Development 1992 Supplement, 113-118 (1992)
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The relationship of gap junctions and compaction in the preimplantation mouse embryo

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Summary

In the mouse embryo, gap junctions first appear at the 8-cell stage as compaction is about to take place. Compaction of the embryo is important for the differentiation of the first two cell types; the inner cell mass and the trophectoderm. Our studies examine the contribution of gap junctional communication at this stage of development. We have characterised the normal sequence of appearance of gap junction protein and its distribution. The extent of communication as shown by the passage of dye between cells has been recorded in both normal embryos and embryos treated with drugs that influence gap junctional communication. Comparisons have been made with embryos that express a lethal gap junction defect and attempts were made to rescue such embryos by increasing their gap junction communication.

Key words: mouse embryo, gap junction, connexin 43, compaction.

Introduction

In placental mammals, gastrulation of the embryo is necessarily preceded by events that ensure the formation not only of the embryo itself, but also the structures involved in implantation and subsequent generation of the supportive tissues; the extraembryonic membranes and the placenta. During this early phase of development, cells generated by cleavage of the fertilized egg are separated into embryonic and extraembryonic lineages. This process constitutes the first differentiative step in the development of the mammalian embryo.

Cells produced by the first three cleavages initially contact each other only loosely and are not linked by any specialised intercellular contacts. However, during the 8-cell stage, cellular inter-relations change dramatically. The cells compact down on each other, minimising the intercellular space. They become polarized as tight junctions form between them at their outer edges and they begin to communicate with each other through the intercellular structure, the gap junction. Both tight and gap junctions are important for polarization and compaction, which ensure the subsequent establishment of the separate identities of outer cells, which will form the trophectoderm, and inner cells, which will give rise to the embryo proper. Since gastrulation is restricted to cells of the embryo, the processes that control polarization and compaction during the preimplantation stages take on crucial importance. This article examines evidence implicating gap junctional communication during these stages. We include also a brief description of recent work suggesting that gap junctions are regulated in response to permeability changes.

How tight junctions and gap junctions contribute to the morphogenetic movements of gastrulation has not yet been investigated for any species. One can predict from the nature of the invagination through the primitive streak that all close cellular interactions must be modified. The tight junctions that maintain the integrity of the epithelium must break down. Transient gap junction formation between cells destined to form the mesoderm layer of the embryo is likely during invagination, but how this might contribute to cellular rearrangements achieved by gastrulation remains to be investigated.

The time course of appearance of gap junctions in the mouse embryo

Prior to the 8-cell stage, cells may communicate via cytoplasmic bridges or mid bodies remaining after cleavage. Such communication is characterised by the ability to pass large molecules, such as Horse Radish Peroxidase, between cells. In contrast, intercellular communication through gap junctions will only allow the transfer of small molecules such as the dye Lucifer Yellow. Gap junctional communication begins for the first time at the 8-cell stage, as the embryo begins to compact (Lo and Gilula, 1979; Goodall and Johnson, 1982; 1984). From this time onwards gap junctional communication is found between all cells of the preimplantation embryo, except during division, when gap

junctions (and other junctions) dissociate and communication ceases until division is over (Goodall and Maro, 1986). mRNAs for gap junction protein connexin 43 are first expressed at the 4-cell stage and gap junction plaques appear during the 8-cell stage (Dulcibella et al., 1975; Barron et al., 1989; Nishi et al., 1991). Gap junctions are constructed from a highly conserved family of proteins, termed connexins (Beyer et al., 1990), with the basic structure and assembly being the same for all members. A hexameric arrangement of the connexins creates a central channel, through which communication can take place when two hexamers in opposing membranes are linked. The evidence so far suggests that the first gap junctions in the early mouse embryo are constructed from connexin 43.

Gap junctions first appear in the compacting 8-cell-stage embryo as punctate spots between the cells at points of contact. Fig. 1A shows a confocal optical section through an early 8-cell embryo stained with an antipeptide antibody to an amino acid sequence unique to connexin 43 (Harfst et al., 1990; Gourdie et al., 1990). This illustrates the punctate intercellular staining, characteristic of gap junctions. Control competition experiments on heart sections indicate that gap junction labelling with this antibody is eliminated by the addition of the corresponding free peptide. Antibodies to peptides unique to other connexins (e.g. connexin 32: Evans and Rahman, 1989; Rahman and Evans, 1991; Evans, W.H., Green, C.R. and Warner, A.E., in preparation) do not stain at this early stage, indicating that connexin 43 is the predominant gap junction protein. Fig. 1B, C show projections of two compacting 8-cell embryos, reconstructed from a series of confocal optical sections. One embryo (B) has not yet begun to compact and no gap junctions are present. The second embryo (C) has begun to compact and punctate junctions, located in the central region of the embryo, are clearly visible. At this stage, functional communication, mapped with Lucifer Yellow transfer, may occur between as few as two cells or as many as eight, depending on the accretion rate of gap junction protein into plaques in individual samples. As compaction takes place, adjacent cell membranes flatten against each other and the junction plaques increase in both size and number. This is accompanied by a shift of gap junction structures from a predominantly central location to a peripheral position within the junctional complex, where tight junctions form (see Fleming, this volume). Fig. 1D shows a surface view of a compacted embryo illustrating how peripheral spots gradually zip up to form a belt surrounding each cell as compaction is completed.

After division to 16 cells, some progeny lie entirely within the embryo and from this time on inner and outer cells have different destinations: inner cells become the inner cell mass which will form the embryo proper, whereas outer cells form trophectoderm which contributes to the extraembryonic membranes. Trophectodermal cells are linked to each other by gap junctions in stripes along the outermost limiting membrane (Fig. 1D), in close association with the tight junctions (Magnuson et al., 1977; Fleming, this volume). Junctions between inner cells and between inner and outer cells remain in the form of punctate spots.

Cavitation of the embryo, marking the appearance of the nascent blastocoele, depends on the integrity of the tight junction, which allows fluid generated by the action of the sodium pump to accumulate in the intercellular spaces. The third intercellular adhesive structure, the desmosome, appears at cavitation (see Fleming, this volume). The differential distribution of gap junctions between trophectoderm and inner cells is retained throughout blastocoele expansion and is most striking in the expanded blastocyst. Fig. 1E and F shows a projection of confocal optical sections through the surface of an expanded blastocyst where the thin trophectoderm cells are clearly linked by belt gap junctions (E). In contrast, a projection of confocal sections taken through the centre of the embryo, reveals numerous punctate junctions within the inner cell mass (F).

Gap junctions and the maintenance of compaction

The importance of tight junctions for the establishment and maintenance of the compacted state is self evident; the separate identity of inner and outer cells is thereby established and a tight adhesive link between cells is clearly an essential prerequisite for the retention of blastocoel fluid. However, a role for gap junctional communication is not so obvious. Nevertheless, two lines of evidence suggest that gap junctions also may be important for maintaining compaction.

The first comes from experiments in which antibodies against gap junction protein were injected into one cell either at the 8-cell stage, after communication had been established or into one cell at the 2-cell stage, well before gap junctions appear (Lee et al., 1987). The antibody prevented the transfer from cell to cell of small molecules and ions, while preimmune serum was without effect. At the 8-cell stage, gap junction antibody injection led to the decompaction and extrusion of the non-communicating cell or cells while the rest of the embryo maintained compaction and continued to develop. At the 2-cell stage, cell division continued without interruption. However, the progeny of the antibody-injected cell failed to take part in compaction while the progeny of the uninjected cell compacted with a normal time course (see Table 1).

The second line of evidence arises from a study of DDK defective embryos. The DDK syndrome is generated when DDK females mate with males from another strain. DDK/DDK progeny are normal whereas 90-95% of DDK/cross embryos express the defect and are destined to die before the expanded blastocyst stage (Wakasugi, 1973; Buehr et al., 1987). The defect is characterized by the gradual decompaction of cells, beginning at the 16-cell stage and continuing through to the expanded blastocyst. Measurements of the efficiency of gap junctional communication were made by injecting Lucifer Yellow into one cell of fully compacted 8-cell embryos and determining the time taken for the last cell to become visibly fluorescent. DDK/DDK embryos showed transfer times that were no different from other strains (median 92 seconds) while DDK/C3H embryos were significantly slower (139 seconds).

One way of testing for a link between gap junctional communication and compaction is to manipulate gap junc-

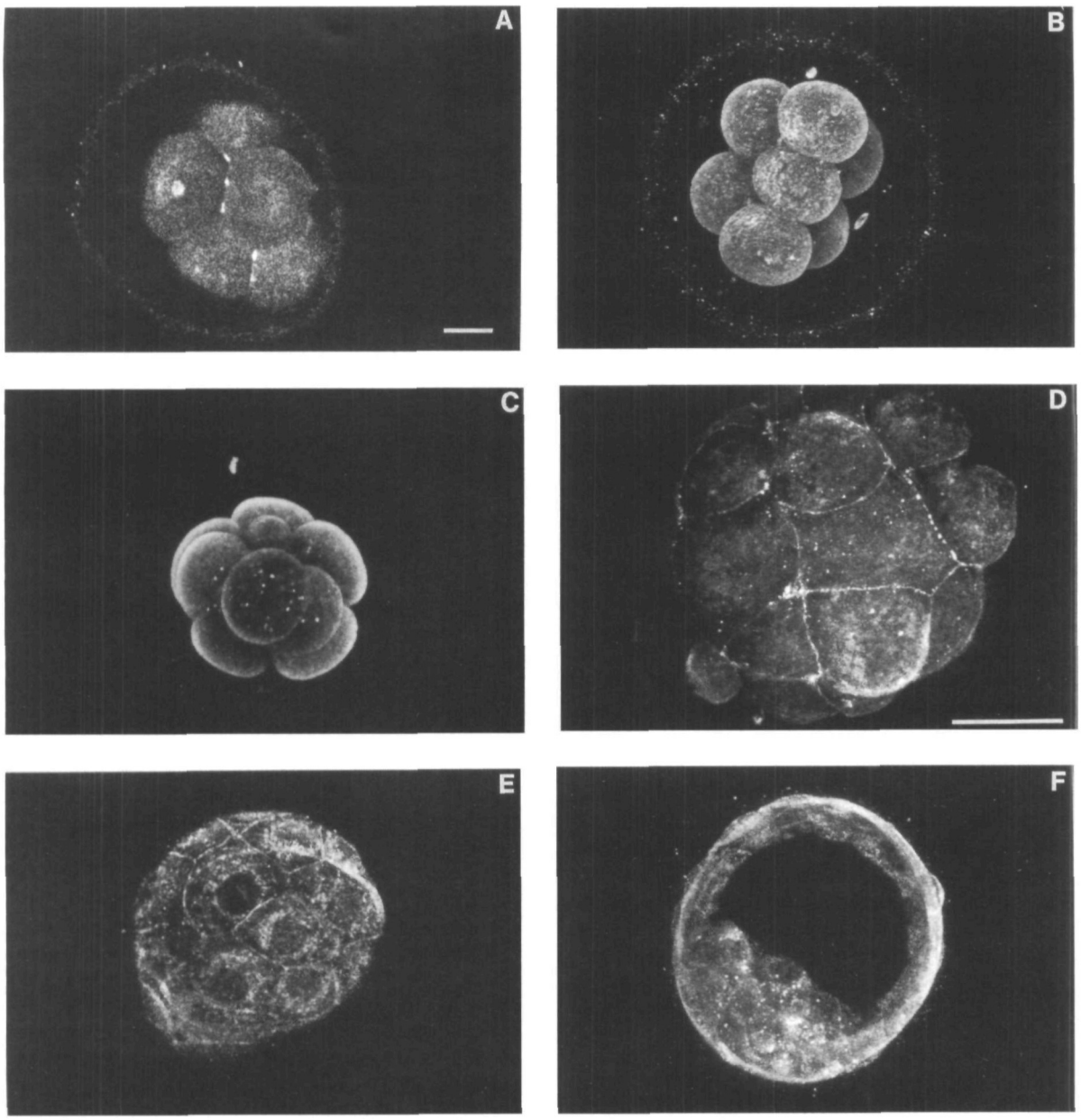

Fig. 1. A-F shows images of mouse embryos immuno labelled for the gap junction protein connexin 43. These were constructed by the use of a laser scanning confocal microscope. (A) Single optical section through the centre of a compacting 8-cell-stage embryo. The punctate intercellular labelling of the gap junction protein is clearly visible. (B) Complete reconstruction of a series of optical sections (34) taken through an embryo that has just reached the 8-cell stage. Note the very round cells and the absence of connexin 43 labelling. (C) Complete reconstruction of a compacting 8-cell-stage embryo showing the punctate labelling of all gap junctions. Note the restriction of junctions to the centre of the embryo. (D) Projection of an almost completely compacted embryo. Note the punctate gap junctions lining up along the outermost region of intercellular contact. As compaction progresses the junctions become smaller and closer together creating linear labelling zipping up the cells. (E) A projection of the top eight optical sections of an expanded blastocyst which shows the linear labelling of the gap junctions between the trophectoderm cells. (F) A projection of 10 optical sections through the centre of the same embryo, to show the inner cell mass where the intercellular labelling is in the form of punctate spots. Scale bars are 25 μm. Bar in A also applies to B,C,E and F.

tion permeability and observe the developmental consequences. Gap junction permeation is highly sensitive to intracellular pH (Turin and Warner, 1977, 1980; Bennett et al., 1978). The effect is both rapid and completely reversible and a relatively small change in pH_i will alter substantially junctional conductance because the relationship between conductance and pH_i is very steep. Incubation in a solution containing mM concentrations of either a weak acid or weak base provides a convenient way to lower or raise intracellular pH and decrease (acid) or increase (base) gap junction conductance.

The weak base methylamine produced a rapid speeding

Table 1. *Gap junction permeability and the maintenance of compaction*

Strain	Treatment	Junctions	Compaction maintained	n/N
B10CF1	None	Normal	91%	39/43
B10CF1	Pre Immune IgGs	Normal	100%	11/11
B10CF1	Gap junction Ab	Blocked	23%	4/17
B10CF1	Butyrate	Reduced	40%	12/30
DDK/DDK	None	Normal	91%	99/109
DDK/DDK	Butyrate	Reduced	63%	25/40
DDK/C3H	None	Reduced	10%	17/188
DDK/C3H	Methylamine	Increased	31%	21/67
DDK/C3H	Cyclic AMP	Increased	21%	14.67

of junctional transfer in both control (DDK/DDK) and defective (DDK/C3H) embryos (Buehr et al., 1987). A relatively short (6 hours) treatment of DDK/C3H zygotes from the 8-cell stage, which is before decompaction begins, improved significantly the survival to the blastocyst stage (see Table 1), but did not affect controls.

An alternative way of improving gap junction conductance is to increase intracellular levels of cyclic AMP (David et al., in preparation). This was achieved either by exposure to a membrane-permeable derivative of cyclic AMP (1 mM) or by treatment with a reagent such as Forskolin (100 μM), which activates adenylate cyclase (Hax et al., 1974) so that cyclic AMP accumulates inside the cell. Both treatments produce a significant increase in gap junction permeation in control and DDK/C3H (defective) embryos, although DDK/C3H embryos respond relatively slowly (> 30 minutes), perhaps because their internal pH is intrinsically low (see below). When DDK/C3H embryos are treated with a membrane-permeable derivative of cyclic AMP for 6 hours beginning at the 8-cell stage, survival to the expanded blastocyst stage is increased (Table 1), although the effect is not as marked as for methylamine. Since cyclic AMP increases junction permeation without a change in intracellular pH, this result supports the view that the efficiency of junctional communication is the dominant factor.

Since improving gap junctional communication in embryos destined to decompact can restore normal development, we asked whether reducing junctional permeability in normal embryos can reproduce the DDK phenotype (David et al., in preparation). The weak acid butyrate lowers intracellular pH by 0.2 to 0.5 of a pH unit in control embryos where pH_i normally lies close to neutrality. When B10CF1 embryos were treated with 10 mM butyrate, the fall in intracellular pH reduced junctional transfer. The degree to which junctional communication slowed was variable, probably because of differing rates of entry of butyrate and intracellular buffering. After 60 minutes exposure to butyrate, 50% (n=12) took more than 25 minutes to transfer dye and were considered to demonstrate complete block. With longer exposure times, the proportion showing complete block increased and after 2 hours all the embryos tested failed to transfer Lucifer yellow. The developmental consequences were tested on embryos at the 8-cell stage and treatment with butyrate lasted 4 to 6 hours, exactly the same interval as for methylamine and cyclic AMP. The embryos were returned to normal medium and scored for maintenance of compaction 24 and 48 hours later. Measurements of intracellular pH showed that even after long incubation periods pH_i was restored rapidly to neutrality. Butyrate-treated embryos reproduced the DDK syndrome; a proportion (Table 1) failed to maintain compaction and did not reach the expanded blastocyst stage.

The finding that lowering internal pH in normal embryos, thereby reducing the permeability of gap junctions, induces the DDK defective phenotype raises the interesting possibility that DDK/C3H embryos might be characterized by naturally low intracellular pH. We used the fluorescent dye BCECF to measure intracellular pH (David et al., in preparation). Normal embryos have an intracellular pH close to pH 7.0. However, pH_i in DDK/C3H embryos is significantly lower (mean pH 6.7). Thus, poor gap junctional communication in DDK/C3H embryos probably derives from low pH rather than any alteration in the properties of the gap junctions. This finding has interesting consequences for understanding the basis of the DDK defect since it suggests that the primary defect may come from defective pH regulating mechanisms.

Table 1 summarizes the various manipulations that influence junctional permeability together with the consequences for the maintenance of compaction. There is a clear correlation between junctional communication and compaction. Reduced gap junctional communication is associated with significantly increased likelihood that compaction will fail. It is important to note that both decompaction of normal embryos and improved compaction in DDK/C3H embryos were achieved by relatively short treatments at the 8-cell stage, almost 24 hours before the developmental consequences were apparent.

The regulation of gap junctions

The consequences of manipulating gap junction permeability are expressed as subsequent alterations in embryonic development, manifested long after junctional permeability has returned to normal. This raises the interesting possibility that the developmental outcome may reflect regulation of gap junctions in response to the imposed permeability change. This possibility is reinforced by the observation that junction permeability must be held at an altered level for a minimum of 4 to 6 hours to see long-term effects. Thus Buehr et al. (1987) noted that rescue of DDK/C3H embryos to the expanded blastocyst stage required at least 6 hours treatment with methylamine and that embryos could only respond if treatment was completed before the morphological consequences of the defect became manifest. We have recently begun to analyse the long-term consequences of such treatments (Becker, D. L., Green, C. R. and Warner, A. E. unpublished data).

Since intracellular pH is naturally low and junction permeability reduced in DDK/C3H embryos, we have begun by examining the distribution of gap junctions in DDK defective progeny. Fig. 2 shows an example of a DDK/C3H embryo at the 16-cell stage stained with an antibody to connexin 43. It is clear that there are many punctate gap junctions, indeed a greater number than normally found in con-

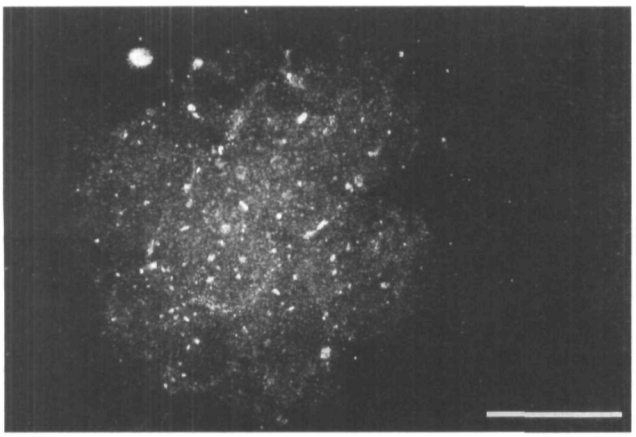

Fig. 2. Projection of a series of optical sections reconstructing a poorly compacted DDK/C3H morula which has been immunolabelled against the gap junction protein connexin 43. The gap junction labelling is heavy and in the form of large punctate spots. Note the absence of linear bands of label between the outermost cells of the presumptive trophectoderm. Scale bar 25 μm.

trols of the same age. Furthermore, the junctions exist entirely as punctate spots apparently randomly distributed along the intercellular opposing membranes. The translocation to form belt gap junctions within the junctional complex between outermost cells has not occurred, implying that cell polarization is lost as the defect is expressed. A small proportion of DDK/C3H embryos showed a normal distribution of gap junctions; presumably these embryos fall into the class of DDK/C3H embryos that are destined to survive and form normal blastocysts (approximately 10%).

We mimicked the DDK syndrome by exposing normal embryos to butyrate for 18 hours and examined gap junction distribution as soon as treatment ended. Most of these embryos fail to compact and the distribution of gap junctions matched that observed in the majority of DDK/C3H embryos. Outer cells failed to develop belt gap junctions between them and all cells were linked by numerous, large, punctate gap junctions. As with DDK/C3H embryos a very small proportion reached the expanded blastocyst stage; these embryos proceed through the preimplantation stages at a slower rate than controls.

The consequences of increasing gap junctional communication have as yet only been studied in normal embryos treated with Forskolin (100 μM), which raises intracellular cyclic AMP and speeds junctional communication. Although the number of embryos so far examined is small, the pattern of gap junction location matches that of normal controls. However, double blind analysis suggests that such embryos develop more rapidly than untreated controls and appear to express fewer gap junctions than normal.

The results of these preliminary experiments are summarized in Table 2 and suggest that gap junctions up-regulate when junctional permeability is low and down-regulate when junctional permeability is high. The mechanism that triggers these alterations in gap junctions remains to be explored.

Table 2. *Gap junction regulation in response to permeability change*

Strain	Treatment	Junctions permeability	Junctions number
B10CF1	None	normal	normal
B10CF1	Forskolin	high	reduced
DDK/C3H	None	low	increased

Future directions

These experiments raise many questions. It will be necessary to follow gap junction protein synthesis to define the minimum interval required to trigger change. One interesting issue that inevitably emerges is the inter-relation between the various intercellular junctions that link cells of the preimplantation embryo. Compaction is normally accompanied by the generation of tight junctions and an embryo that fails to maintain the compacted state must lack functionally effective tight junctions. It is already known that antibody-induced dissociation of tight junctions can induce decompaction without influencing gap junctional communication (Goodall, 1986). One clear message that emerges from our experiments is that the converse is unlikely to be true. With the increasing availability of antibodies and probes for following the consequences of these manipulations for tight junctions and desmosomes as well as gap junctions, it should now be possible to follow all three junctional contacts and see whether they are regulated in a coordinated manner. The extension of such studies into the postimplantation stages that include gastrulation may remain difficult in the mouse because of the constraints imposed by the culture of embryos outside the uterine environment, which is necessary for experimental investigation of these problems. However, work on other vertebrates should prove more feasible and provide information that can illuminate our understanding of the way in which close cellular interactions contribute to gastrulation in the mouse.

We are grateful to our colleagues for allowing us to describe the results of unpublished experiments. This work was made possible by grants from the Wellcome Trust to A. E. W. and to C. R. Green and A. E. W. A. E. W. thanks the Royal Society for their support.

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