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### REVIEW

# Genetics of polarity in mammalian embryos<sup>1</sup>

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**Abstract** This brief review is devoted to the genetic control of polarity and embryonic axes in preimplantation mammalian embryos. Discussion is related to their formation, the considerable variations in gene activity in these early phases of development, and the influence of timers over polarities and related aspects of development. Modern genetic analyses assess vast numbers of genes in outline, and the actions of individual genes in detail. These factors operate within a mixture of inherited maternal controls, gene silencing, bouts of transcription and the actions of mini RNA in controlling gene expression. Within this context, maternal factors regulate the planes of early cleavage divisions and unevenly distribute animal and vegetal characteristics to successive blastomeres by the 4-cell stage. This varied inheritance confers varying combinations of animal and vegetal cytoplasm to single blastomeres in many human 4-cell embryos. The blastomere inheriting animal cytoplasm only may be the trophectodermal stem cell, that with vegetal cytoplasm may be the germline precursor, and the two with full polarity may produce inner cell mass. Some implications of these findings are considered. 

**KEYWORDS:** blastomere, embryo, genetics, polarity

### Introduction

Genetic controls involved in the establishment of polarity and embryonic axes in oocytes and embryos of various species are essential characteristics of developing embryos. Most work reported in this text has been carried out on mammalian oocytes and embryos, together with data from amphibians, *Caenorhabditis elegans* and *Drosophila*. This field has had a relatively long history ever since the uneven deposition of pigment in some amphibian eggs, well recorded more than a century ago, led to terms such as the animal and vegetal poles in oocytes. These poles were determined by an axis passing across the oocyte and early embryo. Papers by Spemann (1927) and Nusslein-Volhard (Driever and Nusslein-Volhard, 1988) led to enquiries into the major aspects of polarities in non-mammalian systems over the 20th century. Today, sophisticated molecular and genetic analyses are applied across species of many Orders and Phyla. These new methods are essential to a fuller understanding of the mammalian preimplantation phase, which involves the actions of numerous genes and highly varied developmental systems.

Polarity is fundamental in imposing regulated differentiation in embryos and also to direct the functions on some individual cells, especially epithelia, including trophectoderm. In embryos, it establishes the background structure for later developmental systems, and involves the concerted, timed and integrated actions of numerous genes. Gene and systems homologies involved in its formation persist across the major phyla, so that knowledge gained from well-characterized models in flies and amphibians can be applied to mammalian systems. Nevertheless, deep differences between phyla still persist today, e.g. in echinoderm embryos where the specification and determination of body axes differs from vertebrates, with their multiple asymmetries on both sides of a bilateral body as in mammals (Jost, 1950). Some echinoderms utilize a single asymmetric structure formed in larvae as a group of cells on the left side of the gut. This produces the future adult, with a left–right asymmetry specified according to the dorsoventral axis (McCain and McClay, 1994).

The opening sections of this paper will cover the immense significance of genetic systems in

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determining embryonic polarities. Later sections cover the roles of specific regulatory genes and their roles in mammalian oocytes and embryos. New investigative systems, including microarrays and novel techniques to study transcription, are assessed. A final section discusses new outlooks in mammalian embryology emerging from all this accumulating knowledge, together with novel techniques still in the process of clarification. References mostly quote recent data, since space is lacking for detailed histories.

## The genetic basis of polarity

### The gene *Cdc42* and the establishment of polarities

The most fundamental question about polarities concerns their origins in oocytes and elsewhere. A major review in this direction published in 2004 revealed how the gene *Cdc42*, a member of the *Rho* family, had a highly significant role in establishing polarities in species varying from yeasts to vertebrates (Etienne-Manneville, 2004). It produces a small GTPase, which controls many systems establishing polarities in *Drosophila*, its expression being precisely controlled temporally and spatially by internal and external factors. *Cdc42* has an 'amazing capacity to coordinate the control' of numerous pathways via multiple signalling in a wide variety of polarized cells, and seemingly in species varying from yeasts to mammals. These properties are exactly what would be expected of regulatory control systems active in developing embryos, which may explain why this gene is conserved from yeasts to mammals.

The role of *Cdc42* is being clarified in studies on *Drosophila* and *Xenopus*. Activated initially at a presumptive control site in yeasts, *Cdc42* is influenced positively by guanine nucleotide exchange factors and negatively by guanine nucleotide dissociation inhibitors. It is also controlled by many diverse regulators and downstream effectors. External stimuli, for example, exert primary regulatory effects, acting via contact receptors (integrins and cadherins) and soluble cytokines. This enables polarizing cells to 'sense' their microenvironment as *Cdc42* orientates and maintains a polarized morphology. Acting via the cytoskeleton, it is active at the leading edge of migrating cells, at cell-contact sites in epithelial cells, and in phagocytic crypts in neutrophils and macrophages (Etienne-Manneville *et al.*, 2004). It regulates the microtubule cytoskeleton, itself organized by the PAR proteins 1–6 and protein kinases. This microtubule cytoskeleton is reorganized, and epithelial junctions formed, under the coordinated effects of several protein complexes and by the formation of tight junctions between adjacent cells. Membranal traffic is then organized one-way. Hence, *Cdc42* is effectively

a molecular switch reactive with many proteins to influence intracellular and extracellular systems.

Such information emerged from numerous studies clarifying the nature of polarities in oocytes of numerous species. Detailed genetic analyses on *Xenopus* clarified the role of *Cdc42* (Sokac *et al.*, 2003). It is involved in organising the cytoskeleton, especially in relation to de-novo actin polymerization, and recruited by exocytosis from membranes of cortical granules in the oocyte. It is closely involved with cytoskeletal F-actin, which associates dynamically with plasma membrane and cortical granules. These systems are fundamental in fertilization, the primary structure of the oocyte cytoskeleton and exocytosis from cortical granules at sperm entry. More studies are essential to relate this knowledge on the roles of *Cdc42* and the *Par* genes in imposing polarities in oocytes of various phyla.

### Basis of genetically regulated polarities in oocytes and embryos

Polarities are clearly highly significant from the earliest stages of oogenesis and embryogenesis in embryos of numerous phyla. They are presumably maintained and persist in adult tissues. One common core mechanism organizes the cytoskeleton to deliver membrane proteins to specific polarized sites in oocytes and early embryos. As discussed above, actin has a significant role in these processes. Its polymerization is involved in the despatch and transport of specific molecules to polarized positions in single cells, including somatic cells, and to control factors regulating cell contact and adhesion. Establishing adhesion involves apical junctions and cadherin-catenin interactions as the identities of different membranal domains are defined. Interference with cell-to-cell adhesion can be disastrous, as illustrated in the actions of *Armadillo* as polarities are established in *Drosophila* oocytes (Nelson, 2003).

Microtubules are essential in establishing polarities and gradients of various compounds known as morphogens (Wolpert, 1969; Gurdon *et al.*, 1999). They carry mRNAs and proteins to specialized locations via their interactions with the 3' untranslated regions in mRNA. Localized mRNA may then divide many times over to increase its local expression or be distributed to several sites in ooplasm to initiate more distant gradients. Established polarities hence signify the existence of concentration gradients of proteins and mRNA and traverse the oocyte and early embryo from the animal to the vegetal pole. Sensing these gradients, neighbouring responsive embryonic cells react to specific morphogen concentrations at particular positions and along embryonic gradients. They may respond individually or in groups to varying concentrations of morphogens at different positions along specific gradients, as during the formation of

the anterior–posterior axis in *Drosophila*. This is how embryos establish their positional information. Once polarities are well determined, later-acting genes function within these established channels.

The existence of morphogens and the exquisite responses of neighbouring embryonic cells indicate the existence of very fine sensitivities to biochemical factors in differentiating cells (Wolpert, 1969; Gurdon *et al.*, 1999). The gene *bicoid* displays such a spatial gradient in *Drosophila*, and its positional information is distributed to downstream genes (Driever and Nusslein-Volhard, 1988). In one sense it is itself a morphogen, and its varying concentrations may invoke threshold responses along the animal–vegetal axis. Examples of responding cells include individual blastula cells in *Xenopus* which react to local concentrations of activin, as witnessed in the expression of the genes *Xbra*, *Xgsc*, *Xeomes*, *Xchordin*, *Mix1*, *Xlim1* and *Cerberus*. They respond in a gradient-related manner according to the local activin concentration and not in relation to their position in regard to adjacent cells (Gurdon *et al.*, 1999).

Among the many genes controlling polarities and axes, the *par* genes have an essential role. Initially identified in *C. elegans*, they are now known to be widespread (e.g. Guo and Kemphues, 1996). Six *par* genes, plus *mex-1* and *mes-1* are involved in the regulation of polarities in early embryos and the orientation of mitotic spindles in differentiating cells. They act in regions of cell contact in epithelia, at tight junctions in *C. elegans*. Homologies have been identified in mammals, and each of them seemed to retain many of the functions they performed in *Drosophila* (Table 1). Other distinct regulatory genes involved in establishing polarities in *Drosophila* include *bicaudal*, *staufer* and *egalitarian*. Some of the genes characterized in *Drosophila* control microtubular development as it expands via the movements of plus ends to the posterior pole and minus ends to the anterior pole (reviewed by Edwards, 2001; Nelson, 2004). *Staufen*, for example, locates in microtubules and in endoplasmic reticulum, moves to the posterior pole and organizes the location of maternal RNA in *Drosophila* oocytes (St Johnston *et al.*, 1991). Its detailed molecular relationships with RNA in oocytes

and embryos characterize its action in most of these species. *Bicaudal-C* is a mammalian homologue of a similar gene in *Drosophila* and *C. elegans* with similar functions in each of these species as it binds to RNA in oocytes and other tissues in each of these species (Wessely *et al.*, 2001).

Gradients can nevertheless have their problems (Houchmanzadeh *et al.*, 2002). Their properties and distributions may vary immensely between individual embryos, and resulting ‘noise’ in more imperfect distributions may be filtered by other genes, e.g. *hunchback* (*hb*), under the influence of *staufer* and its various mutant forms. Some of these mutants can themselves invoke disastrous modifications in the polarized distribution of some products, leading to early embryonic death. Frequent interactions occur between differing gene systems, with *vasa* for example regulating the signalling action of *gurken* in establishing polarities in *Drosophila* oocytes (Styhler *et al.*, 1998; Tomancak *et al.*, 1998). Such systems present in human embryos might explain their varying capacities to develop in widely varying conditions *in vitro*.

## Polarities and gene actions in mammalian oocytes and embryos

### Early history

Studies on polarities in the early twentieth century were mostly done in frogs and flies (reviewed by Edwards and Beard, 1997). Many investigators searched for similar systems in rodents and other mammals, especially from the 1950s, and deep similarities were clarified towards the end of this century. By today, many such homologies are known to include the actions of single genes and complex developmental systems, accompanied by increasing evidence on their control by timing systems. For example, the *par* genes and their roles in establishing polarities via their actions on microtubules are common to species from nematodes to mammals, and *staufer* homologies, its gene domains and its variant forms have been identified in humans, mice, rats, *C. elegans* and *Drosophila* (Table 2).

This review must be restricted to discussing a few of these developments. Driven by earlier findings in amphibians and flies, Dalcq (1951, 1957) identified axes and gradients in rat oocytes. Jones-Seaton (1950) detected evidence of a histological polar axis in rat eggs and Denker (1983) concurred when identifying a bilateral asymmetry in rat oocytes. Yet, detailed histochemistry using X-ray microradiography and ultraviolet microscopy failed to reveal any signs of polarity in human ovarian oocytes (Hedberg, 1953). Other newly invented techniques were also unsuccessful, as when autoradiography was tested

**Table 1** Some homologies between human, mouse and *Drosophila* genes (from Edwards, 2001).

Human	Mouse	<i>Drosophila</i>
FOXL21	<i>Foxl2</i>	<i>Forkhead</i>
POF	<i>Formins</i>	<i>Diaphanous</i>
HS6ST		<i>Egalitarian</i>
<i>Bicaudal</i>		<i>Bicaudal</i>
<i>Staufen</i>		<i>staufer</i>
<i>Oct-4</i>	<i>Oct-4</i>	

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**Table 2** Groups of *par* genes and pattern formation in *C. elegans* eggs (based on work by Kemphues *et al.*, 1988). This table is a modified form of that published by Edwards (2001).

Gene	Structure	Function
<b>Group 1: Major effects on cytoplasmic locations, weak on spindle orientation</b>		
PAR-1	Serine–threonine kinase	Organizer of A/P axes, locate transiently at periphery with PAR-2 as organized by PAR-3. Then moves to a posterior site. Regulates asymmetry of the first cleavage division and colocalizes in germline. Conserved in mammals.
PAR-4	Serine–threonine kinase	Not distributed asymmetrically. Influences distributions of PAR-3 and PAR-6, to establish asymmetry in embryos.
<b>Group 2: Spindle rotation, less effective in cytoplasmic rotation</b>		
PAR-2	ATP-binding site and zinc-binding domain	Locates briefly at posterior periphery with PAR-1, this mutual location restricted by PAR-3.
PAR-3	Encodes 3 repeats of protein domain sited in plasma membrane	Locates at periphery of fertilized egg and then at the anterior periphery of the oocyte. Regulates centrosome–nuclear rotation. Restricts PAR-2 mutually to posterior periphery. With PAR-2 and PAR-4 organizes PAR-1 location at anterior periphery. Complexes with PAR-6 and PKC-3.
PAR-5		Controls centrosome–nuclear rotation. Distributes PAR-1, PAR-2 and PAR-3 peripherally.
PAR-6	Encodes PDZ-domain similar to PAR3	Complexes with PAR-6 and PKC-2. Distribution regulated by PAR-4.

in visualizing gradients in RNA and protein synthesis in mouse ooplasm, but failed to detect clear-cut polarities despite their irregular distributions (Edwards and Sirlin, 1956). Largely neglected over the following years, rare examples of mammalian polarity were noted by some successive investigators and queried by others (Bachvarova *et al.*, 1985). Examples included the positioning of the germinal vesicle to one pole of the mouse oocyte just before it began its maturation, and the obvious polarized position of the meiotic spindle in oocytes of virtually all mammals. Movements of mitochondria in mouse oocytes, reported initially by Gresson (1942), were analysed in detail by Calarco (1995). They migrated to form a distinct cortical band and were more numerous in one hemisphere of the oocyte, indicating they were also polarized.

Another approach to the analysis of early development in mammals related to the controlled nature of cleavage planes in preimplantation embryos. Regularities in the first two cleavage planes in mouse, rabbit and other types of mammalian embryos were noted by investigators in the first half of the twentieth century (e.g. Lewis and Wright, 1935). Time-lapse imaging and other approaches confirmed these events in rabbit embryos. Tightly controlled axes of the first two cleavage divisions included a meridional first cleavage division, succeeded by a second such division in the first-cleaving 2-cell blastomere and an equatorial cleavage in the second-dividing 2-cell blastomere (e.g. Gulyas, 1975). The first-dividing 2-cell blastomere was predicted to form inner cells in 16-cell mouse embryos and then inner cell mass, a property perhaps related to their ability to form

more attachments to sister blastomeres (Graham and Duessson, 1978).

Johnson and his colleagues (1986) probed the nature of differentiation in mouse embryos (Ziomek and Johnson, 1980; Johnson *et al.*, 1986). They investigated the positive role of cell adhesion during the 8-cell stage and how polarizing systems occurred in outer cells in morulae, which then differentiated to form precursors of a highly polarized trophectoderm. Smith (1980) and Gardner (1997) clarified the nature of polarities in oocytes and early embryos, and matched these axes in early stages to those identified in blastocysts and post-implantation stages. Doubts about the existence of animal and vegetal regions in mouse oocytes and embryos were raised by Zernicke-Goetz (1998), who claimed to have applied microsurgery to selectively remove these regions without imposing any specific effects on later development. This claim was rejected by Antzak and van Blerkom, who discovered that attempts to excise gradients in these polar regions were ineffective, since sufficient markers persisted in gradients deep in ooplasm after microsurgery and might sustain existing polarities (see Edwards, 2001). By the end of the 20th century, there was little doubt that polarizing systems were as important to mammals as to other Orders. Attention also concentrated on understanding the events occurring at fertilization and in successive post-fertilization developmental stages utilizing novel experimental tools.

### Polarities and sperm entry into mammalian oocytes

The site of sperm entry might influence polarities in succeeding embryos. It may define the siting of the

polar axis, or *vice versa*. Opinions vary widely as to its exact importance in mammals. Gardner (1997) proposed the animal–vegetal axis of the mouse zygote was aligned to the later axis of bilateral symmetry in the blastocyst, implying it had been specified before cleavage began. Piotrowska and Zernicka-Goetz (2001) then reported how sperm entry had a significant role in forming the plane of the first cleavage division. Controversy continued to this complex debate when Gardner and Davies (2003) stressed that the bilateral symmetry of the blastocyst was aligned with the animal–vegetal axis of the fertilized egg specified before the first cleavage division, so a decisive role for sperm entry was unlikely. These authors queried the work of Piotrowska *et al.* (2001) and Piotrowska and Zernicke-Goetz (2002), claiming it contained statistical weaknesses and problems associated with the use of fluorescent beads and other analytical methods to measure embryonic and cellular polarities. In return, Zernicke-Goetz (2003) defended her techniques, stressing how her team had confirmed the first cleavage predicts the embryonic–abembryonic axis in blastocysts, perhaps aided by a role for the fertilizing spermatozoon. Inspecting this *contretemps*, Johnson (2003) concluded that alternative explanations were possible. He stressed that no definite rights or wrongs had been established since many fundamental questions still remained unanswered, and the use of a single inbred strain may have been restrictive in formulating interpretations.

A new study utilizing video images then claimed to have identified the sites of maternal and paternal pronuclei in fertilized mouse eggs. They also assessed the first cleavage plane in relation to the site of the second polar body (Hiiragi and Solter, 2004). These authors criticized the conclusions of both Gardner and Davies (2003) and Piotrowska and Zernicka-Goetz (2001), since the second polar body was an unreliable marker, moving dynamically and randomly to the first cleavage plane. Its siting in relation to the first cleavage plane varied widely although it sometimes located spontaneously along its length. Their use of video images revealed how the two pronuclei migrated inwards from opposite peripheral poles, and how the first cleavage plane became apparent at entry into the M phase of the first cleavage mitosis. At this time, pronuclei and the mitotic spindle were surrounded by clusters of mitochondria. The site of the plane separating the two pronuclei was identical with the cleavage plane (Hiiragi and Solter, 2004).

Experimental data based on excising and replacing pronuclei confirmed their conclusions. A pronucleus lying close to the other was excised from some eggs and then replaced by a matching paternal or maternal pronucleus from a donor egg (Hiiragi and Solter, 2004). Transplanted to the opposite pole of the egg, the grafted pronucleus formed a new topology, which

then dominated the site of the cleavage plane rather than the original position of two pronuclei in the great majority of operated eggs. Similar conclusions emerged whether maternal or paternal pronuclei were excised and replaced. Parthenogenetic eggs followed similar lines of growth, further querying a role for sperm entry. These authors concluded there was no determined A/V axis in the mouse egg, the cleavage plane was formed as the two pronuclei move together and observable features locating the first cleavage plane were absent (Hiiragi and Solter, 2004). These wide differences of opinion remain unresolved at the present time.

A third explanation of the role of the fertilizing spermatozoon in deciding polarity in human eggs was suggested by Edwards and Beard (1997). It accepted that the oocyte was polarized, since its formation as a primordial oocyte, and the whole ooplasm rotated at sperm entry to match the position of the spermatozoon and especially its expanding microtubular system. This system could cope without change wherever the fertilizing spermatozoon entered the egg. It also explained how polarities established in the oocyte could pass unchanged through the fertilization process. Further studies on this topic are clearly needed.

### **Polarities and cleavage planes in mammalian embryos**

Increasing attention to cleavage stages and mammalian homologies with polarizing systems in amphibians, *C. elegans* and *Drosophila*, led Edwards and Beard (1997) to conclude that in that an animal–vegetal gradient in mouse and human oocytes was distributed differently to specific 4-cell blastomeres via the combination of meridional and transverse cleavage divisions described by Gulyas (1975). The early mammalian embryo thus contained two blastomeres with full polarities, and two others carrying mainly animal or vegetal cytoplasm respectively (**Figure 1**). Analyses on the nature of cell differentiation led them to conclude how the 4-cell blastomere with mainly animal cytoplasm probably formed trophectoderm, that with mainly vegetal cytoplasm was the precursor of germline and the two blastomeres with full polarities formed inner cell mass. A fate map of the mammalian preimplantation development proposed that individual blastomeres were allocated to developmental fates by the 4-cell stage, and traced potential differentiation stages until the blastocyst. Later, indirect evidence was sifted and also indicated that the blastomere inheriting vegetal cytoplasm was the precursor of germline (Edwards and Beard, 1997, 1999).

Direct confirmatory evidence quickly emerged of the existence of polarities and axes in mouse and human embryos, and how they controlled the distribution of labelled maternally inherited proteins to animal

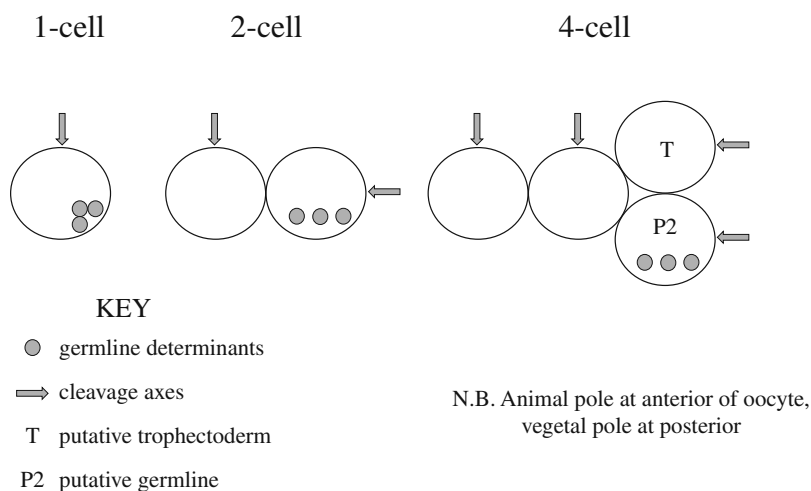


Fig. 1. Diagram based on an earlier illustration by Edwards and Beard (1997) showing the axes of the cleavage planes in 1-, 2-cell and 4-cell mammalian embryos. Note how successive meridional and transverse cleavages distribute animal and vegetal ooplasm unequally in 4-cell embryos. The second cleavage division also varies in different blastomeres; the implications of this cleavage division are not discussed in the present paper.

and vegetal regions (Antczak and van Blerkom, 1997). Initially, the distributions of fluorescently-labelled leptin and STAT3 proteins were traced to the animal pole in all stages from the early-forming oocyte to the blastocyst, concentrating in the cortex and forming gradients in ooplasm. Later, Antczak and van Blerkom (1999) identified several other proteins forming a gradient from the animal pole. These observations revealed close similarities in the formation of axes in oocytes of invertebrates and lower vertebrates, together with close homologies with genetic and developmental systems including polarization in embryos of *Drosophila*, *C. elegans* and *Xenopus laevis* (Edwards, 2001). In a sense, earlier findings by Dalcq (1957) and other investigators into mammals were also confirmed.

Interpretations by Edwards and Beard (1997) that one 4-cell blastomere inheriting animal cytoplasm was the forerunner of trophectoderm received substantial support when Hansis *et al.* (2001) identified a single 4-cell blastomere with molecular characteristics of trophectoderm. They isolated individual blastomeres of 4-cell human embryos, then excised the nucleus from each single cell to assess their chromosomal numbers utilizing fluorescence in-situ hybridization. *Oct-4*,  $\beta$ HCG or  $\beta$ LH mRNAs were identified in cytoplasm in many cells. Only a single cell possessed transcripts for  $\beta$ HCG or  $\beta$ LH alone, and this was defined as the trophectodermal stem cell. Further analyses revealed the expressions of *Oct-4* and  $\beta$ HCG were mutually exclusive (Hansis *et al.*, 2004), implying that inner cell mass had differentiated separately from trophectoderm by the 4-cell stage. Chromosomes counted in excised nuclei identified those embryos that were highly heteroploids and so exposed to abnormal patterns of gene expression.

The developmental implications of these and previous findings were assessed by Hansis and Edwards (2003). The onset of differentiation (sometimes called allocation) among individual blastomeres in cleaving embryos and among cells of the inner cell mass is a

fundamental aspect of early development. Allocation and perhaps commitment, i.e. a commitment to one tissue, may be characteristic of cells in slightly later developmental stages in inner cell mass. Many investigators query these early forms of differentiation, and propose that differentiation of individual cells of inner cell mass is induced by inductive factors released from extra-embryonic ectoderm.

This concept of differentiation by the 4-cell stages has been extended by Edwards and Hansis (2005). They proposed the trophectoderm stem cell could be identified in living human embryos according to its position in the embryo. It would be one of the three blastomeres adjacent to the polar body in many embryos, marking its animal pole with fluorescent beads or other markers. This could be confirmed by placing markers at animal or vegetal poles in individual 2-cell blastomeres to discover how differing regions of polar cytoplasm were distributed to each 4-cell blastomere. Direct evidence identifying the presumptive trophectoderm stem cell would include its high content of maternal leptin and STAT3 proteins, transcripts of HCG $\beta$  and possibly HLA-G, and of other genes identified at the animal pole by Antczak and Van Blerkom (1999).

Support for the theory of cell allocation by the 4-cell stage was also sustained by lineage tracing using *Cre-loxP* as a recombinant activator of  $\beta$ -galactosidase (Fujimori *et al.*, 2003). It marks cells derived from precursors carrying the transgene CAG-CAT-2, and confirmed how descendants of one 4-cell blastomeres produced extra-embryonic layers, trophoblastic giant cells and ectoplacental cone, i.e. the descendants of trophectoderm. Exciting studies on mammalian embryos, and possibly novel clinical application to assess quality in human embryos are promised by these new concepts on early differentiation (Edwards and Hansis, 2005).

These concepts must be related to the nature of ongoing differentiation in 8-cell mouse embryos when cellular polarities are established in addition

to the classic embryonic axes. These systems are driven by two proteins related to *par6* that display differential and asymmetrical locations in mouse oocytes and embryos. Showing no specific affinity in oocytes carrying a germinal vesicle, Pard6a-related proteins localize on the spindle during maturation and migrate to its pole nearest to the cortex, driven by microtubules as the germinal vesicle regresses. In contrast, Par6b-associated protein persists in spindle microtubules until the onset of oocyte maturation, when it switches to cortex at the animal pole through the stages of metaphase 2 arrest. It is claimed as the first identified protein to localize to the animal pole and to contribute to oocyte polarization (Vinot *et al.*, 2004), yet curiously, these investigators do not quote the data of Antczak and Van Blerkom (1997, 1999).

*par*-related genes also function in 8-cell mouse embryos (Plusa *et al.*, 2005). This is the stage when outer cells of the mouse embryo begin to form trophoblast, as distinct modifications arise in their structure and cellular activities and they become asymmetrical and polarized (reviewed by Johnson and McConnell, 2004). Their outer position in the embryo may determine their fate as described by earlier investigators (Tarkowski and Wroblewska, 1967). They also display different types of cell division, with some dividing equally to produce identical daughters and others dividing transversely to produce differing outer and inner cells. These forms of division are regulated by Par 3 and Par6, which complex with atypical protein kinase C, and become localized to the apical plasma membrane and tight junctions by shifting their position during 8- and 16-cell stages (Plusa *et al.*, 2005). Down regulation of this system can result in all descendant cells forming inner cells and producing inner cell mass as described later in this review.

## Microarrays and mammalian embryos

### Modern approaches using microarrays

Microarrays offer a detailed knowledge of gene activity and function in mammalian embryos. They enable genes active at specific developmental stages to be identified and compared with those active in other forms of differentiating tissues. Desires of early investigators for more genetic knowledge on the role of multiple regulators of embryonic development are now more than satisfied as enormous numbers of genes active in virtually every aspect of early differentiation have been identified and classified. Groups of genes are classed into categories such as those influencing polarities, circadian and other timers, the onset of transcription, the role of mitochondria, silencing systems, DNA replication, signalling systems and other means of control. Numerous genes change their expression at successive developmental stages, and

the mass of available information requires deciphering by complex statistical systems.

Data from Hamatani *et al.* (2003), utilizing the NIA 60-mer oligo microarray platform, are among many examples of large-scale microarrays. Procedures of linear gene amplification could be performed on minute pieces of tissue or on pooled embryos to enable thousands of active genes to be identified. Single oocytes and embryos are now being assessed. Sensitivities of ca. 60% and a low percentage of false positives (<4%) verified its findings on the activation and deactivation of numerous maternal and zygotic genes at various embryonic stages. Groups of genes were activated and then deactivated, resulting in wave-like activation patterns. Major waves involving the activation of numerous genes were succeeded by minor patterns, a result confirmed in similar studies using microarrays (Sharov *et al.*, 2004).

Four major transcription waves were identified, the first associated with 2–4-cell stages (zygotic genome activation or ZGA), the next with 8-cell stages (mid-preimplantation development or MGA), then with morulae and finally with blastocysts (Hamatani *et al.*, 2003). Each wave had its own characteristic set of activated genes. Rapid switches between genes active in turn at particular developmental stages revealed a complex succession. Timing factors were significant, the first activation wave of gene transcription possibly being timed by a maternally regulated zygotic clock. This may have delayed the translation of many zygotic RNAs until the 2-cell stage. Active genes influenced polarities, timing, metabolism, transcription and other major regulatory systems as a mixture of maternal and zygotic factors, including cell cycle controls regulating preimplantation growth. Among 30,000 mouse genes identified in other databases, almost 1000 genes were unique to this study (Sharov *et al.*, 2004).

Identifying more than 12,000 active genes in early embryos enabled Hamatani *et al.* (2003) to identify those with early or late expression and so produce patterns of activity at various embryonic stages. These could be compared with similar data gained from differentiating or differentiated mesodermal, neural and other classes of stem cells. Genes were grouped into clusters, each cluster having its own characteristics. Genes in clusters 1, 4, 5 and 8 formed an initial group that was activated from the zygote stage and peaked at 2- and 4-cell stages before declining. Cluster 5 alone contained 2522 genes. Clusters 7 and 9 contained genes active in oocytes then degraded in early development. Clusters 2 and 3 included 2990 genes initiated from maternal RNA that peaked at 8-cell then declined, some of the oocyte transcripts being sustained later by zygotic transcripts. These data offer an impressive means of characterizing specific embryonic pathways in mice.

**Table 3** Analyses on human oocytes using microarrays (Bermudez *et al.*, 2004).**A. Selected gene classes**

Apoptosis	4
Cell cycle regulation	16
Signalling pathways	26
Protein synthesis	18
RNA/transcription	65

**B. Expressed genes of single or multiple metaphase 2 oocytes**

	Total genes expressed	Exclusively expressed genes	Genes in common
Single	1467	106	1361
Multiple <sup>a</sup>	1823	426	

<sup>a</sup> *n* = 5.

Human studies were reported by Bermudez *et al.* (2004) (Table 3). Single or groups of five oocytes were measured and expressed approximately 100 unique genes. Many were already known including *staufen*, a Fanconi anaemia gene, *forkhead box O1A*, *mitochondrial translation initiation factor 2*, *lactate dehydrogenase A* and *translation initiation factor 1A* (*eIF-1A*). Such overlaps between data gleaned from earlier analytical methods with those from microarrays confirmed the high accuracy of the latter. Average signal strength ranged from 295 (*PTPN13*) to >56,000 (*histone family 3B (H3.313)*). Confirmation of the expression of *eIF-1A* followed instantly when it was identified using real-time polymerase chain reaction in preimplantation mouse and human embryos (Lindberg *et al.*, 2004). Its expression declined from the oocyte to the 4-cell stage in human embryos and then increased from the 8-cell stage, in one of the transcription waves identified by microarrays. Human embryos differed from mouse embryos in that their expression increased from the 2-cell stage, seemingly in an earlier wave, a difference requiring future analysis. Such differences in the timing programmes of mouse and human embryos were in a sense forecast by differences in the onset of compaction where each species has its own previous cell divisions and differing numbers of cells at its onset.

**Genetic regulation of multigenic and single gene systems**

Microarrays are by no means the only new approach to understand the regulation of complex genetic systems. Secrets of the action of single or multiple genes will doubtless be revealed using findings from the human genome or elsewhere. Henrique (2001), for example, summarized new concepts on multigenic

function, which possibly apply to mammalian embryos. Groups of genes, called equivalence groups, may act together to select any one of a group of permitted fates. This equivalence can be broken and restored, and is obviously an important concept for studies on embryogenesis. Perhaps this property explains how human embryos can seemingly adapt to widely varying conditions in culture and still implant in their mothers.

Developmental studies in embryos must also cope with the various forms of gene silencing characterizing epigenetic and other timing systems. These include the inactivation of one X chromosome in female embryos and the actions of various developmental genes regulating the activation and deactivation of specific genes. X-inactivation may be conferred before conception, to establish equivalence in gene dosage between the sex chromosomes in male and female embryos (Huynh and Lee, 2003). It may be achieved as XP is silenced in paternal germline and is inert by fertilization, a situation succeeded by regulatory factors operating from 2-cell stages as the transcription wave is activated. *Xist* seemingly takes over in later preimplantation stages. The inactive X segregates to trophoctoderm in blastocysts, and is present in trophoctodermal stem cell lines where X-inactivation and imprinting genes involve *Xist* and associations with the *polycomb* proteins Eed and Enx1 (Matsui *et al.*, 2001; Mak *et al.*, 2002; Chadwick and Willard, 2003).

**Gene silencing**

Common to many Orders, genes are also silenced by small forms of RNA composed of sequences of 21–26 nucleotides in a system named RNAi (interference). It is active in species as varied as plants, worms and mammals (Fire *et al.*, 1998). It involves the use of sense and antisense RNAs to silence specific genes by means as simple as adding these compounds to culture medium. It is also effective in intact animals and has been used in species as far apart as plants, *C. elegans* and mammals, as specific transcripts are degraded and reduced in nuclei and cytoplasm. Double-stranded RNAi was used to down-regulate Par3 in 8-cell mouse embryos, and negative forms of aPKC was injected into 4-cell blastomeres (Plusa *et al.*, 2005). Other RNA forms are also effective in regulating transcription. Small temporal RNAs (stRNA) regulate the timings of various stages of embryonic development in *C. elegans*. They operate via their untranslated regions that are common with those in particular proteins. Larger RNA forms consisting of 70 nucleotides fold into loop structures and also induce silencing as they correspond to each expressed gene (Grishok *et al.*, 2001). They are recognized as temporal controllers of the timing of developmental systems in *C. elegans*. Silencing is also induced by developmental genes such

as *Dicer-t*. It may act by converting double-stranded RNAs (dsRNA) into small RNAs of 22 nucleotides named interfering RNAs (siRNA) or to numerous endogenous, small RNAs (micro RNA). Normal development can be compromised by lesions induced in *Dicer-1* as when a gene vector was used to excise exon 21, which was substituted the neomycin-resistance marker cassette (*neor*). Controlled maternally, this system may interfere with miRNA processing via induced epigenetic effects (Bernstein *et al.*, 2003).

Mutations, deletions, abnormal imprinting and knock-out can induce silence gene action. The gene *lethal giant larvae (Lgl)*, for example, interacts with extensive signalling systems regulated by *Par* and *Notch* genes in mouse embryos (Klezovitch *et al.*, 2004). Abundant in embryos, brain and other tissues by 10–11 days post-fertilization, it produces a protein imposing apical-based polarities and organising epithelia, it exists in two forms in mammals, *Lgl1* and *Lgl2*, as in other Orders. It belongs to a genetic pathway involving membranal domains and protein targeting, and mutations in either gene reduce polarization in fibroblasts, and disorganise normal symmetries in gene expression. Knock-out *Lgl1<sup>-/-</sup>* mice display defects in brain function as polarities are disordered in neural progenitor cells. An apparent normal growth to day 9.5 is followed by an enlarged striatum, dilated blood vessels, enlarged ventricles by day 15.5 and hydrocephalus at birth. Normal asymmetries in protein distributions and cell cycles fail, perturbations occur in *Notch* signalling and cell cycles are scrambled (Klezovitch *et al.*, 2004).

Anomalous imprinting systems leading to abnormal forms of gene expression also result in early death in mouse embryos. One recent example was the early demise of parthenogenetic embryos that fail to develop to full term. This situation was avoided by replacing their pronuclei with nuclei from donor oocytes and deleting 13 kilobases from the gene *H19*. The resulting more appropriate expression of *H19* and *Igf2*, and the reduced expressions of many of the anomalous genes indicated this approach was successful. The parthenotes were now able to survive to full term and reproduce (Kono *et al.*, 2004).

### Timers of gene expression

Many anomalies are also associated with disorders or mutations in timing systems essential for regulating polarities (Edwards, 2002; Johnson, 2002). Timers include circadian clocks, hour glasses and developmental genes. Numerous developmental timers govern early embryonic growth, as in embryonic transcription in the G2 phase in 1-cell embryos and potassium channels in 1-cell embryos. Developmental clocks also control the actions of the gene *Lunatic fringe*. First expressed in early neurulae, the anterior neural plate and mesoderm, it controls mesodermal

segregation, marks the posterior boundary, and regulates timers. It encodes a protein, transmembrane glycosyltransferase, which is expressed in mouse and chick embryos, and in *Drosophila*. It is involved in *Notch* signalling, and with related genes active in forebrain, rhombomeres, neural tube, somites and limb buds (Mazet *et al.*, 2003).

Late-acting effects of polarities and timing systems, especially the left–right axis, induce serious disorders in laboratory animals and human beings. The gene *lefty* regulates this axis and the differentiation of numerous cell types. In humans, variants of this gene are known to exert immense effects of various developmental systems. It inhibits signalling by TGF $\beta$  by preventing the phosphorylation of *Smad-2* and its numerous downstream events (Ulloa and Tabibzadeh, 2001). Its various forms invoke serious clinical conditions in many physiological systems. These include minor and major effects of left- and right-handedness including brain development, through asymmetries affecting stroke, ciliary flow, embryopathies, the structure of amino acids and carbohydrates, relationships between handedness and many other disorders (Cohen, 2001; Gorlin, 2001). Many mutant forms have been identified in human patients (Table 4). They are also active in many other mammals. The *dynein* gene is involved in organizing ciliary flow at the embryonic node and any defects can disturb normal embryonic growth across a wide range of vertebrates (Essner *et al.*, 2002).

### Conclusions: new outlooks on mammalian embryology

As in many other disciplines, studies in mammals have intensified as the human and mouse genomes were clarified. Considerable interest centres on the flow of gene actions in preimplantation embryos, and flow diagrams are being constructed to present these data in three-dimensional forms (Hamatami *et al.*, 2003; Ko, 2005). Based on the activation and then the inactivation of numerous genes, this information is certain to clarify patterns of growth in intact embryos, single embryonic and stem cells and perhaps help to clarify how their differing functions are elaborated. Another approach currently restricted to animals utilizes 'balancing' chromosomes carrying inversions into the genome to isolate a specific chromosomal region covering ca. 2% of the genome (700 genes). Used widely in studies on *Drosophila*, 88 new mutations were isolated on mouse chromosome 11 from screening 735 pedigrees (Kile *et al.*, 2003). Among them, 55 were lethal and others affected nerves, blood tissues and head; one anaemic mutant was related to a known candidate gene.

**Table 4** Clinical aspects of asymmetries (modified from Cohen, 2001 and Essner *et al.*, 2002).**A. Regulation of the left–right axis**

Gene	Chromosomal mapping	Phenotypes
<i>LEFTYA</i>	1q42	Situs ambiguus
<i>ACVR2B</i>	3p21.3-p22	Situs ambiguus
<i>ZIC3</i>	Xq26.2	Situs ambiguus
		Situs inversus among some heterozygous females

**B. Human mutants leading to various clinical situations**

Condition	Definition	Human mutants
Situs inversus	Mirror-image reversal of left/right anatomical structures	
Situs ambiguus	Discordant left/right anatomical structures excluding mirror-image reversal of all left/right structures	<i>LEFTYA</i> , <i>ACVR2B</i> , <i>ZIC3</i>

**C. Sidedness of unilateral morphogenic defects**

Defect	Predominant side (%)		
	Left	Right	Equal
Cleft lip	68		
Hemifacial microsomia		62	
Renal agenesis	56		
Clubfoot	55		
Isolated limb asymmetry	73		
Fibular aplasia	65		
Wilms tumour	50		
Planum temporale	65	11	24
Temporoparietal cortex	88	12	

Attention to the finer details of intracellular systems is now essential to the finer details of intracellular systems and especially the specific functions of discrete regions of polarized cells. Allosteric regulation is a well-recognized system to modify protein function via binding small molecules to sites distant to the active centre of a specific protein. It is used to measure co-operative binding. This mechanism is now known to regulate RNAs, acting via a riboswitch to trigger changes in the recipient RNA. It has been used to study regulator genes controlling glycine metabolism, where the riboswitch locates in non-translated regions and is sensitive to local concentrations of glycine (Famulok, 2004).

Another approach copies natural variation by using ligand binding to nucleic acids using compounds known as aptamers. These are synthetic compounds with a biological structure that confers an ability to activate or repress particular genetic systems. Formed in pockets of the molecule and sometimes composed of only 10 atoms, they recognize huge numbers of small organic molecules with high specificities and affinities. Ligands in these pockets identify operons with various RNA types (Mandal *et al.*, 2004). They also offer examples of the many natural means of regulating gene expression already designed artificially

in laboratories. Nor are these systems confined to bacteria, since they have been recognized in plants and their identification in mammalian systems is awaited with interest.

Similar wide and challenging opportunities are emerging to study early mammalian embryology. They could introduce new concepts into assisted human conception. If the trophectodermal stem cell can be identified in 4-cell embryos as carrying mainly animal cytoplasm, the implications could be considerable as described elsewhere by Edwards and Hansis (2005). It might form trophectodermal stem cell lines *in vitro*, enabling comparisons to be made with similar cell lines prepared from mouse blastocysts (Kunarth *et al.*, 2001). This approach might even lead to the identification of the founder germline cell, although this will be difficult to prove since mammalian embryos do not carry the distinct germline markers characteristic of amphibians and flies. Those blastomeres inheriting full polarity and expressing animal and vegetal markers may be the identifiable precursors of inner cell mass (Edwards and Hansis, 2005). Analysing gene activities in these individual cells using microarrays could lead to some highly interesting projects of scientific and clinical significance.

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## Note added in proof

After an earlier version of this lecture was delivered in Bologna, Magdalena Zernicke-Goetze approached the author to stress she had done similar work in mice by labelling animal and vegetal poles in early blastomeres. In this way, she had been able to trace the fate of differentially marked blastomeres and the fate of embryos deficient in specific types of blastomeres as described in references to their published articles, which were added to the current text as this manuscript was being prepared. This new development is clearly welcome, since the research possibilities raised in this paper are now close to application.