Oocyte polarity and cell determination in early mammalian embryos

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Knowledge on determination and differentiation in the mammalian embryo has not kept pace with discoveries in other phyla. Current concepts overlook well-established pathways leading to polarity in oocytes and embryos of other phyla, modern principles of totipotency in plants and animals, and axis formation in lower vertebrates. Various models derived from invertebrates and frogs could be relevant to the situation in eutherian mammals, and we explore the nature of strict genetic controls in these species and its implications for early mammalian differentiation. Concepts on totipotency and related phenomena in animal and human embryos are examined and the possibility raised that two cell lines are formed in early human embryos from the 2–4 cell stage. Clinical consequences are assessed, including causes of the high incidence of chromosomal mosaicism in human embryos. Our interpretations are obviously speculative, and must be clarified by experimentation.

Key words: axes/determination/early differentiation/mammalian embryos/polarity

Introduction

The regulation of primary embryonic differentiation and the establishment of embryonic axes are fundamental to life. In many species, an initial polarity of the oocyte and early embryo dictates the establishment of antero–posterior (A/P), dorso–ventral (D/V) and left/right (L/R) axes. The three germ layers differentiate, soma and germline form, and inductive systems regulate embryonic development. Comprehensive discussions of early differentiation in the animal kingdom have been presented by Davidson (1986, 1989), Gurdon (1992) and Wilkins (1993).

This review focuses on early development in laboratory mammals and humans, members of Phylum Chordata, subclass Eutheria, as distinct from the egg-laying mammals (Protheria) and marsupials (Metatheria), and attempts to place these analyses in an evolutionary context. Members of ancient and modern phyla share reproductive features (Figure 1), and striking examples of long-lasting homologies include conserved pathways such as the homeobox genes in Drosophila, amphibia and eutherians, and oocyte polarity in all animal species from Caenorhabditis elegans to marsupials (Ohno, 1976; Selwood, 1994).

Oocyte polarity is plainly visible by pigment distribution in Xenopus laevis, and by shape in the fruit fly Drosophila and the nematode C. elegans. It is not obvious in sea urchins and ascidians. Experiments dating from the beginning of this century showed how polarized ooplasmic determinants regulate embryonic differentiation by imposing an ‘animal/vegetal axis’ in developing embryos (Wilson, 1928; Davenport, 1979; Gerhart, 1980; Davidson, 1986; Keller, 1986; Schroeder, 1986; Wilt, 1987; Gardner, 1996a). Conventionally, the first polar body marks the animal pole, and the vegetal pole lies diametrically opposite in the oocyte. This polarity...
establishes the first embryonic axis, A/P in most animals in their oocytes. The D/V axis emerges in amphibian oocytes after fertilization when the gray crescent marks the transition from a radial to a D/V symmetry (Waddington, 1956; Gerhart, 1980). By the 4-cell stage or soon afterwards, organelles and cortical actin distribute asymmetrically in eggs of many species as the D/V axis forms (Davidson, 1986). Formation of the L/R axis in many species depends on the integrity of the A/P axis (Gurdon, 1992; Goldstein et al., 1993; McCain and McClay, 1994; Goldstein, 1995). In *Xenopus*, animal and vegetal poles specify ectoderm and endoderm respectively and mesoderm arises consequentially on interactions between them. Dorsal mesoderm formation is signalled by the Nieuwkoop centre via the Spemann organizer of responding mesoderm (Boterenbrood and Nieuwkoop, 1973).

Current ideas on differentiation in eutherian mammals largely overlook polarity and axis formation, and focus instead on the role of cell position in later cleavage divisions and in blastocysts, an interpretation of the ‘inside/outside’ concept of embryonic differentiation in mice (Tarkowski and Wroblewska, 1967). Oocytes of eutherian mammals seemingly differ from those of virtually all other animals in having no obvious functional polarity (Davidson, 1986; Gurdon, 1992; Wilkins, 1993), and D/V symmetry evidently arises first in blastocysts at an angle to the eccentrically-placed inner cell mass (ICM) where the true embryonic axes develop. We believe that a re-evaluation of mammalian differentiation is needed, and begin with a comparative analysis of early differentiation in *C. elegans*, *Xenopus laevis* and *Paracentrosus lividus*.
Table I. Localization of maternal mRNA in Caenorhabditis elegans embryos

<table>
<thead>
<tr>
<th>Gene</th>
<th>Product/function</th>
<th>Expression</th>
<th>Stage</th>
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<tbody>
<tr>
<td><strong>Oocyte polarity</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>par-1</td>
<td>serine/threonine kinase</td>
<td>posterior cortex</td>
<td>oocyte</td>
</tr>
<tr>
<td>par-2</td>
<td>ATP binding protein</td>
<td>posterior cortex</td>
<td>oocyte</td>
</tr>
<tr>
<td>par-3</td>
<td>novel protein</td>
<td>anterior cortex</td>
<td>oocyte</td>
</tr>
<tr>
<td><strong>Germline differentiation (Type II genes)</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>skn-1</td>
<td>transcription factor</td>
<td>nuclear protein</td>
<td>2-cell embryo</td>
</tr>
<tr>
<td>pie-1</td>
<td>novel protein</td>
<td>P1 blastomere</td>
<td>2-cell embryo</td>
</tr>
<tr>
<td>ced-1F</td>
<td>mRNA translation</td>
<td>germline</td>
<td>1–28 cell embryo</td>
</tr>
<tr>
<td>ced-2</td>
<td>enhancer protein</td>
<td>P1 cell</td>
<td>2-cell embryo</td>
</tr>
<tr>
<td>HSP70A</td>
<td>Heat shock (chaperone) protein</td>
<td>germline</td>
<td>1–30 cell embryo</td>
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<td></td>
<td></td>
<td></td>
<td>1–15 cell embryo</td>
</tr>
<tr>
<td><strong>Multiple functions</strong></td>
<td></td>
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<tr>
<td>Glp-1</td>
<td>Notch-like receptor</td>
<td>AB inducer</td>
<td>4-cell embryo</td>
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<td></td>
<td></td>
<td>MS inducer</td>
<td>8-cell embryo</td>
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<tr>
<td>mex-3</td>
<td>RNA binding protein</td>
<td>AB cell</td>
<td>1–4 cell embryo</td>
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<td></td>
<td></td>
<td>P1–P4 cells</td>
<td>1–4 cell embryo</td>
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<td></td>
<td></td>
<td>P granules</td>
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**Polarity and axis formation in various animal phyla**

**Differentiation in C. elegans**

This nematode has a mosaic (determinate or autonomous) form of primary differentiation. Special features to note in this well-studied species include the nature of polarity in oocytes and temporal and spatial control of gene expression in early development. Rigid controls on cell lineages are imposed by several known genes and orchestrated by ooplasmic determinants and cell-to-cell interactions. The cell lineage map for AB (somatic) and P (germline) blastomeres is shown in Figure 2A.

**Polarity and cleavage planes in oocytes, fertilized eggs and embryos**

Three or more par (partition defective) genes regulate the initial polarity in oocytes, as expressed in the cortical localization of mRNAs and proteins. par-1 encodes a serine/threonine kinase and par-2 encodes a novel protein with an ATP binding site, and their transcripts and proteins localize in the posterior cortex. par-3 transcripts and protein, and those of recently-identified par-6, locate in the anterior cortex (Kemphues, 1989; Kirby et al., 1990; Watts et al., 1996). The mosaic patterning of determinants in oocytes was believed to strictly govern blastomere function and cell lineages, but this is now questioned since inductive pathways have been found in C. elegans embryos.

Sperm entry reorganizes ooplasm, as a new A/P asymmetry arises after membrane ruffling, polarizing flows of cortical and ooplasmic material. The sperm centrosome probably directs anterior flows of cortical material which forms a contractile network. RNA-rich granules (P granules) localize in specific sites where germline will differentiate (Goldstein et al., 1993). P granules and polarity determinants flow in ooplasm to a cortical site, and are briefly anchored there by microfilaments late in the first cell cycle.

Embryonic axes are established by strictly consistent cleavage planes. Large anterior (AB) and small posterior (P1) daughters form after the first division. AB produces soma. It divides parallel to the A/P axis, and the axis of its skewed spindle places its first daughter, ABa, anterior to ABp. AB descendants establish the L–R axis as inductive relationships begin. P1 produces germline and muscle precursor cells during successive cleavages (Figure 2A). It displays a different form of cleavage. One centrosome pivots microtubules, and enables the other to rotate through 90° to a ‘capture’ position near the anterior cortex. P1 daughters are thus polarized at right angles to AB cells (Goldstein et al., 1993). Similar centosome rotations occur in later P cell descendants (see Figure 2B) (Goldstein, 1995).

Cleavage planes are regulated autonomously or by specific cell contacts, e.g. between EMS and P2, or E and P3 (Goldstein, 1995). The initiating blastomere forms attachment sites to recipient blastomeres during contact-dependent mitotic spindle orientation. It attracts one centrosome and reorganizes the recipient’s microtubules to modify its cleavage plane. P2 provides an example, reorganizing EMS in 5–6 min of its cell cycle to polarize and re-orient it via microtubule-organizing centres and so retain germ plasm for its own descendants (Goldstein, 1995). These events seemingly specify fate in successor cells (Figure 2A,B,C).

**Temporal and spatial gene expression during axis formation in embryos**

Maternal mRNA is essential in early differentiation. It persists until gastrulation, during specific patterns of gene expression in AB and P cell lines. Products of type I genes, e.g. tubulin \(\alpha\) and \(\beta\), and RNA polymerase II, are distributed universally throughout development (Seydoux and Fire, 1994). Type II genes specify regulatory proteins (Table I), and their maternal mRNAs persist in germline, degrade rapidly in somatic cells, and regulate transcription (e.g. skn-1, cey1, cey2) or translation.
Figure 2. Cell lineages, cleavage planes and early differentiation in *Caenorhabditis elegans*. (A) Cell lineages of the individual blastomeres. The fertilized egg is called P₀ and divides at first cleavage to form AB and P₁ blastomeres which are the precursors of somatic and germline lineages. The fate of individual cell lines is indicated, and the number of muscle cells in the body wall developing from particular blastomeres is shown in brackets. Data from Goldstein et al. (1993), Schnabel (1994) and Seydoux and Fire (1994). (B) Centrosome movements and cleavage planes at the second cleavage. Centrosomes divide and migrate to opposite sides of the nucleus in AB and P₁. Then nucleus–centrosome complexes rotate clockwise in P₁ to establish a cleavage plane at right angles to that in AB. Similar nuclear rotations occur in P₂, P₃, EMS and E so that while the AB line divides orthogonally, the P lineage divides successively in the same axis (Goldstein, 1995). (C) Distribution of *cey-2* mRNA in somatic (AB) and germline (P) cell lines respectively (Seydoux and Fire, 1994). Maternally transcribed *cey-2* is specifically down-regulated in somatic cells but remains active in the germline in 8–24 cell embryos. Embryos were hybridized with *cey-2* digoxigenin-labelled antisense probes and visualized using anti-digoxigenin antibody coupled to alkaline phosphatase. Courtesy of G.Seydoux and A.Fire (1994) and *Development* and Company of Biologists Ltd.

They stabilize proteins, including the heat shock protein HSP 70A. Maternal *cey-2* mRNA illustrates a localized type II expression as it codes for a germline enhancer protein (Figure 2C), and segregates to each 2-cell blastomere at the first division. Its transcripts persist in P₁, but decline rapidly in AB by the late 2-cell stage and even more after the next division. Type III mRNAs consist of a cluster of poly(A)⁺ mRNA species co-localizing with P granules in early germline, transcribed by unknown genes (Seydoux and Fire, 1994).

Shape and timing are determined genetically. Temporal controls are imposed on genes controlling the asymmetric distribution of P granules. Maternal *glp-1* expression is repressed temporally in oocytes and 1-cell embryos, perhaps to limit its expression in later blastomeres (Crittenden et al., 1997). *glp-1* thereby becomes active in P₁ descendants in 2-cell and older embryos, where it may regulate asymmetry. Other genes control its spatial but not temporal expression, e.g. *par-1, par-6, emb-8,* and *Par(q537)* and so partake in the...
Oocyte polarity and cell determination in early mammalian embryos

<table>
<thead>
<tr>
<th>Table II. Gene functions for mRNA localizing to animal and vegetable poles in Xenopus</th>
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<tbody>
<tr>
<td><strong>Genes</strong></td>
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<tr>
<td><strong>Animal pole</strong></td>
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<tr>
<td>An1</td>
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<tr>
<td>An2</td>
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<tr>
<td>An3</td>
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<tr>
<td>Xlan4</td>
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<tr>
<td><strong>Vegetable pole</strong></td>
</tr>
<tr>
<td>Xcat-2</td>
</tr>
<tr>
<td>Xcat-3</td>
</tr>
<tr>
<td>Xlsirts</td>
</tr>
<tr>
<td>Xlwnt-11</td>
</tr>
<tr>
<td>Vg1</td>
</tr>
<tr>
<td>VegT</td>
</tr>
</tbody>
</table>

CNS = central nervous system; TGF = transforming growth factor.

**Figure 3.** Migratory pathways of various mRNAs through ooplasm. Model for two different pathways localizing maternal RNAs at the vegetal pole in Xenopus. The message transport organizer METRO pathway localizes Xlsirts and Xcat-2 mRNAs. METRO is a region of the mitochondrial cloud where these mRNAs accumulate after leaving the germinal vesicle. They translocate to the apex of the vegetal pole during stages 1 and 2. mRNAs such as Vg1 are localized using the lower Vg1 pathway (modified from Kloc and Etkin, 1995).

**Figure 4.** Cytoplasmic organization in Xenopus. In-situ hybridization using digoxigenin-labelled antisense RNA probe for (A) mitochondrial large ribosomal RNA, and (B) Xcat-2 shows localization of each to the mitochondrial cloud in stage 1 in Xenopus oocytes. Bar = 80 µm. Courtesy of Y. Zhou and M.L. King (1996) and Development and Company of Biologists Ltd.

regulation of anterior-posterior symmetry. Regulatory genes such as skn-1, pie-1, mex-1 and apx-1 do not affect GLP-1 patterns, and instead control blastomere differentiation to specific pathways (Crittenden et al., 1997). The multifunctional mex-1 and mex-3 are active in AB and P cell lines. mex-3 displays complex maternal and transient expression, influences mRNA processing, and is probably an early regulator of AB and P1. Its maternal transcripts are expressed until the 4-cell stage, and may establish A/P asymmetry independently of skn-1 (Draper et al., 1996). Its interactions with and processing of RNA are signified in the repeated RNA-binding sequences in its KH protein domain. Mutations in mex-1 induce inappropriate anterior expression of skn-1 in AB, which then produces muscle.

It now seems that this strict mosaic (determinate) embryonic differentiation in C.elegans, and its strict lineages governing...
cell fate, may not differ entirely from the inductive systems of *Xenopus* and *Drosophila*. Ten or more inductive pathways identified in *C. elegans* embryos now blur this distinction. *C. elegans* may differ only in its number of cells involved in early differentiation (Schnabel, 1994, 1995, 1996). Inductive systems regulating muscle differentiation in body wall provide an example. Founder cells MS, C and D can produce muscle autonomously when isolated *in vitro* yet they enter interactive systems within the embryo. Altogether, 81 body wall muscle cells are produced, 1 from AB, 28 from MS, 32 from C and 20 from D (Figure 2A). Muscle specification in MS is inhibited by D, and in D by C. Inhibitory systems are overridden by these induction systems, e.g. in MS by ABa, and in D by MS descendants (Schnabel, 1994, 1995). Despite their strict lineages, embryonic cells of *C. elegans* can obviously influence specification in neighbouring cells, and acquire transient states independent of their developmental fate.

**Differentiation in *Xenopus laevis***

Primary differentiation in this species evidently differs sharply from that of *C. elegans*, even though an existing polarity in oocytes is modified at sperm entry in both species, and cell fate is specified by cell–cell interactions and inductive pathways (Gurdon, 1992; Schnabel, 1996).

**Polarity in oocytes and fertilized eggs**

Oocyte polarity established during oogenesis is visible in pigment distribution. Polarity is established as maternal mRNAs migrate along specific routes from germinal vesicle to cortical sites. mRNAs of *An1, An2, An3, Xlan4* migrate to the animal pole, and those of *Xcat-2, Xcat-3, Xlwnt-11, Vg1, VegT* and *Xlsirts* to the vegetal pole (Elinson *et al.*, 1993; Forristall *et al.*, 1995; Zhang and King, 1996). Transcripts anchoring at the vegetal cortex specify mesoderm, the dorso–ventral axis and primordial germ cells (see Table II). *Xcat-2* encodes an RNA-binding protein and is homologous with *nanos in Drosophila* (Forristal *et al.*, 1995). *Xlwnt-11* specifies the dorsal/ventral axis. *Vg1* is a primary mesoderm inducer and a member of the transforming growth factor (TGFβ) family.

Vegetal mRNAs follow different routes to cortex. *Xlsirts, Xcat-2 and Xlwnt-11* move from germinal vesicle to mitochondrial cloud in oogenesis stages 1 and 2, transported by messengers transport organizer (METRO) to vegetal cortex within the cloud and anchored there by microfilaments and cytoskeleton (Figures 3, 4A and B) (Kloc and Etkin, 1995). *Vg1* mRNA leaves the germinal vesicle in stage 1 and 2 and translocates to vegetal cortex in stages 2 and 3, to be anchored by microfilaments with other mRNAs until released during maturation (Weeks and Melton, 1987; Kloc and Etkin, 1995). *Xcat-2* RNA may follow the *Vg1* pathway in later migratory stages before homing to mitochondrial cloud at stages 1 and 2 (Figure 5) (Zhou and King, 1996).

The micrometre-thick cortex contains microfilaments, intermediate filaments, cortical granules, pigment granules and mitochondrial clumps. Cytokeratin forms at both poles, and germ plasm may associate with mitochondria and intermediate filaments at the vegetal pole (Elinson *et al.*, 1993). Ooplasmic streaming carries migratory mRNAs to the animal pole, a movement possibly regulated by microfilaments. Movements to the vegetal pole may be controlled by microfilaments. The early pathway via mitochondrial cloud could carry germ lineages vegetally, while the later pathway may localize RNAs regulating somatic cell differentiation (Zhou and King, 1996). The *Xenopus* mitochondrial cloud, with its mitochondria, electron-dense fibrillar material and RNA, and vegetal location, resembles Balbiani’s body in oocytes of other species including mammals (Figure 6) (Balbiani, 1864; Guraya, 1979).

**Establishment of embryonic axes**

At sperm entry, the sperm aster orients the A/P axis as a final embryonic axis forms from a dorsalization centre directly opposite. The oocyte axis rotates through 30°, assisted by the release of cortical granule fluid and strong cortical contractions (Davidson, 1986 p.508). Cortical rotation activates proteins such as vegetally-localized β-catenin and the mesoderm inducer Vg-1. Dorsalization involves interactive pathways between membrane ligand receptors, cell signalling proteins and transcription factors. Regulatory genes include some homologous...
with wingless genes in Drosophila. wnt family members, e.g. wnt11 and Xwnt8, are dorsal inducers acting through wnt membrane receptors. Other ligand/receptors in this pathway include epidermal growth factor and a Notch-like receptor analogous to that in C. elegans (Vize and Thomsen, 1994; Miller and Moon, 1996).

Maternal transcripts encode developmental information. TGF-β has a wide distribution and action in embryos, inhibiting cell proliferation, modulating effects of growth factors, participating in extracellular matrix formation and inducing cartilage (Weeks and Melton, 1987). It may induce mesoderm, interacting with Vg-1. Mesoderm-inducing genes include Steel (also known as stem cell factor and involved in mammalian haematopoiesis), and those homologous to c-kit in mammals (Davidson, 1986; Gurdon, 1992). GATA-2 is maintained as a nuclear complex and differs from GATA-1 with its role in haemopoiesis (Partington et al., 1997). Maternally-encoded Fgf induces homeobox genes to control patterning (Ruiz i Altubo and Melton, 1989), and may impose competence to respond to activin, itself probably activated by Vg1 (Vize and Thomsen, 1994). Maternally coded Vg-1 protein, a weak mesoderm inducer, is activated by post-fertilization cortical movements, binds to a BMP2 family member of regulatory proteases and forms B-Vg1, a strong mesoderm inducer capable of rescue in UV-irradiated embryos (Vize and Thomsen, 1994). Vg1 is homologous to decapentaplegic (dpp) protein, a mesoderm inducer in Drosophila.

Maternal transcripts control body axes and differentiation of germ layers until embryonic transcription begins in mid-blastulae. These transcripts are temporally and spatially regulated. Endoderm differentiates from vegetal transcripts, ectoderm from mRNA localized to the animal pole, and mesoderm is induced from the midregion Spemann organizer by molecules released from the Nieuwkoop centre in dorsal endoderm. Recent work suggests that induction via the Spemann organizer involves BMP4, another homologue of the dpp protein of Drosophila (Ferguson, 1996). Germ plasm subsequently migrates to endoderm (Davidson, 1986; Forristall et al., 1995); its early segregation appears to be less rigid than in C. elegans. Xcat-2 mRNA co-localizes with germinal granules, where its distribution and degree of expression vary in each blastomere. It can locate entirely in a single blastomere, or in all four ‘totipotent’ blastomeres in 4-cell embryos to varying degrees and in various combinations (see Figure 5).

Maternally-encoded β-catenin typifies a gene with a central cell signalling role. It is a member of the cadherin superfamily, which includes cadherin, a cell-surface adhesion molecule, and α-, β- and γ-catenins (Wylie et al., 1996; Miller and Moon, 1996). It interacts with many genes (Figure 7), and cytoplasmic domains of cadherin complex with β- and γ-catenin. β-catenin locates in nuclei, cytoplasm and plasma membranes. Its cytoplasmic levels are regulated by wnt and other factors controlling ligand-receptor binding, to activate a Xdsh product which represses glycogen synthase kinase (GSK) and stabilizes β-catenin. Stable β-catenin may migrate to the nucleus, interact with the homeobox transcriptional factor Xtef-3 (homologous to mammalian LEF-1), and activate this homeobox regulator (Huber et al., 1996). Its targetted depletion, or disruption of the post-fertilization cortical rotation, inhibits the dorsal distribution of β-catenin, formation of dorsal mesoderm and inhibits the D/V axis. β-catenin may thus interact with many animal and vegetal components over the entire dorsal axis (Miller and Moon, 1996; Jessell and Melton, 1992; Wylie et al., 1996). Maternal β-catenin and later-expressed γ-catenin may also participate in regulating primary differentiation in mouse embryos (Ohsugi et al., 1996).

Zygotic gene transcription begins in blastulae after 12 cleavages, as maternally-inherited repressive H1 histones are
substituted by newly-coded histones. The transcription of some promoters may be delayed by the lack of a functional TBP (TATA box binding protein) until the mid-blastula transition (Nothias et al., 1995). Complex interactive pathways involving inducers, growth factors and signalling systems control animal and vegetal halves. These are identified by isolating animal and vegetal halves or by induction assays on ectodermal caps in studies on axis formation and germ layer differentiation.

**Differentiation in P. lividus**

This sea urchin is characterized by a strict oocyte polarity and invariant cell lineages, as in *C. elegans*. Polarity of maternal mRNAs in oocytes and early embryos, combined with meridional or equatorial cleavages, regulate early growth, gene expression and totipotency in blastomeres. Animal and vegetal segments establish the A/V axis before fertilization, and impose an invariant fate on individual embryonic cells and tissues after fertilization (Horstadius, 1937; Wilt, 1987; Davidson, 1989). Maternal mRNAs localized on the A/V axis transiently activate two novel genes, *HE* and *BP10*, from fertilization until the blastula outside the influence of the inductive cascade. These genes produce the earliest-activated zygotic transcripts, are the earliest to be spatially restricted, and are strongly expressed in 16–32-cell embryos. Both are animal-derived, activated autonomously and spatially restricted to presumptive ectoderm in the animal two-thirds of blastulae (Ghiglione et al., 1996).

BP10 protein domains include a signal peptide, a propeptide, epidermal growth factor (EGF)-like sequences, and a catalytic domain found in metalloproteases (Lepage et al., 1992). Products of *BP10* are homologous with those of the *Drosophila* dorso-ventral patterning gene *tolloid* and the human protein BMP-1 (Lepage et al., 1992). It may function as a surface regulatory protease before germ layer formation, to regulate spatial information and cytodifferentiation at the animal pole. Primary patterning is not limited to a small centre, such as the vegetal Nieuwkoop centre in *Xenopus*, but extends along the whole A/V axis. Analogies with other species include the post-translational activation of Vg1 by BMP protein in *Xenopus* (Vize and Thomsen, 1994).

During cleavage, two meridional planes form parallel to the A/V axis. These divisions result in four equivalent totipotent 4-cell blastomeres. Meridionally-sectioned 2- or 4-cell embryos produce near-normal embryos of reduced size. The third cleavage plane is equatorial through all four blastomeres, i.e. horizontal to the first two meridional cleavages. It results in four totipotent blastomeres at the vegetal pole, and four with limited multipotency at the animal pole (Ghiglione et al., 1996). Vegetal halves develop into normal blastulae, whereas animal halves produce larvae with ectodermal defects (Figure 8). Even though oocyte polarity and specific cleavage planes are rigid, and blastomeres have predetermined fates within the 8-cell embryo, they can respond when isolated in *vitro* to animalizing and vegetalizing agents. Their potency is obviously far greater than their usual fate in the embryo, just as in *C. elegans* (Ghiglione et al., 1996).

This interpretation of primary differentiation in *P. lividus* as being strictly regulated by localized determinants has been questioned. Five primary territories could be organized by cytoplasmic determinants: oral and aboral ectoderm, gametogenic mesenchyme, skeletogenic plate and micromeres. Development within each territory could be regulated by induction and cell–cell interactions (Davidson, 1989).

**Conclusions from studies on lower animals**

Our brief reviews on *C. elegans*, *Xenopus* and *P. lividus* draw attention to some salient points about their forms of differentiation. Similar conclusions could have been drawn from other well-studied species. For example, polarity is reorganized in some ascidians following fertilization. Although a pre-fertilization animal–vegetal axis is established before fertilization in the ascidian *Phallusia mammillata*, future bilateral asymmetry coincides with movements of sperm nucleus and a mitochondria-rich domain to the vegetal hemisphere, and with the plane of the first division (Roegiers et al., 1995). These domains in the lower vegetal hemisphere establish the sites of future gastrulation (Figure 9). Oocyte determinants localize to the animal/vegetal axis after fertilization in another ascidian, *Halocynthia roretzi*, where animal ooplasm dominates epidermis differentiation and vegetal ooplasm encodes endoderm and muscle (Yamada and Nishida, 1996). A/P differentiation becomes anomalous when fragments of ooplasm are selectively excised (Nishida, 1994).

_Drosophila melanogaster_ offers a contrast, since the D/V axis is established prior to fertilization as maternal transcripts such as *swallow* and *exuperantia* in the anterior cortex of the oocyte establish primary axes in combination with nurse cells (Melton, 1991; Schleif, 1993; Deng et al., 1997). Oocytes produce the primary regulator, signalling to nurse cells via *gurken* (*grk*) which resembles TGFα (Figure 10). Nurse cells signal back, transmitting *bicoid* mRNA to bind at the anterior pole previously determined by *swallow* and *exuperantia*. *nanos* mRNA is transmitted to binding sites at the posterior pole controlled by *BicD*. Cytoskeletal integrity is important for oocyte polarity. *BicD* protein complexes with Eg1 protein (encoded by *Egalitarian*), evidently by polarizing the microtubule complex (Mach and Lehmann, 1997). The role of microtubules in midoogenesis is also revealed by disrupting them, which prevents localization to the posterior pole (Manseau et al., 1996). Genes identified with...
Oocyte polarity and cell determination in early mammalian embryos

Figure 9. Time lapse images of post-fertilization contractions in the ascidian *Phallusia mammillata*. Mitochondria-rich myoplasm was labelled with the molecular probe [DiOC₅(3)]. After fertilization, the egg starts to contract on its left (white arrow at 15 s), a constriction appears in vegetal pole (black arrows at 60 s), and a bulge appearing at the animal pole (white arrow, 90 and 240 s) indicates the site of first polar body emission and successive periodic contractions which characterize the meiotic period. Bar = 20 µm. Courtesy of F. Roegiers *et al.* (1995) and Development and Company of Biologists Ltd.

Primary roles in establishing the original axis in oocytes could include *gurken* and *torpedo*, *swallow* and *exuperantia* in *Drosophila*, and *par* and *mex3* in *C. elegans*.

Body pattern in *Drosophila* is established as zygotically-regulated genes gradually replace maternal transcripts. This was also evident in the three species described above. Dorsal–ventral patterning is conserved in arthropods and chordates (Ferguson, 1996) even though their first axes are different. Segmentation is also highly conserved (Favier and Dolle, 1997). For example, homeobox genes such as *fushi tarazu* regulate segments, *Antennapedia* specifies part of the thorax, and *bithorax* determines the posterior portion of thorax and abdomen in *Drosophila* (Schleif, 1993), and these elements can be discerned in mammalian differentiation.

Overall, studies on these species help to clarify major embryological principles relevant to eutherian mammals. The role of maternal transcripts is a constantly recurring theme. They persist for distinct periods in most of the species examined above. Localized short- and long-lived maternal mRNAs thus regulate these initial stages of differentiation, as they encode for embryonic and germ cell determinants.

Axis formation, associated with tightly-controlled cleavage planes is one means of determining the formation of cell lines in the embryo. Blastomere fate, and the retention of germ plasm in germ-line precursor cells, can be determined by spindle rotation, which can sometimes be invoked by contact with a neighbour blastomere as in *C. elegans*. Blastomeres are strictly committed to their fate in some species; in others, they respond to inductive agents in later cleavage. It seems that individual blastomeres are capable of a wider potential than that expressed within the embryo. This dual regulation in different environments could be of fundamental importance in many mammalian studies, such as totipotency, inside/outside differentiation and the ability of cleaving embryos to redress the loss of one or more blastomeres.
Early determination and differentiation in mammals

Polarity and axis formation

Extrapolations between lower animals and eutherian mammals must be made cautiously. Apart from wide evolutionary differences, pregnancy and viviparity complicate comparisons with non-mammals. Mammalian embryos develop in a highly sheltered environment, and knowledge on viviparous development in other animals such as pouched or gastric brooding frogs (Del Pino, 1989) or marsupials (Selwood, 1994) is fragmentary. Polarity is first imposed in blastocysts, much later than in non-mammals. Nevertheless, oocyte polarity characterizes the eggs of many species closely related to eutherians, including reptiles, birds, monotremes and marsupials, and especially those with telolecithal eggs (Selwood, 1994).

This discussion opens with evidence of polarity in oocytes and embryos of eutherian mammals, concentrating mostly on mouse and human studies. Succeeding sections cover the distribution and expression of maternal mRNA, zygotic transcription and spatial and cytogenetic aspects of primary differentiation including totipotency. Early cell lineage models and new areas for investigation are proposed.

Cytological evidence

Polarity and spatial patterning have received scant attention in comparison with the time devoted to inside/outside development and cellular totipotency (Tarkowski and Wroblewska, 1967; Gardner, 1996a). Nevertheless, Jones-Seaton (1950) identified a bilateral symmetry in unfertilized rat eggs and embryos, with a basophilic RNA cortical zone dorsally and a vacuolated region ventrally. This situation resembles cytoplasmic polarity in the sea urchin *Plidius*, and the frog *X.laevis*, but it has not been confirmed in mammals. Dalcq (1955, 1957) and Denker (1983) inferred the existence of a dorso–ventral axis from a bilateral symmetry in RNA distribution and an RNA-free vacuolated area to one side of the polar axis. Dalcq (1957) also indicated the existence of a boundary between dorsal and ventral cytoplasm oblique to the A/V axis. Gradients were not found in RNA and proteins in unfertilized and fertilized mouse eggs, cleaving embryos and blastocysts using [14C]-radiolabelled RNA and protein precursors (Edwards and Sirlin, 1956). In pronucleate and cleaving eggs, ~25% of ooplasm, in an area adjacent to the second meiotic spindle in an axis passing through the second polar body may have been less radioactive than in the remaining ooplasm (Edwards and Sirlin, 1956). This ooplasm could have corresponded to the vacuolated area described by Dalcq et al. (1955), but [14C] is an unsuitable isotope for studying fine detail in such small eggs.

**Figure 10.** Polarized expression patterns in *Drosophila* follicle cells. The ovaries of *Drosophila* contain strings of eggs of increasing developmental age. Each oocyte arises from one of a cluster of 16 germ cells, the remaining 15 are nurse cells, closely associated with the oocyte and providing it with material for oogenesis. Each cluster of germ cells becomes surrounded by somatic follicle cells. The oocyte and follicle cells are in frequent communication and this is crucial for the development of the axes of the egg and embryo. ‘Enhancer’ trapping can be used to visualize the kinds of gene expression patterns occurring in oogenesis. A bacterial gene encoding β-galactosidase is inserted randomly into the *Drosophila* genome and its expression visualized with stain. Its expression is governed by nearby enhancers and therefore mimics the expression of the gene located close to it. (A) This figure shows expression of a *torso*-like gene, initially expressed in all follicle cells surrounding the oocyte and nurse cells from early in oogenesis. (B) It then becomes confined to anterior and posterior cells. (C) As the oocyte enlarges, expression is confined to the follicle cells over the oocytes and it is expressed more strongly at the anterior and posterior than in the middle. (D) Later still expression is confined to caps of degenerating follicle cells at the anterior and posterior of the maturing oocyte. We thank M.Bownes for this illustration.
Oocyte polarity and cell determination in early mammalian embryos

Other analyses on polarity were contradictory. Its existence in mouse oocytes was inferred from an absence of oolemmal microvilli adjacent to the first polar body, but microvilli were distributed evenly in human oocytes (Pickering et al., 1988). Santello et al. (1992) then discovered a microvillous-free region in human oocytes, possibly as a consequence of ageing in culture. Another lead came from the movement of the meiotic spindle from medulla to cortex during oocyte maturation, apparently following a pathway imposing a polarized siting of the metaphase (M) II spindle and microvilli at the cortex (Longo and Chen, 1985).

Evidence of polarity in mammals has also been obtained by analysing movements of organelles in ooplasm. Balbiani’s body and mitochondrial clusters move regularly in mammalian oocytes and fertilized eggs (Figure 6) (Gresson, 1942; Hertig, 1968; Guraya, 1979). Annulate lamellae are found in a polarized mitochondrial-rich region adjacent to large pronuclei in primate eggs (Barton and Hertig, 1972; Sutovsky et al., 1996) and may be involved in mRNA transport similar to the mitochondrial cloud in Xenopus (Zhou and King, 1996). Mitochondrial associations alter as mouse oocytes mature. Some associate with the spindle, others locate at the oocyte centre and another group concentrates in the hemisphere containing the metaphase spindle (Figure 11) (Van Blerkom and Runner, 1994; Calarco, 1995). Equivalent associations have not been described in Xenopus oocytes.

**Does ooplasm rotate after fertilization in mammalian eggs?**

A redistribution of organelles and other aspects of polarity after sperm entry are regular features in ascidians and amphibians. Mammalian ooplasm could also redistribute after fertilization. Several proteins display a polarized cortical distribution in maturing human oocytes. A total of 13 membranal polypeptides expressed in differing intensities at various sites of the oolemma changed their positions individually and regularly at different stages of maturation (Ji et al., 1996a). Calcium signalling systems develop during the maturation of mouse oocytes (Jones et al., 1995). Ryanodine receptors regulating calcium discharges initially distribute homogeneously in ooplasm in the germinal vesicle stage, but become exclusively cortical in mature mouse and sea urchin oocytes (McPherson et al., 1992; Ayabe et al., 1995). Post-translational changes following fertilization such as acetylation of α-tubulin (Schatten et al., 1989) may be localized in specific sites of the oocyte. Integrin β1 is distributed asymmetrically on the human oolemma, indicating that sperm receptor sites are polarized (Ji et al., 1996b). This distribution could be artefactual. Fc receptors are present on the oocyte surface (Bronson et al., 1990), and they could undergo non-specific patching as they bind to immunoglobulins and accumulate in groups on the oocyte membrane. Sperm entry sites were evenly distributed in mouse eggs fertilized by radiolabelled spermatozoa (Sirlin and Edwards, 1958). Indications of preferential binding sites opposite the polar body in mouse eggs (Talansky et al., 1991), were not confirmed in human eggs fertilized in vitro (Santello et al., 1992). In general, sperm entry sites do not seem to be polarized in mammals.

An existing polarity in mammalian oocytes could be modified by ooplasmic rotation at sperm entry, regulated by the sperm centrosome and aster. Fertilizing human spermatozoa apparently dictate their own rapid propulsion straight through ooplasm, unaffected by their orientation relative to the MII spindle or to cytoplasmic irregularities (Van Blerkom et al., 1995). Spermatozoa penetrating near the spindle move inward in linear fashion for 10–12 µm within 30 min and approach the spindle after 1.5 h. Those penetrating opposite this spindle move inwardly and centrally over 25 µm in 1.5 h, then approach

**Figure 11.** Mitochondrial polarization in the unfertilized mouse oocyte. Mitochondria were labelled with mitomarker and viewed by confocal microscopy. Before maturation begins, oocytes have no obvious polarity. (A) Oocytes (75 µM diameter) matured in vitro for 5 h. Mitochondria are tightly localized to the nuclear region. Fluorescence noted by the arrowhead indicates discontinuities in the nuclear envelope. gv = germinal vesicle. (B) Ovalated oocytes matured in vivo to metaphase (M) II. The plane of focus is at the cortex. Mitochondria form a distinct cortical band. Arrowheads indicate the distal extent of the mitochondrial hemisphere. Courtesy of P.G.Calarco reproduced from Developmental Genetics (1995), 16, 36–43. Reprinted by permission of Wiley-Liss, Inc., a subsidiary of John Wiley and Sons, Inc.
the spermatozoon. Similar events occur after intracytoplasmic sperm injection (ICSI). Elongating microtubules emerge from the neck of the spermatozoon (which includes the centrosome) within 1.5 h to form an ooplasmic lattice of microtubules within 7 h post-insemination. A cortical layer of actin filaments 3–5 μm in thickness persists throughout, and a circumferential band of translucent ooplasm in some oocytes is apparently formed before sperm entry (Van Blerkom et al., 1995). These details are similar overall to events in non-mammalian species.

The paternal pronucleus seems to impose or collaborate in the formation of polar axes in mammalian eggs. Chromatin located near the sperm tail becomes polarized facing the oocyte interior as the differentiating paternal pronucleus rotates. This occurs even while the sperm head lies distant from the maternal pronucleus (Van Blerkom et al., 1995). Early maternal pronuclei remain relatively fixed in pericortical ooplasm, as their juxtaposition with the paternal pronucleus is co-ordinated in space and time. Chromatin in maternal pronuclei also polarizes, and rotates to face the paternal pronucleus. Chromatin in both pronuclei facing the other pronucleus becomes highly decondensed during apposition (Figure 12). Apposition of the two pronuclei occurs close to the spindle if a spermatozoon has penetrated close by, and in the oocyte centre if the spermatozoon has entered the egg opposite the spindle (Van Blerkom, 1989; Van Blerkom et al., 1995).

Direct evidence of ooplasmic rotation is scarce, although it could re-set a new axis after fertilization. The sperm centrosome could organize contraction waves noted in 95% of mature human MII oocytes over the 2–3 h period prior to second polar body extrusion following ICSI (Payne et al., 1997). Clockwise rotations of a cortical granulated cytoplasm every 20–53 min in the vast majority of these oocytes ended as the second polar body was extruded. Rotations such as this seem to be possible, since ooplasm can rotate freely within its membrane in somatic cells. Second polar body extrusion often occurs widely distant from the position of the first polar body, even to a difference of 120°, and enlarging pronuclei apparently polarize in line with it as they grow (Figure 13). Embryo quality is correlated with longer cytoplasmic waves, which are thus unlikely to be pathological consequences of ICSI.

Direct evidence stresses the importance of the sperm centrosome in establishing polarity at syngamy. In many eggs, it divides to produce two daughters which migrate to a position between the pronuclei, from where they organize a meridional first cleavage plane (Figure 14) (Asch et al., 1995; Sathananthan et al., 1996). In others, pronuclei almost seem to become arranged at right angles to the second polar body; spindle rotation might occur in these eggs as they form their first cleavage spindle. Pronuclear apposition and syngamy fail to occur in human eggs if the sperm aster has an inherent defect preventing microtubule formation (Asch et al., 1995). Damage to centrosomes may restrict the cleavage of mouse embryos to only one or two cleavage divisions when spermatozoa are exposed to ultraviolet or X irradiation before fertilization (Edwards, 1957a,b). Normal microtubules are formed after isolated sperm centrosomes are transferred into human eggs (Van Blerkom and Davis, 1995).

Indirect evidence of ooplasmic rotation and polarity in human oocytes emerges from the use of ICSI. The spermatozoon is usually injected at the 9 o’clock position as the oocyte is held with its first polar body at 12 o’clock. This positioning produces better quality embryos than other placings (Nagy et al., 1995). With this model, better embryos also arose when the injection pipette passed through the oolemma at 5 o’clock and the spermatozoon was injected near the contralateral membrane at 10 o’clock. Injections at 1 o’clock were less

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**Figure 12.** Confocal laser scanning images of a monospermic human egg stained with a DNA-fluorescent probe to show the polarized distribution of chromatin. Arrowhead shows approximate location of the sperm centrosome; time (hours), after insemination in vitro; F = female pronucleus. Courtesy of J.Van Blerkom et al. (1995) and Human Reproduction Update.

**Figure 13.** Suggested forms of ooplasmic rotation and polarity in pronuclei and polar bodies of human eggs. (A) Sequence of time-lapse video recordings of movements in granulated ooplasm and pronuclei in human eggs after intracytoplasmic sperm injection (ICSI) (Payne et al., 1997). We have inserted arrows to show clockwise rotations of a cortical granulated cytoplasm (*) occurring every 20–53 min in the vast majority of oocytes until second polar body extrusion, as identified by the authors (A–B), and lines to show pronuclear polarity in G and H. Note that medullary pronuclear reorientation is anticlockwise in this illustration, which is what would be expected if the clockwise rotation of granulated cytoplasm continued at a low level after second polar body extrusion. This final movement would explain why early pronuclei become gradually polarized in relation to the second polar body. It is remarkable that polarity has been achieved after ICSI, despite the unusual conditions in the oocyte established by sperm injection deep into the oocyte. Arrowheads show the decondensing sperm head in the medulla in C, the metaphase plate in D, cytoplasmic flare in E, and emerging pronuclei in F. The large arrow in E indicates the second polar body. Courtesy of D.Payne et al. (1997) and Human Reproduction. The oocyte was in a fixed position during filming, so the successive stages should comparable. This view is in one plane only, so the pronuclei could be out-of-line in another plane. However, the oocyte was fixed in position for videoing, so it is highly possible that some pronuclear alignment has occurred. (I) Polarity indicated by an inserted line joining two pronuclei and polar bodies after fertilization in vitro (left). Courtesy of H.Sathananthan et al. (1993) and Atlas of Obstetrics and Gynaecology.
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Figure 13
effective (Blake et al., 1996). The benefits of 5 o’clock insertions, diametrically opposite the metaphase spindle, could be due to their coincidence with oocyte polarity and a facilitated Ca$^{2+}$ release. Oocytes with defective ooplasm fail to grow normally after ICSI (Gabrielsson et al., 1996). The clinical success of ICSI itself could thus depend on the rotation of ooplasm by a correctly-positioned sperm centrosome.

A fresh look is needed at some well-known sequences in mammalian fertilization. Credited with forming the major block to polyspermy (Wassarman, 1990; Ducibella, 1996), cortical granules discharge within 10 min of sperm entry in many mammalian species. Cortical granule discharge assists in ooplasmic rotation at sperm entry in C. elegans, Xenopus and other species (Elinson et al., 1993), and could do the same in mammals. Two types exist in mouse and human oocytes, but not in those of other species. Human cortical granules of 450 nm diameter are synthesized from Golgi bodies throughout oogenesis, and they release hydrolytic enzymes, proteases and peroxidases after fusing with the oolemma to ‘harden’ the zona pellucida and establish the zona block to polyspermy. Those of 350 nm diameter, synthesized only during oocyte maturation, have no known function, and persist apparently intact after fertilization (Sathananthan and Trounson, 1982; Sathananthan et al., 1985; Hinduja et al., 1990).

It may be that small cortical granules, and the translucent cortical ooplasm, regulate rotation in mammalian oocytes. Cortical rotation could be far more important to a species than a strong block to polyspermy, which does not seem to be essential in mammals. Fertilization has already occurred when only a dozen or so spermatozoa reach the oviduct. A fast block to polyspermy could exist in the mammalian oolemma, as revealed by the entry of only one spermatozoon into most human eggs after several are injected into the perivitelline space (Jaffe et al., 1983; Gordon and Talansky, 1986). Polyspermy would thus be prevented primarily by a membrane block, supported by a secondary block after cortical granule discharge.

**Polarity and cleavage planes**

Hints of regular cleavage planes in mammalian embryos have circulated for many years. A ‘crosswise’ appearance of blastomeres in 4-cell mammalian embryos convinced classical embryologists of an underlying polarity in the first and second cleavage planes (Austin, 1961). Sobotta (1895), Gregory (1930) and Lewis and Hartman (1933) noted that a meridional mitotic spindle formed in one blastomere of 2-cell mouse and rhesus monkey embryos, followed by a cleavage plane at right angles to the meridian in the other. This situation resembles the regulated cleavage planes in 2-cell C. elegans and not sea urchin embryos (Figures 2 and 15A).

Polarized cleavage planes occur in 2-and 4-cell rabbit embryos, assuming that an oocyte axis runs from polar body to a point diametrically opposite (Gulyas, 1975). The first cleavage plane follows this meridional axis, is holoblastic, bilateral and nearly equal. In most eggs, the first-cleaving 2-cell blastomere forms a cleavage furrow along this meridional axis before division occurs. Three blastomeres accordingly lie side-by-side. Most cleavage planes in second-cleaving 2-cell blastomeres 2–3 h later form transverse to the previous axes (Figure 15B). In slow-growing embryos, this blastomere initially forms a meridional cleavage plane, which rotates as the cleavage furrow is completed (Gulyas, 1975). Rotation in the second 2-cell blastomere apparently results in a transverse cleavage, and explains the ‘crosswise’ appearance of 4-cell embryos of mouse, rabbit, rhesus monkey, mink, pig, baboon and human embryos (Figure 16) (Edwards et al., 1970; Gulyas, 1975). During the third cleavage in rabbit embryos, the first-dividing cells cleave equatorially, whereas later-dividing blastomeres cleave meridionally to the axis established by the second polar body (Gulyas, 1975).

During a meridional first cleavage in mouse embryos, a slight rotation occurs before the final division of the first-dividing 2-cell blastomere (Graham and Deussou, 1978). This rotation places one daughter more adjacent to the polar body, a situation reminiscent of C. elegans embryos. The long axis of the second-dividing 2-cell blastomere rotates in the opposite direction and produces a largely equatorial division, to place these daughters at right angles. Labelling single blastomeres of 2-cell mouse embryos with silicone droplets detected cytoplasmic redistribution between daughters additional to that caused by cleavage rotation (Graham and Duessen, 1978). These cleavage patterns are similar overall, but differ in details from those in rabbit eggs.

Polarization and A/P axes in mouse oocytes and embryos were observed in a mouse strain with a stable attachment of the second polar body to embryonic cells (Gardner, 1997). The site of attachment was constant during successive developmental stages and in cell lineages traced with horseradish peroxidase and other markers. This enabled potential artefacts
Oocyte polarity and cell determination in early mammalian embryos

Figure 15. (A) Cleavage planes in the sea urchin egg involve two successive meridional divisions at right angles to each other, resulting in four similar blastomeres, A, B, C, D. Polar bodies indicated. (B) Cleavage planes in the rabbit egg. The first division (CP-1), forming of two blastomeres AB and CD, and the division of the first-cleaving 2-cell blastomere (CP-2A) leading to the formation of blastomeres A and B, are both meridional, passing through the animal and vegetal poles. CP-2A bisects the plane of CP-1 at 90°. The second-dividing 2-cell blastomere produces blastomeres C and D, and cleavage is equatorial (CP-2B and is perpendicular to both CP-1 and CP-2A). Polar bodies indicated. This form of division leads to the formation of a 4-cell embryo in the shape illustrated in Figure 16. Courtesy of B.Gulyas reproduced from Journal of Experimental Zoology (1975), 193, 235–248. Reprinted by permission of Wiley-Liss, Inc., a subsidiary of John Wiley and Sons, Inc.

Figure 16. A human 4-cell embryo showing the typical crossed appearance of its blastomeres (Edwards et al., 1970) (magnification ×450). Compare this appearance with that of the Xenopus 4-cell embryo in Figure 5.

in interpretation due to occasional shifts in polar body position (Calarco, 1995) to be excluded. The A/P axis of the fertilized egg was orthogonal to the embryonic/abembryonic axis of the blastocyst (Figure 17), and was aligned with the A/P axis of ICM and implanted embryo (Gardner, 1997). It seems that the meiotic spindle marked the animal pole in this mouse strain, just as in H. roretzi (Yamada and Nishida, 1996), and as assumed by Gulyas (1975). In contrast, the first polar body is an unreliable marker, since it can move in the perivitelline space (Gardner et al., 1992; Gardner, 1997).

Evsikov et al. (1994) questioned evidence of regular cleavage planes in mouse eggs. They suggested that polarity and axes in oocytes are obviated by viviparity in favour of cellular interactions in morulae, that 4-cell blastomeres are arranged crosswise to permit tight packaging within the zona pellucida, and noted that cleavage and growth patterns are unaffected when ooplasm in mouse pronucleate eggs is ‘scrambled’. The work of Gulyas (1975) can be questioned, especially over variations between fast and slow-cleaving embryos. Nevertheless, the generalizations of Evsikov et al. (1994) are not justified by their limited data, and would be negated if former axes re-establish after ‘scrambling’.

Gene expression in mammalian eggs and early embryos

A period of tight maternal control over transcription and differentiation after fertilization characterizes virtually all vertebrates and invertebrates. Fundamentally similar from flies to mammals, it extends over 9–12 cell divisions in Drosophila and Xenopus prior to activation of the zygotic genome. The number of such divisions is fewer in mammals (between one and four). Part of this variation between phyla may be explained by wide differences in cleavage rates. Timing has immense embryological significance, e.g. in the exact timings of matura- tion and ovulation after the luteinizing hormone (LH) surge or injection with human chorionic gonadotrophin (HCG) (Edwards, 1965a,b; Steptoe and Edwards, 1970), and the temporal clocks are equally important after fertilization. Telomeric clocks could function in blastomeres of mammalian embryos, as in human fibroblasts and in yeast (Harley et al., 1990; Feng et al., 1995). Telomeres are seemingly active in embryonic germlines and in frog eggs (Wright et al., 1996), but not in human oocytes and spermatozoa where other timers must override the telomeric clock. It is restored in blastocysts (Wright et al., 1996), perhaps to control minor and major zygotic transcription and the onset of blastulation. A close
control over zygotic transcription might be needed to suppress any residual genetic activity, and to allow specific forms of transcription to begin as individual cell lines begin to differentiate. It could also permit maternally-encoded mRNA to establish a critical polarity and spatiality in embryos.

**Maternal and paternal transcripts**

In mice, stores of maternal mRNA accumulate in the oocyte and are produced by the maternal pronucleus before the onset of syngamy (Aoki et al., 1997). RNA stores in mouse oocytes consist of 60% ribosomal RNA, 25% transfer RNA and 15% poly(A) RNA. Poly(A) RNA provides a pool of genetic information although only a small fraction of it is translatable (Davidson, 1986). Fully-grown oocytes accumulate more protein and less RNA. Mature oocytes store $1.7 \times 10^7$ poly(A) RNAs, an amount virtually doubled after fertilization as poly(A)-deficient RNA is polyadenylated (Clegg and Piko, 1987). Many maternal mRNAs encode housekeeping functions or specialized information for factors involved in oocyte growth and maturation. The 400 different oocyte proteins include transcripts for α- and β-tubulin, β- and γ-actin, cell-cycle proteins involved in meiosis, ribosomal proteins and histones, zona proteins, acetylcholine receptors and factors involved in cell signalling (Edwards and Brody, 1995). Protein transcription factors are active in oocytes, for example, one binds to a promoter of 12 bp known as element IV, which regulates the synthesis of zona proteins (Miller, et al., 1991). Levels of individual proteins decline at specific rates during maturation (Wassarman, 1988).

Little is known about the genes regulating polarity and related activities in oocytes. These genes undoubtedly exist, since the proto-oncogene *pp39* which arrests oocytes at MI in *Xenopus* (Watanabe et al., 1989), also exerts this role in mouse and human oocytes (O’Keefe et al., 1989; Pal et al., 1994). Regulatory genes could include *DFFRX* and *DFFRY* in humans, homologous with the *Drosophila* oogenesis gene *fag* (*fat facets*), and human *DAZH*, which has homologies with the *Drosophila* autosomal meiotic-controlling gene *boule* and autosomal *Dazh* (or *Dazla*) in mice (Saxena et al., 1996). Others could be homologues of *C. elegans* (*par*) and *Drosophila* (*BicD, exuperanti, and swallow*) (Melton, 1991). Mutants suspected of impairing meiosis I and causing oocyte degeneration are located on a region of *Xq* which is required for normal ovarian development (Wyss et al., 1982; Jones et al., 1996). Human homologues could even exist with plant genes *aml1, qfl*, and *runl +*, to control commitment to meiosis and entry into leptotene, and with yeast *arg4* and *ded81* involved in meiotic recombination (Schultes and Szostak, 1991; Massey and Nicholas, 1993).

Maternal mRNA stores are translated over the first few cleavage cycles in mammalian embryos. Their levels, 6–8% of total RNA, compare with those in *Xenopus* embryos (2% of total RNA). In mice, most poly(A) maternal RNA is degraded at the late 1-cell or early 2-cell stage, when embryonic transcripts first appear and gradually replace declining maternal mRNA stores (Braude et al., 1979; Telford et al., 1990; Ram and Schultz, 1993). The temporal expression of maternal transcripts is tightly controlled. Maternal mRNA regulates rising hypoxanthine phosphoribosyl transferase (HPRT) activity in pronucleate mouse eggs at a time when the translation of embryonic mRNA is barely detectable (Harper and Monk, 1983). The switch to embryonic HPRT transcription and translation is virtually complete in morulae, when oocyte-encoded enzymes become scarce (Harper and Monk, 1983). Spindlin (*Spin*), an abundant maternal, stage-specific protein, is expressed in unfertilized mouse eggs and 2-cell embryos, but not in 8-cell embryos (Oh et al., 1997). It associates with the meiotic spindle, becomes phosphorylated during the cell cycle and may regulate the transition from oocyte to embryo. Some maternal transcripts avoid degradation (Telford et al., 1990), perhaps existing as long-lived forms and persisting into blastocysts and later, as in *C. elegans* and *Xenopus* (Bachvarova and Moy, 1985). This situation arises with oocyte-encoded mRNA transcripts for *Gpi-1*, which are stable, translated until day 2.5 post-fertilization, decline as embryonic transcription begins, and become exhausted by day 5.5–6.5 (West et al., 1986). Specific *Gpi-1* markers reveal the onset of embryonic transcription on day 4, and a persisting active maternal expression until day 6 (Gilbert and Solter, 1985). E-cadherin, a

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**Figure 17.** (A) Diagram illustrating the A–P axis in the expanding mouse blastocyst. (B) Implantation stage blastocyst. Note oval shape and rounded anterior mural trophoderm. (C) Early mouse blastocyst photographed with its embryonic pole uppermost, showing an oval shape about its embryonic (Em)–abembryonic (ABem) axis and the polar body (arrow) clearly aligned with its greater diameter. Bar = 20 µm. Courtesy of R.Gardner Hum. Reprod. Update, 2, 3–27 and Development and Company of Biologists Ltd (Fig. 17C).
cell-surface adhesion molecule, provides another example of maternal transcripts persisting until the blastocyst stage. This protein may be essential to sustain compaction in morulae, is localized at cell junctions (Ohsumi et al., 1996) and it maintains its expression until the blastocyst in homozous-deficient mutants which die by day 10 in mice (Larue et al., 1994). Antibodies to E-cadherin disrupt both compaction and the establishment of ICM (Johnson et al., 1986).

Maternal mRNAs may assume a specific spatial expression in early blastomeres similar to germ line and vegetal-specific mRNA distributions in C. elegans and Xenopus (see Tables I and II). Maternally-encoded oct genes could offer an example; oct-4 (sometimes called oct 3 or NF3) is a member of the POU family of transcription factors containing an octamer binding homeodomain, and a transcription activator when fused with the DNA-binding domain of c-Jun (Okamoto et al., 1990; Scholer et al., 1990). The nuclear location of oct-4 implies it has a regulatory function. Some oct-4 protein is localized in a single pronucleus (presumably the maternal pronucleus), and in the polar body of 2-cell embryos. Larger amounts are detected in nuclei of 8-cell embryos and blastocysts where expression is specific to ICM (Palmieri et al., 1994). Its expression in primary endoderm becomes greater than in ICM, perhaps a pointer to the location of germ cell precursors in this tissue as discussed below. The down-regulation of oct-4 in some blastomeres of 8-cell mouse embryos, presumably trophectoderm, resembles the situation of the germline in C. elegans, where specific factors persisting in the germline become gradually down-regulated in somatic precursors.

Two specific forms of paternal inheritance have recently been identified. A few mRNA transcripts are present in spermatozoa as small lengths of repetitive sequences (Kramer and Krawetz, 1997; Miller, 1997). Their location in the mid-piece ensures their entry into ooplasm since this organelle follows the movement of the sperm head. There is no current knowledge on the role of these few paternal transcripts, if any, on embryonic growth. Some paternal transcription occurs in paternal pronuclei, but once again the function of these transcripts is unknown.

**Regulation of gene expression in 1-and early 2-cell mammalian embryos**

Regulation of gene expression occurs by both active and repressive mechanisms. Promoters initiate gene activity, and enhancers regulate a specific aspect of gene function. Most information on mammalian systems has been derived from studies of endogenous and exogenous (plasmid) gene expression in mice. Repression of zygotic expression in the mouse appears to be quite different from that in Xenopus, where the activity of a functional TBP (TATA box binding protein) is impaired by a large maternal stockpile of histones preventing the formation of stable transcription complexes over 12 cell cycles (Davidson, 1986; Nothias et al., 1995; Aoki et al., 1997).

Repressors active at the 1-and 2-cell stage in mice can act through changes in chromatin structure. Repressive factors in ooplasm vary by two-fold in oocytes of different inbred strains (Chastant et al., 1996), and genetic factors impose promoter or enhancer repression unidirectionally. Promoter activity of reporter genes is not restored in nuclei transplanted from 2-cell embryos to 1-cell eggs, even if linked to an enhancer, nor can fertilized eggs reprogramme enhancer utilization in nuclei taken from embryos of more advanced age (Henery et al., 1995; Nothias et al., 1995).

As in Xenopus, histones are repressive. They could include maternal and zygotic histones, since the synthesis of H1 histone begins at the 1-cell stage when repression is activated (Wassarman and Kinloch, 1993). Hyperacetylation of H4 histone isoforms is highly correlated with transcriptionally active chromatin (Turner, 1991), but its consequences can vary at different embryonic stages. Histone hyperacetylation decreases transcription in 1-cell embryos, but raises it by 80% in 2-cell embryos (Aoki et al., 1997). The timing and degree of this acetylation thus appears to be very important, since core histones are hyperacetylated in 1-cell embryos and deacetylated in 2–4-cell embryos. Some hyperacetylated isoforms of histone H4 are enriched in the nuclear periphery (Worrad et al., 1995), so the activation of specific gene subsets may require histone acetylas, known to associate with transcription activators and RNA polymerase II (Brownwell et al., 1996; Roth and Allis, 1996; Aoki et al., 1997).

Regulatory components of zygotic activation therefore include TATA boxes and transcription factors, promoter enhancers, RNA splicing and post-transcriptional modifications (Majumder et al., 1993; McPhee et al., 1994). TATA boxes mediate promoter stimulation via an enhancer in differentiated cells, but may not function in cleaving embryos, where enhancer stimulation is undertaken by transcription factors such as Sp1 which probably regulates housekeeping genes (Worrad et al., 1994; Nothias et al., 1995). Enhancers act from the 2-cell stage in mice to stimulate the activity of weak promoters (Majumder et al., 1993; Nothias et al., 1995).

Some RNA splicing is evidently controlled maternally, since embryonic precursor RNAs first transcribed in 2-cell mouse embryos are processed by snRNPs of maternal origin (Telford et al., 1990). In cow embryos, similar snRNPs are associated with RNA splicing, the formation of blebs of chromatin, and the onset of embryonic transcription (Kopceyn and Niemann, 1993; Tesarik, 1993).

DNA replication is another major controlling event, providing a window of opportunity for transcription factors to bind to DNA (Aoki et al., 1997). The prevention of DNA replication by inhibiting DNA polymerases suppresses both transcription in the male pronucleus (Aoki et al., 1997), and the switching-on of the transcription initiation factor e1F-4C (Davis et al., 1996). In contrast, DNA replication in the first cell cycle is repressive for exogenous plasmid gene expression (Wiekowski et al., 1993; Henery et al., 1995). However, as pointed out by these authors, this form of expression of exogenous reporter genes relies on the transcription of non-integrated plasmid sequences with their own promoter and enhancer requirements, and is not necessarily due to the effects of DNA replication. The second round of DNA replication appears to be critical to down-regulate e1F-4C, a switch-off probably preventable by hyperacetylation (Davis et al., 1996; Aoki et al., 1997).

**Minor and major zygotic transcription**

Initially, an almost-total chromatin-mediated repression of promoter activity is imposed on maternal pronuclei, but not on
paternal pronuclei (Van Blerkom, 1981; Tesarik and Kopecny, 1989, 1990; Nothias et al., 1995; Davis et al., 1996). The ongoing rate of transcription by endogenous genes is four to five times greater in the paternal pronucleus (Aoki et al., 1997). Reporter genes were able to detect transcription in the G2 phase in paternal but not in maternal pronuclei of mouse eggs (Ram and Schultz, 1993), and reporter gene expression in maternal pronuclei is virtually totally repressed, unless chromatin is hyperacetylated by treatment with butyrate (Wiekowski et al., 1993). Paternal pronuclei may be able to undertake a limited transcription because protamine/histone transition and DNA hyperacetylation in enlarging sperm heads could enable transcription factors to bind to DNA (Felsenfeld, 1992; Wiekowski et al., 1993; Henery et al., 1995; Nothias et al., 1995); this perhaps explains why human paternal pronuclei can synthesize nucleolar RNA by 4 h after sperm–egg fusion. DNA synthesis follows, at 12 h (Tesarik and Kopecny, 1989, 1990). Paternal Y linked transcripts are expressed in paternal pronuclei in human embryos (Ao et al., 1994).

The zygotic genome is activated more rapidly in mammals and in sea urchins than in other animals. Zygotic transcription in mouse is activated in two unequal parts, first a ‘minor’ phase and then a ‘major’ phase after the first cleavage division. It is controlled by a cascade of factors including a zygotic clock, chromatin-mediated repression and an enhancer-mediated activation of transcription acting via promoters and transcription factors. The actions of these regulators may be gene specific, effective over very short periods, and capable of imposing a unidirectional sequence on early differentiation (Nothias et al., 1995; Davis et al., 1996; Aoki et al., 1996).

Different systems regulate the two phases of zygotic activation in 1–2-cell mouse embryos (Flach et al., 1982; Bolton et al., 1984; Aoki et al., 1997), and a similar situation could arise in human embryos one cleavage later (Braudel et al., 1988). ‘Minor’ activation is independent of the first DNA replication and depends on the time elapsed from fertilization, i.e. 20 h in the mouse as controlled by the ‘zygotic clock’. Another embryonic clock appears to function later in controlling blastocyst formation. The zygotic clock may regulate general transcription factors required for all three RNA polymerases, and probably functions through post-translational modifications (Van Blerkom, 1981; Nothias et al., 1995). It may delay most embryonic transcription until chromatin has been remodelled from a meiotic state in the oocyte and from a protamine-dominated state in spermatozoa. This process could suppress all promoters in the zygotic genome until specific forms are activated in differentiating cell lines by particular enhancers or transactivators (Nothias et al., 1995). Illegitimate transcription (synthesis of a few copies of a large number of genes) could be a factor in the initiation of zygotic gene expression (Chelly et al., 1989).

The heat shock protein hsp 70.1 is among the products produced during the first minor burst of transcription in 1-and 2-cell mouse embryos. Its restricted expression to the first 6 h of the second cell cycle is a landmark of early zygotic activity, marking the onset of minor transcription from the S phase of the second cell cycle (Christians et al., 1995), and it ends after the second round of DNA replication. This short burst of transcription in 2-cell embryos marks the onset of embryonic regulatory mechanisms (Chastant et al., 1996).

Other regulatory genes such as translation initiation factor (eIF-4C) and transcription requiring factor (TRC) are also transcribed transiently in 2-cell mouse embryos, and resemble the transitory regulators synthesized in nematodes and amphibians (see Tables I and II). The expression of TRC in late 1-cell embryos is enhanced by cytoplasmic cyclic AMP (Latham et al., 1991). These genes function for a very short period, for their transcription is sharply reduced in 4-cell embryos by further modifications in chromatin structure during the second cleavage division (Davis et al., 1996). Their brief activity might signal the onset of a second phase of organization after maternal factors have imposed polarity on the embryo, reminiscent of the transient embryonic regulators HE and BP10 in P. lividus (Ghiglione et al., 1996), and cey-1 and mex-3 in C. elegans (Seydoux and Fire, 1994).

Major zygotic transcription begins in the S and G2 phases in 2-cell mouse eggs. Its onset depends on the first round of DNA replication, and is marked by a burst of synthesis of 40 proteins 2–4 h after the first cleavage is completed. The major phase of transcription is characterized by a marked transition in polypeptide synthesis, and the production of translation products which become visible 4 h later. Plasmid-linked promoters previously injected into pronuclei and repressed at first cell division are also activated at this time (Henery et al., 1995). This phase of transcription is marked by a switch in regulation to include a selectiveness for RNA polymerase II, a requirement for enhancers, and declining genomic methylation (Monk et al., 1987).

An impressive array of genes are expressed in early cleavage stages in the mouse. As in other phyla, these genes may be multifunctional and active over brief periods of development (for examples see Table III). Regulatory genes encoding DNA binding proteins, such as rig, oct-4, Hst and Hex, are expressed at intervals from early cleavage onwards (Taylor and Piko, 1991; Palmieri et al., 1994; Cross et al., 1995; Verlinsky et al., 1995). Some characterize the cell line differentiating into inner cells in morulae and then into ICM. Others regulate trophoectodermal differentiation. Numerous genes control growth factors, cytokines and receptors, adhesion and cell signalling proteins involved in cell–cell communications and inductive pathways. Genes regulating general housekeeping, cytoskeletal and surface secretory functions are also activated during these stages (for reviews see Latimer and Pedersen, 1993; Kinloch and Wassarman, 1993; Kidder, 1993; Kane, 1997).

Totipotency and cloning

Totipotency and cloning in mammals help to clarify several aspects of regulation. Full or cellular totipotency is the ability of a cell other than an oocyte to develop into an entire offspring including the germ line. Nuclear totipotency involves the same ability in a nucleus after its transfer into an enucleated oocyte.

Cellular and nuclear totipotency

The embryological significance of cellular totipotency is unclear. It is unlikely to have evolved to enable an embryo to
Oocyte polarity and cell determination in early mammalian embryos

Table III. Genes expressed in developing mouse embryos

<table>
<thead>
<tr>
<th>Category</th>
<th>Gene</th>
<th>Product/function</th>
<th>Stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gene regulation</td>
<td>Histone H3</td>
<td>modification of chromatin</td>
<td>4-cell</td>
</tr>
<tr>
<td></td>
<td>rig (rat insulinoma)</td>
<td>DNA binding protein</td>
<td>oocyte–8-cell</td>
</tr>
<tr>
<td></td>
<td>Oct 4</td>
<td>DNA binding protein</td>
<td>oocyte–blastocyst</td>
</tr>
<tr>
<td></td>
<td>Hox</td>
<td>DNA binding protein</td>
<td>oocyte–blastocyst</td>
</tr>
<tr>
<td></td>
<td>Hox (positional spec.)</td>
<td>DNA binding protein</td>
<td>oocyte–blastocyst</td>
</tr>
<tr>
<td>Oncogenes</td>
<td>c-myc</td>
<td>DNA binding protein, active in cell division</td>
<td>oocyte–blastocyst</td>
</tr>
<tr>
<td></td>
<td>c-fos</td>
<td>DNA binding protein</td>
<td>oocyte–blastocyst</td>
</tr>
<tr>
<td></td>
<td>c-mos</td>
<td>DNA binding protein</td>
<td>oocyte–blastocyst</td>
</tr>
<tr>
<td>Housekeeping</td>
<td>HPRT</td>
<td>hypoxanthine phosphoribosyl transferase</td>
<td>4–8-cell</td>
</tr>
<tr>
<td></td>
<td>GP1</td>
<td>glucose phosphate isomerase</td>
<td>oocyte–morula</td>
</tr>
<tr>
<td>Cytoskeletal</td>
<td>α, β, actin</td>
<td>DNA binding protein</td>
<td>4–8-cell</td>
</tr>
<tr>
<td></td>
<td>α-tubulin</td>
<td>DNA binding protein</td>
<td>4–8-cell</td>
</tr>
<tr>
<td>Cell adhesion/</td>
<td>E cadherin</td>
<td>compaction</td>
<td>4-cell</td>
</tr>
<tr>
<td>signalling</td>
<td>connexin</td>
<td>DNA binding protein</td>
<td>oocyte–blastocyst</td>
</tr>
<tr>
<td></td>
<td>WNT-1</td>
<td>compaction</td>
<td>4-cell</td>
</tr>
<tr>
<td></td>
<td>Stromelysin</td>
<td>collagenase</td>
<td>blastocyst</td>
</tr>
<tr>
<td></td>
<td>Collagenase</td>
<td>collagenase</td>
<td>blastocyst</td>
</tr>
<tr>
<td>Surface proteins</td>
<td>Alkaline phosphate</td>
<td>transforming growth factor</td>
<td>2-cell</td>
</tr>
<tr>
<td></td>
<td>Stromelysin</td>
<td>collagenase</td>
<td>2-cell</td>
</tr>
<tr>
<td></td>
<td>Collagenase</td>
<td>collagenase</td>
<td>2-cell</td>
</tr>
<tr>
<td>Growth factors,</td>
<td>TGFβ</td>
<td>collagenase</td>
<td>2-cell</td>
</tr>
<tr>
<td>cytokines and</td>
<td>FGF</td>
<td>fibroblast growth factor</td>
<td>4-cell</td>
</tr>
<tr>
<td>receptors</td>
<td>PDGF</td>
<td>platelet-derived growth factor</td>
<td>4-cell</td>
</tr>
<tr>
<td></td>
<td>IGF1, II</td>
<td>insulin-like growth factor</td>
<td>2–8-cell</td>
</tr>
<tr>
<td></td>
<td>LIF</td>
<td>blastocyst implantation</td>
<td>blastocysts</td>
</tr>
<tr>
<td></td>
<td>EGF</td>
<td>epidermal growth factor</td>
<td>2–8-cell</td>
</tr>
<tr>
<td></td>
<td>WNT-2</td>
<td>growth factor similar to FGF</td>
<td>4-cell</td>
</tr>
</tbody>
</table>

MPF = maturation promoting factor; LIF = leukaemia inhibitory factor.

survive when all but one or two of its blastomeres have been destroyed. Mammalian embryos develop in highly sheltered environments and such damage must be rare. All but one blastomere in an embryo might be destroyed after cryopreservation.

Cellular totipotency might be better understood as a cellular response to external stimuli during specific developmental stages. Best studied in plant cells, its induction requires complex molecular events. To achieve it, liquid cultures of single somatic plant cells are starved of growth factors, e.g. 2,4-D 2,4-dichlorophenoxyacetic acid, and grown at low cell densities (Nuti-Ronchi, 1995). Phytohormones, e.g. auxin and cytokinin, and enzymes, e.g. endochitinases and a peroxidases, induce growth in the isolated cells (Schell et al., 1993). Attachment to a solid substrate is necessary for some plant species undergoing embryo regeneration. Cytological phenomena associated with a reversion to totipotency include ‘somatic embryogenesis’, ‘somaticoclinal variation’ and ‘homeotic transformation’ (Nuti-Ronchi, 1995) as isolated cells transform from a mitotic to a modified meiotic state. The onset of reversion may be signalled at particular cell cycle stages, or by the appearance of a dense cytoplasm and conspicuous cell walls (details in Nuti-Ronchi, 1995). Commitment to meiosis might involve aml1 (ameiotic) or afd (affecting meiosis-1) during chromosome synapses in G2 and at prophase-I of meiosis, as in maize. Genes homologous with rum1+ in yeast, which may regulate entry to S phase by interacting with cyclin-dependent kinases and initiation proteins such as cdc6 (Jallepalli and Kelly, 1996; Rudner and Murray, 1996; Rowles and Blow, 1997), could induce DNA synthesis uncoupled from mitosis. This unusual DNA pattern is typical of meiosis, which is apparently essential for plant cells to revert in vitro. Reversion begins <7 h after isolation of specialized endodermis or stem cells lying close to vascular tissue in carrot seedling hypocotyls. DNA synthesis is not required for totipotency. Transforming cells express new forms of genetic variation.

Early mammalian blastomeres can revert to totipotency. One cell of a 2-cell rabbit blastomere is able to sustain full development if the other is destroyed (Seidel, 1952). Isolated 2-cell mouse blastomeres (Tarkowski, 1959; Hoppe and Whitten, 1972), and 4-cell cow blastomeres (Johnson et al., 1995) are totipotent. The isolated 4-cell cow blastomeres were cultured in host zonae pellucidae, and transferred with trophoblastic vesicles to the uterus. Without these precautions, only three out of four cow blastomeres revert (Johnson et al., 1995). Other studies suggest that totipotency may be incomplete during the first two cleavage divisions. Biopsies of 4-cell mouse embryos can result in poor blastocyst formation and implantation, while biopsied 8-cell embryos develop normally (Kryminska et al., 1990). Mechanical ablation of 1/2 mouse blastomeres can decrease the proportion of ICM in blastocysts (Rands, 1985, 1986). Totipotency wanes: in mice, isolated 4-cell blastomeres produce miniature blastocysts, while isolated 8-cell blastomeres produce trophoblastic vesicles (Gardner,
1996a). Cellular totipotency may not be an all-or-none property, and sister blastomeres could express it to different degrees. Cellular totipotency clarifies the limits of embryo repair, and fades as blastomeres initiate transcription and become allocated to specific pathways.

As totipotency declines, blastomeres of cleaving mammalian embryos are able to replace the loss of one or two blastomeres. This modified form of cellular totipotency arises in isolated inner cells in cleaving embryos, ICM, and ES cell lines. They can colonize all somatic and germinal tissues in fusion chimeras, and this reversion may be a consequence of cell density, or imposition of polarity and axes by the recipient embryo. This property presumably lasts while blastomeres are allocated to, but not committed to specific pathways. It is utilized during the diagnosis of genetic disease in human embryos, which requires the excision of one blastomere from 4-cell embryos (forming a 3/4 embryo), or one or two from 8-cell embryos (7/8 and 6/8) (Handyside and Delhanty, 1993). 4-and 8-cell mouse embryos accomodate the excision of one or two blastomeres and re-establish a normal embryo (Monk et al., 1990), and 6/8 human embryos seem to develop virtually identically to intact embryos in vitro (Handyside and Delhanty, 1993). Blastomere excision could risk embryonic death by reducing the number of cells available for differentiation or by the excision of blastomeres essential to form a particular cell line. The removal of too many blastomeres risks the formation of a small ICM and embryo death (Hardy et al., 1990; Tarín et al., 1992; Hardshorne et al., 1993). Abnormal growth in these circumstances may be imposed as development is regulated by strict embryonic clocks, and not by the number of cells. The capacity of an embryo to regulate itself when blastomeres are added or removed is not shared by all mammals, since the destruction of 1/2, 1/4 or 1/8 cell in marsupial embryos results in incomplete development (Selwood, 1994).

Nuclear totipotency involves the transfer of a donor nucleus into an enucleated oocyte. It is highly experimental in comparison with cellular totipotency. Both situations may reflect fundamental but different aspects of cytodifferentiation, and both forms of cloning become less successful as cells become highly differentiated.

Mammalian studies on nuclear cloning followed initial work in amphibians by Briggs and King (1952) and Gurdon (1964). Nuclei from various mammalian tissues have been transferred by surgery or fusion with oocytes (Baranska and Koprowski, 1970; Li et al., 1973; Bromhall, 1975; Hoppe and Illemensee, 1977; Tarkowski and Balakier, 1980). Those from late cleavage stages and ICM revert and encode for intact offspring (Keefer et al., 1994). Some nuclei from older fetuses, e.g., male bovine gonial cells at 175 days post-coitum, sustain fetal development at least to day 35 (Delhaise et al., 1995; Moens et al., 1996). Granulosa nuclei encode only for blastocysts (Collas and Barnes, 1994), and even this limited development could be supported primarily by pre-existing maternal transcripts. The recent birth of a cloned sheep from a mammary cell nucleus (Wilmut et al., 1997) seems to resemble the situation in plants. Specific ‘starvation’ media, a modified cell cycle and growth factors establish a G0 phase as cells exit the growth cycle. Success rates are low, at 11.7%, and fetal losses high (Wilmut et al., 1997). Totipotent nuclei evidently persist in some cells of adult plants and animals, a property previously considered as restricted to mammalian germ-line cells or to those expressing genes such as oct-4.

Cellular totipotency can sometimes be predicted from the relationship between oocyte polarity and the planes of the first cleavages. Two meridional and one equatorial divisions in *Xenopus* and *P.lividus* (see Figure 8) distribute vegetal ooplasm to four totipotent blastomeres and animal ooplasm to four multipotent daughters. This observation implies an element of control via vegetal determinants. The situation in mice is not too different from this, since only the first division and the first-dividing 2-cell daughter cleave meridionally. This form of cleavage could restrict cellular totipotency to the first two blastomeres. A different situation arises in species with a mosaic pattern of development, such as *C. elegans*. The ablation of single blastomeres at the 2-cell stage onwards leads to incomplete development of the embryo (Davidson, 1986; Schnabel, 1996). Many of the remaining blastomeres can develop autonomously, to produce their normal complement of muscle cells, yet they undergo inductive relations with neighbouring cells within the embryo. Development in this species is thus regulated by a combination of maternal determinants and inductive pathways. In some mammals, blastomeres seem to be even more flexible, having the capacity to compensate for the loss of a sister, presumably by reverting to an earlier differentiated state. As suggested by Gueth-Hallonet and Maro (1992), mouse blastomeres may behave differently in intact and disrupted embryos. Inside cells, which do not normally contribute to trophectoderm, become totipotent if the integrity of the embryo is disturbed.

Reversion of blastomeres or nuclei is seemingly widespread among many species, and mammals are not highly exceptional in this respect. Cellular totipotency might require a simple form of polarity, a low level of transcription, or a modified chromatin structure in the blastomere. An ability to repair the loss of blastomeres, or to colonize fusion chimeras, could occur in cells that are allocated but not committed to a particular tissue. A totipotent nucleus may be in its final stage of differentiation compatible with cloning. Methylation and acetylation could be involved in the induction of cloning, as particular cell states are terminated, differentiation interrupted and gene expression reprogrammed (Latham et al., 1991).

**Stress and heat shock proteins in totipotency**

Cells and nuclei reverting to totipotency undergo complex behavioural modifications. These include stress-like responses during tissue disaggregation and cell isolation. The synthesis of small heat shock proteins, similar to those characterizing meiosis in yeast, plants and *Drosophila* (Kurtz et al., 1986), could help cells to overcome various forms of stress and are apparently ancestral to plants and animals evolving rapidly in vents of high-temperature water erupting in mid-ocean fissures (Nisbet and Fowler, 1996). The genetic control of heat shock proteins in bacteria involves sigma (σ) subunits. These initiate their production via promoter sites, and subunits dissociate.
from polymerase as transcripts reach 2–10 nucleotides (Schleif, 1993). Different \( \sigma \) subunits, especially \( \sigma^{70} \), control the transcription of different gene classes including heat shock proteins, those produced under oxidative stress and developmentally-regulated bacterial genes. The large family of heat shock proteins are highly conserved from yeasts to mammals (Kurtz et al., 1986; Gaunt, 1987; Herr et al., 1988). Multifunctional heat shock proteins act as chaperones, stabilize protein structure, grant certain proteins access to mitochondria, affect translation, and stabilize transcription activation complexes (Parsell and Lindquist, 1993; Schirmer et al., 1996). Heat shock proteins are diverse and numerous, and could offer alternative means of genetic expression to a cell. Their large-scale synthesis could indicate the adaptation of a cell to a more primitive state able to mimic natural developmental triggers, or substitute or stabilize normal zygotic expression.

Stress, a modified meiosis and an essential need for polarity are preliminaries to totipotency in plants (Jelaska, 1994). Nematodes are exceptional since embryos grow abnormally in 1/2 C. elegans embryos. Heat shock proteins nevertheless characterize the onset of zygotic transcription in this species, and maternal hsp70A transcripts localize to germline (Seydoux and Fire, 1994). Small 20–30 kDa heat shock proteins are conserved to a less extent, do not accumulate in oocytes of Drosophila and Xenopus, are not induced by heat, and may have a developmental function (Kurtz et al., 1986; Gordon et al., 1997). Heat shock proteins are absent in mature mouse oocytes as described earlier, but are expressed as minor transcription begins (Tesarak, 1993; Christians et al., 1995). Stress responses in oocytes exposed to mild elevations in temperature involve the transcription and synthesis of the heat shock proteins hsp84 and hsp70 among others in Xenopus (Kurtz et al., 1986) and hsp68 in mice (Curci et al., 1991). Constitutively-expressed transcription activators are activated post-translationally, as heat shock factor proteins such as HSF1 mediate the rapidly-induced gene expression of heat shock proteins. HSF2 binds to these proteins as some cells differentiate (Gordon et al., 1997). HSF proteins contain a helix–turn–helix class DNA binding domain, and their binding state and the reversible activation of stress responses are each regulated by free hsp70 family proteins.

Stress responses are not uncommon in intact mammalian embryos. Those newly placed in culture have a heightened uptake and synthesis of lactate, a reduced oxidative metabolism and enhanced aerobic glycolysis (Gardner and Leese, 1990). Human blastocysts exposed to glucose-deficient medium increase their pyruvate consumption (Conaghan et al., 1993). 2-cell mouse embryos growing \textit{in vitro} synthesize five times more hsp70.1 transcripts than in those developing \textit{in vivo} (Christians et al., 1995). The phenotypic expression of liver proteins was modified in two out of 40 mouse blastocysts growing \textit{in vitro} (Reik et al., 1993). In-vitro culture and addition of factors such as the reducing agent superoxide dismutase influence oxidative stress and the transcriptional inhibition of hybrid transgenes in early development. Micro-manipulation and disaggregation may induce this form of stress and activate gene expression (Vernet et al., 1993).

Nuclear cloning could impose even greater modifications on the transferred nucleus, as witnessed by its extensive remodeling, gene reprogramming and premature chromosome condensation, nuclear swelling and disappearance of nucleoli. Success in nuclear cloning involves the precise coordination of cell cycle stage, state of differentiation of the transferred nucleus and source of ooplasm (Gurdon, 1964; Chastant et al., 1996). Inactive genes are activated, possible including a transcription requiring complex (TRC) in mice (Conover et al., 1991). Four gene classes, non-specific alkaline phosphatase, \textit{TNap}, the transcription factors \textit{Oct3/4}, \textit{c-kit proto-oncogene} and DNA methyltransferase \( \textit{Mt} \), characterize early pluripotent embryonic cells derived from mouse gonial cells, and may be essential for these nuclei to retain totipotency (Urven et al., 1993).

Perhaps cellular cloning in mammals involves stress responses and release of heat shock proteins, as in plants and lower animals. They may even display homoeotic transformation and other phenomena, since many cell-cycle genes display homologies from fission yeasts to sea urchins, amphibians and mammals.

**Differentiation of ICM, germline and trophectoderm**

**Cell numbers in embryonic cell lines**

At least three distinct cell lines, ICM, germline and trophectoderm, have differentiated by the late blastocyst in mammals. Their lineages are shown in Figure 18. The timing, maternal or zygotic control and nature of differentiation pose major questions for an understanding of early development in mammals. The first two blastomeres in mammalian embryos are clearly distinct as shown by size (Lewis and Wright, 1935), the meridional division of the first-dividing blastomere as compared with an equatorial division in its sister, and by the tendency of descendants of the first-cleaving blastomere to colonize ICM preferentially (Kelly et al., 1978). Distinct patterns of calcium discharge in the two blastomeres may represent signals from two distinct cell lines, or merely be a consequence of their asynchronous division (Figure 19) (Sousa et al., 1996). We suggest below that this separation of function at the 2-cell stage is an essential step in primary differentiation and formation of a cell line differentiating into germline and trophectoderm.

Preimplantation development takes 5–8 days in mice and slightly longer in humans. By day 3.5 post-coitum, >50% of mouse embryos are early blastocysts, 25% are expanding blastocysts and others are still morulae (Handyside and Hunter, 1986). Diploid embryos have passed through a mean of 5.5 cleavages (Table IV) (Edwards, 1958). Mouse trophectoderm grows quickly, with an extra cell division as compared with ICM. Cell cycles may be as brief as 6 h in trophectoderm of 16–32 cell embryos (Handyside and Hunter, 1986; Fleming, 1987). The mean duration of the cell cycle is 17.5 h on average, while ICM cells retain a 24 h cleavage interval over this entire period (Handyside and Hunter, 1986; Fleming, 1987). Fleming’s (1987) estimates of relative cell numbers in mouse embryos between the 30-and 39-cell stage suggest that trophectoderm has a cell cycle of ≤8 h, whereas cycle length
leukocyte antigen (HLA)-F region (Xu et al., 1993), but is not specific to trophectoderm in mice. The Qa-2 antigen product is bound to the cell membrane of mouse embryos by glycosylphosphatidylinositol (GPI) linkage (Tian et al., 1992).

In human embryos, blastulation begins during the fourth and fifth cleavage divisions. Newly-expanded human blastocysts possess $58.3 \pm 8.1$ cells on day 5, with a minimum of 24–27, rising to $125.5 \pm 19.0$ on day 7. Exceptional blastocysts can possess 130 or more blastomeres by day 5.5 (Steptoe et al., 1971) and 283 by day 7 (Hardy et al., 1989).

Cleavage rates seem to be higher in human trophectoderm than in ICM, just as in mouse embryos. Trophectoderm doubles on day 5 then remains constant on days 5 and 6 (~40 per embryo). ICM doubles ~12 h later, between days 5–6 (to ~40), and is unchanged on day 7 (Figure 20) (Hardy et al., 1989). During these stages of growth, trophectoderm thus increases more rapidly than ICM, by at least one-half of a cleavage division. Cleavage rates slow down as cell death and giant cell formation begin on day 7 in later human blastocysts developing in vitro (Hardy et al., 1989).

**The inside/outside hypothesis: blastomere position and compaction**

Early differentiation in eutherian mammals has seemed to differ from that of all lower animals. According to the inside/outside hypothesis, inner and outer cells initially separate at the 8-cell stage, at least in mice, preparatory to the formation of ICM and trophectoderm (Tarkowski and Wroblewska, 1967). Individual 8-cell blastomeres polarize at this stage, and are allocated to ICM or trophectoderm although not finally committed until the 16–32-cell stage or later. Blastomeres remain totipotent until the morula.

Analyses on blastomere fate from the earliest stages of differentiation in eutherian mammals are quoted in support of this hypothesis. Inner cytoplasm of 2-cell embryos may colonize inner cells, and outer cytoplasm trophectoderm (Wilson et al., 1972). The first-dividing 2-cell mouse blastomere contributes preferentially to inner cells at 16-cell stages, and possibly to ICM in blastocysts (Graham and Duessen, 1978;
Surani and Barton, 1984; Garbutt et al., 1987). Since all blastomeres have similar cleavage rates after the 2-cell stage, the leading blastomere sustains its initial advantage over neighbouring cells during succeeding cycles (Kelly et al., 1978). Early dividing cells contribute up to 75% of the total of inner cells when mouse embryos are reconstructed (Garbutt et al., 1987), and a second wave of colonization from outer cells at the 32-cell stage is reported to complete ICM. Allocation to inner cells, and possibly to ICM, may be reserved to the four 8-cell blastomeres which establish most contacts with sister blastomeres, as assessed by labelling individual 2-cell blastomeres with oil droplets (Graham and Duessen, 1978). Single blastomeres of 2-cell, 8-cell, and 16-cell embryos and late morulae (22–32 cells) labelled with horseradish peroxidase colonize inner and outer cells, and ICM and trophectoderm in blastocysts. Such dual contributions were found in 95, 58, 44 and 35% of the resulting blastocyst in yolk sac sac epithelium near base of allantois. 9–12 days, gonadal ridges, promordial germ cells migrate via dorsal gut mesentery and now number 5000.

The validity of studies utilizing reconstructed embryos (Garbutt et al., 1987; Kelly, 1978) was questioned by Sutherland et al. (1990), who used time-lapse cinemicrography of intact embryos to affirm that cleavage planes in the fourth cleavage are not connected with lineages from the 2-and 4-cell stages. Conflicts have also arisen in interpreting cell lineages of 16-cell and later mammalian embryos. After this stage, inner cells in morulae are, on the one hand, widely believed to produce inner and outer cells, while outer cells form trophectoderm (Fleming, 1987). These allocations apparently begin during the fourth and fifth divisions (Handyside, 1981), when ICM doubles and trophectoderm increases four-fold. Other investigators insist that some inner cells contribute to trophectoderm, and some outer cells to ICM (Cruz and Pedersen, 1985; Pedersen, 1986; Winkel and Pedersen, 1988). Conclusions on cross-colonization have been questioned by Gardner (1996b), since measurements of cell movements probably interfered with normal cell division. Virtually no cross-colonization was found by Dyce et al. (1987) using fluorescent microspheres as tracers in mouse embryos containing 44–65 blastomeres.

Vital dyes have been widely used to label blastomeres and trace cell fate. The marker must be restricted to the original cell and its descendants. Full video recordings and clear-cut data are essential to ensure that silicone or horseradish peroxidase are stable, do not interact with embryonic tissue, and are not redistributed to other cells. Silicone droplets could enlarge a blastomere, alter normal development or distort division planes and embryonic fate.

A second aspect of the inside/outside hypothesis concerns the emphasis placed on the third and fourth cell cycles in mouse embryos, when surface polarization occurs prior to compaction. Polarization and its significance has been studied in intact embryos and in single or pairs of blastomeres which were disaggregated cells in vitro. During the fourth cell cycle in mice, i.e. from the 8-cell stage, blastomeres ‘seem to be programmed to to change their morphological and functional phenotype from radially symmetric to polarized’ (Johnson and Maro, 1986). Terms such as ‘covert polarity’ (Johnson and Maro, 1986) describe conditions in presumptive apical regions as cells polarize in 8-cell blastomeres 3–5 h after their entry into the third cleavage division. Polarized cell divisions control the distribution of various cell types into inner and outer regions of 16-cell mouse embryos, ready to form inner and

### Table IV. Differentiation stages in mouse embryos

<table>
<thead>
<tr>
<th>Time</th>
<th>Stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 h</td>
<td>spermatozoa in perivitelline space</td>
</tr>
<tr>
<td>4.5 h</td>
<td>pronuclei form</td>
</tr>
<tr>
<td>25 h</td>
<td>first cleavage division²</td>
</tr>
<tr>
<td>1–3.5 days</td>
<td>successive cleavage divisions</td>
</tr>
<tr>
<td>3.5 days</td>
<td>diploid embryos average 5.5 cleavages², 20% morulae, 50% early blastocysts, 25% expanding blastocysts</td>
</tr>
<tr>
<td>4–4.5 days</td>
<td>enlarging blastocyst in uterine crypt, primitive endoderm differentiates in epiblast and forms parietal and visceral endoderm of the visceral yolk sac</td>
</tr>
<tr>
<td>5 days</td>
<td>egg cylinder, embryonic and extraembryonic ectoderm, proximal and distal endoderm</td>
</tr>
<tr>
<td>5–6 days</td>
<td>proamniotic cavity, trophoblast giant cells, embryo elongating</td>
</tr>
<tr>
<td>6.5 days</td>
<td>mesoderm, primitive streak, anteroposterior axis in egg cylinder and proamniotic cavity, amniotic folds, exocoelom</td>
</tr>
<tr>
<td>7–7.5 days</td>
<td>allantoios, yolk sac, &lt;100 promordial germ cells in yolk sac epithelium near base of allantois</td>
</tr>
<tr>
<td>9–12 days</td>
<td>genital ridges, promordial germ cells migrate via dorsal gut mesentery and now number 5000</td>
</tr>
</tbody>
</table>

²Edwards and Gates (1959); Snell and Stevens (1966).

---

**Figure 20.** Histogram relating total cell number in human blastocysts growing in vitro at the fifth, sixth and seventh days post-fertilization to the numbers (± SD) of inner cell mass cells (□) and trophectoderm (□) (modified from Hardy et al., 1989).
outer cell lines in morulae and blastocysts (Johnson and Ziomek, 1981a,b, 1983; Ziomek and Johnson, 1982).

Polarization at the 8-cell stage depends on Ca$^{2+}$ and cell contacts. Contact-dependent membrane polarization reflects earlier polarities in cells of 2-, 4- and early 8-cell embryos. It may also involve the exclusion of myosin, actin and an endoplasmic reticulum antigen from subcortical cytoplasmic zones adjacent to membrane apposition, which influence calcium-dependent cell adhesion (Graham and Duessen, 1978; Handside, 1981; Johnson and Ziomek, 1981a). Other factors determining polarity, identified by applying various inhibitors, include cytoskeleton, actin, clathrin and endosomes spreading from regions of intracellular contact. Isolated cell pairs divide at right angles to the position of cell contact and cross the axis of polarity (82%) (Figure 21) (Johnson and Maro, 1986). Cytocortex initiates polarization, and gap junctions are not involved. Microtubules maintain polarity once established. Differences between polar and apolar cells are pronounced as 16-cell embryos form zonular tight junctions and desmosomes (Ducibella et al., 1975). Daughter cells can rapidly re-polarize after the fourth cleavage, but only if they inherit some polar cortex from their 8-cell progenitor. At this stage, actin, clathrin and endosomes accumulate apically, cell nuclei migrate basally and outer cell surfaces express microvilli, probably sustained by the greater adhesion at the basolateral surface. Acetylated microtubules accumulate preferentially in inner cells (Houliston and Maro, 1989). Compaction is timed by an embryonic clock which is independent of DNA synthesis and cleavage times, and is possibly regulated by proteins synthesized in 2- and 4-cell stages. Trophoblast cells pass through three differentiation stages: non-polar, establishment of cell polarity and formation of a true epithelium, and cell flattening in this tissue may be related to polarity.

Later investigations on intact embryos showed how the nature of polarized cell divisions was more complex than had been described earlier. Video films of intact cleaving mouse embryos revealed three types of cleavage plane, periclinal, oblique and anticlinal, and early-dividing 8-cell blastomeres tended to periclinal or oblique divisions (Sutherland et al., 1990). Periclinical cleavage would instantly separate inner and outer descendants, as in 8-cell P. lividus embryos, but it is uncommon in mice so that blastomeres of 16-cell embryos retain their variability (Sutherland et al., 1990). It is not clear if a specific pattern characterized particular blastomeres. One model based on the frequency of these cleavage planes predicted too few inner cells.

We analyse the implications of the inside/outside hypothesis at the conclusion of this review. It is important to stress that all these observations on cleaving embryos and blastocysts could equally well be interpreted in alternative hypotheses of early mammalian differentiation. These large amounts of data provide clear insights into the properties of embryos and their constituent cells in vitro, or about cells passing through compaction. There is no certainty that these properties are causative in establishing embryonic axes and cell lines prior to compaction. The central embryological question concerns the manner and means of organizing early differentiation, and this is not answered in these studies.

Genetic regulation of ICM

At least two groups of transcription regulators, the oct-4 and hox families, seem to effect the differentiation of ICM. Both contain DNA binding homeodomains, with wide homologies as illustrated by the POU domains consisting of 150–160 amino acids present in unc-86 in C. elegans and in oct genes in mammals. These DNA-binding proteins regulate transcription by recognizing the octamer ATGCAAAT (Herr

Figure 21. Membrane polarization visualized by fluorescein isothiocyanate–concanavalin A (FITC–Con A) binding in mouse 1/8 blastomeres which were aggregated in vitro. Polarization has occurred 7 h after aggregation in aggregates of (A) two and (B) three blastomeres. Note the relationship between the orientation of polarity and the regions of cell contact (magnification ×580). Courtesy of M. Johnson and C. Ziomek and reproduced from Journal of Cell Biology (1981), 91, 303–308.

Figure 22. Oct-4 expression in mouse embryos. (A and B) Staining of oct-4 protein using an immunofluorescent antibody. (A) A 2-cell and (B) 8-cell mouse embryo of 48.9 µm showing oct-4 protein expression in 5/8 blastomeres. (C) Morula stage showing labelled and unlabelled cells. (D) A blastocyst at day 3.4, and (E) blastocyst, day 3.6 both showing many labelled and unlabelled nuclei. (F) Blastocyst, day 4.5 post-fertilization, showing higher oct-4 expression in newly-formed primitive endoderm than in surrounding embryonic disc; trophoderm is negative. (Courtesy of Palmieri et al. (1994) and Developmental Biology. (G) Brightfield image of a sagittal section of a genital ridge of an 11-day embryo stained for germ cells using alkaline phosphatase. (H) Similar dark field section of the genital ridge hybridized for oct-4 expression. Courtesy of H.R. Scholer et al. and reproduced from EMBO Journal (1990) 9, 2185–2195.

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The gene fgf4 is also an important regulator of ICM, since its gene targeting impairs the formation of this tissue (Feldman et al., 1995); it may activate homeobox genes in frog embryos (Ruiz i Altaba and Melton, 1989).

Oct-4 expression provides fundamental information about the origins of ICM, germline and trophoderm. Oct-4 is transcribed in fertilized oocytes and early cleavage stages. It differs from oct-6 which first appears in late cleavage human embryos.

Figure 22.
and blastocysts (Abdel-Rahman et al., 1995; Scholer et al., 1989a,b). The oct genes map with the I locus in mice, and it is well known that homozygous \( t_{12} \) and \( t_{22} \) mouse embryos fail to form blastocysts on day 3 (Magnusson and Epstein, 1987).

An examination of published illustrations (Figure 22) (Palmieri et al., 1994) indicates that \( oct-4 \) protein is expressed in five blastomeres of 8-cell mouse embryos and many cells of ICM, but is completely down-regulated in putative trophectoderm (Table V). Equally fascinating, \( oct-4 \) protein expression becomes restricted to a few cells of the ICM at day 3.5, and to nuclei of primary (primitive?) migratory endoderm at day 4.5 post-fertilization (Figure 22E and F). Its expression appears to be upregulated in these endodermal nuclei as compared with those of embryonic disc, which has differentiated from ICM (Palmieri et al., 1994). Later in development, \( oct-4 \) transcripts are restricted to primary ectoderm and then to primordial germ cells in extra-embryonic mesoderm (see Figure 21G and H) (Scholer et al., 1990).

Among the homeobox genes expressed in non-mammals including Drosophila, eight \( Hox \) genes clustered in the complexes Antennapedia (Ant-C head and thorax) and bithorax (Bx-C abdomen) control specific segmentation along the A/P body axis. Each gene contains a homeobox and regulates the spatial control of successive body segments (Favier and Dole´ , 1997). Corresponding \( Hox \) genes, containing an Antennapedia-related homeobox, form 13 groups in vertebrates. Anterior \( Hox \) genes (\( Hox A \) and \( Hox B \)) regulate specific hindbrain segments and neural crest derivatives, while posterior genes (e.g. \( HoxA \) and \( HoxD \)) regulate growth and morphogenesis of skeletal structures along proximo-distal axes of the developing limb. \( Hox \) genes establish spatially-ordered patterns of gene expression along body axes by generating the expression of regulatory transmission factors (Slack et al., 1993; Davidson et al., 1995). They are remarkable in their successive but restricted activation and expression along the rostrocaudal axis in Drosophila and mice. They also apportion undifferentiated, mitotically-active, uncommitted cells into zones for specific embryonic fields, illustrated by the actions of \( HOX-1 \) in haemopoietic cells (Vieille-Grosjean et al., 1992; Shen et al., 1993; Suavageau et al., 1995; Duboule, 1995). In mammals, \( Hox \) transcripts identified in human oocytes might have a primary role in embryonic differentiation (Verlinsky et al., 1995). In embryos, transcripts may be expressed initially in differentiating extraembryonic endoderm, as in outer cells of embryoid bodies derived from mouse EC cells (Labovsky et al., 1993). They are expressed at gastrulation and in the primitive streak, perhaps under the control of a signal (‘morphogen’) responsible for the first-onset \( Hox \) genes (Gaunt, 1987; Deschamps and Wijgerde, 1993; Gaunt and Strachan, 1994; Favier and Dole´ , 1997). Their molecular structure gives no indication of any link to factors regulating primary axes (P.Dole´ , personal communication).

---

### Table V. \( oct-4 \) expression in mouse embryos

**A. \( oct-4 \) protein in cleaving mouse embryos, morulae and blastocysts**

<table>
<thead>
<tr>
<th>Stage</th>
<th>Unstained cells (n) (^a)</th>
<th>Cells stained strongly (n)</th>
<th>Location and intensity of staining</th>
<th>Blastomeres</th>
<th>Inner cells/ICM</th>
<th>Primitive endoderm</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-cell</td>
<td>3(^b)</td>
<td>5/8</td>
<td>weak</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>8-cell</td>
<td>8(^b)</td>
<td>10/18</td>
<td>5/8</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Morula</td>
<td></td>
<td></td>
<td>8</td>
<td>8–9</td>
<td>8–9</td>
<td>8</td>
</tr>
<tr>
<td>Blastocyst</td>
<td></td>
<td></td>
<td>10</td>
<td>2–3</td>
<td>2–3</td>
<td>9–10</td>
</tr>
</tbody>
</table>

\(+ + = strong; + = weak; – = absent.\)

\(^a^\)Negative cells assumed to be trophectodermal.

\(^b^\)May include a few inner cells.

Data based on cell counts from illustrations in Palmieri et al. (1994).

**B. Transcripts and \( oct-4 \) protein at various embryonic stages**

<table>
<thead>
<tr>
<th>Stage</th>
<th>Transcripts (^a)</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oocyte</td>
<td>weak (^b)</td>
<td>weak, diffuse ( DE-oct-4 ) in inner cells and ICM</td>
</tr>
<tr>
<td>1–32 cell and morulae</td>
<td>( DE-oct-4 ) in inner cells and ICM</td>
<td>some nuclei down-regulating in ICM strong staining in primitive endoderm</td>
</tr>
<tr>
<td>Expanding blastocyst</td>
<td>( PE-oct-4 )</td>
<td>down-regulation</td>
</tr>
<tr>
<td>Genital ridge</td>
<td>( DE-oct-4 ) promordial germ cells only</td>
<td></td>
</tr>
</tbody>
</table>

\(^a^\)Data from Yeom et al., 1996.

\(^b^\)Relative to \( oct-5 \) (Scholer et al., 1990).
Natural and experimental factors regulate many aspects of embryonic growth. Imprinting is an essential part of early embryonic growth (Rappollee et al., 1992). The targetting of the imprinted gene IGFII reduces birth weight in mice (De Chiara et al., 1990). Embryonic expression of IGFII depends on methylation of chromatin during oocyte growth (Kono et al., 1996). Disorders in embryonic growth have been induced experimentally, to disturb timing or allocation in early embryos. In mice, growth in pronucleate eggs is not impaired by grafts of synchronous cytoplasts, whereas those from dictate or metaphase II oocytes disrupt the cell cycle (Levron et al., 1996). Substituting maternal pronuclei by the donation of a maternal pronucleus of a different genotype causes transcriptional repression, DNA methylation and fetal growth retardation in recipient embryos even though they are diploid and contain one paternal and maternal pronucleus (Norris et al., 1990; Reik et al., 1993). Exchanges of male and female pronuclei between pronucleate eggs can distort growth in the resulting mouse androgenones and gynogenones (Norris et al., 1990). Biparental androgenesis distorts the growth of embryonic tissues but not trophoblast by day 10 post-fertilization. The ICM but not trophoblast is evidently normal in biparental gynogenones. The conclusion that paternal transcripts regulate trophectoderm and maternal transcripts regulate ICM can be questioned since these results may be affected by the stress of the operation or by slight developmental differences between donor and recipient. Fusions of synchronous nucleoplasts and unipronucleate human eggs sustain growth to diploid blastocysts (Azambuja et al., 1996). 

Genes affecting embryonic axes can be detected through the actions of teratological agents such as lithium invoking heritable alterations in timing or positional markers in mouse 2- or 8-cell embryos carrying Brachyury (Rogers and Varmuza, 1996). Mutations in regulator genes containing RNA binding domains invoke similar developmental defects in widely-separated species, e.g. KH domains of mex3 in C. elegans and fragile X in humans. Fragile X syndrome involves a reduced RNA binding activity in FMRL mutants (Smioti et al., 1994) while FMR1 regulates tissue-specific processing of transcripts from gene(s) involved in development (Gibson et al., 1993).

**Formation of germline**

Debate has been protracted on the allocation of germline and its developmental restriction to primordial germ cells. Some authorities champion a very early origin, even in early cleavage stages, based on analyses of the expression of natural or induced gene mutations in mosaic animals, or the relative proportions of two cell lines in tissues of fusion chimeras. Mintz and Russell (1957) utilized mutation frequencies to calculate that germline precursors numbered as few as ten, and Mintz (1974) later postulated the existence of 2–9 precursor cells from analyses on fusion chimeras. Similar conclusions were drawn by Wilkie et al. (1986) from studies on transgenic mice established by pronuclear DNA injections.

The numbers of founder cells allocated to ICM, trophectoderm and germline, and the timing of allocation, were derived from analyses on mouse embryos infected in vitro between the 4- to 16-cell stage with variants of a recombinant retrovirus (Soriano and Jaenisch, 1986). The incorporated viral sequences genetically marked individual blastomeres, and did not interfere with development, especially as the virus remained silent in most tissues. Descendants of individual founder cells labelled with distinctive viral markers could be identified in tissues of the offspring (Table VI). This approach overcomes difficulties encountered with disaggregation and in-vitro techniques or the use of fusion chimeras to estimate numbers and fate of founder cells. In chimeras, the two parental cell lines may not mix freely, and complications can arise due to effects on embryo size, the selective survival of cells of one parental genotype, and the potential effects of stress during embryo disaggregation and tissue culture of germlinal and somatic cells (Soriano and Jaenisch, 1986). This study would have been best carried out at the 2-cell stage, to mark the very earliest blastomeres. The duration of provirus integration over 1–2 days introduces some bias into the estimates.

Soma and germline, and ICM and trophectoderm, were shown to originate from different founder cells in the early embryo (Soriano and Jaenisch, 1986). The respective origins of germline and trophectoderm were not compared. The relative contribution of virally labelled cells in various tissues estimated their number of founder cells (Figure 23). At most, the embryo proper was formed from eight founder cells which intermingled freely and lost equal proportions of their descendants through cell death. Enough descendants were produced before allocation to ensure an equal contribution to all tissues. Germline was established from two or three cells, set aside before allocation to somatic cell lines (Soriano and Jaenisch, 1986). This study was criticized by Lawson and Hage (1994), since multipotent primordial germ cells in extraembryonic mesoderm could have been overlooked and may have contained some extra viral markers. This criticism seems to be unjustified because germline and placenta were shown to originate independently of soma (Soriano and Jaenisch, 1986).

Other investigators propose a much later differentiation of germinal cells, and suggest that they arise de novo from other primary tissues. Thus, McMahon et al. (1983) reported that the combined precursor pool for mouse germline and germ layers numbers just below 200 at 4.5–5.5 days post-coitus. A late onset of restriction of germline was deduced from analyses on fusion chimeras derived from injections of single cells into the blastocoelic cavity of mouse blastocysts. At least two cells

<table>
<thead>
<tr>
<th>Founder animal</th>
<th>Somatic mosaicism</th>
<th>Germine mosaicism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female B</td>
<td>17</td>
<td>18</td>
</tr>
<tr>
<td>Female C</td>
<td>0</td>
<td>19</td>
</tr>
<tr>
<td>Male J</td>
<td>31, 32, 33</td>
<td>29, 30</td>
</tr>
<tr>
<td>Female N</td>
<td>39</td>
<td>40</td>
</tr>
</tbody>
</table>

Numbers refer to provirus numbers identified in each animal. For 14/37 proviruses, there was no correlation between contribution to the germline and somatic tissues. The above four animals showed the most notable divergence in which genetically transmitted viruses were not present in somatic tissues. The germline of animal J must have been derived from at least three founder cells, one infected with virus 29, one with virus 30 and one uninfected (Soriano and Jaenisch, 1986).

Table VI. Virally-induced mosaicism and primary differentiation in mouse embryos
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Figure 23. Retroviral mosaicism in mice: mouse 4–16 cell embryos were infected with different retroviruses by co-cultivation with virus-producing cells. The intensities of 30 different proviruses detected in mosaic animals were compared with the same provirus found in heterozygous offspring. Relative intensities (‘molarities’) in mosaic tissues varied from 1.0 to 0.12 and control experiments indicated that a relative intensity of 0.06 per tissue was detectable by autoradiography. This evidence indicated a minimum contribution of 1/8 virally infected cells to mosaic tissues. A given virus had equal intensities in all somatic tissues of 14/16 animals. Since placentas were not analysed, this data could have been derived from infection and incorporation of virus into a single cell until the 16-cell stage. It suggests that somatic tissues develop from eight founder cells in the embryo. (Courtesy of P.Soriano and R.Jaenisch and reproduced from Cell (1986), 46, 19–29. Copyright held by Cell Press.)

were identified in day 5 primitive ectoderm as being multipotent and capable of differentiating into both somatic and germinal cells (Gardner et al., 1985). Likewise, the use of a fluorescent dextran marker to mark individual cells in organ cultures of mouse epiblast cells at 6.0 or 6.5 days post-coitum demonstrated that germinal cells could produce germline and extra-embryonic mesoderm after 40 h in culture (Beddington and Lawson, 1990; Lawson and Hage, 1994). This experiment demands that the fluorescent marker was injected into only a single cell, is stable over several cell cycles, distributes equally to daughter cells at mitosis, and that differentiation patterns are fully normal in organ culture. It is debatable whether these conditions were met.

Oct-4 provides essential information on the formation and maintenance of germline. Its differential expression in precursors of germline and ICM is achieved by the actions of different enhancers in the early mouse embryo (Figure 22E and F). Factors regulating its expression in undifferentiated stem cells, germline and other tissues of early embryos, and in ES (resembling ICM), EC (epiblast) and EG (germ) cell lines, have been analysed in transgenic mice carrying oct-4 fragments of varying lengths. Individual plasmids were constructed consisting of diverse oct-4/lacZ transgenes containing two different enhancer regions, a distal enhancer (DE) and a proximal enhancer (PE). Each enhancer was active in specific cell lines and at particular developmental stages (Yeom et al., 1996), although both of them operated through the same promoter in ES and EC cells (Yeom et al., 1996).

Analyses of DE-regulated oct-4 expression revealed its action in cleaving embryos, morulae, blastocysts, EG and ES cells, and also in the germline. When deletions were inserted at identified sites in DE, its expression was totally abolished in ES cells. DE also regulated oct-4 expression in germline, and its final restriction to primordial germ cells in the genital ridge. This enhancer is therefore a line-specific regulator of oct-4 expression in the germline. Curiously, it was apparently inactive over a brief period in germline of pre-gastrulae, despite the presence of primordial germ cells in this tissue (Yeom et al., 1996). These authors write ‘perhaps a very small population of cells still uses the germline enhancer at this stage, i.e. soon after implantation, but we cannot detect them’ (Yeom et al., 1996). They propose that a third enhancer region, omitted in their constructs, may control oct-4 expression at this stage. Alternatively, oct-6 could be active in germline during this and other stages, since it is expressed in human embryos between day 3 and the blastocyst stage (Abdel-Rahman et al., 1995).

In contrast, PE has no obvious role in cleaving embryos morulae and blastocysts. Its primary function is to enhance oct-4 expression in epiblast, where DE is apparently inactive. The ablation of PE extinguishes oct-4 expression in epiblast and EC cells. PE is thus stage-specific at day 6.5, and its activity together with the expression of HOX genes in the gastrula may be regulated by retinoic acid. The later downregulation of oct-4 transcription and expression in epiblast could be due to the down-regulation of its promoter (Yeom et al., 1996).

Oct-4 protein has been proposed as a regulator of germ cell migration through primitive endoderm along the trophoblastic surface (Palmieri et al., 1994). This role would be totally distinct from its actions in primitive endoderm where it may be regulated by the PE enhancer (Scholer et al., 1990). DE-regulated oct-4 is germline-specific, although not necessarily a primary determinant, since it may regulate functional requirements in germ cells. Clearly, oct-4 may have various functions at different embryonic stages, with both DE and PE being active simultaneously in different cell lines at certain developmental stages.

The presence of oct-4 in germline cells in primary endoderm accords with the identification of germline precursors in epiblast (Gardner and Papaioannou, 1975), and with the later presence of mammalian germinal cells in extra-embryonic mesoderm, as identified by alkaline phosphatase staining and the expression of oct-4 (Ginsburg et al., 1990; Scholer et al., 1990). Mouse primordial germ cells arrive at the base of the allantois in 8.5 day embryos and in the genital ridge of the developing gonad by day 11, still expressing oct-4 (Scholer et al., 1990). At this stage, all primordial germ cells are linked by long cytoplasmic threads to form an extensive network of cells which cluster as they enter the genital ridge (Gomperts et al., 1994).

Initially, 100 alkaline phosphatase positive primordial germ cells were estimated to be present in epiblast at 7.2 days postcoitum, proximal to extraembryonic ectoderm (Ginsburg et al., 1990). This estimate was later reduced to 45, since many alkaline phosphate-positive cells were evidently not germ cells (Lawson and Hage, 1994). Germline has therefore undergone approximately five to six cell cycles by this stage. Assuming
a cell cycle of 17.5 h, and a foundation population of 50 germ line cells by day 7.2, a single germline cell would begin dividing at the 128-cell stage (~4 days post-coitum). These estimates lack precision since division rates are variable, and germline may originate from more than one cell. A cell cycle as short as 5 h is needed to explain the presence of 500–600 cells in the egg cylinder at gastrulation, and further increases to 12–15,000 in 24 h and to 26,000 at day 13 when doubling time becomes 16 h (Snow, 1977).

**Regulation of trophectoderm**

Analyses of virus-induced mosaicism and of marked blastomeres of early mouse embryos, discussed previously, show how trophectoderm and ICM originate from different founders (Graham and Duessen, 1978; Soriano and Jaenisch, 1986). Trophectoderm could thus differentiate very early in cleavage. Once differentiated, putative trophectoderm cells must accomplish a totally different task to that of ICM. They must differentiate rapidly to prepare for implantation, by translating many genes regulating membrane polarity and cell architecture, growth factors, basement membrane substances, integrins and other cytokines and their receptors (Edwards, 1995; Edwards and Brody, 1995).

The rapid differentiation of trophectoderm may be initiated by a mixture of maternal and embryonic transcripts at the onset of major embryonic transcription in 4- or 8-cell mouse embryos (Braude et al., 1988). Hxt codes for a helix-loop-helix transcription factor and could be such a regulator, expressed in differentiating mouse trophectoderm during early cleavage. It may invoke changes in cell adhesion and the formation of giant cells. Maternal Hxt transcripts are found in 4- or 8-cell mouse embryos where they may commit some blastomeres to trophectoderm (Cross et al., 1995). It would be of interest to know if Hxt is expressed in the three blastomeres in 8-cell embryos which do not express oct-4 protein (Palmieri et al., 1994), since this would enable a sharp demarcation to be made between ICM and trophectoderm precursors. Hxt transcripts are increasingly expressed in morulae, trophectoderm and placenta (Cross et al., 1995).

Early regulatory genes could provide valuable markers to trace the origins of this cell line. The expression of various genes in developing trophectoderm is shown in Table VII. Most are concerned with household genes, and/or display no lineage specificity. Amongst those with some specificity, TGF-β2 is expressed in mouse embryos from the 4-cell stage, tends to stain outer cells in morulae and segregates into trophectoderm where it stains intensely while ICM is negative (Slager et al., 1991). It could be an excellent marker of differentiation in 8–16-cell stages and in morula, but has not been examined in any detail for this purpose. Post-implantation, it is expressed in embryonic and extra-embryonic visceral endoderm but not in embryonic or extraembryonic ectoderm. The gene Pem is a homeobox gene which is X-linked, and first expressed in compacted morulae and blastocysts where it is not lineage-specific. It is specific for ectoplacental cone by 7–8 days as it down-regulates elsewhere (Lin et al., 1994). The gene Ped has two alleles which affect cleavage rates in preimplantation mouse embryos, fast and slow, linked to the Q region of the MHC class I b histocompatibility genes, and which differ in the expression of the Qa-2 antigen (Warner et al., 1998). Its differential expression in individual blastomeres is not known.

Functional genes regulating trophectodermal activities are activated in early cleavage. A gene encoding for endo A, a trophectodermal cytokeratin, is expressed in 8-cell mouse embryos then down-regulated in ICM precursors (Duprey et al., 1987; Chisholm and Houliston, 1987). The HCG gene is transcribed in 8-cell human embryos, presumably from embryonic transcripts (Bonduelle et al., 1988), and the hormone is secreted from blastocysts from day 8 (Fishel et al., 1984).

Scant information is available on other maternal potential regulators of trophectoderm. Maternal transcripts of rad51 are expressed before the morula stage, and it may regulate many aspects of early embryogenesis, including gamete formation, the loss of totipotency and X-inactivation, although once again no cell-type specificity has been shown (Hayakawa et al., 1996). Zygotic transcripts may encode for xist regulation of preferential X-inactivation in trophectoderm (Goto et al., 1997). This gene is active on the inactive X, and not the active X, in female mouse blastocysts, and it regulates X chromosome activity in embryos (Brown et al., 1991). The 5′CpG island of the X-linked androgen receptor gene is methylated on the inactive X chromosome in chorionic villus of female conceptusses aged 10–12 weeks and hypomethylated on the active X in somatic cells (Goto et al., 1997). Rag genes induce rearrangements in T cell receptor and immunoglobulin (Ig) genes in immature T or B lymphocytes. First expressed in morulae, Rag-1 but not Rag-2 can modify chromatin structure and invoke genetic recombination (Hayakawa et al., 1996), but its expression could not be localized to either ICM or trophectoderm. The non-polymorphic HLA incompatibility antigen HLA-G, widely accepted as conferring immunological neutrality on trophoblast, is expressed in migrating extravillous cytotrophoblast where it may avert maternal immune responses from maternal natural killer (NK) cells (Hunt et al., 1991).

The formation of syncitia in cleaving embryos may indicate the onset of a premature trophectodermal differentiation. Their similar origin is confirmed when two nuclei in a blastomere or several in a syncitium each contain the same chromosomal complement as normal blastomeres (Munne and Cohen, 1993; Hardy et al., 1996). Syncitiotrophoblast is normally formed by the fusion of cytotrophoblast cells in implanted human embryos, and once formed the syncitial nuclei do not divide. Connexins apparently facilitate fusion via the ability of gap junctions to bring membranes close together, and HCG can up-regulate gap junctions and facilitate fusion (Cronier et al., 1994). Perhaps some human preimplantation embryos form connexins or secrete HCG prematurely.

**Cytogenetics of human preimplantation embryos: another sign of early trophectodermal differentiation?**

The diverse chromosome complements identified in preimplantation human embryos in vitro also indicates that the differentiation of trophectoderm could be initiated in the 2-cell stage. Among the considerable numbers of heteroploids, the formation of chromosomal mosaics in astonishingly high
Table VII. Reports of differential gene expression in early mammalian embryos

<table>
<thead>
<tr>
<th>Species</th>
<th>Gene</th>
<th>Unfertilized egg</th>
<th>2-cell</th>
<th>4-cell</th>
<th>8-cell</th>
<th>morula</th>
<th>blastocyst</th>
<th>ICM</th>
<th>TE</th>
<th>later expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>PDGF-A</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td>TGF-β</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td>E-cadherin</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td>TGF-β2</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>endoderm</td>
</tr>
<tr>
<td>Mouse</td>
<td>α-E cadherin</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>abembryonic pole</td>
</tr>
<tr>
<td>Mouse</td>
<td>fibronectin</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>ectoplacental cone</td>
</tr>
<tr>
<td>Mouse</td>
<td>Mem</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>endoderm</td>
</tr>
<tr>
<td>Mouse</td>
<td>cytot.endoA</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>abembryonic pole</td>
</tr>
<tr>
<td>Mouse</td>
<td>oct-4</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>ectoplacental cone</td>
</tr>
<tr>
<td>Mouse</td>
<td>hox</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>cleavage rate</td>
</tr>
<tr>
<td>Mouse</td>
<td>ped</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>trophoblast</td>
</tr>
<tr>
<td>Human</td>
<td>hcg</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Notes:
4. Torres et al. (1997), mutant form of E-cadherin which binds to and disrupts trophoderm leading to blastocyst collapse and death.
5. Schultz and Armani (1995), activity found at abembryonic pole where it associates with trophoderm and translocates integrins β1 and β3 to the apical surface.
6. Lin et al. (1994), unrelated to Hox genes, Mem is X-linked and shows nonspecific expression in blastocysts and later expression in ectoplacental cone at 7.5 days post-coitum.
7. Duprey et al. (1985), an X-linked cytokeratin endoA expressed in 8-cell embryos and later specific to endoderm and amnion. Associated with an active maternal X.
8. Palmieri et al. (1994), enhancers control its differential expression in cleaving embryos, inner cell mass, endoderm and germline.
10. Warner et al. (1997), fast and slow alleles regulate cleavage rates in preimplantation embryos.

Table VIII. Simple mosaicism in day 4 human embryos. Figures in parentheses are percentages

<table>
<thead>
<tr>
<th>Ages (years)</th>
<th>20–34</th>
<th>35–39</th>
<th>40–47</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Arrested embryos</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Limited DM 7 (23)</td>
<td>3 (7)</td>
<td>2 (8)</td>
<td>12 (12)</td>
<td>34 (34)</td>
</tr>
<tr>
<td>Extensive DM</td>
<td>3 (10)</td>
<td>25 (60)</td>
<td>6 (23)</td>
<td>34 (34)</td>
</tr>
<tr>
<td>Total DM</td>
<td>10 (32)</td>
<td>28 (67)</td>
<td>8 (31)</td>
<td>68 (46)</td>
</tr>
<tr>
<td>Total no. embryos</td>
<td>31</td>
<td>42</td>
<td>26</td>
<td>99</td>
</tr>
<tr>
<td><strong>Slow embryos</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Limited DM</td>
<td>12 (29)</td>
<td>17 (29)</td>
<td>9 (28)</td>
<td>38 (29)</td>
</tr>
<tr>
<td>Extensive DM</td>
<td>12 (29)</td>
<td>13 (22)</td>
<td>4 (13)</td>
<td>29 (22)</td>
</tr>
<tr>
<td>Total DM</td>
<td>24 (57)</td>
<td>30 (52)</td>
<td>13 (41)</td>
<td>67 (38)</td>
</tr>
<tr>
<td>Total no. embryos</td>
<td>42</td>
<td>58</td>
<td>32</td>
<td>132</td>
</tr>
<tr>
<td><strong>Good embryos</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Limited DM</td>
<td>17 (36)</td>
<td>13 (30)</td>
<td>16 (19)</td>
<td>46 (26)</td>
</tr>
<tr>
<td>Extensive DM</td>
<td>3 (6)</td>
<td>9 (20)</td>
<td>10 (12)</td>
<td>22 (12)</td>
</tr>
<tr>
<td>Total DM</td>
<td>20 (43)</td>
<td>21 (48)</td>
<td>26 (30)</td>
<td>68 (38)</td>
</tr>
<tr>
<td>Total no. embryos</td>
<td>47</td>
<td>44</td>
<td>86</td>
<td>177</td>
</tr>
</tbody>
</table>

DM = diploid mosaicism.

DM is defined as the occurrence of more than three out of eight cells in a diploid embryo being chromosomally abnormal. Limited mosaicism refers to the rest of the diploid mosaic embryos. Polyplloid and haploid embryos excluded from these figures.
frequencies in human embryos is particularly relevant. One example is the high frequency of monosomy or trisomy for chromosomes XY, 13, 18 and 21, as assessed in >500 embryos. Extensive diploid mosaicism was detected in 22/177 (12%) of ‘good’ diploid embryos, 29/132 (22%) of slow-growing embryos and 34/99 (34%) of arrested embryos (Munné et al., 1995). Total mosaicism, including embryos with fewer than three chromosomally abnormal blastomeres, arose in 38–46% of all embryos (Table VIII). Another analysis for mosaicism of chromosomes X/Y, 1 and 17 mosaicism in a total of 69 good-quality 6–10 cell embryos revealed 15% (5/34) sex chromosome mosaics, 30% (10/35) autosomal mosaics containing mostly diploid cells, and 15% (5/35) highly abnormal autosomal mosaics (Harper et al., 1995). A third analysis, on 47 human embryos of which 36 carried one or more multinucleated blastomeres, revealed 24 diploid mosaics (51%) and other more complex mosaics (Kligman et al., 1996). Overall, 15–40% of the embryos were diploid mosaics carrying a monosomic or trisomic cell line when classified on four of the 23 chromosome pairs. Of the normal 8-cell embryos, ~40% contained up to three cells with non-diploid chromosomes (Table VIII), but many more mosaic embryos, even approaching 80%, would be expected if all chromosome pairs were analysed.

Nuclear replication without cytokinesis, fragmentation of nuclei, defective chromosome movements at anaphase (Munné and Cohen, 1993) or the possible expression of rad51 or other recombination genes in ICM and trophectoderm could raise the risks of chromosomal non-disjunction. Putative trophectoderm cells may retain a persisting short G1 phase typical of early blastomeres (Hardy et al., 1990; Delhanty and Handyside, 1995), and display a failure of spindle regulation or other anomalies. Checkpoints at spindle formation detect anomalies of spindle structure or the alignment of chromosomes (Rudner and Murray, 1996). Non-diploid cells are normally destroyed at cell cycle checkpoints established by genes such as p53, Gadd45 and Bcl-xL. p53 is a sequence-specific transcription factor, acting via several genes including p21, a cyclin-dependent kinase inhibitor partially responsible for arresting cells in G1. Gadd45 stimulates DNA repair, produces a checkpoint in G1 and probably in G2/M, and is involved in apoptosis. These genes regulate DNA repair or invoke apoptosis (El-Deiry et al., 1993; Harper et al., 1993; Smith et al., 1994; Murray, 1995). Damage or mutations in p53 could raise cellular thresholds to destruction, while inappropriate bcl-2 expression may reduce sensitivity to apoptosis (Minn et al., 1996). A short G1 phase or a rapid rate of division could impair p53 and bcl-2 functions, lead to checkpoint failure and enable nondiploid multinucleated and fragmented cells to survive in ICM or trophectoderm (Hardy et al., 1990; Delhanty and Handyside, 1995).

Diploid cells characterize most human mosaics, with the frequency of aneuploid blastomeres varying from a few to ~50%. The diploid line apparently cleaves normally from the 2-cell stage, whereas a second cell line might risk becoming aneuploid at each successive division. The latter could be a precursor of trophectoderm, since many aneuploid cells characterize trophoblast in confined placental mosaicism (Ford, 1969; Warburton et al., 1978; Simoni and Fraccaro, 1992). In this case mosaicism could be a regular feature of normal development, and not an indication of severe anomalies in overwhelming numbers of human embryos.

Mosaicism could also be a consequence of multinucleation in many blastomeres of 2-cell and older embryos (Table IX; Figure 24) (Tesarik et al., 1987b; Hardy et al., 1993; Winston et al., 1991, 1993; Munné and Cohen, 1993; Munné et al., 1994, 1995; Pickering et al., 1995; Balakier and Cadesky, 1997). Easily distinguished from apoptotic cells in 8-cell embryos and blastocysts (Hardy et al., 1996), multinucleated cells synthesize less nucleoplasmic RNA and display fewer ultrastructural changes associated with transcription in comparison with single-nucleated blastomeres (Tesarik et al., 1987a). One-quarter of multinucleated cells in later cleavage stages arise from multinucleated precursors (Pickering et al., 1995), and disordered cytokinesis and cytoplasmic fragmentation progress to severe forms 48 h later (Zaninovic et al., 1996). Fragmentation or apoptosis in one cell line (Jurisova et al., 1996) need not be lethal to an embryo if a diploid core persists. Abnormal cells could be shed into the perivitelline space, discarded with the zona pellucida during hatching (Edwards and Surani, 1978; Hardy et al., 1989) or preferentially colonize trophectoderm (James and West, 1994).

Environmental factors or conditions in vitro may cause multinucleation and mosaicism (Pickering et al., 1995; Munné and Cohen, 1993; Munné et al., 1997). A low oxygen tension in follicular fluid could be another cause, as found in a study of >1000 embryos in which multinucleation was associated with oocytes taken from follicles with a low oxygen content (Van Blerkom et al., 1997). Nevertheless, certain multinucleate forms are evidently compatible with further development. Implantation fails only with those lowest-quality embryos having very few normal blastomeres (Plachot et al., 1989). Rare fully-multinucleated 2-cell embryos might develop to full term. Multinucleation afflicts many more (67%) 2-cell than 4-cell embryos (25%) (Balakier and Cadesky, 1997), and multinucleated day 1 embryos can become mononucleate on day 2 (Joris et al., 1997). Multinucleation occurred temporarily in 14 out of 36 (38.6%) 2-cell embryos examined for chromosomes X/Y and 18/18 at 24–48 h, and afflicted both blastomeres in 22 of the remaining embryos, which became chaotic mosaics. The other 14 embryos possessed at least one XX or XY,18/18 cell (Staessen et al., 1996). Multinucleation in 8-cell and older embryos does not involve abnormal forms of non-diploid development (Kligman et al., 1996; Pickering et al., 1995). There is no evidence that multinucleated cells are trophectoderm precursors.

Cytogenetic analyses of human embryos, and cytological, genetic and embryological evidence of an early separation of two cell lines, suggests that ICM and trophectoderm diverge early in cleavage. Excellent opportunities exist to verify this situation (see Addendum). Studies on human embryos in vitro are criticized because infertile patients may have various afflictions which could cause anomalous embryonic development. This objection cannot be sustained since the disorders are almost all entirely unconnected to ovary and embryo, and some fertile patients attend clinics.
Table IX. Proportions of embryos with one or more multinucleated blastomeres, and the proportions of blastomeres that were multinucleate. Figures given in parentheses are percentages

<table>
<thead>
<tr>
<th>Culture medium</th>
<th>Embryos Day 2</th>
<th>Blastomeres</th>
<th>Embryos Day 3</th>
<th>Blastomeres</th>
</tr>
</thead>
<tbody>
<tr>
<td>EBS + HIS</td>
<td>7/20 (35)</td>
<td>14/59 (24)</td>
<td>13/20 (65)</td>
<td>26/111 (23)</td>
</tr>
<tr>
<td>T6 + HIS</td>
<td>7/22 (37)</td>
<td>10/90 (11)</td>
<td>5/7 (71)</td>
<td>9/45 (20)</td>
</tr>
<tr>
<td>Universal IVF</td>
<td>7/32 (22)</td>
<td>10/109 (9)</td>
<td>9/23 (39)</td>
<td>15/144 (10)</td>
</tr>
</tbody>
</table>

Embryo type Day 4

- Arrestedd: 70/250 (30.4) 97/845 (11.5)
- Arrestedd: (47.2) (23.9)
- PGD rejects: 14/21 (66.7) 21/76 (27.6)
- Slowb: (45.6) (12.0)
- Goodb: (34.4) (9.9)

EBS + HIS = Earle’s balanced salts medium + 10% heat-inactivated patient’s serum; T6 + HIS = modified T6 medium + 10% heat inactivated patient’s serum; PGD = prenatal genetic diagnosis.

dData from Pickering et al., 1995; Munné and Cohen, 1993; Munné et al., 1995.
bData from Munné et al., 1995.
cData from Munné and Cohen, 1993.
dP <0.01 and dP <0.005 for comparisons with EBS + HIS.

Figure 24. Multinucleation in human embryos that developed from monospermic eggs. (A) 2-cell stage embryo containing a mononucleated and a binucleated blastomere (magnification ×400). (B) 2-cell stage embryo with two heavily multinucleated blastomeres (magnification ×400). Transfer of embryos such as A may lead to pregnancy and livebirth. Transfer of embryos such as B is not usually undertaken in in-vitro fertilization (IVF) clinics, and the majority arrest in culture. Courtesy of H.Balakier and K.Cadesky (1997) and Human Reproduction.

for the diagnosis of genetic disease in their embryos. Implantation rates per embryo are similar among these patients (Edwards and Brody, 1995).

Cell lineages in the mammalian embryo

We close this review by integrating information on mammalian development into a comprehensive model. Each early cleavage division will be considered, and a lineage map combining many of the aspects of early development will then be outlined.

Cell determination and differentiation in early cleavage stages

Fertilization and the first cleavage division

Among several non-mammalian species discussed in this review, ooplasm is segregated in various ways by meridional or equatorial cleavage planes during the first cleavage divisions. What is the significance of these cleavage planes? Unlike C. elegans, in which the first equatorial plane segregate P granules to the germline, two meridional cleavage divisions in Plividus and Xenopus results in four totipotent daughters (Figure 25). These blastomeres may have an equivalent totipotency, but they are different in other ways, for example in the uneven distribution of the X-cat-2 germline determinants (see Figure 5). This evidence raises questions about totipotency in relation to germline markers. Two forms of cleavage, polar and radial, characterize marsupial embryos; polar patterns involve the emission of yolk or yolk vesicles in a polar fashion, whereas radial cleavage involves emission over the whole embryo surface, although polarity is still distinct. Embryonic and extraembryonic cell lines separate early in development in monotremes and marsupials with radial cleavage, e.g. at the 2-cell stage in Virginia opossum (Selwood, 1994).

An initial meridional cleavage also occurs in mouse and
C. elegans

P granules C. elegans,

* Xcat-2 in Xenopus

○ germine determinants in mammals

Figure 25. Proposed distribution of determinants in early mammalian embryos, based on comparative data from Caenorhabditis elegans, Paracentrotus lividus and Xenopus laevis. P₀ = unfertilized egg; AB, P₁ = first blastomeres of somatic and germlines in C. elegans. P₁ divides to give EMS and P₂. P₁ and P₂ = putative germine precursors; T = putative trophectoderm in mammals. Shaded areas represent vegetal regions, and dotted lines represent cleavage planes.

Second cleavage division
As in C. elegans, the first-cleaving 2-cell daughter of mouse embryos divides meridionally to produce ICM precursors (Graham and Duessen, 1978). Cleavage rotation and equatorial division of the second-dividing 2-cell blastomere presumably segregates different factors to its daughters (see Figure 25). It is possible that germline separates from trophectoderm at the end of this equatorial division. 4-cell embryos would thus have three totipotent cells, i.e. two inner cells and one germline cell, and one trophectoderm cell (Figure 25). Two cell lines could have emerged in this way, coinciding with the onset of major zygotic transcription in 2-cell mouse embryos.

Rotating cleavage planes, unequal cytoplasmic distributions, the down-regulation of transcripts in one daughter cell, and translational modifications may all be involved in segregating developmental information by the stage of growth. Non-rigid cleavage planes and varied distribution of cytoplasm may allow a degree of adaptability, and blastomeres may readily revert to full totipotency prior to the onset of zygotic transcription. This model explains why totipotency is restricted to the 2-cell stage in mice, but can persist to the 4-cell stage in cow embryos in which zygotic transcription, and formation of junctional complexes, also occur one day later.

Third cleavage division
An ability to induce membrane polarity via cell contact develops from the 2-cell stage in mouse embryos. The formation of numerous cell contacts by the 4-cell stage indicate that signalling and induction rather than cleavage planes increasingly determine cell lineages. There is nothing as yet to suggest that one blastomere may regulate cleavage planes in a sister blastomere, as in C. elegans (Goldstein, 1995).

Cleavage planes now vary in mouse embryos (Graham and Duessen, 1978). In C. elegans, the germline cell (P2) again rotates its spindle and retains germine P granules, and induces polarization in its sister which develops into soma. Parallels can be drawn with the Nieuwkoop centre in Xenopus which controls the development of neighbouring cells via inductive pathways (Davidson, 1986). Similar germline control in mammals would produce five ICM or germine precursors, and three trophectoderm cells, in 8-cell embryos. This fits with oct-4 expression in 8-cell mouse embryos (five positive, three negative) (Figure 22B). This model would not conform to inside/outside hypothesis, or to cell allocation studies suggesting cross-colonization. These concepts originate from studies on embryos in vitro under sometimes severe experimental conditions, and may therefore reflect what can happen but not necessarily what does happen.

Differentiation after the 8-cell stage in mice
At this stage of growth, cell lineages with highly asynchronous divisions are presumably regulated by spatial or ‘quantitative’ factors at this stage of growth. The original A/V axis persists,
revealed in a constant relationship of the second polar body with embryonic axes (Gardner, 1997). Trophoectoderm now has a higher cleavage rate than ICM; one of its cell cycles is as short as 5 h as it becomes more numerous than ICM.

By 82 h post-fertilization, mouse blastocysts display a morphological asymmetry involving a rounded anterior and pointed posterior blastocoele (see Figure 17). They have ‘a sense of right and left’ (Smith, 1980), oct-4 expression again proves invaluable to identify ICM, trophoectoderm and primitive endoderm. It is initially distributed uniformly among ICM, but soon down-regulates, beginning in trophoectoderm. A simultaneous oct-4 up-regulation in primitive endoderm (see Figure 22) suggests that ICM is now differentiating rapidly.

**Lineage map of mammalian regulation and differentiation**

A lineage map of early mammalian differentiation is proposed in Figure 26. It is based on the concept of the P line, with the oocyte called P0 and successive germline precursor cells called P1, P2, P3 etc, as in *C. elegans* (Figure 2A) (Goldstein, 1993; Goldstein *et al.*, 1995). Germline precursors could be important regulators in early cleavage, for example in controlling early cleavage planes. They are allocated in the first cleavage in our model, but not committed until later since isolated ICM cells transferred into blastocysts can contribute to germline in fusion chimeras. Germline has been generally believed to originate in mesoderm (Wilkins, 1993; Ginsburg *et al.*, 1990; Motta *et al.*, 1997). Allocation to trophoectoderm begins at the 4-cell stage, as indicated by the down-regulation of oct-4 in some cells of 8-cell mouse embryos.

Markers are needed to show what happens in 4-cell embryos, and to confirm that oct-4 labelled cells include precursors of germline and soma. *Oct-4* may perform a similar function to germ-line specific transcription factors in *C. elegans* (*skn-1, pie-1, celF, cey1*) and maternal mRNAs for Xcat-2 and *Xlsirts* in Xenopus. Blastomere commitment has begun in the 8-cell stage, shown by differential oct-4 expression. By compaction at the 16-cell stage, biochemical differences such as the acetylation of microtubules between inner and outer cells at compaction indicate that differentiation is now advanced.

The model could explain the repair of specific forms of damage in one cell line and not in the others, e.g. CGG amplification in somatic but not germinal cells of male fetuses with fragile X, so that the full mutation is not expressed in spermatozoa (Reyneirs *et al.*, 1993), or mosaicism for Y deletions in the germline and not in soma (Kent-First *et al.*, 1996; Edwards and Bishop, 1997). It could also explain too why genes can be hypermethylated in soma and underestimated in extraembryonic tissues and spermatozoa.

This model conflicts with the inside/outside hypothesis. It substitutes the concept of determinative events occurring in unfertilized and fertilized eggs, during the maternal control of early determination and in the formation of cleavage planes. It challenges concepts of position in 8-cell embryos as the primary regulator of the early embryo. It is firmly based on molecular, genetic and embryological studies. The inside/outside hypothesis has depended heavily on experimental methods involving stress artefacts or other shortcomings which can modify biochemical profiles such as the synthesis of heat shock proteins. Isolating blastomeres *in vitro* modifies developmental characteristics in other species, e.g. in *C. elegans* and *P. lividus*, and presumably does the same with mammalian blastomeres. Totipotency is a complex matter undefined in molecular terms and of uncertain significance in undisturbed regulation in mammals. Membrane polarization is a characteristic of differentiating cells when determination must be far advanced, and focusing on it obscures the significance of earlier molecular events. As a result, there is virtually no molecular basis to support the inside/outside hypothesis (Latimer and Pedersen, 1993; Edwards and Brody, 1995).

Reservations also arise about the applicability of an inside/outside form of differentiation to all eutherian mammals. Different species exhibit various forms of early embryonic differentiation. Membrane polarization at the 8-cell stage exists to a very limited extent in rabbit morulae (Ziomek *et al.*, 1990; Latimer and Pedersen, 1993). The common shrew, elephant shrew, lemur and tenrec evidently have no morula stage and develop into unilaminar blastocysts, a situation common to several marsupials (Selwood, 1994). In multilayered pig and goat blastocysts, large outer cells accumulate at one pole, which develop into embryonic tissues. Inner or outer positions in the embryo cannot determine cell fate in these eutherians (Selwood, 1994). Smith (1980) finds it difficult to conceive how late-acting or environmental factors can regulate early differentiation within the confines of a stable uterine existence, and we agree with this attitude.

The concept of a regulatory oocyte (Po) controlling adjacent cells could explain how the oocyte imposes a strict timing on follicle formation, utilization from the pool and growth according to an imposed ‘production line’ (Henderson and Edwards, 1968; Edwards, 1980). This control might be exerted in primary oocytes at the onset of meiosis as they organize follicles into specific cortico–medullary patterns (Byskov *et al.*, 1997). Oocytes may also control local follicle systems such as granulosa cell proliferation, steroid biosynthesis and formation of cumulus cells (Eppig *et al.*, 1997). Oocytes and granulosa cells might ‘talk-back’ in mammals, as with *gurken*, *torpedo* and *bicoid* in *Drosophila*. Granulosa cells synthesize kit ligand to maintain meiotic arrest in oocytes, and those cells immediately adjacent to the oocyte synthesize the largest amounts of it (Ismail *et al.*, 1996). The high concentrations of kit ligand in these cells could be regulated by the proximity of a controlling oocyte. Mouse cumulus cells secrete meiosis-inducing sterols (Byskov *et al.*, 1995).

As we conclude this review, it is essential to stress the weaknesses of our model. There is no direct evidence that trophoectoderm and germinal cells originate from the same 2-cell blastomere, although they both have a different origin from soma (Soriano and Jaenisch, 1986). There is no evidence on trophoectoderm evolution, which is presumably linked with
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Figure 26. Proposed model of cell lineages in mammalian embryos, based on the following assumptions: (i) the oocyte and embryo are polarized; (ii) the first cleavage, and the first-dividing 2-cell blastomere have a meridional division; (iii) the second-dividing 2-cell blastomere has an equatorial division; (iv) maternal factors can be distributed unevenly in 2- and 4-cell stages; (v) germline and trophectoderm have a different allocation from soma in the 2-cell embryo; (vi) germline and trophectoderm are allocated from the same 2-cell blastomere; (vii) individual blastomeres are not committed until the 8-cell stage; (viii) germline originates in two or three founder cells; (ix) expression of oct-4 protein marks totipotent, highly multipotent and germline cells until the blastocyst; (x) trophectoderm divides more rapidly than inner cell mass after the 8-cell stage in mice; (xi) interactions between blastomeres increasingly regulate differentiation by the 8-cell stage; and (xii) a population of 45–100 primordial germ cells exists by gastrulation. I = inner cell mass; E = endoderm; U = undifferentiated; P = germline precursors; T = trophectoderm precursors.

Table X. Genes from different phyla with known sequence homologies

<table>
<thead>
<tr>
<th>Category product</th>
<th>Mammals</th>
<th>Amphibians</th>
<th>Drosophila</th>
<th>Nematodes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Regulation</strong></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>DNA binding protein</td>
<td>Hox</td>
<td>HMG</td>
<td>HOM</td>
<td>unc 86</td>
</tr>
<tr>
<td>DNA binding protein</td>
<td>Oct-1-4</td>
<td></td>
<td></td>
<td>mex-3</td>
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</tr>
<tr>
<td>RNA binding protein</td>
<td>Xcat-2</td>
<td>HSP70</td>
<td>HSP70</td>
<td></td>
</tr>
<tr>
<td>Heat shock proteins</td>
<td>HSP70</td>
<td></td>
<td>HSP70</td>
<td></td>
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<tr>
<td><strong>Cell signalling</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Membrane/intracellular proteins</td>
<td>β-catenin</td>
<td>armadillo</td>
<td></td>
<td>glp-1</td>
</tr>
<tr>
<td>Phosphoprotein</td>
<td>Xdh</td>
<td>dishevelled</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Receptors</td>
<td>W-1</td>
<td>Xnt 8</td>
<td>Wingless</td>
<td></td>
</tr>
<tr>
<td>Kinases</td>
<td>GSK</td>
<td></td>
<td>Zeste white 3</td>
<td></td>
</tr>
<tr>
<td><strong>Growth factors</strong></td>
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<tr>
<td>Transforming growth factors</td>
<td>Vg-1</td>
<td>dpp</td>
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</tbody>
</table>

viviparity. Homologies between eutherian mammals, marsupials and marsupial frogs remain to be identified or proven. Nevertheless, several lines of evidence point to a distinct maternal control of determination in oocytes and fertilized eggs of mammals. The long-existing notion that polarity and embryonic axes are regulated differently in mammals to all other animals seems to be highly improbable in view of their wide genetic homologies (Table X). Regulation and early differentiation in human embryos must be understood, since embryonic regulatory factors must be sustained throughout life, to maintain established characteristics of form and shape. They are often oncogenes, perhaps encoding or directing pathogenic events in later life. And an emphasis on germline may be warranted, since it regulates early differentiation in
some species and is entrusted with the carriage of genetic programmes to the next generation in all of them.

Addendum. Investigations needed on human embryos

Various studies could be designed to investigate early development in the human embryo. These studies fall into various classifications:

Search in human oocytes for homologues of the ooplasmic genes active in other phyla. This approach has been highly rewarding in the past, e.g. with pp19mos which is involved in metaphase arrest during MII. Several genes clearly showing animal/vegetal polarity have been identified in other phyla, e.g. BicD, exuperantia, swallow (Drosophila), par genes in C. Elegans as well as other well-characterized genes involved in regulation: Xcat-2, Vgl (Xenopus) which have cross species homologies. It should not be too difficult to find them in mammalian embryos.

Study isolated single blastomeres from 2-cell and older human embryos, to assess metabolic characteristics, cell cycle controls, transcription and its initiating factors, and their developmental fate. The object would be to find variations between blastomeres, and could include the following analyses:

Culture single blastomeres from 2 cell human embryos to find out if one line remains diploid, and the other is prone to chromosomal modifications such as trisomy or monosomy.

Perform similar analyses on single blastomeres from 2-cell embryos to find out if the fastest line to reach the 4-cell stage produces intact blastocysts whereas the slowest displays apoptosis (Juriscova et al., 1996) and differentiates into trophoblastic vesicles or miniature blastocysts.

Mark the fastest-cleaving blastomere of a 2-cell embryo to see if it differentiates into ICM.

Assess the expression of the factors regulating transcription, e.g. Rad51, Hxt, RAG1 and 2, DFFRX and Y, eIF-4C, oct-4 and oct-6 in single cells of the embryo. These factors have been examined in intact rodent and human embryos, but not in isolated blastomeres or tissues of morulae and blastocysts.

Attempt to identify different mRNA species in isolated cells from cleaving embryos, ICM and trophectoderm. Different mRNA species have been identified in meromeres, macromeres and micromeres of 8-cell sea urchin embryos (Raff and Kaufman, 1983). Examine the distribution of mRNA in relation to the localized expression of Xcat-2 and Vgl as found for Xenopus and to mitochondrial organization.

Examine metabolic activity in single cells isolated from cleaving embryos, morulae, ICM and trophectoderm to find out if a ‘totipotent’ metabolism is common to several cell types.

Examine single cells and tissues of preimplantation human embryos to find out when transcription begins, and its relationship to the actions of regulatory genes.

Examine the properties of isolated blastomeres in studies on totipotency to find out if they display similar phenomena as in totipotent plant cells (Nuti-Ronchi, 1995). This would involve the formation of heat-shock proteins and perhaps the expression of genes which regulate meiosis.

Measure metabolism in individual cells isolated from 2-cell or 4-cell embryos, ICM and trophectoderm, and in individual cells of these tissues. The purpose would be to find out if ICM and its individual constituent cells retains the consumption of lactate and pyruvate, whereas glucose is metabolized in trophectoderm and its precursors. Lane and Gardner, (1996) have described assay methods. The onset of glycolysis may signify the onset of differentiation as in other tissues.

Use time-lapse photography and other methods to identify the pattern of post-fertilization reorganization in the egg, and regulation of the axis of first cleavage plane in relation to the position of the second polar body.

Examine isolated cells from ICM or embryonic discs for variations in gene expression or metabolism. Assess karyotypes of individual ICM cells to calculate the incidence of mosaicism. If the cells are largely diploid, then preimplantation diagnosis may have to utilize the extraction of single ICM cells for diagnosis to avoid problems of mosaicism in trophectoderm.

More complex experiments could involve exciting cytoplasm from regions of the human egg using the position of the second polar body as a spatial marker. Injections of cytoplasts taken from various sites in the ooplasm might then impose abnormal forms of polarity or segmentation. Perhaps such epigenetic disorders arise in some cleaving embryos in vitro, as in healthy implanting blastocysts which possess trophectoderm but no ICM (Hardy et al., 1996).

Assess whether these conditions existing in the early embryo persist into later embryos, for example, whether the action of oct genes, or the low oxygen tension required by preimplantation embryos persist into the first weeks of post-implantation development. Assess the expression of Hox genes in embryonic tissues, especially in relation to the onset of transcription in the various cells and tissues of the embryo.

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References


Duprey, P., Morello, D., Vasseur, M. et al. (1985) Expression of cytokeratin...


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Partington, G.A., Bitwistle, D., Nicolas, D. et al. (1997) GATA-2 is a maternal transcript present in Xenopus oocytes as a nuclear complex which is maintained throughout early development. Dev. Biol., 181, 121–143.


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