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Several of the new reproductive technologies have been cultivated from our current understanding of the genetic programming and cellular processes that are involved in the major morphogenetic events of mammalian preimplantation development. Research directed at characterizing the patterns of gene expression during early development has shown that the embryo is initially under maternal control and later superseded by new transcriptional activity provided by the activation of the embryonic genome. Several embryonic transcripts encoding (*i*) growth factors, (*ii*) cell junctions, (*iii*) plasma membrane ion transporters, and (*iv*) cell adhesion molecules have been identified as contributing directly to the progression of the embryo through the preimplantation interval of development. In this brief review, we have outlined the patterns of expression and the integral roles that these gene families play in the morphogenetic events of compaction and cavitation. Research of this type has greatly facilitated our understanding of the control processes that underlie preimplantation development and represent but one area of this exciting and vigorous field of research.

Key words: Na,K-ATPase, cavitation, compaction, preimplantation development, embryonic transcription.

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Plusieurs des nouvelles techniques de reproduction ont été cultivées d'après nos connaissances courantes de la programmation génétique et des processus cellulaires impliqués dans les événements morphogénétiques du développement lors de la préimplantation mammalienne. La recherche voulant caractériser les profils de l'expression génique au début du développement a montré que l'embryon est d'abord sous contrôle maternel remplacé plus tard par une nouvelle activité transcriptionnelle fournie par l'activation du génome embryonnaire. Nous avons identifié plusieurs transcrits embryonnaires codant : (*i*) facteurs de croissance, (*ii*) jonctions cellulaires, (*iii*) transporteurs ioniques de la membrane plasmique et (*iv*) molécules d'adhésion cellulaire qui contribuent directement à la progression de l'embryon à travers l'intervalle de préimplantation du développement. Dans cette brève revue, nous exposons les profils d'expression et les rôles intégraux que ces familles de gènes jouent dans les événements morphogénétiques de la compaction et de la cavitation. Une telle recherche a grandement facilité notre compréhension des processus de contrôle qui sous-tendent le développement lors de la préimplantation et elle représente rien de moins qu'un domaine de recherche vigoureux et excitant.

Mots clés : Na,K-ATPase, cavitation, compaction, préimplantation, transcription embryonnaire.

[Traduit par la rédaction]

Introduction

It is 13 years since Louise Brown was born. She was the first reported full-term birth to result from the technique of *in vitro* fertilization and embryo transfer in humans. Today, hundreds of infertility clinics worldwide carry out this procedure and thousands of children have been born because of the application of *in vitro* techniques involving manipulation of oocytes or preimplantation embryos. Likewise, cattle breeders have been quick to incorporate and exploit these technological developments and capabilities. In this way, nonsurgical collection and transfer of embryos, storage and deep freezing of embryos for export or domestic use, and embryo splitting for cloning of animals have become routine in the cattle industry (Wilmut *et al.* 1991). More recently, embryo biopsy coupled with polymerase chain reaction technology has been used to sex human (Handyside *et al.* 1990) and bovine embryos (Peura *et al.* 1991) and may also soon be utilized for the early diagnosis of genetic abnormalities. All of these developments have become possible because of several decades of study applied to mammalian oocytes and preimplantation embryos. For practical purposes of laboratory convenience, the mouse embryo has been the animal model of choice for the bulk of the experimentation, but the extension of these technological advances to the human and domestic species has led directly to the current vigorous research activity in these embryo systems. In this brief review, we will outline the critical events in the early developmental program of the mouse embryo up to the blastocyst stage, which just precedes embryo implantation to the uterine wall. Research of the type reviewed below has allowed the acquisition of a sufficient understanding of the metabolic and genetic properties of these preimplantation embryos to result in the development of the culture and manipulative procedures that have so much social and economic impact on our lives today.

ABBREVIATIONS: TGF-α, transforming growth factor-α; PDGF-A, platelet derived growth factor A chain; IGF-II, insulin-like growth factor II; RT-PCR, reverse transcription – polymerase chain reaction; EGF, epidermal growth factor.

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Genetic control of early mammalian development

The process of development begins during gametogenesis within the ovaries and testes of the parental animals. The spermatozoon contributes one set of chromosomes to the embryo at the time of fertilization. Since the sperm chromosomes are densely packaged in the form of nucleoprotamines, they are initially transcriptionally inactive and genetically silent until activated later in embryonic development. The maternal chromosomes of the ovulated oocyte are arrested at metaphase II of meiosis and are also transcriptionally inactive. The oocyte, however, contains a stockpile of maternal ribosomes, mRNAs, tRNAs, and other macromolecules that is sufficient to direct protein synthesis and cleavage until the embryonic genome is activated. At this point, the maternal components are complemented and eventually superseded by new macromolecules encoded by transcripts from the embryonic genome (for reviews see Schultz 1986; Kidder 1993).

In the mouse embryo, only the first cleavage of the fertilized ovum is entirely under maternal control. Development beyond the two-cell stage is dependent upon the resumption of transcriptional activity that is associated with the activation of the embryonic genome (Kidder and McLachlin 1985). During this transition from maternal to embryonic control at the two-cell stage, the majority of maternal mRNA molecules accumulated during oogenesis are degraded and gradually replaced by new mRNA molecules (Piko and Clegg 1982; Clegg and Piko 1983*a*, 1983*b*; Giebelhaus *et al.* 1983). There is a major turnover of mRNA populations during this phase of early development and these changes are both qualitative and quantitative. In a small cDNA library constructed from late two-cell embryos, Taylor and Piko (1987) observed that roughly half of the transcripts present in ovulated oocytes was still represented within the cDNA library, but those of the other half were clones complementary to new gene transcripts synthesized from the embryonic genome during the maternal to embryonic transition period. These fluctuations in mRNA populations are also reflected in marked changes in protein synthetic patterns between the one- and two-cell stages (reviewed in Schultz 1986; Kidder 1993). The most recent study utilized the QUEST system for quantitative analysis of two-dimensional protein gels (Garrels 1989) to examine changes in polypeptide synthesis (Latham *et al.* 1991). The vast majority of the polypeptides analyzed exhibited a twofold or greater change in abundance during the one- to two-cell embryo stages. Roughly 40% showed a marked decline at the two-cell stage, whereas the other 40% was observed to increase in synthesis through the two-cell stage (Latham *et al.* 1991). The former almost certainly reflects the loss of maternal mRNAs owing to decay and the latter is likely due to the appearance of new mRNAs from the embryonic genome's transcriptional activity. The rather abrupt change in polypeptide synthesis pattern occurring during the maternal–zygotic transition does not necessarily mean that all oogenetic products disappear from the conceptus at this time and have no further role to play. In the mouse there is evidence for the persistence of some oogenetic mRNAs and proteins through the transition period (Taylor and Piko 1987; Barron *et al.* 1989; Brenner *et al.* 1989; West and Flockhart 1989). This is an issue that merits further attention, since it is possible that some oogenetic proteins persist and could be functional throughout preimplantation development.

Similar transitions in maternal to embryonic control occur in preimplantation development of embryos from other eutherian mammals, but the timing of the switch is delayed. In human and pig embryos it occurs at the four-cell stage, whereas in sheep and cow embryos the transition begins at the eight-cell stage (reviewed in Telford *et al.* 1990).

The precise mechanisms necessary for the stage-specific activation of embryonic transcription are not known. Members of the POU-domain family of transcription factors (oct-3 and oct-4) are important for DNA replication and initial cleavages of the mouse embryo (Rosenfeld 1991; Scholer *et al.* 1990). In addition to its role in DNA replication it has been suggested that oct-3 might also direct the activation of a set of regulatory genes in the one-cell embryos that could stimulate transcription in the early embryo (Rosenfeld 1991). Indeed, it has been demonstrated that enhancers are required for transcription after the first cleavage of the mouse embryo, but not before (Martinez-Salas *et al.* 1989*a*, 1989*b*).

Many of the transcripts expressed at the two-cell stage of mouse development continue to be expressed and accumulate as development proceeds to the blastocyst stage. However, there are a number of additional transcripts important for cell proliferation, compaction, and blastocoel formation (cavitation) that are not expressed until after the two-cell stage (Schultz 1986; Kidder 1993). The morphological changes in embryos that are necessary for blastocyst formation are, in fact, tightly coupled to ongoing transcriptional events (Kidder and McLachlin 1985; Lee *et al.* 1987). The variation in the patterns of newly synthesized polypeptides observed on two-dimensional gels between the four-cell to blastocyst stages is not as marked as that between the one- and four-cell embryo stages, but several new polypeptides undergo a transcriptionally dependent increase during this interval (Braude 1979).

One set of gene products important for successful blastocyst formation is that encoding the polypeptide growth factors and their receptors. Transcripts for TGF-α, PDGF-A, Kaposi's sarcoma type fibroblast growth factor, and IGF-II have all been detected in cleavage stage mouse embryos by the compacted eight-cell stage through the use of the sensitive RT–PCR method (Rappolee *et al.* 1990). Transcripts for insulin receptors, IGF-I receptors, EGF receptors, and the PDGF-α subunit receptors have also been detected in preimplantation mouse embryos by the RT–PCR method (Rappolee *et al.* 1990; Wiley *et al.* 1992). The same ligand and receptor transcripts are detectable by RT–PCR in RNA isolated from preimplantation cow embryos (Watson *et al.* 1992). To investigate function, a number of studies have been conducted either by the addition of growth factors to the culture medium or by the blockage of growth factor expression by genetic or biochemical methods. These studies have shown that growth factors do, indeed, play a significant role in the stimulation of metabolic and synthetic activities in early mouse embryos, as well as in increased cell proliferation and cell number in blastocysts (Harvey and Kaye 1988, 1991; Heyner *et al.* 1989; Paria and Dey 1990; Beebe and Kaye 1991; Dardik and Schultz 1991; Tamada *et al.* 1991; Rappolee *et al.* 1991).

Another set of important genes is that encoding the con-

nexins, which form the protein component of the gap junction channels. Gap junctions are assembled at the eight-cell stage of mouse development, during the process of compaction (see next section for the morphological aspects of compaction) and provide channels for embryo wide intercellular coupling (for review see Kidder 1987). If gap junction formation is perturbed in early mouse embryos through the microinjection of either antibodies or antisense RNA, the embryos decompact and development is arrested (Lee *et al.* 1987; Bevilacqua *et al.* 1989). Recent studies by Valdimarsson *et al.* (1991) and Nishi *et al.* (1991) have shown that connexin43 is one member of the connexin family that accumulates and is incorporated into functional gap junctions at the time of compaction. The accumulation of connexin43 transcripts is due to embryonic transcription, beginning with low levels at the two- to four-cell stages and increasing to high levels by the blastocyst stage.

During the process of cavitation, one of the key gene products is the Na,K-ATPase that has been localized to the basolateral plasma membrane domain of the mural trophectoderm cells (Watson and Kidder, 1988; Watson *et al.* 1990*a*; Wiley *et al.* 1990). It is an important mediator of fluid transport across the trophectoderm epithelium into the nascent blastocoelic cavity. The Na,K-ATPase consists of an α and β subunit and it is the α1 and β1 isoforms, products of two small multi-gene families, that are expressed during cavitation (Watson *et al.* 1990*b*; Gardiner *et al.* 1990*b*). The α1 subunit is transcribed and accumulates from the two-cell stage onwards, but the β1 transcript is present in extremely low amounts until the morula stage when its translation may be required for the maturation and membrane insertion of the α subunit (Watson *et al.* 1990*a*, 1990*b*; Gardiner *et al.* 1990*b*; Kidder 1993).

There are many other genes (some of them for the usual housekeeping functions in cells) that have been studied during mouse preimplantation development, but we have restricted our discussion to those cited above because they provide excellent examples of gene sets that are temporally regulated in the early embryo and that play an important role in the formation of the blastocyst.

Morphological components of compaction

The first three cleavage divisions of the mouse embryo serve to subdivide the ooplasmic constituents into smaller equipotent compartments or blastomeres. No increase in embryo volume or diameter occurs. Following the third cleavage division, morphological processes are initiated that lead to the polarization of the embryonic blastomeres. These events encompass the morphogenetic event of compaction. Compaction is a common feature of preimplantation development within all eutherian mammalian embryos, although the timing of this event varies greatly, arising later within the preimplantation embryos of the domestic species (McLaren 1982). Compacting embryos undergo an increase in interblastomeric contact that obscures the distinct individual cell boundaries and continues until the embryo ultimately appears as a uniform cell mass called a morula (Ducibella and Anderson 1975; Ziomek and Johnson 1980; Ziomek 1987; Wiley *et al.* 1990). The cellular events associated with compaction include (*i*) the development of Ca^{2+}-dependent cell adhesion, (*ii*) the establishment of gap-junction mediated interblastomeric cell communication, (*iii*) the initiation of cell-contact-induced cell polarization, and (*iv*) the appearance of focal tight junctions which eventually divide the plasma membrane of the outer blastomeres into discrete apical and basolateral membrane domains (Hogan *et al.* 1986; Ziomek 1987; Fleming and Johnson 1988).

Interblastomeric adhesion is mediated by the redistribution of the Ca^{2+}-dependent cell adhesion molecule uvomorulin within the basolateral membrane surfaces of compacting eight-cell mouse embryos. The increase in interblastomeric contact provided by uvomorulin plays an important role in blastomere polarization and the formation of the focal tight junctions (Wiley *et al.* 1990; Watson *et al.* 1990*a*). As interblastomeric contact increases, the formation of free and apposed plasma membrane regions occurs, and by the 16-cell stage the embryo is composed of an outer layer of polar cells that completely encloses an inner group of four to seven apolar blastomeres. The degree of cell contact associated with cell position is thought to provide the necessary developmental cue for the maintenance of polarity within the outer blastomeres (for review see Ziomek 1987). The free or apical membrane surfaces of the outer blastomeres develop a cytochalasin-D-resistant microvillus cap (Fleming and Johnson 1988), Na^+-dependent amino acid transport systems (Miller and Schultz 1985), a Na^+,glucose-cotransporter (Wiley *et al.* 1991) and Na^+ channels (Manejwala *et al.* 1989). The basolateral surfaces of the outer blastomeres remain free of microvilli, but become distinguished from the apical surfaces by the localization of the tight junction ZO-1 polypeptide (Fleming *et al.* 1989), gap junctions (Kidder 1987), and uvomorulin (Vestweber *et al.* 1987). The polarity exhibited by the outer blastomeres is displayed within the cell cortex by the appearance of an apical actin cap and also within the cytoplasm by the asymmetric distribution of lipid vesicles, mitochondria, and the nucleus towards the basal poles of the cells (Wiley 1987). This cell polarity is also very stable, as it is still maintained even in the presence of inhibitors (such as cytochalasin B or D) that block the cell flattening, the development of cell adhesion, and the establishment of cell coupling via gap junctions (Ziomek *et al.* 1982). Although asymmetric cell contact certainly plays an integral role in the generation of cell polarity, the presence of asymmetrical ion currents between the free and apposed cell surfaces of the outer blastomeres may also contribute to this process (Nuccitelli and Wiley 1985). These events of compaction, particularly the development of asymmetrical cell contact and polarity within the outer blastomeres, are essential processes that directly contribute to the formation of the blastocyst. By the late morula stage, the embryo acquires the remaining macromolecules that are necessary for it to proceed into the process of cavitation, which is the second major morphogenetic event of preimplantation development.

Cavitation and blastocyst formation

Preimplantation embryogenesis represents, primarily, a period of preparation for eventual implantation to the uterine wall. This process culminates in the development of the blastocyst. Cavitation is required for implantation, since it is through this process that the first epithelium, the trophectoderm, develops (for review see Wiley *et al.* 1990).

The trophectoderm initiates implantation via direct contact with the uterus and eventually contributes to the trophoblast giant cells and extraembryonic membranes such as the chorion (Hogan *et al.* 1986). This epithelium also encloses a small cluster of cells called the inner cell mass (the progenitors of the embryo proper) within the fluid-filled confines of the blastocyst. The differentiation of these two cell types has direct ties to compaction, since the trophectoderm is derived from the polar outer cells and the inner cell mass is derived from the apolar inner cells of the morula (Hogan *et al.* 1986). The mechanism of cavitation is tightly coupled with trophectoderm cell differentiation.

During the process of epithelial polarization, the trophectoderm develops the capacity to initiate and regulate the events of cavitation by the acquisition of the gene products required to generate the blastocoelic fluid, as well as those that prevent the uncontrolled leakage of this fluid from the blastocoel cavity. To understand the mechanism of blastocyst formation we must focus our attention on the trophectoderm ion transport systems, the development of the epithelial junctional complex, and the role of cell adhesion in the maintenance of the polarized state. Since the blastocoelic fluid is largely composed of water, the trophectoderm ion transport systems are thought to play a central role in the establishment of ion concentration gradients across the epithelium, which could facilitate the osmotic accumulation of water into the blastocoelic space to form the nascent blastocoelic fluid (for review see Biggers *et al.* 1988). Isotope flux measurements, as well as electron probe microanalyses of Na^+, Cl^-, K^+, Ca^{2+}, and Mg^{2+}, have shown that all of these ions are concentrated within the blastocoelic fluid (Powers and Tupper 1975, 1977; Borland *et al.* 1977; Manejwala *et al.* 1989; Van Winkle and Campione 1991). Active transport would be required to move these ions against their concentration gradients. The vectorial transport of Na^+ and Cl^- (but not K^+) from the medium is essential for the onset and progression of cavitation (Manejwala *et al.* 1989). The Na^+ ions likely enter the trophectoderm cells through apically localized Na^+ channels and (or) via various Na^+ cotransporters such as the Na^+,H^+-exchanger (Manejwala *et al.* 1989) or the Na^+,glucose-cotransport system (Wiley *et al.* 1991). The Cl^- transport is not carrier mediated and is therefore paracellular, and must be regulated by the epithelial junctional complex (Manejwala *et al.* 1989). To complete the Na^+ gradient across the trophectoderm would require the localization of an active Na^+-transport system confined to the basolateral membrane domain.

Experimentation conducted over the last 15 years has shown conclusively that mouse and rabbit blastocysts express an active Na,K-ATPase that becomes confined to the basolateral plasma membrane domain of the trophectoderm (Dizio and Tasca 1977; Vorbrodt *et al.* 1977; Wiley 1984; Watson and Kidder 1988; Benos *et al.* 1985; Overstrom *et al.* 1989; Gardiner *et al.* 1990*a*). At least one isoform of the α subunit of this enzyme becomes detectable by immunofluorescence at the late morula stage and, by the onset of cavitation, the enzyme is localized exclusively in the basolateral surfaces of the trophectoderm (Watson and Kidder 1988). Na,K-ATPase has also been detected within earlier preimplantation embryo stages (Gardiner *et al.* 1990*b*). This was expected because of the essential housekeeping role that this enzyme plays. The immunofluorescence results, however, suggest that the Na,K-ATPase increases in abundance within the mouse embryo and achieves its polarized distribution just prior to and during the events of cavitation. The polarized distribution of this enzyme within the trophectoderm would provide a mechanism for the completion of the trans-trophectoderm Na^+ gradient by the active transport of Na^+ into the blastocoelic cavity. The water that would form the blastocoelic fluid would be osmotically driven across the epithelium into the nascent blastocoelic cavity.

The epithelial junctional complex has several roles to play during cavitation, including the regulation of paracellular transport and the maintenance of the plasma membrane apical and basolateral membrane domains (Fleming *et al.* 1989). The regulation of paracellular transport facilitates the Cl^- transport into the blastocoelic cavity and perhaps more importantly also regulates the leakage of the nascent blastocoelic fluid. The development of the junctional complex is initiated within the eight-cell embryo, but requires an additional two cleavage divisions to become complete in the blastocyst (Magnuson *et al.* 1977; Fleming *et al.* 1989). The impermeable seal does not form until after cavitation has begun and it is only after the formation of this seal that the leakage of blastocoelic fluid is regulated, allowing the embryo to expand in size owing to fluid accumulation (McLaren and Smith 1977). The epithelial junctional complex, along with the membrane cytoskeleton, almost certainly contributes to the maintenance of the polarized Na,K-ATPase distribution within the trophectoderm (Watson *et al.* 1990*a*). If the junctional complex and the cortical actin cytoskeleton are disrupted by the incubation of mouse blastocysts in medium containing cytochalasin B or D, the Na,K-ATPase polarized basolateral distribution is also disrupted and the enzyme becomes confined to the apical membrane domain (Watson *et al.* 1990*a*). These embryos can recover and proceed normally through the cavitation process if they are subsequently cultured in medium without cytochalasin. This experiment demonstrates that cavitation is dependent upon the epithelial junctional complex and the cortical cytoskeleton, perhaps owing to their role in maintaining the basolateral distribution of the Na,K-ATPase.

Further experimentation has shown that the cell adhesion provided by uvomorulin also plays an important role in the formation of the epithelial junctional complex and the stable polarized integration of Na,K-ATPase into the basolateral membrane surfaces of the trophectoderm (Watson *et al.* 1990*a*). Embryos treated with an antibody to uvomorulin do not undergo compaction and also do not proceed with a normal cavitation. Instead of a single fluid-filled cavity, several smaller intracellular cavities form within each of the blastomeres of the embryo. Immunofluorescence has demonstrated that each of these fluid-filled cavities is encircled by Na,K-ATPase (Watson *et al.* 1990*a*). Under these conditions, the epithelial junctional complex also does not form. It appears that uvomorulin, possibly working in concert with the membrane cytoskeleton, contributes to the establishment of the epithelial junctional complex and the insertion of Na,K-ATPase into the basolateral membranes.

Experimentation of the type reviewed above has clearly identified the roles of the epithelial junctional complex, the membrane cytoskeleton, Na,K-ATPase, and uvomorulin in the process of blastocyst formation. We are only now beginning to understand how the culture environment may

influence this morphogenetic event. The addition of TGF-α and (or) EGF to the culture medium results in a dramatic increase in blastocoel expansion rates (Dardik and Schultz 1991). These growth factors are likely exerting their effects via the EGF receptor which is expressed in the blastocyst (Wiley *et al.* 1992). A possible pathway for this effect is through an EGF-receptor-mediated increase in intracellular cAMP levels, which, in turn, is thought to have a stimulatory effect upon Na,K-ATPase activity (Manejwala *et al.* 1986). Only further investigation will clarify whether this pathway is being utilized in the blastocyst.

Cavitation is thus a complex cellular process that requires the precise coordination of several cellular events. Several of the crucial gene products have been identified including the tight junction (ZO-1 protein), gap junctions (connexin43), uvomorulin, several ion transporters such as the Na^+,H^+-antiporter, Na^+,glucose-cotransporter, Na,K-ATPase, and also growth factors such as TGF-α and EGF. These gene products represent important molecular markers for cavitation and a further clarification of their roles will lead to a greater understanding of the overall mechanism of blastocyst formation.

Summary and future prospects

Our knowledge of the components and events involved in blastocyst formation is but one example of how vigorous research within the last few years has led to an increased understanding of the cellular processes that promote early mammalian development. The application of molecular and recombinant DNA methods to the study of preimplantation development has resulted in the advancement of several powerful techniques such as transgenesis and gene targeting by homologous recombination. These approaches provide the mammalian developmental biologist with the ability to study the expression pattern and function of virtually any gene during the process of embryogenesis. Additionally, the improvement of embryo culture methods now allows the reliable production of relatively large numbers of preimplantation stage embryos from the agricultural domestic species. This new source of research material should ensure that the investigation of mammalian preimplantation development remains an exciting and productive area of research long into the future.

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