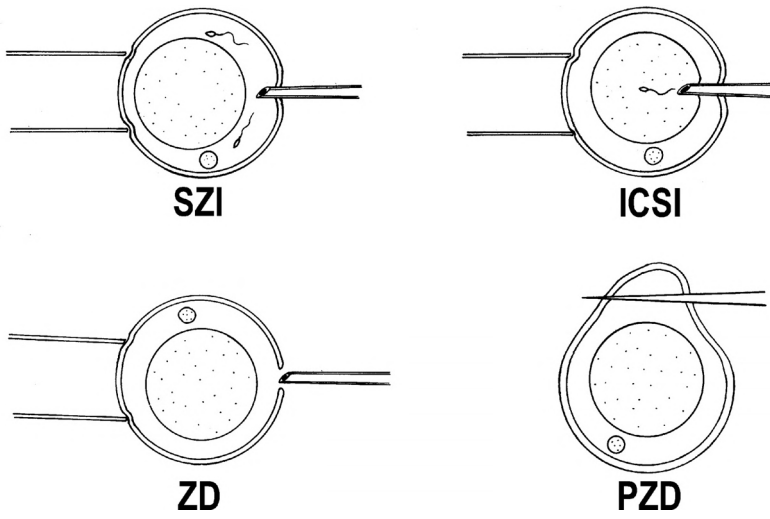

Figure 13.1 Micromanipulation techniques include subzonal injection (SZI), intracytoplasmic sperm injection (ICSI), zona drilling (ZD) and partial zona dissection (PZD).

for collecting sperm samples from the epididymis and directly from the testis, so that the whole spectrum of male infertility can now be treated, from suboptimal ejaculate samples or ejaculatory failure, to obstructive and nonobstructive causes of azoospermia. However, an increasing number of genetic defects have been found to be associated with male infertility: a higher incidence of numerical and structural chromosomal aberrations is found in infertile and subfertile men than in the general population, in particular karyotypes 47XXY, 47XYY, 46XX, 46X, derY, Robertsonian translocations, reciprocal translocations, inversions and additional marker chromosomes. Between 12% and 18% of men with azoospermia or severe oligospermia (less than 300 000 sperm in the ejaculate) have deletions in intervals 5 and 6 on the long arm of the Y chromosome. Microdeletions of the q11 region of the Y chromosome are related to the dysfunction of Deleted in AZoospermia (DAZ) and RNA-binding motif (RBM) genes, and androgen receptor (AR) gene mutations have also been reported in infertile men. In a population of approximately 3000 infertile men, the pathological (nonpolymorphic, phenotype associated) microdeletions rate in at least one of four critical regions on the Y chromosome was found to be as high as 22%, with an additional (as yet unknown) percentage being attributed to cryptic mosaicism. Furthermore, it appears that microdeletions will be transmitted in at least 10% of unselected father/son pairs. Three to ten per cent of infertile men present with congenital bilateral absence of the vas deferens (CBAVD), and approximately 65% of these individuals carry the gene for cystic fibrosis (CF), with defects in the cystic fibrosis conductance regulator (CFTR) gene. Many are compound heterozygous for the CFTR mutation, with an increased risk of having children with CF or CBVAD.

Although the genetic risk for couples who require ICSI treatment has yet to be fully defined, karyotyping, and preferably also Y-microdeletion analysis is recommended as part of the pretreatment screening process for all men referred for ICSI (Qureshi *et al*., 1996; Simoni *et al*., 1998). The couple should also have access to professional genetic counseling to discuss potential risks, and appropriate informed consent must be obtained before treatment.

Surgical sperm retrieval

In cases of obstructive azoospermia, samples can be aspirated from the epididymis. The original "open" microsurgical technique of microepididymal sperm aspiration (MESA) was superseded by the simpler procedure of percutaneous epididymal sperm aspiration (PESA), which can be carried out by fertility specialists without microsurgical skills, and can be performed under local anesthetic or mild sedation as an outpatient procedure. Aspiration is carried out using a 25-gauge butterfly needle connected to a syringe. If sperm cannot be aspirated from the epididymis, a modification of the technique using wide-bore needle aspiration of the testis, testicular sperm aspiration (TESA) or testicular fine needle aspiration (TEFNA) often harvests sufficient testicular spermatozoa to carry out an ICSI procedure. In nonobstructive azoospermia, spermatogenesis is

impaired. The epididymis is devoid of sperm, but the testis usually contains focal areas of spermatogenesis. Multiple biopsies may be required in order to identify these areas. The focal nature of spermatogenesis in such patients makes diagnosis based upon a single biopsy unrealistic, but prepared testicular samples can also be cryopreserved for a future ICSI procedure at the time of diagnostic testicular biopsy. The biopsy is carried out either by multiple needle aspirations (TEFNA or TESA) or by open biopsy (testicular sperm extraction: TESE), and both procedures may be safely carried out with local anesthetic or mild sedation.

Indications for ICSI

The ICSI procedure involves the injection of a single immobilized spermatozoon directly into an oocyte, and therefore not only can it be used for cases in which there are extremely low numbers of sperm, but in bypassing gamete interaction at the level of the zona pellucida and the vitelline membrane it can also be used in the treatment of qualitative or functional sperm disorders.

1. Couples who have suffered recurrent failure of fertilization after IVF-ET may have one or more disorders of gamete dysfunction in which there is a barrier to fertilization at the level of the acrosome reaction, zona pellucida binding or interaction, zona penetration, or fusion with the oolemma. ICSI should be offered to patients who have unexplained failure of fertilization in a previous IVF-ET cycle.
2. Severe oligospermia can be treated with ICSI; in patients where as many normal vital sperm can be recovered as there are oocytes to be inseminated, fertilization can be achieved in approximately 90% of cases. In extreme cases of cryptozoospermia, where no sperm cells can be seen by standard microscopy, centrifugation of the neat sample at a higher than usual centrifugal force (1800 *g*, 5 minutes) may result in the recovery of an adequate number of sperm cells.
3. Severe asthenozoospermia, including patients with sperm ultrastructural abnormalities such as Kartagener's syndrome, or "9 + 0" axoneme disorders can be treated by ICSI.
4. Teratozoospermia, including absolute teratozoospermia or globozoospermia.
5. In cases of CBAVD, vasectomy or postinflammatory obstruction of the vas, sperm samples can be retrieved by PESA, TESA or TESE.
6. Samples can be recovered by needle or open biopsy of the testis in cases of nonobstructive azoospermia.
7. In cases of ejaculatory dysfunction, such as retrograde ejaculation, a sufficient number of sperm cells can usually be recovered from the urine.
8. Paraplegic males have been given the chance of biological fatherhood using electro ejaculation and IVF (see www.multicept.dk); they may also be successfully treated using a combination of PESA/TESA/TESE and ICSI.
9. Immunological factors – couples in whom there may be antisperm antibodies in female sera/follicular fluid, or antisperm antibodies in seminal plasma following vasectomy reversal or genital tract infection can be successfully treated by ICSI.
10. Oncology – male patients starting chemotherapy or radiotherapy should have semen samples frozen for use in the future; ICSI offers the patient an excellent chance of achieving fertilization following recovery from their disease and treatment. Testicular biopsy specimens may also be cryopreserved for these patients as a further back-up when the quality of the ejaculate is inadequate for freezing.
11. For preimplantation genetic diagnosis where PCR is used, ICSI should be used as the means of fertilization to prevent sperm contamination of the sample.

Although the main indication for ICSI was originally for the treatment of male factor infertility, its use has become far more widespread, with an increasing trend to use it routinely for indications that include moderate male subfertility, advanced maternal age, low responder patients, donor oocytes or sperm; indeed, some clinics now use ICSI as a routine for all indications. European data for 2005 showed that the proportion of ICSI cycles in different countries ranged from 58% to 67%; in the USA, 62.2% of fresh non-donor cycles used ICSI during 2006. However, several randomized controlled studies have compared the efficacy of IVF versus ICSI in couples with non-male factor infertility, with results that showed no difference in fertilization or pregnancy rates. A Cochrane review (van Rumste *et al.*, 2004) concluded that the use of ICSI for non-male factor infertility remains an open question, and further research should focus on live birth rates and adverse events. In their 2008 report on good clinical treatment

in assisted reproduction, the European Society for Human Reproduction and Embryology (ESHRE) published an Executive Summary that concludes: "ICSI should be considered in the presence of severe sperm abnormalities or a history of fertilization failure in conventional IVF attempts. It must be emphasized that ICSI does not represent the most suitable treatment for female pathologies such as poor ovarian response or previous implantation failure."

Practical aspects

When scheduling patients for ICSI, it is important that the whole IVF team should appreciate the extra dimension of time and effort required for every case, and make an effort to schedule the laboratory workload accordingly. ICSI demands the same meticulous attention to detail that is needed in all IVF manipulations, but the number of details requiring attention is dramatically increased. Successful results with ICSI can only be achieved with the dedication of concentrated time, effort and patience.

Location of ICSI set-up

The laboratory should preferably be on a ground floor, near a structural frame or wall to minimize vibration interference, and must be kept dust-free. The equipment must be installed on a substantial bench top, away from distractions of traffic such as people or trolleys, etc. Any vibration will seriously interfere with the injection procedure, and it is essential to make sure that the equipment is completely stable, using anti-vibration equipment if necessary. Subdued lighting is helpful for microscopy. Well in advance of any ICSI procedure, ensure that the microscope is set up optically and checked. Ensure that the tool holders and all other parts of the micromanipulation system are correctly fitted and adjusted for optimal range of movement, and that the microtools can be accurately aligned.

Micro-injection equipment

All the major microscopy companies now supply micro-injection set-ups ready for use. The essential element is an inverted microscope with ×10, ×20 and ×40 objectives, Hoffman modulation contrast optics in order to visualize the cells on plastic Petri dishes (Nomarski optics uses polarized light and cannot be used through plastic). The micromanipulators consist of two coarse motorized manipulators and two fine mechanical, electrical or hydraulic joysticks, together with micro-syringes capable of delivering minute quantities of liquid. Tables 13.1 and 13.2 compare the different types of equipment currently available. For training purposes it is advisable to have a camera attached to one of the microscope optical outlets.

Microtools

Two types of microtools are used:

1. Holding pipettes to hold and immobilize the oocyte
   Outer diameter: 0.080–0.150 mm
   Inner diameter: 0.018–0.025 mm
   Fire-polished aperture
2. Injection pipettes to immobilize, aspirate and inject the sperm cell
   Outer diameter: 0.0068–0.0078 mm
   Inner diameter: 0.0048–0.0056 mm
   Beveled tip, sometimes tipped with a spike.

Both microtools are bent to an angle of approximately 30° at the distal end in order to facilitate horizontal positioning and manipulation adjustment within culture dishes. Aspiration pipettes (of different diameters) may also be used to aspirate anucleate fragments, or to biopsy blastomeres for preimplantation diagnosis. A third type of microtool may be used for piercing or cutting the zona pellucida in assisted zona hatching techniques. Uniform microtool quality is crucial for consistent results, and specifically tooled, sterile, ready-to-use holding and injection pipettes are commercially available. A blunt injection pipette can damage the oocyte by compression, whereas a pipette with too large a diameter will damage the oocyte by injecting too much fluid.

Supplies

Most manufacturers of tissue culture media supply all the components necessary for micromanipulation techniques.

Polyvinyl pyrrolidine (PVP)

A viscous solution of 10% polyvinyl pyrrolidine is used to reduce sperm motility prior to immobilization and aspiration into the injection pipette. Experienced operators can carry out the procedure without the use of PVP, but it is helpful in the initial stages of learning and practice. Experimental evidence has shown that PVP can interact with acrosomal and mitochondrial

Table 13.1 Micromanipulator

Manipulator type	Pros	Cons	Examples
Manual			
Fluid-filled: oil, fluorinert, distilled water	Easy control	Air bubbles render control difficult. Leakage of fluid can be messy, requires careful priming. Time-consuming to set up and flush system. Glass syringes expensive	Narashige, Eppendorf
Air-filled	OK for holding pipette	Good control only if set up carefully, with a bubble of oil between air and media	Research Instruments, Narashige
Pneumatic	Injection controlled by foot pedal	Pressure leakage	Tritech
Piezo Drill	Less disruption to the cytoplasm	Difficult learning curve; possible use of mercury	PrimeTech

Comments contributed by M. Blayney, A. Burnley, L. Devlin, D. Kastelic, M. van den Berg and B. Woodward.

Table 13.2 Joysticks

Joystick type	Pros	Cons	Examples
Hanging joystick	Comfortable to use for short cases	Elevated hand position can be uncomfortable during long ICSI cases	Narashige
Standing joystick	All-in-one 3-D control	May fall aside, with possible oocyte damage	Narishige, Eppendorf
Mechanical	Separate coarse and fine manipulators. Very little to go wrong	Usually fixed to the microscope, with possible vibration transmission; 2-D control separate to "up-down" control	Research Instruments
Hydraulic	Smooth	System fails if there are any leaks, the oil degrades in sunlight	Narashige
Motorized	Very convenient if positions can be stored in memory. Movements are quick, decreasing ICSI procedure time	Some have a delay, pipette continues to move over a small distance. Potential for breakdown, difficult to repair in-house	Eppendorf, Narashige

Comments contributed by M. Blayney, A. Burnley, L. Devlin, D. Kastelic, M. van den Berg and B. Woodward.

membranes, as well as cause chromatin deterioration after prolonged exposure, and questions have been raised about the wisdom/safety of injecting this artificial agent directly into ooplasm. Although no adverse effects have as yet been reported, it should be used cautiously, with attention to the time that the sperm is exposed to the polymer, and with efforts to minimize the amount that is injected into the oocyte cytoplasm.

Hyaluronic acid (HA)

Hyaluronic acid (HA), a natural component of the cumulus–oocyte complex, can be used as an alternative to PVP. HA has a relatively high negative charge and a high hydration capacity, so that viscous solutions can be prepared which can be used to slow sperm motility for the ICSI procedure; a commercial preparation using recombinant HA is available (Sperm Slow, Medicult). The motility of spermatozoa in a hyaluronate solution resembles that in the extracellular matrix of mature cumulus cells, and spermatozoa resume normal motility once returned to culture medium. Binding to HA has also been used as a marker of sperm maturity, and this offers added benefit to its use as an alternative to PVP for sperm impedance (van den Bergh *et al.*, 2009); a further advantage is that it degrades to natural sugar molecules that can be readily metabolized by cellular pathways.

Hyaluronidase

This enzyme is used to loosen and disperse cells of the cumulus and corona, prior to their removal from the oocyte by dissection. Preparations of sheep or bovine origin were commonly used in the past, but human recombinant hyaluronidase is now available (e.g., Cumulase, Origio; Hyase, Vitrolife), and its use is preferable in order to minimize risks of disease transmission from the animal sources.

ICSI – step by step

Selecting sperm for injection

As discussed in Chapter 10, there is evidence that sperm with DNA damage can have an adverse effect on the outcome of ART. Efforts have been made to develop techniques that will enhance sperm preparation methods that can be used to identify and select sperm with lesser levels of chromatin or DNA damage. Technologies that have been applied include magnetic-activated cell sorting (MACS, Said *et al.*, 2008), electrophoretic separation of sperm on the basis of their charge and size (Fleming *et al.*, 2008), binding to hyaluronic acid as an indication of sperm maturity (Huszar *et al.*, 2007), using PICSI (Petri-dish ICSI) dishes containing hyaluronic acid bonded to the Petri dish (www.midatlanticdiagnostics.com), assessment of sperm head birefringence (Gianaroli *et al.*, 2008), and the use of high-magnification microscopy (Bartoov *et al.*, 2003). To date, intracytoplasmic morphologically selected sperm injection (IMSI) appears to be the technique that shows the most promise in sperm selection for ICSI, and will be discussed in more detail below.

Oocyte preparation and handling

Patients for ICSI have oocyte retrieval scheduled after programmed controlled ovarian hyperstimulation (COH), according to protocols described in Chapter 2. Oocyte identification is carried out immediately after follicle aspiration, using a dissecting microscope with heated stage. Take care to maintain stable temperature and pH of the aspirates at all times. At the end of the oocyte retrieval, note quality and assess the maturity of the oocytes, and preincubate them at 37°C in an atmosphere of CO_2 in air until preparations are ready for cumulus–corona removal.

Cumulus–corona removal

1. Prepare a culture dish containing one drop of hyaluronidase solution and 5 wash drops of a HEPES-buffered medium, covered with an overlay of equilibrated mineral oil (denudation may also be carried out in Nunc four-well dishes). Incubate at 37°C for 30–60 minutes. *Note*: HEPES-buffered medium has been adjusted to pH 7.4 and usually 5 mM bicarbonate, and exposure to a CO_2 atmosphere will cause the pH to drop; therefore culture dishes that contain HEPES-buffered media should be warmed to 37°C in a warming oven, and not in a CO_2 incubator.
2. Prepare a thin glass probe and select denudation pipettes.
3. Remove the oocyte and hyaluronidase dishes from the incubator. Place one to four oocytes together into the enzyme drop, agitating gently until the cells start to dissociate. Do not leave them in the enzyme for more than 1 minute. Carefully aspirate the oocytes, leaving as much cumulus as possible behind. Wash by transferring them through at least five drops of culture medium, and change to a fine-bore tip for aspiration in order to remove all of the coronal cells. Remaining corona cells may hinder the injection process by blocking the needle or obscuring clear observation of the cytoplasm and sperm.
4. Assess the quality and maturity of each oocyte under an inverted microscope. Use the glass probe to roll the oocytes around gently in order to identify the polar body, and examine the ooplasm

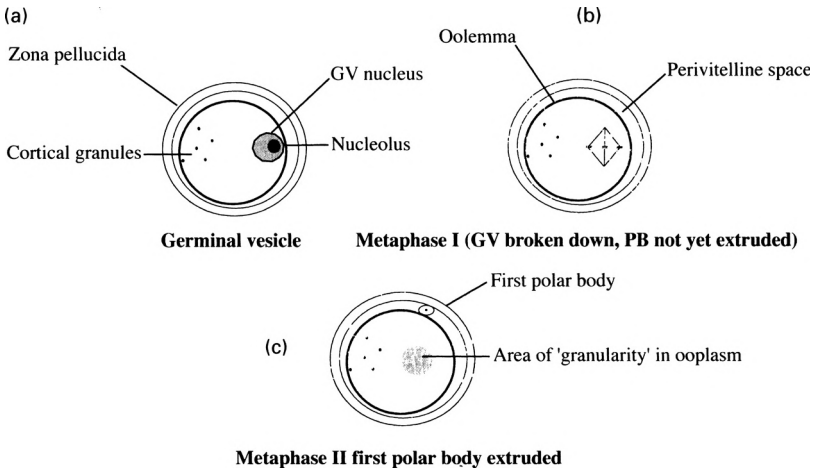

Figure 13.2 Variations in egg maturity found after hyalase treatment and corona dissection. (a) Germinal vesicle; (b) metaphase I; (c) metaphase II.

for vacuoles or other abnormalities. Separate metaphase I or germinal vesicle (GV) oocytes from metaphase II oocytes, label them, and return to the incubator until ready for the injection procedure.

5. Examine the oocytes again before starting the injection procedure to see if any more have extruded the first polar body. ICSI is carried out on all morphologically intact oocytes with first polar body extruded.

Figure 13.2 illustrates different stages of egg maturity that are revealed after hyalase treatment and corona dissection.

Preparation for injection

1. Prepare injection dishes with 4–8 droplets of 2–5 µL of HEPES-buffered culture medium for each individual oocyte, and a 5 µL droplet of PVP for the sperm. The droplets can be arranged in a circle with sperm/PVP in the center, or in parallel groups, but must be positioned so that they are not too close to the edge of the dish, where manipulation will be difficult. The oocyte droplets should not be too close to the sperm/PVP, in order to avoid mixing – use an arrangement that allows quick and easy distinction between sperm and oocyte droplets, with numbers etched on the bottom of the dish. Small volumes of media evaporate very quickly, and they should be covered immediately with a layer of oil. Equilibrate the dishes in the incubator for at least 20–30 minutes, and keep them in the incubator until you are ready to begin the procedure. If Falcon 1006 dishes are being used with HEPES-buffered medium, the lids must be tightly fixed if they are to be equilibrated in a CO_2 incubator. Dishes with HEPES medium and no lids should be warmed to 37°C without CO_2 atmosphere.
2. A prepared and pre-equilibrated traditional culture dish should be available, to transfer and further culture the oocytes after injection.

Micromanipulator

1. Make sure that the microscope heating stage is at a temperature that will maintain droplet temperature in the dishes at 37°C, ensure that all controls are set to neutral and can be comfortably operated, and that you are confident that all parts function smoothly before you begin. It is essential to check that you can smoothly carry out very small movements. This involves not only the equipment itself, but its position on the bench in relation to your (comfortable) seating position.
2. Insert holding and injection pipettes into the pipette holders, tighten well, and if an oil-filled system is being used, make sure that there are no air bubbles in the tubing system. Bubbles interfere with sensitivity when attempting to control movement with fine precision.
3. Align the pipettes so that the working tips are parallel to the microscope stage. First align the holding pipette under low magnification, then align the injection pipette, again under low

magnification. Check the position of both under high magnification. It is important to begin with pipettes in accurate alignment, with both working tips sharply in focus. If a part of the length is out of focus, the pipette is probably not parallel to the stage, but pointing upwards or downwards.

4. Adjust the injection controls: if using an oil system, the oil should just reach the distal end of the pipette; do not try to fill the needle with oil, this will work only if you leave a tiny 5 mm gap of air between the oil and the medium. Briefly touch the tips of both pipettes in oil, and then in medium, so that the ends fill by capillary action (a drop of oil behind the drop of medium will act as a buffer). The injection dish is still in the incubator, so you should be using a "blank" dish for this!

Transfer of gametes to the injection dish

1. Carefully add a small aliquot of sperm suspension (0.3–0.5 mL, depending on the concentration of prepared sperm) to the edge of the central PVP droplet. The viscous solution should facilitate sperm handling by slowing down their motility, and also prevents the sperm cells from sticking to the injection pipette during the procedure. Be careful of sperm density: too many sperm will make selection and immobilization more difficult.
2. After the sperm droplet has been carefully examined for the presence of debris or any other factors that might cause technical difficulties, examine all the denuded oocytes again for the presence of a first polar body; wash them gently with HEPES-buffered medium and transfer one oocyte into each oocyte droplet on the injection dish, taking care to avoid too much handling or cooling of the oocytes. Keep the oocytes in the incubator until you are confident that the injection procedure can proceed smoothly. Until sufficient experience of the procedure has been gained, it may be advisable to keep sperm and oocyte dishes separate, avoiding overexposure of the oocytes while selecting and immobilizing sperm.
3. Place the injection dish with central sperm droplet on the microscope stage. Using the coarse controls of the manipulator, lower the injection pipette into the drop.

Sperm selection and immobilization

1. Select sperm that appear morphologically normal. Sperm can be selected and stored in the PVP drop for a limited period of time (be aware that prolonged exposure to PVP can cause damage to sperm membranes) before starting the injection procedure; this is an advantage in cases of extreme cryptozoospermia, and reduces oocyte exposure time. If SpermSlow is being used for immobilization, the sperm become rigid and stick to the dish after approximately 30 minutes.
2. Immobilize motile spermatozoa by crushing their tails: select the sperm to be aspirated, and lower the tip of the injection needle onto the midpiece of the sperm, striking down and across, and crushing the tail against the bottom of the dish. This "tail crushing" impairs motility and destabilizes the cell membrane; the latter may be required for sperm head decondensation. If the resulting sperm has a "bent" tail, it will be difficult to aspirate into the needle, and will stick inside it. When this happens, abandon that sperm and repeat the procedure with another sperm. Do not strike too hard, or the sperm will stick to the bottom of the dish, also making aspiration into the needle difficult. After some practice, sperm immobilization in routine ICSI cases can be carried out quite quickly. If the preparation contains only a few sperm with barely recognizable movement and a large amount of debris, this part of the procedure can be very tedious and require great patience!
3. Aspirate the selected immobilized sperm into the injection pipette. Sperm were traditionally aspirated into the pipette tail-first, but they can be aspirated head-first (Woodward *et al*., 2008a). Position the sperm approximately 20 μm from the tip.
4. Lift the injection needle slightly, and move the microscope stage so that the injection pipette is positioned in the first oocyte drop. If the sperm moves up the pipette (due to the difference in density between culture medium and PVP) bring it back near the tip before beginning the injection procedure.

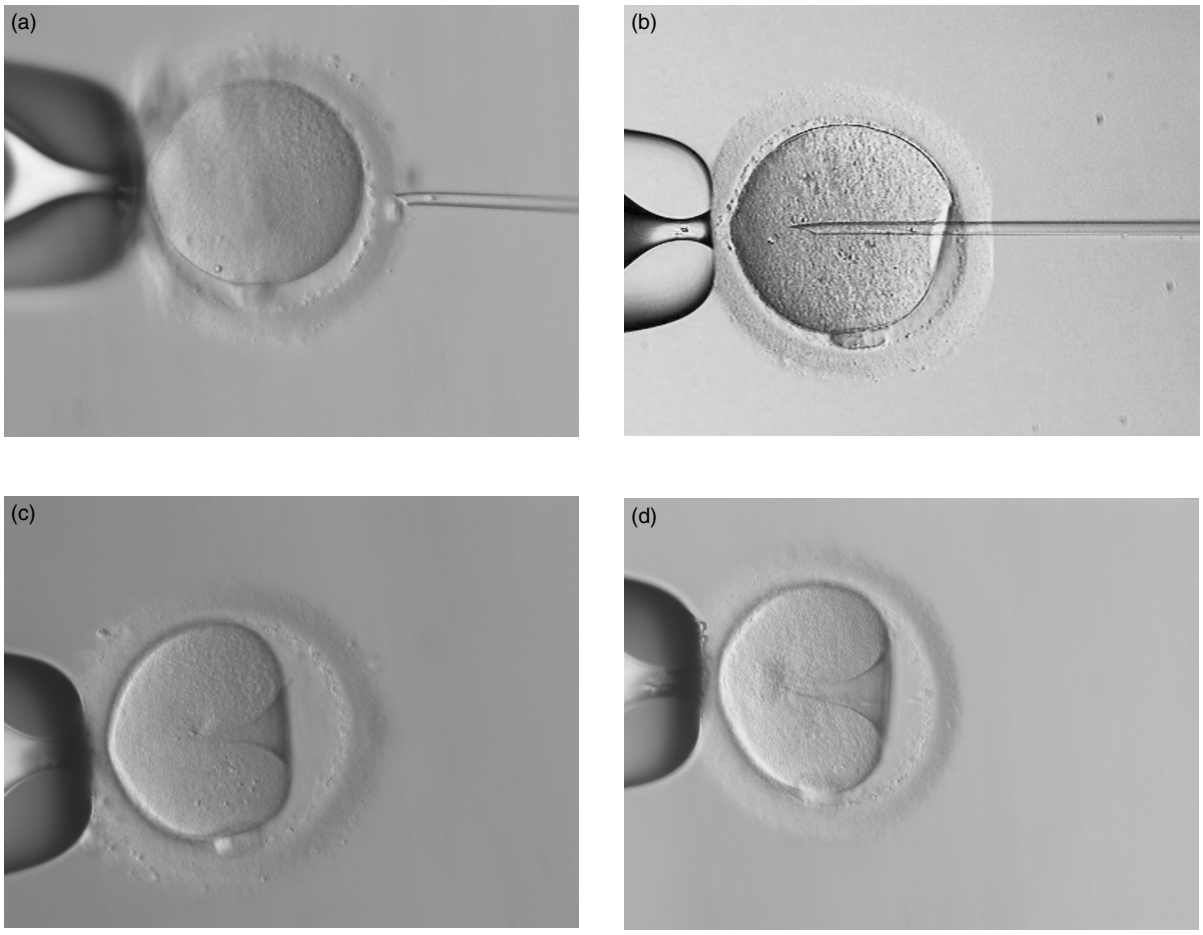

Figure 13.3 ICSI. (a) Metaphase II oocyte with injection needle in position prior to injection. (b) Injection needle within the cytoplasm, prior to release of sperm. (c) Post-injection illustrating the typical track left following withdrawal of the needle. (d) Post-injection, oocyte with a very elastic membrane. (With thanks to Agnese Fiorentino.) (See color plate section).

Injection procedure

1. Lower the holding pipette into the first oocyte droplet, and position it adjacent to the cell. Using both microtools, slowly rotate the oocyte to locate the polar body. Aspirate gently so that the cell attaches to the pipette. The pressure should be great enough to hold the oocyte in place, but not so strong that it causes the oolemma to bulge outwards.

   Polar body positioning at 6 or 12 o'clock in order to minimize the possibility of damaging the meiotic spindle was thought to be important, but later evidence using polarized microscopy to visualize the spindle itself suggests that polar body positioning is of less benefit than minimizing the duration of the ICSI procedure (Woodward *et al.*, 2008b).
2. Move the injection pipette close to the oocyte, and check that it is in the same plane as the right outer border of the oolemma on the equatorial plane at the 3 o'clock position. Check that the sperm can be moved smoothly within the injection needle, and position it near the beveled tip.
3. Advance the pipette through the zona pellucida until the tip almost touches the oocyte membrane at the 9 o'clock position (Figure 13.3). If the pipette is in the wrong plane, entry into the cell will be difficult. The membrane may rupture spontaneously, or may require negative pressure,

sucking the membrane into the pipette before expelling the sperm. When it breaks, there will be a sudden flux of cytoplasm into the pipette. Inject the sperm slowly into the oocyte with a minimal amount of fluid (1–2 picoliters). The sperm should be ejected past the tip of the pipette, to ensure a tight insertion among the organelles, which will hold it in place while the pipette is withdrawn. Some surplus medium may be re-aspirated to reduce the size of the breach created during perforation. If the plasma membrane is elastic and difficult to break, it may be necessary to withdraw the pipette from the first membrane invagination and slowly repeat the procedure.

4. Gently remove the injection pipette, and examine the breach area. The membrane should be funnel-shaped, pointing in towards the center. If the border of the oolemma is everted, cytoplasm may leak out, and the oocyte may subsequently cytolyse. Release the oocyte from the holding pipette.
5. Repeat the sperm aspiration and injection until all the selected metaphase II oocytes have been injected.
6. Wash all the oocytes in culture medium, transfer to the prepared, warmed culture dish, and incubate overnight in the CO_2 incubator.

Injection procedure: important points

1. All conditions must be stable: temperature, pH, equipment properly set up, adjusted, aligned, and checked for leaks and air bubbles. Check everything, including secure and comfortable operating position, before you begin.
2. Correct immobilization of sperm.
3. Advance far enough into the ooplasm with the injection pipette.
4. Ensure that the plasma membrane is broken (immediate membrane rupture after introducing the injection needle results in a lower probability of fertilization).
5. Inject a minimal volume.
6. If the sperm comes out of the ooplasm into the perivitelline space, reinject.

Assessment of fertilization and cleavage

16 to 18 hours after injection, assess the number and morphology of pronuclei through an inverted microscope, rolling the oocyte gently with a glass probe. Polar bodies should also be counted, with reference to digynic zygotes or activated eggs; polar bodies may fragment, even in normal monospermic fertilization. Rapid cleavage (20–26 hours post-injection) can occur in ICSI zygotes (see also the discussion of "silent fertilization" in the "Causes of early embryo arrest" section in Chapter 5).

Evaluate normally fertilized, cleaved embryos after a further 24 hours of culture. Embryo transfer is usually performed approximately 48–72 hours after microinjection. Suitable supernumerary embryos may be cryopreserved either on Day 1 (pronucleate stage) or after the transfer procedure on Day 2 or Day 3 (early cleavage stage).

No fertilization after ICSI

Complete failure of fertilization is rare after ICSI; most of these cases involve semen containing no motile sperm, or round-headed sperm. Some cases of failed fertilization may be attributed to low oocyte number, abnormal oocyte morphology, or fragile oocytes which are easily damaged by the trauma of the injection. Fertilization rates using epididymal and testicular sperm are equivalent to those for ejaculated sperm, but the use of immature sperm cells results in a dramatic decrease in fertilization and pregnancy rates. The most common cause of failed fertilization in ICSI is due to highly elastic plasma membranes and difficulty in rupturing the oocyte membrane: essentially you have not placed the spermatozoon in the cytoplasm of the oocyte. Figure 13.4 shows the variations that can be seen on Day 1 after ICSI.

Transport ICSI

In the same manner that nearby peripheral hospitals or clinics can use a central IVF laboratory to offer assisted conception treatment, a central ICSI laboratory can offer this specialized technique to peripheral hospitals that do not have the equipment or expertise. Preovulatory oocytes and prepared sperm from patients are transported from the peripheral unit by the male partner immediately after the oocyte recovery procedure. Culture tubes containing the gametes are transported in a portable incubator, as described for transport IVF in Chapter 11. On arrival at the central unit, the oocytes can be transferred to culture dishes and prepared for the ICSI procedure, and sperm preparation assessed and adjusted if necessary. Fertilized

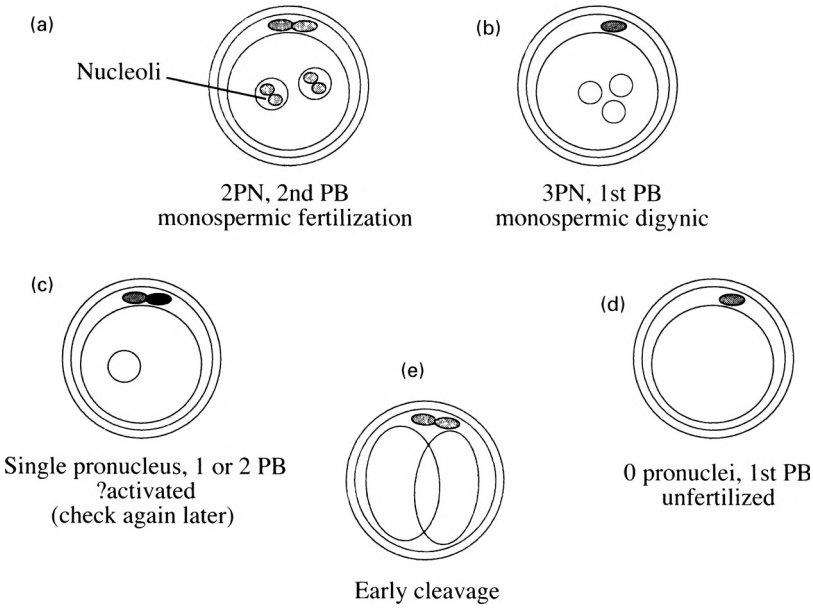

Figure 13.4 Diagrams showing variations of fertilization after ICSI. PN: pronuclei; PB: polar body; (a) two pronuclei, two polar bodies; (b) three pronuclei, one polar body; (c) one pronucleus, two polar bodies; (d) no pronuclei, one polar body; (e) early cleavage (with thanks to Gian franco Coppola).

oocytes are cultured to the early cleavage stage, and the embryos may then be transported by the male partner back to the peripheral unit for the embryo transfer procedure. Supernumerary embryos may also be cryopreserved at the central unit if appropriate. As with transport IVF, cooperation between participating units is particularly important in order to provide an effective service. Well-planned protocols are essential for selection, consultation and counseling of patients, the handling, preparation and transport of gametes, and communication of treatment cycle details/transport arrangements between units.

IMSI – intracytoplasmic morphologically selected sperm

Sperm morphology has long been accepted as one of the best indicators for a positive outcome in human fertilization, whether via natural fertilization, IUI, IVF or ICSI. More recently, IMSI, a more strict technique for selecting "normal sperm," has been shown to improve pregnancy rates and decrease abortion rates, at least in some patient categories. This procedure, originally promoted by Bartoov and colleagues (2003), consists of real time, high magnification, motile sperm organellar morphology examination (MSOME) that uses 24 characteristics to define the normal morphology of seven sperm organelles: acrosome, post-acrosomal lamina, neck, mitochondria, tail, outer dense fibers and the nucleus. MSOME is performed with an inverted light microscope equipped with high-power Nomarski optics enhanced by digital imaging that allows the embryologist to magnify sperm up to 6000 times, compared to the traditional 400 times with ICSI. Figure 13.5 shows IMSI photographs of a single normal human spermatozoon and a selection of dysmorphic spermatozoa.

Patient selection for IMSI

Potentially all males with severe oligospermia and samples dissected from the testicles (TESE) can benefit from application of IMSI.

Equipment and materials for IMSI

IMSI requires a standard ICSI micromanipulator with two modifications:

1. In order to use a higher magnification objective, ranging from ×60 in air to ×100 with oil immersion, sperm observation and selection must be carried out using glass-bottomed dishes or slides of 0.17 mm thickness.
2. Since the specimens must be observed on a glass surface, the Hoffman modulation contrast optics used in ICSI is replaced by Nomarski optics.

The optical signal is then enhanced by a video zoom and digital imaging system, giving a final magnification of up to ×10 000. The image is observed, stored

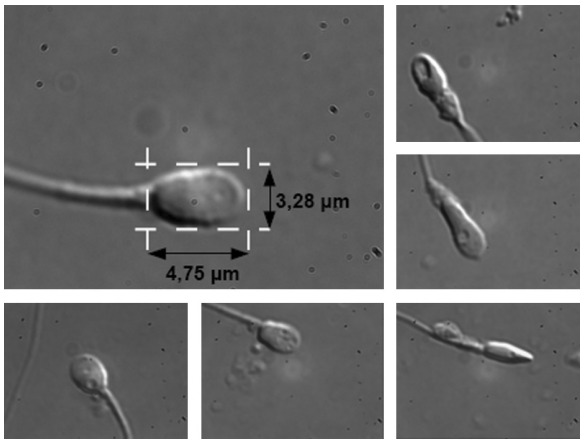

Figure 13.5 IMSI: A single normal human spermatozoon showing true dimensions in µm, and a selection of dysmorphic spermatozoa. Nomarski optics at ×6000 magnification.

and analyzed using specific software supplied by the microscopy company.

Procedure

The type of injection dish is the most important criterion for IMSI. Since sperm identification and selection is made on glass with Nomarski optics, and the injection procedure is usually carried out in plastic dishes with Hoffman optics, special glass-bottomed dishes have been designed so that the two procedures may be carried out in the same dish. In this case, the operator uses Nomarski Differential Interference Contrast (DIC) for the injection procedure. Tissue culture medium, PVP, paraffin oil and microtools remain unchanged.

Assisted hatching (AH)

Cohen and colleagues postulated in the 1990s that the inability of blastocysts to hatch from the zona pellucida may be one of the factors involved in the high implantation failure rate of human IVF procedures. The human zona becomes more brittle and loses its elasticity after fertilization, and spontaneous hardening also occurs after in-vitro and in-vivo aging. The zona also changes in its sensitivity to low pH, so that it is easier to create a hole with acid Tyrode's solution in a zygote than in an unfertilized oocyte. Early observations from video cinematography studies suggested that embryos with a thick, even zona pellucida on Day 2 had a poor prognosis for implantation (see Chapter 11). In addition, embryos produced as a result of microsurgical fertilization had a higher implantation rate, and hatched one day earlier than expected (Day 5 instead of Day 6) after in-vitro culture. Original trials on AH were performed on Day 2 embryos, but studies by Dale and colleagues in Naples (Dale *et al.*, 1991; Gualtieri *et al.*, 1992) on the formation of intercellular junctions and, in particular tight junctions and desmosomes, indicated that Day 3 was a more suitable time for AH. Following these observations, a series of experiments in a mouse embryo system led to the development of a clinical protocol, with the following notable features:

1. Large holes are more efficient in supporting hatching than small holes: if the hole is too small, the embryo can become "trapped" and fail to hatch. Zona drilling using an acid Tyrode's solution prevented "trapping" which occurred as a result of mechanical partial zona drilling: optimal hole size is approximately half the size of a single blastomere, 15–20 µm.
2. Embryos with these large gaps in their zonae should be transferred after the onset of compaction, on Day 3: if embryo transfer is traumatic, blastomeres may escape through the gap in the zona. Embryo transfer must therefore be gentle and atraumatic.
3. Embryos must be preselected for assisted hatching, based upon previous IVF history (repeated failed implantation), maternal age, basal FSH levels, cleavage rates and morphology of the embryos with attention to zona thickness or variation.

Laser assisted hatching

Successful assisted hatching can be carried out with the use of a 1.48 mm diode laser (FertilaseTM, MTG, Germany); a few milliseconds of laser irradiation instantly makes an opening in the zona pellucida, and apertures ranging from 3 to 25 µm can be obtained. The procedure has been shown to be safe, simple and rapid. However, the equipment is expensive, and zona drilling with acid Tyrode's solution can also be used (Figure 13.6).

Acid Tyrode's protocol

The protocol below for assisted hatching by acid drilling of the zona pellucida is adapted from J. Cohen (2007).

Tyrode's solution is acidified by titrating to pH 2.3–2.5 with HCl.

1. Perform zona drilling with acid Tyrode's (AT) solution approximately 72 hours after oocyte retrieval, with embryo transfer 5–7 hours later

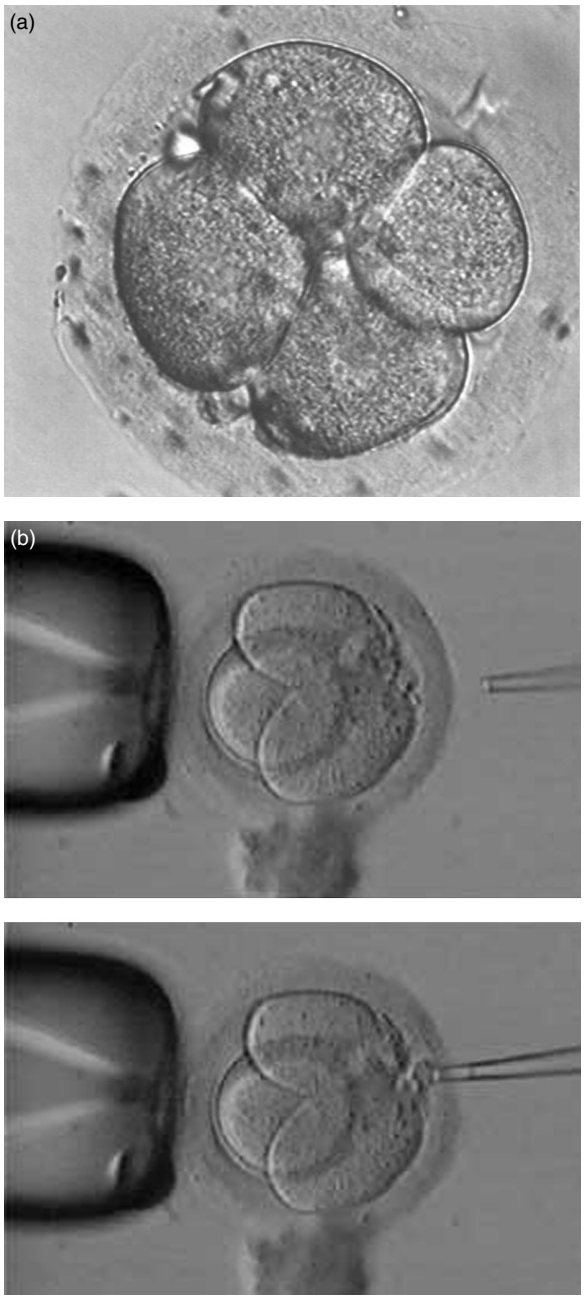

Figure 13.6 Assisted hatching. (a) Laser-hatched four-cell embryo. (See color plate section) (b) Assisted hatching with acid Tyrode's.

(i.e., drill before the formation of intercellular connections, but transfer after they have been established).

2. Perform the procedure in HEPES-buffered human tubal fluid (HTF) medium with 15% human serum albumin or other protein supplement (e.g., SPS, Sage).
3. Use a straight microtool with an aperture of 10–12 µm.
4. Embryos are held in small microdroplets (25 µL) under mineral oil in a depression slide or shallow Falcon 1006 dish containing one droplet of AT solution and four wash droplets.
5. Micromanipulate each embryo individually, and immediately wash each embryo three to four times to remove the acidic medium.

AH with acid Tyrode's, step-by-step

The key to successful assisted hatching is to produce a gap in the zona without exposing the embryo to acidified solution. Aspirating AT solution through a needle of very small diameter leaves considerable negative pressure within the needle after it is removed from the AT droplet, and this will result in culture medium being aspirated as soon as the needle enters the embryo culture droplet. This will dilute the AT solution, making it ineffective for dissolving the zona pellucida. In order to avoid this technical problem, the system should be prepared well in advance, so that the hatching needle will be precisely in the correct position relative to the embryo as soon as it is lowered into the droplet of medium. Applying slight positive pressure as soon as the needle breaks the surface of the drop will also help to counteract the residual suction.

1. Front load the microneedle with AT solution before each hatching event; the meniscus of the acidic fluid is difficult to control, and therefore precisely controlled suction is important.
2. Pre-align the embryo onto the holding pipette (syringe suction system) so that the AT-filled microneedle at the 3 o'clock area is exposed to empty perivitelline space or to extracellular fragments.
3. When the embryo is in position on the holding pipette, lower the AT-filled needle into the medium and bring it next to the target area as quickly as possible. In order to avoid diluting the AT solution with medium of normal pH, there should be no more than a 2-second delay before the hatching begins: releasing fluid that is not sufficiently acidic will damage the embryo, without affecting the zona pellucida.

4. Expel acidic medium gently over a small (30 μm) area by holding the needle tip very close to the zona; using small circular motions can avoid excess acid in a single area. The circular motions should create a hole that is nearly rectangular.
5. If thinning is not immediately obvious, stop the procedure immediately: the total time required to breach the zona should not exceed a couple of seconds; most zona will yield within less than 5 seconds.
6. The inside of the zona is more difficult to pierce, and the expulsion pressure may need to be increased. The optical system should be optimized for this part of the procedure, as the stream of acid may be relatively invisible, and the piercing of the inside of the zona may be almost imperceptible. Zona breakthrough should cover an area of at least 20 μm, and not a single small point.
7. As soon as the zona is breached, reverse the flow through the AH needle immediately. All of the expelled acid solution must be aspirated, paying particular attention to any solution that might have entered the PVS. Move the embryo to another area of the droplet, away from excess AT.
8. A small "inside" hole may be widened mechanically by moving the microneedle through the opening in a tearing motion while continuing gentle suction.
9. Carefully transfer the embryo through the wash droplets, and return to culture for incubation prior to embryo transfer.

Polar body or blastomere biopsy

The next chapter will discuss the genetic and chromosomal analysis of cells biopsied from oocytes (the polar body), cleavage stage embryos and blastocysts. The standard equipment used for embryo biopsy is a slightly modified ICSI apparatus, with specific microtools. Biopsy is carried out in two stages: the zona pellucida must be breached, and then the cells are aspirated.

The optimal time for embryo biopsy is before strong intercellular junctions are formed, which is usually Day 3. Biopsy is extremely difficult with compacted embryos, and cleavage stage embryos should be incubated in Ca/Mg-free media to facilitate the biopsy procedure. To avoid contamination with extracellular sperm that could be attached to the zona, it is important that the oocytes are fertilized using ICSI. A variety of techniques can be used to carry out the biopsy, as described below. A hole in the zona can be created mechanically (as in PZD), using a laser, or by drilling with AT solution. If using AT drilling, the AT should first be prewarmed to 37°C; a pipette of 8–10 μL containing AT is placed adjacent to the zona pellucida and the solution expelled from the pipette until the zona thins and eventually breaches. The cell is then moved away from any excess AT. For biopsy, a second micropipette, fire-polished with an inner diameter of 30–40 μm) is used to aspirate the blastomere. The pipette is pushed through the breach in the zona and, by gentle aspiration, the cell drawn up into the pipette. When the cell is clearly free from the embryo, the pipette is moved sideways and the cell expelled. If the cell lyses, another cell should be aspirated.

Cleavage stage biopsy techniques

The totipotency of cleavage stage blastomeres ensures that removal of a small number for diagnosis does not affect the ability of the remainder to compensate and thus adequately differentiate to all required cell lineages on development (see more on this subject in Chapter 7).

Aspiration (Figure 13.7a)

The biopsy micropipette is used like a needle to puncture the zona pellucida of an embryo held in place with a holding micropipette. A blastomere is then removed by suction into an aspiration micropipette. The zona may also be punctured by administering AT solution to a limited spot on the surface or by using a sharpened dissection pipette or a laser to drill a hole in the zona.

Extrusion

(a) Stitch and pull (Figure 13.7b)

This method involves initial zona drilling with either a laser or acid Tyrode's to create a hole. A stitching movement with the microneedle is then used to displace and remove a blastomere which is then pushed out of the incision by pushing the zona with a microneedle at a distance from the hole.

(b) Displacement (Figure 13.7c)

A beveled pipette is used to make the first incision in the zona. A second incision is then made through which one blastomere is displaced by applying a gentle flow of medium, causing the blastomere to emerge from the first incision site.

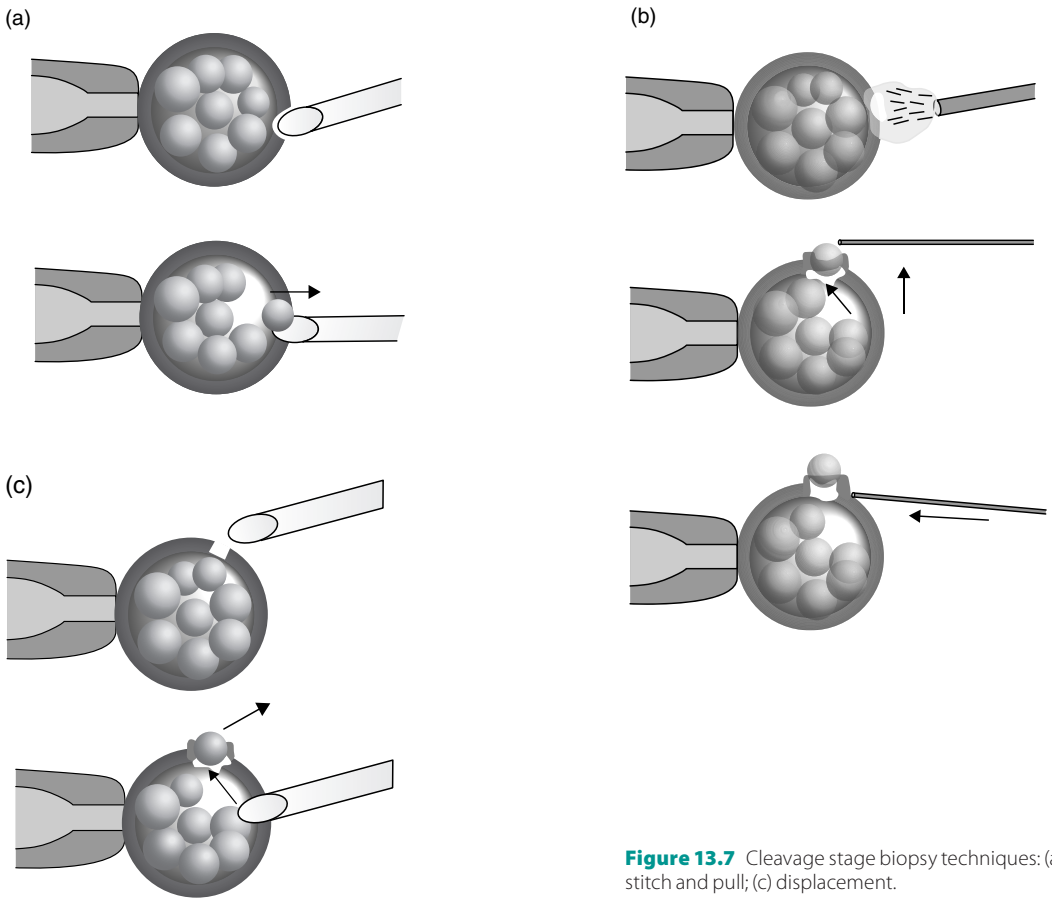

Figure 13.7 Cleavage stage biopsy techniques: (a) aspiration; (b) stitch and pull; (c) displacement.

Typical clinical protocol for cleavage stage embryo biopsy for PGD

Day 1 (1 day after oocyte collection)

1. Set up culture dishes (one for each normally fertilized embryo): Label a four-well dish (Nunclon) with the patient's name and embryo number on the base of the dish and on the front panel. Put 0.5 mL of cleavage stage or blastocyst culture medium in each well and cover with a monolayer of washed oil. Place the dishes in the incubator to equilibrate overnight.

Day 2 (2 days after oocyte collection)

2. After scoring the embryos, transfer each embryo into blastocyst culture medium in well 1 of the appropriately labeled four-well dish, wash and transfer to well 2 and transfer to the incubator for overnight culture. The timing of the switch between cleavage stage and blastocyst culture media may differ depending on the medium used.
3. In the warming oven, place 10 mL of HEPES-buffered biopsy medium (Ca^{2+}/Mg^{2+}-free) and Falcon dishes (1006) for the biopsy. In the incubator, place enough washed oil for the biopsy procedure (allow 4 mL per embryo).

Day 3 (day of embryo biopsy)

4. Half an hour before the biopsy:
   (a) Set up a biopsy dish (1006 Falcon) for each embryo and label it with the patient's name and embryo number. Take a Gilson pipette set at 10 µl and a sterile yellow tip and flush the tip (×10) with the HEPES-buffered biopsy medium. Pipette three drops of HEPES-buffered biopsy medium and one drop of AT as shown in Figure 13.8 – it is important that the dish is oriented as shown in relation to the "bumps" on the outside of the dish. Immediately cover the dish with 4 mL of washed and pre-equilibrated oil to avoid evaporation and put the prepared dishes in the warming oven until required.

(b) Set up a four-well dish for transferring the embryos into biopsy medium with 0.5 ml of HEPES-buffered biopsy medium in each of the wells, cover with oil and place in the warming oven.

5. About 15 minutes before each biopsy, take the appropriately labeled biopsy dish and the four-well transfer dish from the warming oven and carefully wash successive embryos through each well of HEPES-buffered medium, transferring minimal medium between wells. Leave the embryo in each well for at least 1 minute: it is essential to completely remove the divalent cations from the culture medium to promote the reversal of any compaction. Place the embryo into the middle of the three droplets in the biopsy dish (the two other droplets are spare in case of difficulties during biopsy).
6. Biopsy cells.
7. At the end of the biopsy procedure, transfer the embryo into well 3 of the four-well culture dish – this is a washing stage to remove the HEPES-buffered biopsy medium. Finally, transfer the embryo with minimal medium to well 4 and return to culture.
8. Return the biopsy dish with the isolated blastomeres to the flow hood for sample preparation.
9. Repeat until all of the embryos have been biopsied.
10. When the PGD analysis result is available, assess the morphology of each embryo and count the number of cells as accurately as possible to get an indication of division post biopsy.
11. In consultation with the other members of the PGD team and finally with the couple themselves, select a maximum of two genetically unaffected embryos with the best morphology for transfer.

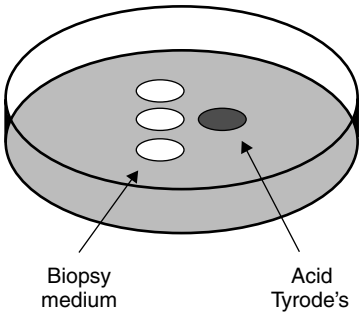

Figure 13.8 Biopsy dish for PGD.

Blastocyst stage biopsy

The advantage of blastocyst biopsy is that a larger number of cells can be removed from the outer trophectoderm layer without affecting the inner cell mass from which the fetus later develops. However, trophectoderm cells (TE) may have diverged genetically from the inner cell mass (ICM), as confined placental mosaicism, observed in at least 1% of conceptions: the chromosome status of the embryo differs from that of the placenta. Recent studies have indicated that blastocysts may have high levels of chromosomal mosaicism. Preferential allocation of abnormal cells to the TE may be a mechanism of early development; in this case the TE would not be representative of the rest of the embryo, which would complicate and compromise PGD. Blastocyst biopsy may be performed using stitch and pull, aspiration, and herniation techniques.

Aspiration (Figure 13.9a)

A number of cells are sucked from the mural trophectoderm cell layer opposite to the inner cell mass, through the aspiration micropipette. The microneedle is also used to break cell–cell contacts as the TE cells herniate through the hole.

Extrusion technique (Figure 13.9b)

Partially dissolve a section of the zona with AT solution or with a laser, and use two siliconized glass microneedles to extrude mural TE cells with a stitch and pull motion through the zona.

Herniation

Make a tear in the zona opposite the inner cell mass, and leave the embryos for several hours until a herniation of the mural TE can be seen. Remove the emerging cells by cutting with a glass needle or scalpel blade. The incubation time required means that this method is less useful for transfer in the same cycle.

Polar body biopsy

Since the oocyte of a carrier contains either the abnormal or normal gene for a particular genetic disorder, analysis of the status of the gene from the polar body (PB) will indirectly determine which gene is present in the oocyte. However, for analysis of single gene and chromosomal defects, recombination events during

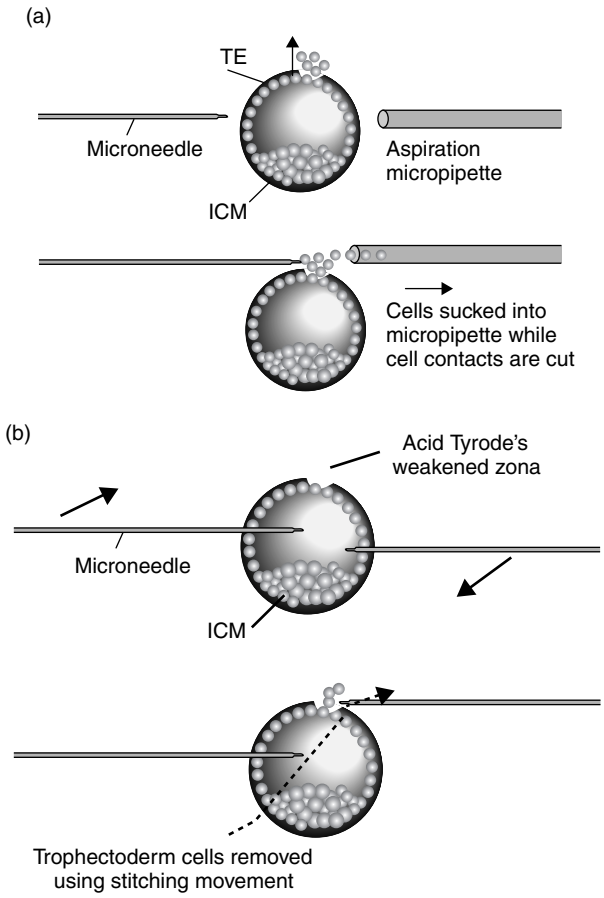

Figure 13.9 Blastocyst biopsy techniques: (a) aspiration; (b) stitch and pull.

meiosis I require biopsy and analysis of PB2 to confirm which allele is present in the zygote. Both polar bodies can be removed together after fertilization (Figure 13.10), or PB1 can be removed before, and PB2 after fertilization.

Method

1. Immobilize the oocyte using a holding pipette.
2. Insert a beveled micropipette (12–15 μm diameter) through the zona to the perivitelline space and aspirate the polar body into the pipette as it detaches from the ooplasm; a breach in the zona can also be created with the use of a laser. The two polar bodies are distinguished by morphology, the first having a crinkly appearance and the second being smooth, possibly with a visible interphase nucleus under interference contrast.

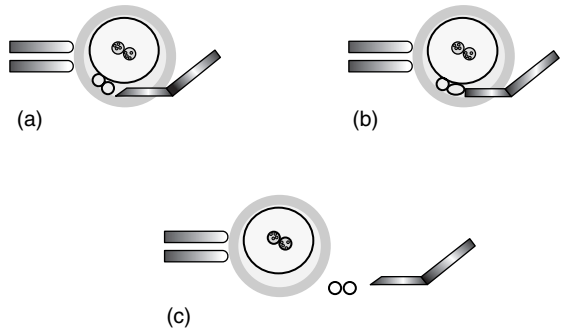

Figure 13.10 Polar body biopsy. (a) Position the polar bodies at 5–6 o'clock position, and penetrate the zona with the biopsy pipette. (b) Gently aspirate the polar bodies from under the zona pellucida. (c) Expel the polar bodies into the medium and remove the biopsied zygote from the biopsy medium.

Appendix

Equipment for ICSI

For microinjection

Dissecting microscope with heated stage
Inverted microscope with heated stage, attached to micromanipulators

- ×4 objective for locating oocytes and drops
- ×20 or ×40 objective for microsurgery
- ×15 eyepiece
- Hoffman modulation contrast optics
- Video monitoring facility

Bilateral micromanipulators for manipulation in three dimensions
Microtool holders
Two suction devices with steel syringes (80–100 mL) filled with light mineral oil (BDH), or appropriate alternative device for controlling holding and injection micropipettes
Incubator
Supply of 5% CO_2 in air.

Supplies

- Shallow Falcon Petri dishes (Type 1006)
- Culture medium
- Culture medium + HEPES
- Human serum albumin (HSA)
- Hyaluronidase solution
- Mineral oil
- PVP solution
- Pasteur pipettes
- Hand-pulled polished glass pipettes

Pipette bulbs
Holding pipettes

ICSI needles.

Adjustment of manipulators for ICSI (with thanks to Terry Leonard)

This guide refers to the Nikon/Narishige system; the same principles apply to an Olympus system, but the details are slightly different. Before attempting to fit or adjust the micromanipulators, first adjust the microscope optically, ideally using an oocyte in the same type of Petri dish to be used for ICSI. The microscope settings will influence the working distance of the microtools. The final position of the micromanipulators on the microscope will depend upon:

(a) the angle at which the tool holder is fixed
(b) the combined length of the tool holder and needle from the point where it is held in the tool holder attachment to the center of the light source.

Before finding the ideal position for the micromanipulators, they should be fitted correctly to the microscope, and final adjustments made later.

Micromanipulator parts

1. Mounting bar: this joins the coarse manipulator to the microscope.
2. Coarse manipulator: consists of three parts, each controlling one of the three dimensions of movement. There are two types: manually operated and motor driven.
3. Fine manipulator: consists of two parts:
   (a) the driving section: attached to the coarse manipulator, controls fine movements directed by joystick.
   (b) ball joint/tool holder attachment: attached to the driving section, used both to hold the microtool holder and to vary the angle at which it is held.
4. Joystick: links to the driving section via oil-filled tubes. The movement of the joystick is scaled down and transferred to the fine manipulator.

The mounting arrangement which attaches the coarse manipulator to the microscope is L-shaped. In the Nikon/Narishige system, the mounting is fitted to the illumination pillar.

- The mounting bar position is marked with a white L-shape.
- The mounting bars have tracks into which the coarse manipulator fits, and the position of the coarse manipulator can be adjusted along these tracks. The entire mounting bar can be adjusted up and down.

Adjusting the coarse manipulators

1. Set the mounting bars at 90 degrees from the microscope stage or bench top.
2. Set the second part of the mounting at 90 degrees to the first, and ensure that the track is set flat.
3. Attach the coarse micromanipulator and adjust the lower section (left/right movement) so that it is parallel with the mounting bar. Set the other two sections of the coarse manipulator at right angles to each other.

Attaching the fine manipulator

The fine manipulator is attached to the coarse manipulator by a small metal rod (coupling bar). This can be screwed into one of the two holes on the driving section of the fine manipulator, depending on the side on which it is to be used. In order to fit the fine manipulator on the right hand side, find the "R" mark on the driving section and screw the coupling bar into the hole directly behind this mark. Attach the coupling bar/driving section to the coarse manipulator and tighten. Position the driving section so that it is parallel with the microscope or 90 degrees to the bench. Attach the left hand side in the same manner, screwing the coupling bar into the hole directly behind the "L" mark. Arrange the joysticks so that the "R" and "L" marks are facing the operator on the appropriate sides.

Attaching the ball joint/tool holder attachment

The ball joint/tool holder attachment has a black metal bar projecting from it. This is fitted into the V-groove of the driving unit. The ball joint is then rotated to an appropriate angle so that when a needle is held in it, the tip of the microtool will be parallel with the microscope stage. The extent to which it is rotated will depend upon the angle at which the microtool is bent. For example, for a microtool which is angled at 35 degrees from the plane of the needle, the ball joint must be rotated 35 degrees anticlockwise from the vertical position.

The manipulators should now be attached in the correct manner, but they may not be in the optimal working position. The ideal position will vary depending upon the angle of the needle and the combined length of the microtool and tool holder. It is therefore

best to have a fixed tool angle, a fixed length of projection of the tool from the tool holder, and a fixed (marked) point where the tool holder is clamped to the tool holding attachment. If any of these three factors change, the fine adjustments will require resetting.

Finding the best position for the micromanipulators

- Adjust the three coarse manipulators so that they are in the middle of their range of movements.
- Adjust the range of movements of the three fine manipulators on the joystick so that they are in the middle of the scales.

Forward/backward movement

First ensure that the hole in the stage is positioned directly in the middle of the light path. View the micromanipulator from the side of the microscope, then align the tool holder attachment with the middle of the hole in the microscope stage. This can be adjusted by changing the position of the coupling bar on the fine manipulator, the projection from the ball joint, or the two screws on the very top of the coarse manipulator which controls the forward/backward movement.

Left/right movement

Place a microtool holder together with a microtool in the tool holder attachment. Attach it at the very tip of the tool holder, and gently slide it along towards the light source. If it will not reach the light source, the entire manipulator should be moved to the left. Likewise, if it reaches it too soon (before the marked point) the entire manipulator should be shifted to the right. This adjustment is performed by loosening the bolts that attach the coarse micromanipulator to the mounting bar. After this adjustment, ensure that the micromanipulator is still parallel to the bar.

Up/down movement

Adjust the manipulator so that the microtool is approximately 0.5 cm from the surface of the stage. For large adjustments loosen the bolts that attach the mounting bar to the microscope and move up or down. Remember to ensure that it is still 90 degrees from the bench after adjustment. Fine up/down adjustments can be made by moving the small sliding section above the ball joint.

Alignment of the ICSI needle in the manipulator

When using a Narishige tool holder, push the needle in so that 4–5 cm of the needle is outside the holder. Place the tool holder in the right hand micromanipulator with the needle tip over the light source. The angle at which the tool holder is held should be such that the angled tip of the needle is parallel with the stage of the microscope. Under a very low power (×4 objective), place the tip of the ICSI needle in the field of vision of the microscope. Loosen the tool holder attachment and rotate the tool holder so that the bent portion of the needle appears to be straight. Ensure that you can focus on a good portion of the needle (from the tip). The portion of the needle after the bend will be out of focus. If the microscope has a graticule, make sure that the movement of the needle from right to left does not vary. Place the ICSI needle along the middle of the microscope field with the tip almost in the center. Align the holding pipette in the same manner so that the tips of the needle and pipette are facing each other, and then raise them up away from the stage.

Perform the final adjustment of the angle at which the tool holder is held within a sperm droplet. Find a nonmotile sperm and then bring the ICSI needle into the same optical plane. Raise the ICSI needle off the surface of the Petri dish very slightly, move it over the top of the sperm, and try to touch the sperm by lowering the needle. If it is impossible to touch the sperm, the needle is not parallel with the surface. The end of the needle will be lower than the tip. To rectify this, raise the needle and rotate the ball point anticlockwise slightly, lower the needle, and try to touch the sperm again. If the tip of the needle can touch the sperm, but the rest of the needle is not in focus, the tip of the needle is lower than the bend. To rectify this, raise the needle and rotate the ball joint slightly in a clockwise direction.

Ratio of movement and joysticks

1. Focus on the tip of the microtool.
2. Loosen the two screws on the movement adjustment rings.
3. Ignore the ratios written on the side of the joystick, and rotate the adjustment rings anticlockwise until you are satisfied with the movement as observed down the microscope.

When all of the adjustments and alignments are made, the need to repeat any of these should be minimal, unless the style of the needle or tool holder is changed. When removing the tool holder from its attachment, keep the ball joint at the same angle so that when a new needle is inserted, it will be at approximately the right

angle for use. Fine adjustments will be necessary for each manipulation.

Further reading

Useful websites

ICSI, follow-up and risks: www.ivfctrstl.org/icsi-safe.htm, www.advancedfertility.com/icsirisks.htm

Micromanipulation images: www.ivf.net/ivf/micromanipulation-b338_0-en.html

Micromanipulation tools: www.zdlinc.com/icsi_tools.htm

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Chapter 14

Preimplantation genetic diagnosis

Joyce Harper

Preimplantation genetic diagnosis (PGD) is an early form of prenatal diagnosis, developed in the late 1980s to help couples who are at risk of transmitting an inherited disease to their offspring. If such couples wish to have a healthy family, the main option open to them is prenatal diagnosis by amniocentesis or chorionic villus sampling (CVS). The disadvantage of prenatal diagnosis is that if the diagnosis shows the fetus to be affected, the couple have to decide whether they wish to terminate the pregnancy or carry on with the knowledge that their child is going to be affected by the genetic disease. PGD offers some of these couples an alternative, as the diagnosis is performed on the preimplantation embryo and only unaffected embryos are transferred to the patient. The pregnancy is therefore initiated with the knowledge that the fetus is free from the disease.

The majority of patients referred for PGD are those who have already experienced several terminations of affected pregnancies, those with moral or religious objections to termination, patients who have experienced repeated miscarriages due to unbalanced chromosome arrangements in the fetus and infertility patients who are also carrying a genetic or chromosomal abnormality.

PGD covers several fields: IVF, genetics, embryo biopsy and single cell diagnosis. Before discussing embryo biopsy and single cell diagnosis, it is important to outline the inheritance of genetic disease and the principles of prenatal diagnosis.

The genetics of inherited disease

Three groups of inherited disease can be diagnosed by PGD:

1. Single gene defects
2. Triplet repeat disorders
3. Chromosomal abnormalities.

Single gene defects may affect the autosomes (chromosomes 1–22) or the sex chromosomes (X and Y). Single gene defects are inherited by autosomal recessive, autosomal dominant and X-linked (sex-linked) modes. The triplet repeat disorders are a group of diseases caused by an expansion of a triplet repeat of base pairs on a chromosome. Chromosomal abnormalities, such as translocations and inversions, can lead to a fetus with an unbalanced chromosome complement.

Since chromosomes exist in pairs (one of each pair inherited from each parent), genes are present in two copies, with the exception of those carried on the sex chromosomes in males: they have only one X and one Y chromosome. The majority of genetically inherited diseases are caused by a mutation within a specific gene, which causes the gene to be inactive or faulty. Whether the disease is expressed when both or only a single copy of the gene carries a mutation depends upon the mode of inheritance. The mutations that lead to a disease can be caused by a single change, or by more complicated changes in the bases within the gene. This change may be a deletion, substitution or insertion in the base sequence. Within a single gene, there are "hot spots" prone to mutation. For example, over 800 cystic fibrosis (CF) mutations have been identified, but, in the UK, 70% of individuals who carry CF have the same mutation, deltaF508, caused by a deletion of three base pairs in exon 10 of the CF gene.

Age-related aneuploidy is also an important factor that may lead to chromosomally abnormal offspring. This is not an inherited disease, and it can occur in any pregnancy.

In-Vitro Fertilization: Third Edition, ed. Kay Elder and Brian Dale. Published by Cambridge University Press.

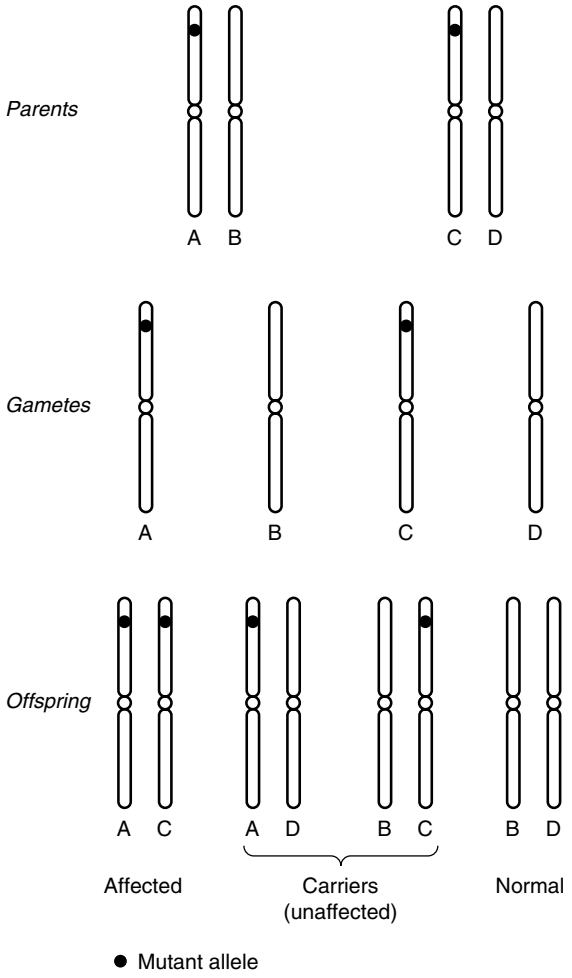

Figure 14.1 Autosomal recessive inheritance.

Autosomal recessive disease

The inheritance of autosomal recessive disease is shown in Figure 14.1.

Autosomal recessive inheritance accounts for the majority of genetic disease. If an individual has one normal gene and one abnormal gene, he or she is a carrier of the disease and will usually be unaffected. The individual will be affected by the disease if the genes inherited from both the mother and the father carry the mutation. For example, if both the mother and the father are carriers of CF, the offspring have a 1 in 4 chance of being affected, a 1 in 4 chance of being unaffected, and a 2 in 4 chance of being a carrier.

The most common autosomal recessive single gene defect is beta-thalassemia, which is caused by a mutation in the beta-globin gene. However, there are many different mutations for beta-thalassemia, especially between different ethnic groups: the majority of couples carry different mutations (compound heterozygotes) and this complicates PGD (see section on PCR diagnosis).

Cystic fibrosis is the most common autosomal recessive single gene defect for which PGD has been applied. Since deltaF508 is such a common mutation, there are many couples in whom both partners carry this mutation, making PGD relatively simple.

Table 14.1 shows the major autosomal single gene defects that have been diagnosed by PGD.

Table 14.1 PGD for autosomal recessive diseases

Cystic fibrosis (various mutations)
Sickle cell anemia
Tay–Sachs disease
Spinal muscular atrophy
β-Thalassemia
Adrenogenital syndrome
Hypophosphatemia

Autosomal dominant disorders

A single copy of the mutated gene will lead to the disease in disorders that are dominant in their inheritance (Figure 14.2). These diseases are not as life-threatening as some recessive diseases, and therefore affected individuals can still reproduce and transmit the disease to their offspring. Many dominant disorders are late in onset, such as Huntington's disease and some inherited cancers.

PGD has been developed for dominant diseases, such as myotonic dystrophy, Marfan's syndrome, polyposis coli, Charcot–Marie–Tooth disease and Huntington's disease (Table 14.2).

X-linked diseases

X-linked diseases affect genes that are carried on the X chromosome, and more than 400 such diseases have been identified. They can be inherited in a recessive or dominant manner, but almost all severe types have recessive inheritance. Males inherit the X chromosome from their mother, and if this inherited X chromosome is abnormal they will be affected with the disease (Figure 14.3). Therefore, carrier mothers transmit the disease to half of their male offspring, and half of her daughters will be carriers. With PGD, sexing can be

Table 14.2 PGD for autosomal dominant diseases

Marfan's syndrome
Familial adenomatous polyposis coli
Huntington's disease
Myotonic dystrophy
Osteogenesis imperfecta

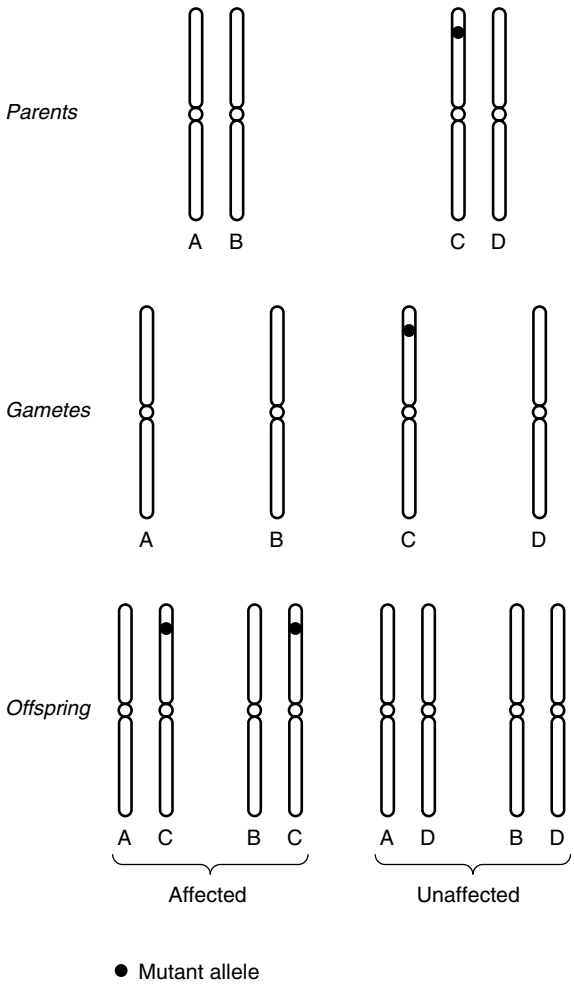

Figure 14.2 Autosomal dominant inheritance.

performed at the cleavage embryo stage and female embryos selected for transfer or specific diagnosis can be carried out using PCR, which allows the transfer of non-affected males and non-carrier females.

X-linked diseases reported as diagnosed by embryo sexing are listed in Table 14.3a. The X-linked diseases where a specific diagnosis has been performed are listed in Table 14.3b.

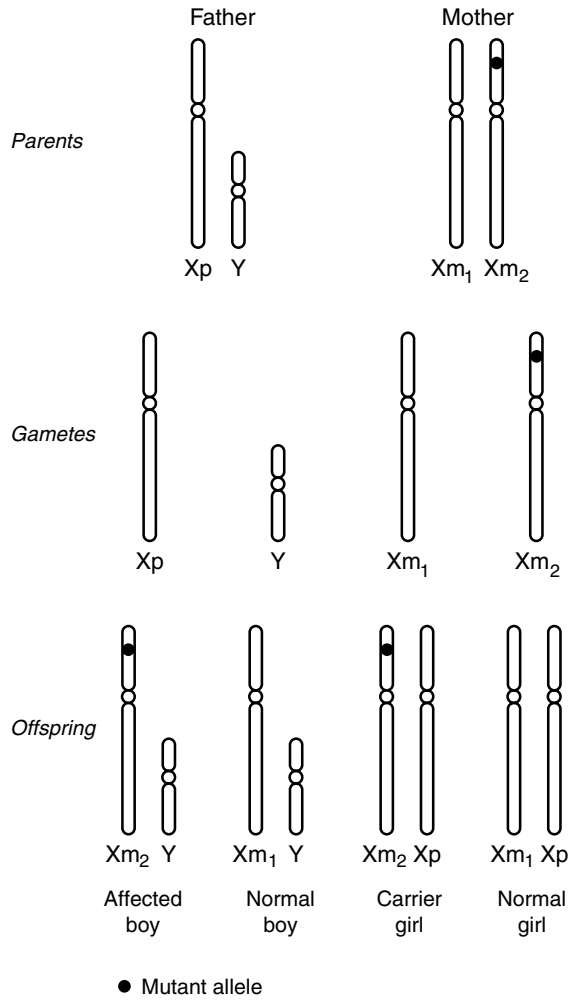

Figure 14.3 X-linked inheritance.

Triplet repeat disorders

A new class of genetic disorders was classified during the 1990s: the triplet repeat disorders are caused by the expansion of a triplet repeat of bases within a gene, and are usually associated with neurological disorders. Each disease has a range of repeats associated with a spectrum from normal individual to affected individuals. For example, for the triplet repeat responsible for fragile X syndrome, a normal individual will have from 6 to 54 triplet repeats; those having the "premutation" will carry between 54 and 200 repeats, and those affected with fragile X will have over 200 repeats.

Fragile X was originally thought to be an X-linked disease, as males are generally affected, but it has been reclassified as a triplet repeat disorder. It is caused by

Table 14.3 PGD for X-linked diseases

(a) Sexing
Duchenne muscular dystrophy
Becker muscular dystrophy
Chronic granulomatosis
Hunter's syndrome
Hemophilia
Adrenoleukodystrophy
Barth syndrome
X-linked hydrocephalus
X-linked ataxia
X-linked incontinentia pigmentosa
X-linked mental retardation
X-linked Wiscott–Aldrich
X-linked spastic paraplegia
Laber's optic atrophy
Sensory motor neuron disease
Retinitis pigmentosa
Bruton's disease
Menkes disease
(b) Specific diagnoses
Duchenne muscular dystrophy
Hemophilia
Lesch–Nyhan syndrome
Charcot–Marie–Tooth

the unstable expansion of a CGG repeat in the 5′-untranslated region of the FMR1 gene, which is on the X chromosome. This triplet expansion results in mental retardation. Females carrying the premutation are at risk of transmitting the full mutation to their offspring, and, since males inherit the X chromosome from their mothers and have a single X chromosome, their male offspring are at a 50% risk of inheriting fragile X. Females who inherit the expanded gene from their mothers will also inherit a normal X chromosome from their father, and show variable disease manifestations. Males carrying the premutation are at risk only of transmitting the premutation to their female offspring, who will be carrier females.

Huntington's disease (HD) is a progressive neuropsychiatric disorder of late onset that is inherited in a dominant fashion. The gene is on chromosome 4, and involves a CAG triplet repeat where expansion beyond 36 results in HD. The age of onset is about 40 years and patients often die by their mid 50s. PGD has been performed for HD, but it draws some ethical discussion: many potential carriers of HD know that they have a 50% risk of being affected because one of their parents is affected, but they do not wish to know their own HD status. For prenatal testing and PGD, an exclusion test can be offered, where patients are given a risk factor without being told their actual status.

Myotonic dystrophy (DM) or Steinert's disease is a progressive muscular dystrophy. The gene is on chromosome 19 and DM is caused by expansion in a CTG repeat at the 3′-untranslated part of the DM kinase gene. Normal individuals have between 5 and 37 repeats, and affected individuals may have anything from 50 to several thousand repeats. Intermediate numbers of repeats can give rise to a premutation. PGD can be performed for DM.

Chromosomal abnormalities

Abnormalities that involve whole chromosomes are usually lethal. Those compatible with life involve the sex chromosomes, such as Turner's syndrome (XO), Klinefelter's syndrome (XXY) or Down's syndrome (three copies of chromosome 21).

The most common chromosome abnormality is a chromosome translocation, where two chromosomes have broken and rejoined to the opposite chromosome. If the chromosomes are still balanced, i.e., all the genetic material is still present, the patient is described as having a balanced translocation. The majority of patients carrying a balanced translocation do not realize they have abnormal chromosomes until they try to reproduce. During meiosis, the segregation of the chromosomes becomes confused, and unbalanced chromosome complements are formed in the gametes, leading to the formation of an embryo with abnormal chromosomes (unbalanced translocation). Therefore patients carrying balanced translocations may experience infertility, repeated miscarriage or the birth of a child with abnormal chromosomes.

Robertsonian translocations involve breakages around the centromere of the "acrocentric" chromosomes (13, 14, 15, 21, 22). These chromosomes contain a satellite region on their short arm, and loss of this area

has no effect on phenotype. Since two of the acrocentric chromosomes join together, the patient has only 45 chromosomes. Robertsonian translocations can be diagnosed using PGD, as only the number of chromosomes present needs to be identified.

Reciprocal translocations involve breaks at any location on two chromosomes and thus can involve any chromosomes. The fact that every couple has different chromosome breakpoints makes PGD difficult.

Chromosome abnormalities can also be caused by chromosome inversions, insertions, deletions or rearrangements (such as ring chromosomes).

Age-related aneuploidy

Women over the age of 35 are known to be at increased risk of having a fetus with a chromosome abnormality. However, only 20% of Down's syndrome babies are born to women over the age of 35. Screening methods have been developed to help identify those pregnancies at risk, as the use of age alone as an indication for prenatal diagnosis of age-related aneuploidy will miss the majority of affected pregnancies. Biochemical (plasma alpha-fetoprotein, hCG, unconjugated estriol) and ultrasound screening methods are therefore used to determine which pregnancies are at risk. Patients found to be at risk undergo prenatal diagnosis, with a karyotype performed to ascertain the status of the fetus. The chromosomes most commonly involved in age-related aneuploidy are 13, 16, 18, 21, X and Y.

Serum screening

During the second trimester of pregnancy (16–17 weeks) a number of markers have been found to help identify pregnancies with chromosome abnormalities. Down's syndrome pregnancies show lower maternal serum alpha-fetoprotein (AFP) and unconjugated estriol, whereas human chorionic gonadotropin (hCG) levels are two times higher than normal. Taking into account the patient's age, the use of the triple test to measure AFP, free beta-hCG and unconjugated estriol can increase the detection rate to 70%, thereby reducing the number of women who require invasive prenatal diagnosis.

Ongoing research is directed towards trying to identify first trimester markers for aneuploidy. Pregnancy-associated plasma protein A (PAPP A) is reduced in Down's syndrome pregnancies.

Ultrasound

During the first trimester of pregnancy, ultrasound detection of nuchal translucency measuring greater than 3 mm is associated with a chromosome abnormality. This is caused by fluid accumulation at the back of the fetal neck. In conjunction with maternal age, studies have shown this to give a detection rate of 86% with a false positive rate of 4.5%. When used in combination with first and second trimester serum screening, a detection rate of over 90% was reported.

In the second trimester, ultrasound markers such as cardiac malformations, duodenal atresia, hydrops, choroid plexus cysts, nuchal edema, renal pyelectasis, omphalocele, hypoplastic midphalanx of the fifth finger, and short femur and humerus can be used to screen for aneuploidy. The addition of digital imaging and computer analysis of two-dimensional ultrasound pictures allows three- and four-dimensional images to be built up, which allow more in-depth assessment of cardiac and neural abnormalities, and show a clearer picture of what specific abnormalities look like.

Prenatal diagnosis of inherited disorders

Couples who have already had an affected pregnancy or child, or have a family member affected with the disease are aware that they are at risk of transmitting an inherited disease. Prenatal diagnosis is the main option open to such couples and to those who have a positive serum or ultrasound screen. Chorionic villus sampling (CVS) and amniocentesis are the methods of choice for prenatal diagnosis; techniques such as fetal blood sampling are rarely used.

Chorionic villus sampling (CVS)

CVS can be performed transcervically between 10 and 12 weeks of gestation, or from 12 weeks onwards by the transabdominal route. A sample of cells is removed from the placenta and used for diagnosis of chromosomal, metabolic and DNA analysis. The disadvantages of the procedure are that it cannot be used for neural tube and other congenital abnormalities, and some studies have suggested a risk of limb reduction deformities if it is performed too early, or by inexperienced operators. There is also a 1–2% risk of miscarriage, which is a little higher than the risk after amniocentesis. In 1.5% of cases, the karyotype of the placenta is

found to be different from that of the embryo (confined placental mosaicism).

Amniocentesis

This is the most common method used for prenatal diagnosis usually performed in the second trimester, from 15 weeks onwards. Under ultrasound guidance, 15–20 mL of amniotic fluid is aspirated; this can be used for the diagnosis of chromosome abnormalities, measurement of specific substances, detection of inborn errors of metabolism such as Tay–Sachs disease, measurement of enzyme activity and diagnosis of neural tube defects. Its disadvantages include the potential of causing fetal loss (1%) and rarely there may be continued leakage of the amniotic fluid. The main limitation of this technique is that results are available only very late in the pregnancy (17–20 weeks), so that a second trimester termination has to be induced.

Fetal blood sampling, cordocentesis or PUBS (percutaneous umbilical blood sampling)

This is used less frequently than CVS or amniocentesis; samples can be taken from 18 weeks' gestation to term. Fetal blood is taken from the cord or intrahepatic umbilical vein and used for fetal karyotyping (quick result), evaluation of fetal status (if an infection is thought to be present) and hematological abnormalities (Rh or immune hemolytic disease). The most common indication is karyotyping for single or multiple congenital abnormalities and mosaicism.

Fetal tissue sampling

Using ultrasound guidance it is possible to biopsy skin, liver, muscle and fluid collections from the urinary tract, abdomen, thorax and cystic hygroma.

Diagnostic testing

Following CVS or amniocentesis, the sample must be diagnosed in order to identify the status of the fetus. Two tests are used: the polymerase chain reaction (PCR) or karyotyping. PCR is used for the detection of single gene defects, the triple repeat disorders, and identification of sex. A karyotype is performed for any diagnosis which involves chromosome identification, i.e., in those patients carrying chromosome abnormalities or who are at risk of age-related aneuploidy. In some situations, fluorescent in-situ hybridization (FISH) is used to complement the karyotype result. More recently DNA microarray-based technology has been developed.

PCR

PCR is a technique whereby a portion of DNA is amplified thousands of times, and it is probably the most important technique used for genetic testing. For prenatal diagnosis, PCR can be used to detect the normal or mutated gene by amplifying the region around the mutation. This is achieved using primers which have a complementary sequence to a region of the gene. Primers are selected which bind to either side of the mutation, they bind to their complementary sequence, and copies are generated for the region in between by a number of cycles of heating (to denature the DNA) and cooling (to allow synthesis). This is achieved with the use of an enzyme that can connect bases together and can also withstand the high temperatures needed for denaturation. The first such enzyme to be used for PCR was *Taq* polymerase.

Once the DNA sequence of interest has been copied, the PCR products are analyzed using a number of different techniques. The simplest method, which can be used to detect an insertion or deletion, is to separate the PCR products by polyacrylamide gel electrophoresis; more recently techniques such as single-stranded conformational polymorphism (SSCP), amplification refractory mutation system (ARMS) and heteroduplex analysis have been developed.

Using these techniques even just a single base change within a gene can be detected. PCR will be discussed in more detail in the section concerning PGD diagnosis.

Karyotyping

Karyotype analysis is the ideal method to examine the chromosomes of a cell. For prenatal diagnosis, the sample obtained by CVS or amniocentesis is cultured to increase the number of cells, and mitotic inhibitors are used to arrest some of the cells in metaphase. Agents which elongate the metaphase chromosomes are also used. Slide preparations of the nuclei are treated with Giemsa stain, which results in a specific banding pattern for each chromosome. Using this method, missing or extra chromosomes, translocations, inversions, etc. can be identified. Occasionally the results of a karyotype may be inconclusive, and FISH can be used to help elucidate the diagnosis.

Karyotyping is also used to check the number of chromosomes in diagnosis of age-related aneuploidy.

FISH

FISH uses DNA probes that bind to complementary sequences on specific chromosomes. There are three types of FISH probes:

1. Repeat sequences or alpha satellite probes, which can be used in interphase and metaphase chromosomes. They bind to repeat sequences, usually to the centromeres (with the exception of chromosomes 9 and Y) and can be used directly labeled with fluorochromes. They require only 1 hour for hybridization and have been cloned in plasmids and cosmids. Probes for 13/21 and 14/22 cross-hybridize.
2. Locus-specific probes can be used in interphase or metaphase chromosomes and bind to a unique sequence. They require 6–12 hours for hybridization and have been cloned in cosmids or YACs (yeast artificial chromosomes).
3. Chromosome paints can only be used in metaphase chromosomes, and they paint the entire chromosome.

There are several stages to the FISH technique:

1. Cell spreading.
2. Pepsin digestion. This is required to remove any protein from around the nuclei and is especially important for blastomeres.
3. Fixing. A paraformaldehyde fixative is used to ensure that the nuclei are stuck onto the slide.
4. Denaturation. This makes the nuclear and probe DNA single-stranded.
5. Hybridization. Allows the probes to find and bind to the complementary sequence.
6. Posthybridization washes. Removes any unbound probe.
7. Detection – for use with indirect probes.
8. Visualization.

Arrays

Two types of arrays are used in molecular diagnostic testing: array comparative genomic hybridization (A-CGH) and single nucleotide polymorphism (SNP) arrays. SNP arrays can examine the chromosome content as well as look at individual genes. The problem has been in interpreting these arrays as so much information is obtained, especially in relation to copy number variants. A-CGH only examines the chromosomes and is easier to interpret. It is possible that array technology will replace FISH, PCR and karyotyping in the future.

Preimplantation genetic diagnosis

PGD is comparable to prenatal diagnosis. The embryo biopsy is equivalent to the CVS or amniocentesis and the single cell diagnosis equates to the prenatal diagnosis. Since two techniques are involved, biopsy and diagnosis, a PGD team is made up of an IVF and a genetics team. The embryo biopsy technique should be performed by a trained embryologist, but the diagnosis must be performed by a genetics department.

Embryo biopsy

Cell biopsies can be taken from oocytes/embryos at three different stages:

1. Oocyte/zygote (polar body biopsy)
2. Six- to eight-cell cleavage stage embryo (cleavage stage biopsy)
3. Blastocyst stage embryos (blastocyst biopsy).

The majority of PGD centers have been using cleavage stage biopsy for PGD. However, in recent years polar body biopsy has been commonly used in countries where manipulation of the embryo is not allowed (e.g., Germany). Blastocyst biopsy has only recently been reported for clinical PGD but its use may increase with the introduction of array technology into the PGD arena.

As outlined in Chapter 13, embryo biopsy is performed using micromanipulation equipment used for ICSI, and all of the biopsy techniques involve two stages: zona drilling, and aspiration (or herniation in the case of blastocyst biopsy). Table 14.4 outlines an overview of embryo biopsy methods; the techniques are discussed in detail in Chapter 13.

Polar body biopsy

Biopsy of the first polar body was developed in order to overcome ethical objections to embryo biopsy. Some individuals opt for PGD in order to avoid termination of pregnancy, and performing the test on the preimplantation embryo may be as objectionable as termination of pregnancy. Polar body biopsy was first used for the detection of CF, but due to crossing-over

Table 14.4 The three methods of embryo biopsy used in preimplantation genetic diagnosis. Reproduced with permission from Harper 2009.

	Day performed	Types of cells removed	Indications	Zona drilling	Cell removal	Limitations
Polar body	First PB Day 0 Second PB Day 1 Or simultaneously on Day 1	First and second polar bodies	PGS Monogenics carried by mother	Laser Mechanical Beveled pipette	Aspiration	Only maternal chromosomes/ genes
Cleavage-stage	Day 3	Blastomeres	PGS Monogenics Sexing Chromosome abnormalities	Laser Mechanical Acid Tyrodes	Aspiration Displacement	Postzygotic mosaicism
Blastocyst	Day 5	Trophectoderm	PGS Monogenics Sexing Chromosome abnormalities	Laser Mechanical Acid Tyrodes	Herniation	Postzygotic mosaicism Some embryos will arrest prior to biopsy Short time for diagnosis

events the second polar body was required in some situations. Biopsy of the first and second polar body is recommended if polar body biopsy is going to be used for PGD.

Cleavage stage biopsy

Biopsies performed at earlier stages (four cells) may alter the ratio of inner cell mass to trophectoderm cells, which may be detrimental to embryo development. Therefore, the main strategy used for embryo biopsy has been to biopsy embryos at the six- to eight-cell stage, on Day 3 post insemination.

Several difficulties arise from cleavage stage embryo biopsy: the first is that human embryonic cells are very fragile and easily lyse. If this occurs during the biopsy procedure, the nucleus may be lost and another cell will have to be removed. Compaction occurs between the eight-cell and morula stage, and during compaction the cells of the embryo can no longer be distinguished as they flatten out over each other to maximize intercellular contacts. If the biopsy is performed at this stage, it is very difficult to remove a blastomere, as it has established strong contact with adjacent blastomeres. Trying to remove a cell from a compacted embryo may also result in lysis of the cell. The thickness and dynamics of the zona pellucida also vary between patients and can lead to some problems during the biopsy procedure. In many cases, numerous sperm are associated with the zona pellucida and therefore intracytoplasmic sperm injection (ICSI) should always be used with PCR techniques to reduce the risk of sperm contamination.

In the human, the cryopreservation of embryos in IVF treatment cycles is routine, and from frozen embryo replacement cycles up to 50% of blastomeres can be destroyed and the embryo is still capable of producing a viable fetus. No increase in fetal abnormalities has been reported following transfer of cryopreserved embryos in which some cells have been destroyed by freezing/thawing. Studies examining the effect of embryo biopsy have shown that at the eight-cell stage, removal of two cells was not detrimental to embryo metabolism or development and is an efficient process with more than 90% of the embryos surviving. Data from the ESHRE PGD consortium show that 97% of embryo biopsies were successful.

Pregnancies obtained from biopsied embryos after PGD have been studied. Biochemical and ultrasound measurements showed that there were no significant developmental differences between these pregnancies and controls. Deliveries, including infant birth weight and Apgar scores, were considered to be normal.

Blastocyst biopsy

Blastocyst biopsy can be performed on Day 5 or Day 6 post insemination, and has the advantage that a larger number of cells can be removed from the outer trophectoderm layer without affecting the inner cell mass from which the fetus later develops. Analysis of a larger number of cells is of benefit in diagnosis of monogenic diseases. However, trophectoderm cells (TE) may have diverged genetically from the inner cell mass (ICM) as confined placental mosaicism is observed in at least 1% of conceptions, where the chromosome status of the embryo is different from the placenta. Studies indicate that blastocysts may have high levels of chromosomal mosaicism. Vitrification as a method of cryopreserving blastocysts has recently shown excellent results; vitrifying after blastocyst biopsy allows more time for the diagnosis.

The main limitation of blastocyst biopsy is that a limited number of embryos will reach the blastocyst stage. Although improvements in culture conditions have increased the numbers of blastocysts available, for PGD to be successful a large number of embryos are required to ensure that normal embryos are available for transfer; this may not be the case when embryos are cultured to the blastocyst stage.

Single cell diagnosis

Cells removed from the embryo after biopsy are used for diagnosis, as outlined in Table 14.5. PCR is used for the single cell diagnosis of single gene defects, triplet repeat disorders and embryo sexing. Karyotyping requires a metaphase spread of chromosomes, and therefore this cannot be used on single embryonic cells; these cells do not divide well in culture, and it is difficult to obtain metaphase spreads. In cases where a metaphase spread is obtained, the chromosomes are short and difficult to band. Therefore FISH is used to examine chromosomes in embryos for embryo sexing, chromosome abnormalities and aneuploidy. Array technology has been applied in PGD.

Molecular diagnosis

PCR diagnosis from a single cell is used for the diagnosis of single gene defects, triplet repeat disorders and embryo sex, but it is not a simple procedure. The two major problems encountered are contamination and allele dropout (see below), which both complicate the procedure. If a diagnosis is available on whole DNA, it should be possible to make such a diagnosis sensitive at the single cell level. However, some modifications of the procedure may be required. A common method of making the PCR procedure more sensitive is the use of nested PCR, where an inner set of primers amplifies the original PCR product. Since amplification failure can occur, it is essential that PGD does not rely on a negative result. To ensure that a single cell PCR method is accurate and sensitive, a preliminary workup is usually performed on single cells, such as buccal cells, from normal, carrier and affected individuals. The analyses of PGD PCR products have been performed by heteroduplex analysis, SSCP, ARMS and restriction endonuclease digestion. Fluorescent PCR is a quantitative PCR method that can also be used. For the diagnosis of some diseases, such as fragile X, polymorphic markers may be used that identify which chromosome the embryo has inherited; i.e., the normal or at-risk chromosome.

Contamination

Single cell PCR is so sensitive that it will amplify any DNA that may contaminate the PCR reaction, such as a stray cumulus or sperm cell that may have been released from the zona during the biopsy, cells from the atmosphere or DNA found in the air or medium. To reduce these problems to a minimum, steps have to be taken to eliminate contamination. These include working in a positive pressure PCR room, performing ICSI for all PCR diagnosis and examining PCR products in a separate laboratory. Misdiagnoses reported after PGD probably arise from contamination. These problems can be reduced with the use of a multiplex PCR with markers that can identify all four parental alleles to ensure that the amplified product is of embryonic origin.

Allele dropout

Allele dropout (ADO), or preferential amplification, refers to the situation where one of the two alleles preferentially amplifies over the other. For example, for a heterozygous cell, the normal allele may preferentially amplify so that the diagnosis would only identify the normal allele – the embryo would be diagnosed as normal instead of heterozygous. This would not cause a problem for recessive conditions where both partners carry the same mutation, but would create problems for dominant disorders, or in cases where the couple carry different mutations for a recessive disorder, as affected embryos could be

Table 14.5 Methods used for preimplantation genetic diagnosis. Reproduced with permission from Harper 2009.

	Indications	Cell preparation	Protocol	Limitations
FISH	Sexing Chromosome abnormalities PGS	Spreading cells using methanol:acetic acid or Tween HCl	Fix Denature Hybridization Wash off unbound probe Visualize	Cumulus contamination Mosaicism Overlapping signals Failure of probes to bind
PCR	Sexing Monogenic disorders	Tubing cells into lysis buffer	Lyse cell Cycles of denaturing, annealing, elongation Detect products	Cumulus contamination Sperm contamination (use ICSI) Other contamination Amplification failure Allele dropout
Metaphase CGH	Sexing Chromosome abnormalities PGS	Tubing cells into lysis buffer	Lyse cell, whole genome amplification Co-hybridization with control sample on to metaphase spread Analysis of each chromosome using CGH software	Contamination Mosaicism Procedure takes several days and so currently embryos are frozen Requires many skills, PCR, and cytogenetics

FISH, fluorescent in situ hybridization; PGS, preimplantation genetic selection; PCR, polymerase chain reaction; ICSI, intracytoplasmic sperm injection; CGH, comparative genomic hybridization.

diagnosed as normal. To reduce this problem, methods can be built into the diagnosis to ensure that both alleles can be identified.

FISH diagnosis

Fluorescent in-situ hybridization (FISH) allows every nucleus within an embryo to be examined, but the number of chromosomes that can be analyzed at one time is limited. Using two rounds of FISH on a single nucleus allows a panel of seven to nine chromosomes to be screened (commonly including X, Y, 13, 16, 18, 21, 22), but repeated denaturation leads to DNA degeneration and decreases the efficiency of the procedure. Repeat and locus-specific probes can be used for cleavage or blastocyst biopsies as the embryonic nuclei are in interphase, but chromosome paints can be used on polar bodies.

FISH has been used since 1991 to sex embryos for PGD in cases of X-linked disease. It has advantages over PCR sexing as the copy number is identified: the difference between XO and XX can be determined, and there is no risk of contamination. Usually, probes for chromosomes X, Y and 18 are used and only embryos showing normal female chromosomes are transferred. However, the best method to perform PGD for X-linked disease is a specific diagnosis of the disorder using a molecular method. This will differentiate between the affected male, unaffected male, carrier female and non-carrier female.

With the development of a wider range of locus-specific probes, FISH can be used for the detection of chromosome imbalances in patients carrying chromosome abnormalities. In these situations, a normal and balanced chromosome pattern cannot be distinguished. If the female carries the translocation, polar body biopsy can be performed and chromosome paints used. For Robertsonian translocations, probes for any area of the chromosome involved can be used, but for reciprocal translocations probes are used either side of the break points. A-CGH is being developed to diagnose chromosome imbalances in embryos.

PGD of age-related aneuploidy (preimplantation genetic screening, PGS)

Preimplantation genetic screening (PGS) has been used extensively in an attempt to select the best embryo to transfer for patients going through IVF. Indications have included those patients with advanced maternal age, repeated implantation failure, repeated miscarriage with a normal karyotype and severe male factor. Initial studies reported an improvement in pregnancy rate when using PGS with FISH to detect the common aneuploidies (13, 16, 18, 21, 22, etc.). To date ten randomized controlled trials using cleavage stage biopsy and one using blastocyst biopsy have shown no increase in delivery rates when using PGS for a variety of indications. Some studies showed a significant decrease in delivery rates in the treatment group. For PGS to be used successfully, the negative effect of the biopsy must be compensated for by the advantage of the genetic testing. Using FISH at cleavage stages does not do this due to limitations in the FISH procedure (a limited number of probes can be used and the technique is not 100% efficient) and the high levels of mosaicism seen at cleavage stages which will result in false positives and negatives.

Current work is looking at polar body or blastocyst biopsy and the use of array technology to determine if PGS has a future in the field of ART.

Chromosomal mosaicism

FISH, metaphase and array CGH have been used for the analysis of chromosome patterns in human preimplantation embryos which have been donated for research purposes. Studies on abnormally fertilized embryos (such as polyspermic embryos) have shown, as expected, that these embryos were highly abnormal, in agreement with the karyotype data obtained from such embryos. In the majority of cases, mosaicism was observed and, in some cases, normal diploid embryos were found from supposedly polyspermic embryos; this may have been due to misidentification of a vacuole as a pronucleus. As expected, embryos from older women show high levels of chromosome abnormalities but interestingly normally fertilized, normally developing embryos also show high levels of chromosomal abnormalities. In order to categorize these abnormalities, the patterns have been divided into four groups. Embryos are either:

(a) uniformly diploid,
(b) uniformly abnormal, such as Down's syndrome or Turner's,
(c) mosaic, where usually both diploid cells and aneuploid, haploid or polyploid nuclei are present or
(d) chaotic embryos, where every nucleus shows a different chromosome complement.

The data from FISH and CGH analysis show a higher rate of abnormalities than has previously been reported from karyotyping data. However, since mosaic and chaotic embryos are common, if only one or two cells are analyzable from an embryo, then karyotyping would underestimate the level of chromosome abnormalities.

Normal, abnormal and mosaic embryos have all been observed in fetal development. It has been estimated that in 1% of conceptions the placenta has a different karyotype from the fetus (confined placental mosaicism – CPM). CPM was first detected when first trimester fetal karyotyping after chorionic villus sampling showed discrepancies between chorionic cells and the embryo proper. The presence of two cell lines could arise due to an abnormal chromosome arrangement caused by a postzygotic event, or the chromosome loss from a trisomic embryo, which restores the diploid state (trisomic rescue). Several mechanisms would indicate that these abnormal cells are more likely to be found in the trophectoderm, and hence the placenta. First, only a few cells from a blastocyst give rise to the embryo and it would be unlikely that the abnormal cells would be found in the embryo; second, in most cases a fetus with abnormal chromosomes will not be compatible with life. The chaotic group of embryos was an unexpected finding, as such embryos have not been observed in later stages of embryonic development, probably because these embryos would arrest and fail to implant.

Multinucleated blastomeres have been reported from both karyotyping and FISH analysis. The presence of such blastomeres may be more common in arrested embryos, and may occur more readily in some patients. Binucleate blastomeres have been observed in mouse embryos at the morula stage, and it has been suggested that these blastomeres might be the precursors for mural trophectoderm giant cells. However, in human embryos the binucleate cells appear at cleavage stages before trophectoderm differentiation. Binucleate cells may arise from asymmetrical cytokinesis so that

one daughter cell contains two nuclei and the other is anucleate.

Embryos containing tetraploid cells may be a normal part of development of the trophectoderm. Such cells have also been found in cattle, pig and sheep.

Overall, extrapolation of this data would suggest that few embryos are completely chromosomally normal at early cleavage stage. However, various models for which there are experimental data may help to explain the observation that pregnancies following IVF do not result in an increased incidence of chromosomally abnormal infants. Few cells (possibly a single cell from an eight-cell embryo) differentiate to the embryo proper – the majority contribute to the cytotrophoblast and fetal membranes. Data accumulated on the chromosomal constitution of surplus nontransferred embryos from PGD cycles have revealed that, despite the fact that these embryos are from women of proven fertility, the incidence of postzygotic chromosomal anomalies is similar to that in embryos from routine IVF patients. This finding may provide one explanation for the apparently poor success rate of IVF procedures. A second significant finding is that the incidence of the most bizarre type of anomaly, chaotically dividing embryos, is strongly patient-related. In repeated cycles, certain women regularly produced "chaotic" embryos while others did not, although the frequency of diploid mosaics was similar in both groups.

All the studies performed in recent years to analyze chromosomes in embryos have been carried out using embryos generated by IVF, which may not be representative of in-vivo development. However, the classic studies of Hertig *et al*. (1956) showed that embryos from natural cycles also showed high levels of nuclear abnormalities, and from the studies of normally conceived pregnancies, we know that 60% of abortions are chromosomally abnormal.

Mosaicism and PGD

Chromosomal mosaicism may cause a problem in PGD for some diseases, namely dominant disorders and chromosome abnormalities. A misdiagnosis of embryo sex would be unlikely to occur as an XX cell would have to be found in an XY embryo. XO cells have been identified in male embryos, but XO embryos should never be considered for transfer; if the offspring have Turner's syndrome, they would have the same risk of suffering the X-linked disease as would a male. For recessive disorders, the presence of extra chromosomes or a haploid cell would not lead to a misdiagnosis. A carrier embryo with a haploid cell would be diagnosed as normal or affected depending on which gene was present in the cell: this would be the same situation if allele dropout had occurred. For dominant disorders, a haploid cell could lead to a misdiagnosis; if a cell from an affected embryo carried only the unaffected gene, the cell would be diagnosed as normal. Therefore the same precautions as for allele dropout would have to be applied. For chromosome abnormalities, mosaic embryos containing some normal and some abnormal cells have been identified, such as in cases where a few normal cells arise in an embryo which otherwise carried trisomy 21. If the normal cells are biopsied, the embryo would be diagnosed as normal, resulting in misdiagnosis. As with confined placental mosaicism, this problem cannot be solved. Patients undergoing PGD for chromosomal abnormalities have to be aware that chromosomal mosaicism can lead to a misdiagnosis, but that this is a rare event. The probability can be reduced if two cells are used for the diagnosis, but this is not always technically feasible.

Ethics and laws

The law governing PGD varies worldwide. Some countries have legislation regulating PGD, or PGD and embryo research, and others have no legislation. However, a few countries have banned cleavage stage biopsy – which is astonishing in view of the fact that the aim of PGD is to eliminate termination of pregnancy. The main arguments against PGD are that it may be abused, as in the case of embryo sex selection for family balancing, or for choosing certain characteristics, the so-called "Designer Baby." Prenatal diagnosis has been abused for fetal sex selection in several countries for many years, but, as with all medical practices, the good should outweigh the bad: such practices should not be banned just because they could be abused. Legislation governing the use of PGD should eliminate such problems. In the UK, the Human Fertilisation and Embryology Authority (HFEA), which licenses all IVF practices, has banned embryo sexing for family balancing and they license all PGD centers. It must be remembered that it is no easy task to undergo PGD, mainly because the IVF procedure is so invasive and the pregnancy rate is low. If couples wished to select their baby, the cheaper and simpler route would be prenatal diagnosis, where many diseases could be diagnosed at one time.

The future of PGD

PGD is more complicated than originally thought: the concept that a single cell would be representative of the rest of the embryo has been confused and compounded by the discovery of high levels of chromosomal mosaicism in human embryos. Unfortunately, misdiagnoses do occur, mainly due to chromosomal mosaicism, allele dropout or contamination (possible from cumulus or sperm cells).

Cost is also an important consideration, as some single cell diagnoses are expensive techniques and array technology is currently very expensive. For example, the cost of FISH probes can equal the cost of an IVF treatment cycle, which makes PGD a very expensive technique. In the UK, some health authorities have paid for PGD cycles, and in other countries government or health insurance funds are available; if patients have to meet the cost they may opt for prenatal diagnosis.

Data from the ESHRE PGD consortium shows that the delivery rate per cycle for PGD is similar to that seen in IVF, even though PGD patients are normally fertile. Therefore, any center or patient embarking on PGD has to be aware that the diagnosis may not be 100% accurate due to mosaicism and allele dropout, and that the chance of an unaffected baby after one cycle is low. The patients also have to decide whether undertaking an IVF cycle is more or less traumatic than natural conception and prenatal diagnosis. We have found that the natural pregnancy rate for patients registered for PGD is very high, as most of them have the alternative of prenatal diagnosis.

The most logical, and highly motivated group of patients to treat by PGD are those who are infertile or who have experienced repeated miscarriages. Patients who carry chromosome abnormalities are one of the most difficult groups of patients to treat, due to limitations of available probes and the high levels of abnormal embryos they produce.

Additional techniques for single cell diagnosis include whole genome amplification, where the whole genome is randomly amplified to allow several diagnoses to be performed on a single cell. This allows examination of several mutations for a disease from one cell, and haplotyping can also be performed to ensure that the diagnosed DNA is embryonic. Products of whole genome amplification may also be used to examine chromosomes using array CGH. For CGH, nick translation is used to label embryonic DNA with a fluorochrome (e.g., red), and some control DNA is labeled with a green fluorochrome (control samples may be provided by the manufacturer in order to eliminate a source of error). Both samples are cohybridized onto a control metaphase plate (metaphase CGH) or an array platform (array-CGH) and the ratio of red to green labeling indicates which chromosomes or parts of chromosomes are in excess or missing from the embryo. The advantage of array technology is that multiple defects can be assessed in a single test, compared to FISH, which requires a specific probe for each chromosome or region, and PCR, where a specific primer is required for each sequence of interest.

Many couples around the world opt for PGD as an alternative to prenatal diagnosis, and several thousand babies have been born. Although it is a demanding technique that requires a fully equipped molecular biology laboratory, highly skilled molecular biologists and close collaboration with clinical geneticists, technical breakthroughs over the past decade continue to make PGD a very real alternative for couples at risk. Hopefully, with improvements in IVF and single cell diagnosis, the range of diseases that can be diagnosed at the single cell level and the number of patients who can be treated will increase.

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Chapter

15 Epigenetics and assisted reproduction

John Huntriss

Introduction

During mammalian development the growth of the fetus is regulated by genetic information that is inherited from both the sperm and the oocyte. Apart from the clear differences that are associated with the X and Y chromosomes, the parental genetic contributions to the embryo also differ via a system of "epigenetic" marks. This functional non-equivalence between the parental genomes, how gametes and preimplantation embryos are reprogrammed, and how these delicate processes may be affected by assisted reproductive technology and infertility will be described in this chapter. A full understanding of the cellular and molecular biology of human reproduction must include a study of epigenetics and genomic imprinting.

Epigenetics

Epigenetics is an additional "layer" of information that complements the information in the genomic DNA sequence; it is essentially a marking system that regulates gene expression and hence the phenotype of the cell. An epigenetic mark is a chemical modification of the DNA bases themselves, or a modification that leads to a change in chromatin structure. These modifications affect the way that sequence information is read by the cell by influencing interaction with transcription complexes and other regulatory factors. This level of gene expression may be termed the "epigenotype"; an incorrect representation of the information, such as occurs in an epigenetic disease, is an "epimutation."

The biological mechanisms that are epigenetically regulated include X-chromosome inactivation, parent-of-origin effects of genomic imprinting, as well as tissue-specific and age-dependent DNA modification. Epigenetic information is also responsible for the phenotypic variability of somatic cells within an organism, controlling how tissues and cells in the body define themselves. For example, within a human individual, the genotype (the gene complement) is the same for different types of cell (e.g., a muscle cell or a liver cell) yet the cellular functions and phenotypes are very different. Although the DNA sequence within these different cells is identical, the repertoire of expressed genes differs greatly between cell types, a difference that is essential in determining the specific functions of different cells.

Epigenetic marks

The mechanisms that contribute to imprinting include DNA methylation, histone modification and RNA-mediated (transcriptional) mechanisms. DNA methylation is stable but reprogrammable, heritable, and affects the regulation of gene expression. This type of epigenetic mark is believed to involve methylation of CpG dinucleotides around certain genes. For example, a gene whose active expression is required in a liver cell may be unmethylated across the majority of CpG sites within the vicinity of its genomic sequence, whereas a gene that needs to be silenced in the liver may be heavily methylated, repressing transcription by "locking" the genes within an inaccessible heterochromatin structure.

Histone proteins are important in DNA packaging; they can be covalently modified by a number of post-translational modifications that significantly affect whether chromatin conformation is open or closed. "Open" chromatin is accessible to DNA replication and transcription (gene expression), and "closed" chromatin is not accessible. Chromatin conformation is affected by modification of histone tails via methylation, acetylation, phosphorylation etc., especially those of H3 and H4 histones. Each different modification or combination of modifications affects chromatin structure, and thus gene expression, differently. In many

In-Vitro Fertilization: Third Edition, ed. Kay Elder and Brian Dale. Published by Cambridge University Press.

situations, both DNA methylation and the histone modification "code" probably contribute to the overall process of epigenetic regulation.

Histone nomenclature

Histone modifications are named by using:

- the name of the histone (e.g., H3)
- the abbreviation for the amino acid and its position within the modified histone protein (e.g., K9 for lysine at position 9)
- the type of epigenetic modification (e.g., "me" for methylation)
- the extent of the particular modification (e.g., mono, di or tri-methylation: me1, me2, me3).

Genomic imprinting

Genomic imprinting is the exclusive expression of only one of the parental alleles of a gene, a unique mode of gene expression that affects the growth and development of the fetus according to whether an allele of a particular imprinted gene is inherited paternally or maternally. In normal circumstances, imprinting exerts fine control over the growth of the developing conceptus via the placenta. A number of human diseases involve abnormal regulation of imprinted genes or "parent of origin" effects, and disruption of imprinting can lead to cancer (Walter and Paulsen, 2003; Holm *et al.*, 2005). Genomic imprinting is particularly susceptible to disruption during early preimplantation development, and is therefore vulnerable to potential aberrations that may be introduced through certain assisted reproductive technology (ART) procedures.

Approximately 80 mammalian genes that show imprinted (monoallelic) expression have been described to date, although more have been predicted by bioinformatic analysis. Genomic imprinting is regulated by imprinting control regions or imprinting centers (ICRs or ICs) that acquire epigenetic marks such as DNA methylation upon passage through the germline. Imprinted genes have different methylation patterns (differentially methylated regions, DMRs) on the maternal and paternal alleles that play an important regulatory role. DMRs typically consist of stretches of differentially methylated CpG sites that are close to an imprinted gene, and this epigenetic information regulates allele-specific gene expression. A germline DMR will therefore have a different methylation pattern in the sperm than in the oocyte, and this differential marking will be recognized in the zygote and preimplantation embryo. The majority of imprinted genes are located in clusters within the genome, and are regulated by ICRs that regulate all or most of the imprinted genes in a cluster.

Figure 15.1 shows a simplified representation of differential allele marking of an imprinted gene in the germline, which leads to mono-allelic expression in somatic tissue of the offspring. The paternal allele is not methylated in sperm (left), whilst the maternal allele is methylated in the oocyte (right). This differential methylation imprint persists after fertilization and early development. The epigenetic mark (methylation) placed on the allele during oogenesis silences the maternal allele in the offspring and only the paternal allele is transcribed to mRNA (bent arrow).

Imprinted genes are also believed to play a role in the parent–offspring conflict model (Moore and Haig, 1991), a theory proposing that the parental alleles of these loci have different interests with respect to regulation of fetal, placental and neonatal growth; i.e., the paternal genome will fight for the biggest size and health of the current litter (his litter), whereas the maternal genome will try to counter or moderate this effect in order to reduce current nutritional strain on herself and ensure that she will be able to raise future litters. This hypothesis is reinforced by the fact that many imprinted genes regulate growth of the fetus and placenta. Interestingly, disruption of certain imprinted mouse genes by knock-out experiments also affects maternal nurturing behavior. Imprinting may also prevent parthenogenesis, since it ensures that both parental genomes are necessary for an embryo to develop to term (Kono, 2009).

Epigenetic reprogramming in the germline

The mature oocyte and sperm are highly specialized cells, and this means that their cellular specialization needs a significant amount of epigenetic information. Information inherited from the previous generation must be erased in primordial germ cells, so that new epigenetic information may be subsequently added according to whether the primordial germ cell is destined to become an oocyte or a sperm cell (Figure 15.2). Extensive epigenetic reprogramming is therefore required in the primordial germ cells (erasure) and during gametogenesis (establishment) (see Morgan *et al.*, 2005 for review). The most complete information available is for reprogramming via DNA methylation, but other epigenetic marks (e.g., the histone code) are

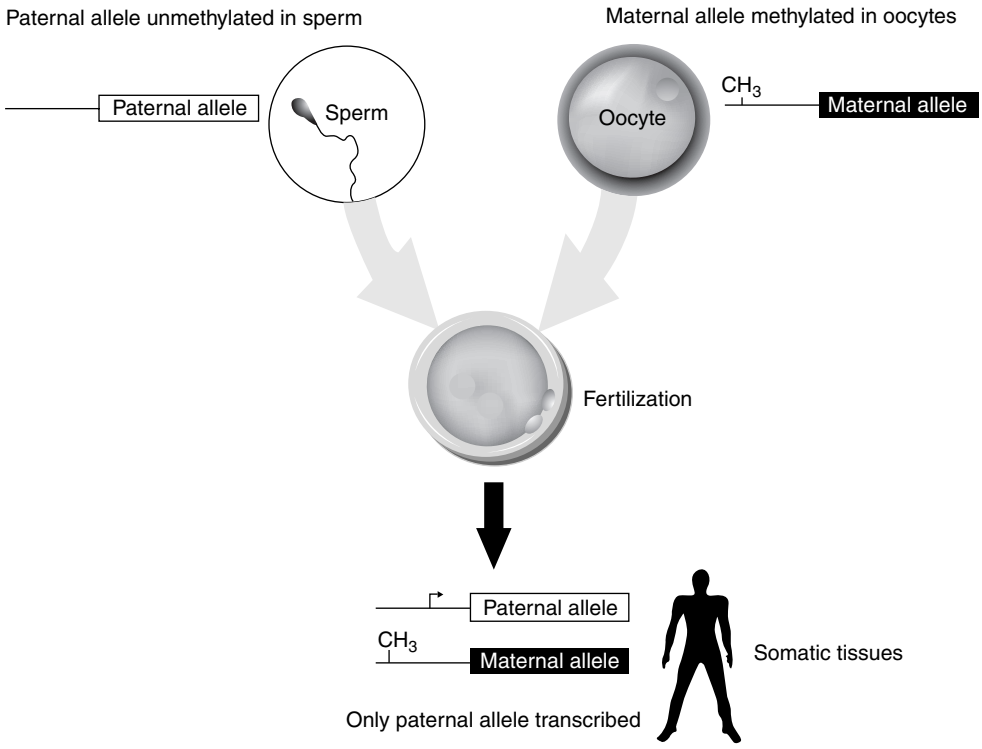

Figure 15.1 Epigenetic information from the germline regulates genomic imprinting.

also reprogrammed in primordial germ cells and during gametogenesis.

Erasure

Reprogramming events have been studied extensively in the mouse (reviewed in Constância *et al.*, 1998). Mouse primordial germ cells (PGCs) are identifiable by embryonic day E7.5, and they then migrate to the genital ridge by day E10.5–11.5, where they form the gonadal primordia. At this stage, primordial germ cells still retain methylation patterns derived from the oocyte and sperm, and any of their respective modifications from early development. At day E13.5, male germ cells enter into mitotic arrest and female germ cells enter meiotic prophase. Between days E10.5 and E13.5 DNA is globally demethylated in the PGCs of both sexes, at imprinted genes and also at many DNA elements including IAPs (retroviral elements containing long terminal repeats), Line 1 sequences (long interspersed nucleotide elements), direct repeats and non-CpG island genes. Each element displays a unique erasure profile, and different degrees of demethylation occur between the various elements (see Lees-Murdock and Walsh, 2008 for review).

Establishment

After methylation has been erased, the germ cells that are still diploid undergo *de novo* methylation. In mouse gametogenesis, the majority of DNA remethylation occurs around day E15.5 in both sexes, although timing does vary between the various elements; timing also differs between the male germline and female germline (Lees-Murdock and Walsh, 2008). IAPs, Line 1 sequences and other elements are fully methylated in mature sperm. Certain genes escape this remethylation, for example those containing CpG islands (genomic regions with high densities of CpG nucleotides, which are distinct from the smaller differentially methylated CpG sites of imprinted genes).

Imprinting marks are established according to the sex of the individual (i.e., whether the germ cell is an oogonium or spermatogonium), and most of the imprinted genes acquire methylation in the female germline. The *H19* imprinted gene is one that is

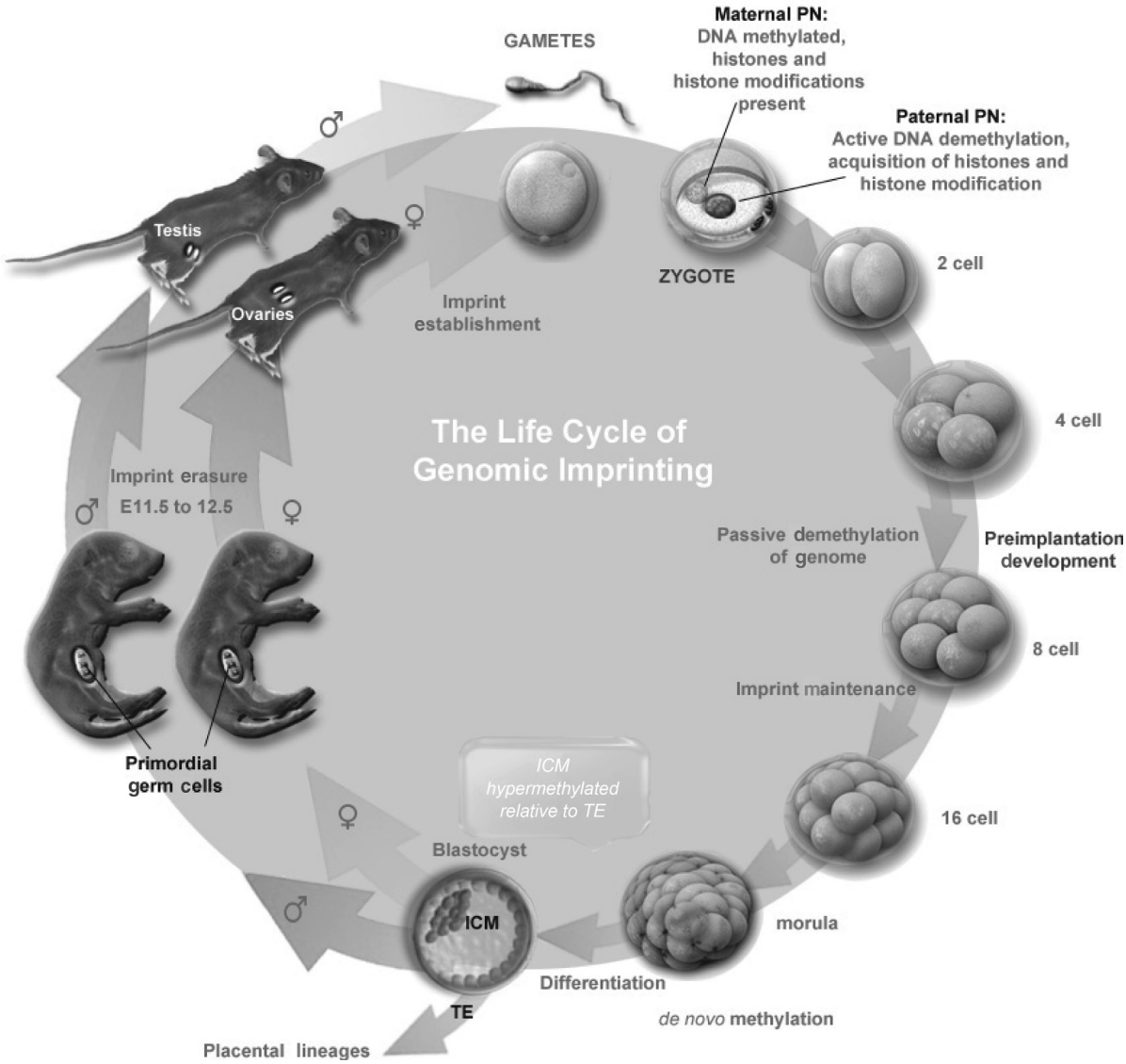

Figure 15.2 The life cycle of genomic imprinting (adapted from Morgan *et al.*, 2005). Imprint erasure, establishment and maintenance are all features of the epigenetic reprogramming cycle that is required for genomic imprinting. In this figure, the imprint is via DNA methylation. Extensive reprogramming occurs in the primordial germ cells, erasing the imprints from the previous generation. Imprints are re-established during gametogenesis according to the sex of the embryo, maintained in the embryo, and translated into stable functional differences between the parental alleles in the developing conceptus. Extensive epigenetic reprogramming of the paternal pronucleus occurs in zygotes.

methylated in the male germline. Despite the fact that epigenetic information has been erased in the primordial germ cells, sufficient underlying epigenetic information remains so that the parental origin of all of the alleles can still be distinguished: for *H19* in the male germline, the DMR on the paternal allele is completely remethylated by E15.5, whilst the maternal DMR is remethylated around birth.

In the female germline, maternal DMRs remain hypomethylated until the pachytene stage of meiosis I in the postnatal growing oocyte. Maternal methylation imprints are acquired during oocyte growth, and the DMRs for different imprinted genes appear to be remethylated at different times. Thus imprinted genes *Snrpn*, *Znf127*, *Ndn* are methylated by the primary follicle stage, *Peg3* and *Igf2r* genes are methylated by the

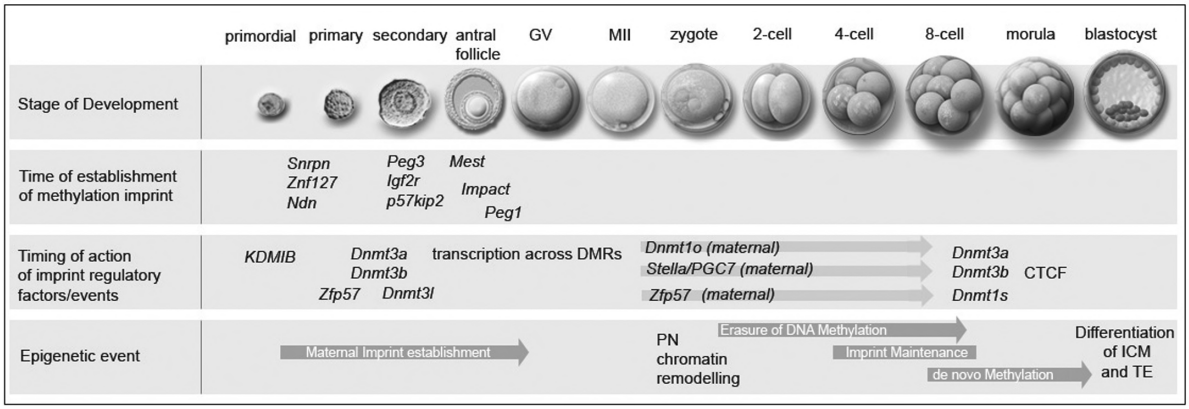

Figure 15.3 Regulation of imprinting in the mouse female germline. The approximate sequence of gene-specific imprint establishment in the mouse female germline is illustrated, showing factors that are relevant to the reprogramming events. Imprinted genes receive methylation imprints at different times during the oocyte growth phase. The lower panel shows key reprogramming phases that occur at imprinted genes and in the genome as a whole. GV = germinal vesicle; MII = metaphase II; PN = pronuclear; ICM = inner cell mass; TE = trophectoderm.

secondary follicle stage, and the *Impact* gene is methylated by the antral follicle stage. As in the male germline, some underlying epigenetic signal is retained since the maternally inherited alleles of *Snrpn*, *Zac1* and *Peg1* genes are methylated before the paternally inherited alleles (Obata and Kono, 2002; Lucifero *et al.*, 2004; Hiura *et al.*, 2006).

The DNA methyltransferases (Dnmts) play a major role in the establishment of methylation imprints during gametogenesis; the *de novo* methyltransferases Dnmt3a, Dnmt3b and the related protein Dnmt3L are expressed coordinately and work together to establish methylation imprints during oogenesis. Methylation at imprinted genes in oocytes appears to be dependent on the size of the oocyte, and it has been suggested that methylation is linked to the accumulation of DNA methyltransferases during the growth phase. In oocytes, histone H3K4 must be demethylated (via KDM1B histone demethylase) before the DNA methylation imprints are established; transcription through imprinted gene DMRs keeps chromatin domains open and accessible for methylation (Figure 15.3).

Parental genomes are packaged differently in the gametes and during the first stages of fertilization. The maternal genome from the oocyte is nucleosomal, whilst the paternal genome from the sperm is condensed and packaged by protamines, which are quickly lost and replaced by histones after sperm entry into the oocyte. During pronuclear maturation, the paternal pronucleus undergoes significant chromatin reorganization, with active demethylation during the transition from PN0 to PN5 and metaphase, followed by histone acquisition towards the end of PN maturation. The male pronucleus gathers epigenetic marks such as H3K9me1 and me2, and H3K27me2/3, whilst the maternal pronucleus, largely rich in histone epigenetic marks, remains relatively unchanged from PN0 to PN5. This difference between male and female pronuclei is referred to as "epigenetic asymmetry." By the end of pronuclear maturation (PN4/PN5), DNA methylation (5MeC) is removed from the male pronucleus. Further differential histone modifications between the parental genomes are observed in the early embryo (reviewed in Corry *et al.*, 2009).

DNA methylation is again reprogrammed during preimplantation development (reviewed in Reik *et al.*, 2001), with initial erasure and then *de novo* DNA methylation towards the end of the preimplantation period as differentiation occurs. Epigenetic differences are seen between the inner cell mass (ICM) and trophectoderm of the blastocyst: H3K27me1, me2 and me3 are present predominantly in the ICM (reviewed in Morgan *et al.*, 2005). The ICM and trophectoderm cell lineages of the mouse blastocyst are differentially marked by histone H3 lysine 27 methylation at key developmental genes.

Species-specific differences in mammalian reprogramming

Assessment of 5-methylcytosine immunostaining in different mammalian zygotes shows:

- The paternal pronucleus undergoes demethylation in mouse, human and bovine zygotes, but not in sheep and rabbit zygotes.

- Demethylation of the male pronucleus occurs more slowly in rat than in mouse.
- The male pronucleus is completely demethylated within 4 hours of fertilization in the mouse, and passive loss of methylation in the embryo continues up to the morula stage.

Imprint maintenance during preimplantation development

Dynamic changes take place in the level of genomic DNA methylation during preimplantation development. The imprints that were established in the male and female germline must be recognized and maintained during global DNA demethylation in the early embryo, so that the imprinting mark may be propagated during later development in order to allow expression of the appropriate allele.

Factors involved in imprint maintenance

Proteins essential for early development that are expressed by the mammalian oocyte (maternal effect genes) perform essential functions in maintaining methylation at imprinted loci:

- DNA methyltransferase protein has two forms, Dnmt1s (somatic form) and Dnmt1o (an oocyte-specific form).
- Dnmt1 maintains genomic imprints at a time when there is genome-wide erasure of DNA methylation.
- Maternally-derived Dnmt1 proteins (Dnmt1o and/or Dnmt1s) probably maintain methylation imprints at imprinted genes during the first cell cycle.
- Zygotic Dnmt1s maintains the imprints from the two-cell stage onward.
- Stella/PGC7 is required to maintain methylation at several maternally expressed imprinted genes, and also to maintain the paternally derived methylation imprints at *H19* and *Rasgrf1*.
- The zinc finger protein Zfp57 maintains both paternal and maternal methylation imprints at multiple imprinted regions after fertilization.
  - Depletion of Zfp57 from oocytes and embryos results in loss of methylation imprints at a number of paternally methylated and methylated DMRs.
  - Zfp57 is also required to establish the germline methylation imprint at the *Snrpn* gene during oogenesis, but at no other tested loci.

Exactly how some unmethylated CpG sites (such as unmethylated DMRs) are protected from remethylation events towards the end of preimplantation development is unclear. However, mice lacking the CpG binding protein CGBP (a transcriptional activator protein that binds unmethylated CpG sites) die during the peri-implantation stages, and embryonic stem cells that lack this protein are hypomethylated. CGBP or related factors may therefore be involved.

Finally, the imprints must be "read" or recognized so that the alleles can be appropriately expressed; for example, the zinc finger protein CTCF regulates genomic imprinting by binding differentially methylated regions in a manner that is dictated by the methylation pattern.

Epidemiological data in ART

Handling human gametes and embryos outside the human body could potentially introduce stresses that might later be manifested during development. In addition, ART procedures are performed during a period when dynamic and essential epigenetic reprogramming events are occurring on the genome of the gamete or embryo during normal development. Not surprisingly, data surrounding the subject is fragmented and incomplete, fraught with differences in sample size and selection criteria. At the time of writing, more than 10 million healthy children have been born after ART, bearing testimony to this remarkable technology of the twentieth century. However, developmental abnormalities must be rigidly monitored, and research on the epigenetic regulation of human gametes and embryos is fundamental. A summary of some of the syndromes that have been noted in ART children is presented below, together with examples of potential ART pathways that might possibly induce epigenetic mistakes.

Beckwith–Wiedemann syndrome (BWS) and ART

BWS is caused by faulty expression of the imprinted genes on chromosome 11q15.5, and occurs sporadically after natural conception at an approximate rate of 1 in 15 000 births. The syndrome is associated with large pre/postnatal growth (approximately 160% increase), childhood tumors (commonly Wilms' tumor), macroglossia, exomphalos, organomegaly, hypoglycemia and hemihypertrophy. Approximately 20% of cases show paternal uniparental disomy of the 11q15.5 chromosome, and overexpression of the imprinted gene *IGF2* is found in 80% of cases.

BWS registries reveal that the syndrome has been observed in children conceived after assisted

reproduction. The major epimutation identified in these children is hypomethylation of the KvDMR1, a methylated imprinting control element on maternal (oocyte) chromosome 11p15.5, at the promoter region of the *KCNQ1OT1* gene; 24 out of 25 ART children with BWS presented with hypomethylation at KvDMR1 (Lim *et al.*, 2009). The mechanism behind this methylation defect is currently being investigated.

To date, over sixty ART-conceived BWS cases have been reported (Maher *et al.*, 2003; DeBaun *et al.*, 2003; Gicquel *et al.*, 2003; Halliday *et al.*, 2004; Sutcliffe *et al.*, 2006). The cause of the BWS epimutation could not be linked to any particular aspect of ART or infertility, but a study that identified nineteen ART-conceived children from a BWS registry found that the only common parameter that could be identified was the use of ovarian stimulation (Chang *et al.*, 2005). This aspect will be covered in more detail later in this chapter.

A more generalized epigenetic defect may be associated with ART (such as inefficient maintenance of imprints in the preimplantation embryo), since epigenetic defects outside of KvDMR1 at the DMRs for the imprinted genes *IGF2R*, *SNRPN* and *PEG1/MEST* have been identified in a small number of ART-conceived BWS patients (Rossignol *et al.*, 2006). However, widespread epigenetic errors are also seen in naturally conceived BWS patients. Hypomethylation at KvDMR1 has also been observed in three out of eighteen normal children that were conceived by ART (Gomes *et al.*, 2009), supporting the idea that there may be more "global" epigenetic defects associated with ART. Maternal hypomethylation syndrome has been recently recognized as an imprinting syndrome that features hypomethylation at multiple loci (Mackay *et al.*, 2006, Boonen *et al.*, 2008). The study by Rossignol *et al.* (2006) therefore suggests that this hypomethylation syndrome, or related causes, might be associated with assisted reproduction.

Angelman syndrome and ART

Angelman syndrome (AS) is a rare disease that affects approximately 1 in 15 000 newborns, caused by a spectrum of genetic defects, one of which is a defect at the chromosome 15 imprinting center. An increased incidence of AS has been reported following the use of ICSI (Cox *et al.*, 2002; Ørstavik *et al.*, 2003; Ludwig *et al.* 2005; Sutcliffe *et al.*, 2006); it has been suggested that some aspects of the ICSI technique might be responsible for the epigenetic abnormality, for example introduction of the sperm acrosome and its digestive enzymes into the ooplasm, ICSI-induced mechanical stress on the oocyte, or disruption of cellular factors or structures required for correct imprinting of chromosome 15. Paternal RNA-mediated mechanisms must also be considered. Loss of methylation at the *SNRPN* imprinting control region was observed in three cases (normally accounting for less than 5% of all AS cases, occurring in only 1 in 300 000 newborns). Conversely, no *SNRPN* methylation defects were observed in a study of 92 children born after ICSI (Manning *et al.*, 2000).

Retinoblastoma and ART

Retinoblastoma is a childhood tumor of the retina. The *RB1* gene acts as a tumor suppressor, and inactivation of the *RB1* gene by epigenetic mechanisms (such as hypermethylation of the promoter region of the *RB1* gene) can lead to tumor development. Retinoblastoma has also been observed after ART in several different studies, one of which found a significantly increased risk of retinoblastoma in children that were conceived by IVF between 1995 to 2007; however, all of the cases arose between 1995 and 2002 and not thereafter (Marees *et al.*, 2009). Until very recently, the *RB1* gene was not thought to be imprinted, and so the incidence of retinoblastoma after ART may have been overlooked in some of the surveys of ART and imprinting disorders. However, a recent report has demonstrated that the *RB1* gene is preferentially expressed from the maternal allele (Kanber *et al.*, 2009a). It is possible that the ART-associated retinoblastoma cases may be caused by aberrant imprinting of the *RB1* gene, although there is no data available at present.

Russell–Silver syndrome and ART

Russell–Silver syndrome (RSS) features growth retardation and learning disabilities, and has been observed in several cases after ART. A girl conceived via IVF was reported to have hypermethylation at the *PEG1/MEST* DMR, although it is not clear whether this methylation defect is responsible for the syndrome (Kagami *et al.*, 2007). In this case, there was normal methylation at the *H19* DMR, a region that is typically implicated in Russell–Silver syndrome. Partial hypermethylation at the *PEG1/MEST* DMR was present in the father, and the authors suggested that IVF treatment may have exacerbated this pre-existing paternal methylation error. Several other cases of RSS in children conceived by

ART have been reported, but more studies are required to establish whether there is a true association.

ART induced epigenetic mistakes

Culture media

Certain cell culture medium compositions have been shown to affect epigenetic mechanisms and thus the regulation of imprinted genes in mammalian preimplantation embryos. Non-imprinted genes may also be susceptible, indicating that "global" epigenetic changes may be manifested during cell culture. Specific chemicals that are present in culture media may also affect the epigenetic state of the in-vitro embryo; e.g., ammonia may accumulate in culture media as a by-product of embryonic metabolism and from breakdown of the culture medium. Mouse blastocysts cultured in the presence of 300 μM ammonium showed significant upregulation of the *H19* imprinted gene (Lane and Gardner, 2003). Optimizing culture media is therefore critical, in order to minimize the risks of introducing epigenetic disease to the in-vitro cultured cell (see Ménézo *et al*., 2010).

Human embryonic stem cells (hESCs) have been used as a model for understanding epigenetic events in early human development. Like the preimplantation embryo, hESCs are exposed to culture media and micromanipulation procedures that have the potential to alter the epigenetic state of the cell line. Research to date has focused on the epigenetic stability of hESCs, which is an important factor to consider if these cells are to be used to generate differentiated cell types for therapeutic application. Detailed studies of imprinted gene expression in hESC lines have identified variable imprinting between different cell lines. Global DNA methylation analysis in six hESC lines identified numerous unstable loci (Allegrucci *et al*., 2007) with methylation changes occurring during cell culture, after the stem cell lines were derived and during adaptation to a serum-free media.

It is not clear whether the variability between hESCs is due to inter-individual differences in imprinting that were inherited from the founder embryo, or whether these are truly epigenetic disruptions introduced when the stem cells were derived or during subsequent culture. Other mammalian cell types also undergo epigenetic changes when cultured in vitro, suggesting that the in-vitro culture environment might induce cellular stress. For example, in-vitro culture of mouse embryonic fibroblasts downregulates the expression of six imprinted genes via methylation of the respective gene promoters, and the silencing was specific to the imprinted genes (Pantoja *et al*., 2005). It is not known whether this exact event occurs during culture of gametes and embryos, but similar gene expression changes have been described. Culture-induced effects in mouse preimplantation embryos caused an aberrant expression bias towards the maternal allele of the imprinted *Igf2* gene (Ohno *et al*., 2001). There is also evidence for altered *IGF2* expression (Blondin *et al*., 2000) and for reduced methylation at the imprinted *SNRPN* locus (Suzuki *et al*., 2009) in bovine fetuses following in-vitro culture. Immunofluorescence staining for 5-methylcytosine has revealed higher DNA methylation in both mouse and rat embryos developed in vitro compared to in-vivo controls (Zaitseva *et al*., 2007). Animal studies have shown that the addition of serum to media can result in culture-induced effects that persist beyond the preimplantation period, in particular the large offspring syndrome (LOS), an overgrowth syndrome observed in ruminants conceived following IVF. This syndrome is caused by aberrant methylation and upregulation of the *IGF2R* gene after embryo culture in serum-containing media (Young *et al*., 2001). Serum-induced imprinting defects have also been detected after culture of mouse embryos in medium containing fetal calf serum (FCS).

Animal models: imprinting and specific culture media compositions

Mouse embryo culture

Whitten's medium

- Mouse embryos cultured from the two-cell stage to the blastocyst showed aberrant expression of the *H19* gene, with activation of the normally silent paternal allele; the imprint for this gene may be especially labile (Doherty *et al*., 2000).
- Aberrant biallelic expression of five imprinted genes has been observed in the placenta after in-vitro culture (Mann *et al*., 2004). The authors suggested that culture-induced disruption of epigenetic mechanisms may be more severe in the placenta, as this tissue may not be able to restore imprints that are lost during preimplantation development in vitro.

M16 medium/G1/G2

Mouse gametes manipulated and fertilized in M16 medium followed by embryo culture in G1/G2 medium showed downregulation of around two-thirds of placental gene transcripts (Fauque *et al*., 2010b). Imprinted gene expression was also significantly affected. The authors suggested that a memory

of the epigenetic insult that was experienced during embryo culture was maintained, with an impact upon the placenta later in development.

HTF (Quinn's Advantage)

- Mouse embryos showed aberrant *H19* expression when compared to in-vivo derived embryos (Li *et al.*, 2005). Embryonic stem cells derived from these embryos also had defective imprinting of *Igf2/H19 genes* and histone modifications at a regulatory ICR.

Bovine embryo culture

- Culture of bovine embryos in Charles Rosenkrans 1 (CR1aa) upregulated expression of the imprinting regulator *Dnmt3a* methyltransferase.
- Culture in KSOM containing amino acids caused the upregulation of the *Igf2r* gene (Sagirkaya *et al.*, 2006).

ICSI

There is an association between male infertility and epigenetic problems, and the ICSI procedure bypasses natural barriers to fertilization. Injecting epigenetically abnormal sperm could potentially transmit abnormalities to the offspring, and, as described above with respect to the Angelman syndrome, the injection procedure also carries the risk of disturbing epigenetic processes in the oocyte (Manning *et al.*, 2000; Qiao *et al.*, 2009).

Immature gametes

In view of the evidence that gametes progressively acquire epigenetic information, it is possible that immature gametes do not have the full and correct complement of epigenetic information necessary for normal development. Round spermatids (ROS) are transcriptionally active, and introducing their RNAs into the oocyte via ICSI may lead to altered gene expression, either directly or through RNA interference mechanisms (Borghol *et al.*, 2008). Ejaculated spermatozoa, elongated spermatids and round spermatids all have the correct paternal imprint on chromosome 15q11–13, suggesting that this specific paternal imprint is established in immature testicular spermatids. However, other imprinted regions must be assessed before round spermatid injection may be considered safe.

In-vitro maturation (IVM) and in-vitro growth (IVG) of oocytes

Nuclear transfer experiments from staged mouse oocytes indicate that maternal imprints are laid down progressively throughout oocyte growth, and that genes acquire their imprint at different times; these observations have been supported by further molecular studies. This raises the question as to whether in-vitro culture conditions used during IVG and IVM protocols are suitable for the correct establishment of maternal imprints in the oocyte. For example, the use of synthetic serum substitute (SSS) instead of FCS during bovine oocyte maturation led to reduced expression of the DNA methyltransferase *Dnmt3a*, an important regulator of imprinting in the germline and during early development (Sagirkaya *et al.*, 2007).

Clearly the technologies of oocyte in-vitro growth and manipulation must be fully researched with respect to maternal imprint establishment and stability, especially where these methods are applied for human fertility treatment. Some genes may be more susceptible to defects in imprint establishment during in-vitro growth/maturation of human oocytes, and there may be variable stability of imprints once established.

Controlled ovarian hyperstimulation (COH)

Evidence from both animal and human studies suggests that superovulation can cause epigenetic errors in assisted reproduction. Ovarian stimulation may induce oocyte maturation during an inappropriate time frame, or in a cellular and developmental context that is incompatible with achieving complete epigenetic programming of the oocyte, thus pushing lower quality oocytes to maturity. As indicated earlier, maternal imprints are established at different times during the oocyte growth phase, and there is further evidence that imprinted gene methylation in oocytes is size-dependent. Superovulation could override this stepwise, progressive growth-dependent process and instead recruit young follicles that have not correctly established their imprinting during maturation.

Infertility and epigenetics

Infertility in certain cases is due to defects in gametogenesis; since gametogenesis requires coordination of numerous cellular and molecular events, including epigenetic programming, it follows that that some cases of infertility may be associated with epigenetic defects. Imprinting defects in infertility may be a result of defects in gene expression, or due to mutation in the DNA methylation machinery of the gametes. Global DNA methylation analysis techniques have indicated that poor quality human sperm may have defective

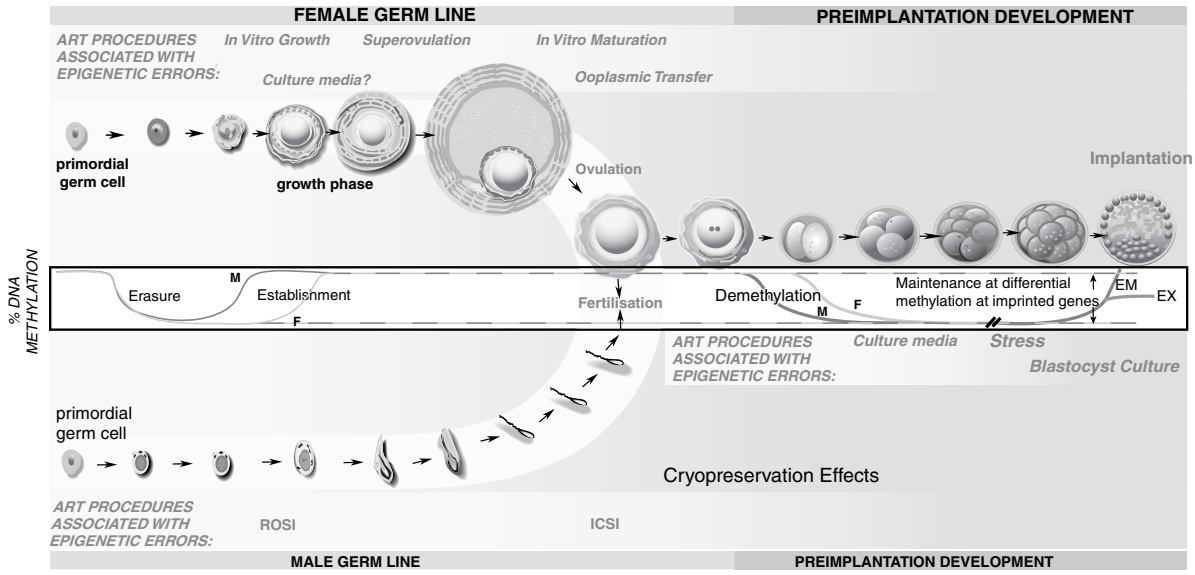

Figure 15.4 Major DNA methylation reprogramming events during mammalian gametogenesis and preimplantation development shown together with a summary of the assisted reproductive technology (ART) procedures that are associated with epigenetic errors. The methylation reprogramming panel (center panel, adapted from Reik *et al.*, 2001) indicates the level of methylation in male (M) and female (F) gametes (left side), and also in the paternally inherited (M) and the maternally inherited genomes (F) after fertilization, during preimplantation development (right side). The timing and nature of these reprogramming events varies between species. The dashed lines indicate the maintenance of differential methylation at imprinted genes during preimplantation development. The genome is remethylated differentially within the blastocyst in the embryonic (EM) and extraembryonic lineages (EX). Figure reprinted with permission from Huntriss and Piction, 2008.

erasure of methylation (Houshdaran *et al.*, 2007). The epigenetic status of the sperm may affect the outcome of results after assisted reproduction, as high "global" sperm DNA methylation has been correlated with improved pregnancy rates (Benchaib *et al.*, 2005). Methylation defects at several imprinted genes have been reported both in oligozoospermic patients and in patients with abnormal protamines (Hammoud *et al.*, 2009). Whether epigenetic disorders are actually inherited from infertile patients who harbor epimutations is not clear. However, it has been reported that DNA methylation defects observed at imprinted loci in ART concepti are associated with mutations in the gene encoding *DNMT3L*, a gene that is important in imprint establishment, and these were also present in the parental sperm (Kobayashi *et al.*, 2009).

Summary

An association between ART and an increased risk of at least some epigenetic disorders must clearly be considered (Figure 15.4), and sophisticated molecular studies that compare the "global" epigenetic status of children born in vivo and those born in vitro are now emerging (Katari *et al.*, 2009; Palermo *et al.*, 2008; Tierling *et al.*, 2009). Studies such as these will help us to understand the molecular processes that are affected by ART, and the potential risks associated with each technique. Research techniques must be developed that allow technologies to be rigorously tested, to precisely gauge their effect upon the epigenetic development of human gametes and preimplantation embryos, so that ART protocols can be adapted in order to avoid potential problems. Animal studies are useful in highlighting problems associated with particular ART methods, but there are significant differences between humans and other mammals with respect to epigenetic regulation, and in the regulation of imprinting during gametogenesis and early development. Further research on the epigenetic regulation of human gametes and preimplantation embryos is urgently needed, as well as further research on the safety aspects of existing and emerging ART treatments.

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