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INTERCELLULAR COMMUNICATION IN PREIMPLANTATION DEVELOPMENT: THE ROLE OF GAP JUNCTIONS

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1. ABSTRACT

Gap junctions are sites where intercellular membrane channels are clustered that allow neighboring cells to pass small molecules directly between them. Gap junctional intercellular communication has been implicated in a variety of human diseases. Gap junction channels are assembled from a large family of proteins called connexins with each type of channel having some unique properties. Preimplantation mouse and rat embryos express multiple connexins and thus potentially contain many types of gap junction channels. Based on experiments focussing on connexin43, gap junction assembly in the mouse begins during compaction in the 8-cell stage and is post-translationally regulated. Gene targeting has been used to create mice lacking individual connexins that are expressed in preimplantation embryos, but none of these experiments has yet revealed a necessary role for any single connexin before implantation. Experiments with anti-connexin antibodies and pharmacological blockers of gap junctional coupling have provided conflicting evidence as to the importance of gap junctions for preimplantation development. However, connexin knockouts have revealed important roles for gap junctional coupling in early postimplantation development. It is proposed that expression of multiple connexins in the blastocyst could prepare the implanting conceptus for rapid diversification of cell types during gastrulation and development of the placenta.

2. INTRODUCTION

As in any developmental process, intercellular communication is required to order the many changes that occur during preimplantation development. Paracrine factors, either secreted by the embryo itself or by the reproductive tract, have been shown to influence preimplantation embryogenesis in a variety of ways (reviewed in reference 1). Another, more direct, avenue of intercellular communication within the embryo is provided by gap junctions. Gap junctions are specialized regions of contact between cells where intercellular membrane channels are concentrated. Gap junction channels provide direct conduits for sharing of metabolites and other small molecules, including second messengers, between cells thus functionally coupling them as in a syncytium

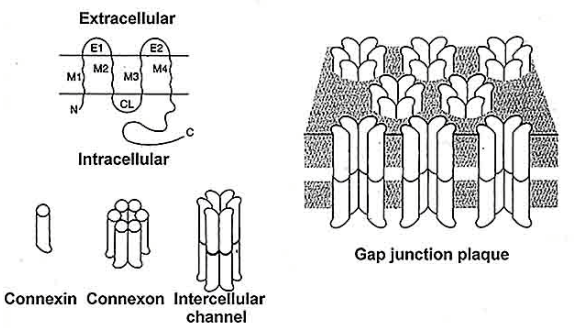

Figure 1. Organization of connexins into connexons, intercellular channels, and gap junction plaques (modified from reference 5).

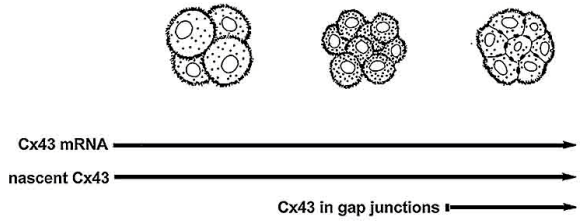

Figure 2. Gap junction assembly during preimplantation development of the mouse as revealed by analysis of Cx43. Transcripts encoding Cx43 accumulate and are translated through the early cleavage divisions. Nascent Cx43 is restricted to an intracellular compartment (small dots) until compaction, when it translocates into plasma membranes and into gap junction plaques (solid bars) in areas of cell apposition. The latter step, which is sensitive to inhibitors of protein trafficking, coincides with the onset of intercellular coupling.

(reviewed in 2-5). Gap junctional coupling is established very early in mammalian development; in the mouse it occurs during compaction in the 8-cell stage (reviewed in 6). Once gap junctions have formed, the embryo remains coupled throughout until "communication compartments," areas of spatially restricted intercellular coupling, begin to appear around the time of implantation (7-10).

3. CONNEXINS, CONNEXONS, AND GAP JUNCTIONS

A gap junction is an array of paired hemichannels called connexons (2-5). The end-to-end docking of connexons from closely apposed plasma membranes creates intercellular channels. Each connexon is a cylinder constructed of six subunits called connexins (Figure 1). Connexins are encoded in mammals by a large multigene family; sixteen of them have been cloned and characterized thus far from rodent cDNA or genomic libraries. All have the same basic structure, with cytoplasmic N- and C-termini, four membrane-spanning domains (at least one of which lines the channel), one cytoplasmic loop, and two extracellular loops that mediate docking between connexons from opposing membranes (Figure 1). Connexins vary in mass from 26 to 60 kD, most of the variation arising from differing lengths and amino acid compositions of the C-terminal cytoplasmic tail. There are two nomenclatures in current use for connexins. The one most often used is based on the predicted mass of the protein, e.g. connexin26 (Cx26) is the only known connexin with an approximate mass of 26 kD. The alternate nomenclature divides connexins into separate groups based on sequence similarity (and presumed evolutionary relatedness) and numbers them in order of discovery: connexin43 (Cx43) was the first connexin cloned of the alpha group, hence it became alpha-1 connexin. In this review we have used the molecular mass nomenclature throughout but have included the phylogenetic nomenclature in Table 1.

In addition to their different but frequently overlapping expression domains (Table 1), connexins differ in the properties that they confer on the channels that they constitute (reviewed in 11). These differing properties include single channel conductance, permeability (to tracer dyes as well as natural metabolites such as nucleotides and second messengers), sensitivity to pH and transjunctional voltage, and ability to be gated (closed) by the action of protein kinases. Because of these differences, it has been proposed that each type of channel may play a unique role in cellular regulation (12). This complexity is compounded when one considers that different connexins can oligomerize to form heteromeric connexons, and connexons composed of different connexins can dock with one another, creating heterotypic intercellular channels (4). A variety of human congenital diseases have been linked to connexin gene mutations, confirming the importance of gap junctional intercellular communication for normal cellular function and indicating that different connexins do indeed perform distinct developmental or physiological functions (reviewed in 13). To discriminate between unique and shared functions of several connexins, Plum *et al*. (14) generated "knockin" mice in which Cx32 or Cx40 replaced Cx43. The two knockin connexins were able to "rescue" some aspects of the Cx43 null mutant phenotype but not others, confirming that particular channel types do have unique functions in some contexts, whereas other functions may be shared with other members of the family.

4. GAP JUNCTIONS AND CONNEXINS IN PREIMPLANTATION DEVELOPMENT

4.1. Connexin expression and gap junction assembly

Following the discovery that assembly of functional gap junctions occurs in the mouse during compaction (15-17), newly available antibodies and cDNAs for specific connexins were used to study this process at the molecular level. The first connexin identified as being expressed during preimplantation development was Cx43 (18,19). The gene encoding Cx43 is active from the 2-cell stage, but nascent Cx43 remains in an intracellular location until an as yet unknown signal triggers its assembly into gap junctions, an event that is sensitive to protein trafficking inhibitors (20). The time course of these events is illustrated in Figure 2. Experiments with an inhibitor of DNA synthesis revealed that the timing of gap junction assembly is linked to DNA

Table 1. Classification of rodent connexins

Connexin	Phylogenetic Nomenclature	Organs with prominent expression
26	beta-2	liver, skin, kidney
30	beta-6	skin, brain
30.3	beta-4	skin
31	beta-3	skin
31.1	beta-5	skin
32	beta-1	liver, brain, kidney
33	alpha-6	testis
36	alpha-9	brain, eye (retina)
37	alpha-4	vasculature
40	alpha-5	heart, vasculature
43	alpha-1	heart, skin, brain, ovary, testis, uterus
45	alpha-7	heart, vasculature
46	alpha-3	eye (lens)
47	alpha-11	brain, spinal cord
50	alpha-8	eye (lens)
57	alpha-10	weakly expressed in many organs

The possibility that the alpha group of connexins should be subdivided into two or even three subgroups is under consideration by the international gap junction community.

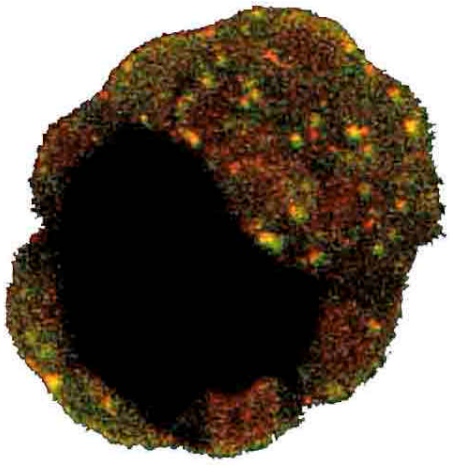

Figure 3. Co-localization of Cx43 (red) and Cx31 (green) in gap junctions of a blastocyst (confocal micrograph). The yellow spots indicate where both fluorescent signals are eminating from the same gap junction plaque.

replication in the second cell cycle after fertilization, as though a developmental "clock" controlling compaction (cell flattening and gap junction assembly) were activated at that time (21). Thus the assembly of Cx43 into gap junctions is post-translationally regulated to occur within a precise temporal window during compaction. Based on these findings, it was hypothesized that Cx43 plays an essential role in embryogenesis.

This hypothesis was proved incorrect when it was discovered that mouse embryos lacking Cx43 develop into normal blastocysts that implant and give rise to full-term pregnancies (22,23). Null mutant morulae do suffer a severe reduction in intercellular dye coupling and the channels remaining differ in their permeability properties from those of wild-type embryos, implying that other connexins are present in Cx43-deficient embryos. Indeed, immunogold electron microscopy of wild-type embryos revealed some gap junctions lacking Cx43 (23). Screens for expression of additional connexins using RT-PCR identified eight transcripts (encoding connexins 30, 30.3, 31, 31.1, 36, 40, 45 and 57) in wild-type preimplantation mouse embryos (24,25). At the protein level Cx31, Cx31.1, and Cx45 (in addition to Cx43) have been identified in gap junctions in preimplantation embryos (9,10,23,24), with Cx31 and Cx43 being present in the same junctional plaques (Figure 3). It is reasonable to assume, then, that embryos lacking Cx43 utilize these other connexins to retain a level of intercellular coupling sufficient to support development. In other words, Cx43 does not have a unique or even essential role in preimplantation development. The same can be said for connexins 30, 31, 36, 40, and 45, all of which have been inactivated by gene targeting with no apparent effects on preimplantation development (26-30; K. Willecke, personal communication). Multiple connexins have been detected in gap junctions of rat preimplantation embryos as well (25), as summarized in Figure 4.

4.2. Functional analyses of gap junctional coupling

The precise temporal control of gap junction assembly, together with its association with a critical morphogenetic process such as compaction, imply that gap junctional coupling plays an important role in preimplantation development. A variety of approaches, not specific to individual connexins, have been used to test this hypothesis. Early experiments involved attempts to disrupt gap junctional coupling in preimplantation mouse embryos by means of connexin antibodies (31,32) or antisense RNA (33). In each of these three studies, the injected agent blocked intercellular dye transfer with the result that affected blastomeres underwent "decompaction." Thus it was concluded that the maintenance of compaction and, as a consequence, blastocyst development depend on gap junctional coupling. These results conflict with those of the Cx43 knockout (23): when intercellular communication was severely reduced by the loss of Cx43, there was no apparent effect on either compaction or blastocyst formation. One explanation for this discrepancy, at least in two of the earlier studies (31,33), might be that the agent used for disrupting gap junctional coupling was not specific to a single connexin and thus could have interfered with several or all of the connexins present, producing a more complete blockade than that of the knockout. On the other hand, the third study (32) used peptide-specific antibodies designed to be highly specific for Cx43. Thus we are left with the implication that when Cx43 function is blocked by a specific antibody, compaction is affected, but when synthesis of Cx43 is prevented by mutational inactivation of the gene, there is no effect. As suggested by Becker *et al.* (32), this may reflect up-regulation of one or more

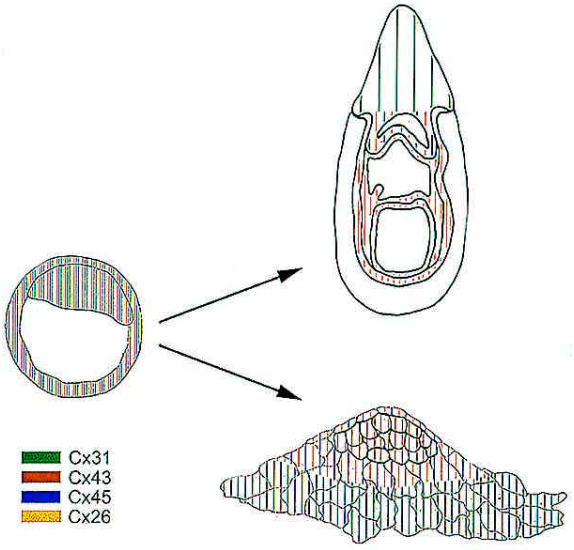

Figure 4. Segregation of connexins, co-expressed in the rat blastocyst, between "communication compartments" during peri-implantation development *in vivo* and in blastocyst outgrowth *in vitro*.

additional connexin genes in the absence of Cx43, a phenomenon that might not occur when Cx43 channels are blocked directly by an antibody. Our data indicate, however, that this up-regulation, if it occurs, does not involve Cx31, Cx31.1, Cx40, or Cx45 (23,25). Perhaps a better explanation lies in the ability of different connexins to co-assemble to form heteromeric (mixed) connexons (34-36). If the multiple connexins expressed during preimplantation development normally co-assemble, then an antibody against Cx43 might have a much more pervasive effect on gap junction formation than a Cx43 null mutation.

Recently, another line of evidence was presented that suggests that gap junctional coupling is not an essential aspect of preimplantation development. Pharmacological inhibitors of gap junctional coupling can potentially abolish coupling through whatever channels are present. This approach was taken by Vance and Wiley (37), who treated mouse preimplantation embryos with the gap junction blocker, 18-α-glycyrrhetinic acid (AGA) using a concentration sufficient to block intercellular dye transfer completely. Treatment of developing embryos with AGA beginning in the 4-cell stage (before the onset of gap junction assembly) and continuing into the blastocyst stage had no effect on blastocyst development, total cell number, or the ratio of cells in the trophectoderm and ICM. These data indicate that gap junctional coupling may in fact be dispensible for preimplantation development of the mouse.

5. AN HYPOTHESIS CONCERNING THE SIGNIFICANCE OF MULTIPLE PREIMPLANTATION CONNEXINS

Why do preimplantation embryos express multiple connexins and assemble them into gap junctions? One possible explanation, supported by analyses of connexin expression domains in postimplantation conceptuses and in blastocyst outgrowths *in vitro*, is that expression of multiple connexins before implantation is a prerequisite for rapid segregation of gap junction channel types in the embryonic and extraembryonic regions of the conceptus which arise during and after implantation (Figure 4). For example, a rapid differentiation program establishes the first functional organ, the placenta. Directly after implantation, the mouse conceptus establishes two major communication compartments, embryonic and extraembryonic, as indicated by dye injection experiments (8). Cx43 and Cx31, both of which which are abundantly expressed in preimplantation embryos, segregate into these two postimplantation compartments (embryonic and extraembryonic, respectively), in mouse (9) as well as in rat (38,39). Since Cx31 connexons are apparently unable to form functional heterotypic channels by docking with other types of connexons (12), this distribution pattern may establish the communication boundary between these two compartments. This segregation of connexin expression domains can occur *in vitro* in blastocyst outgrowths, hence it is not under maternal influence but represents an endogenous program associated with the development of the ectoplacental cone and its derivatives (25). Later on in placental development, Cx31 expression is maintained in the spongiotrophoblast (30,38). Mice lacking Cx31 demonstrate decreased embryonic survival due to transient placental dysmorphogenesis (30). Before their death at 9.5 dpc, the placentae of homozygous conceptuses are smaller and have reduced labyrinth, chorionic plate, and almost no spongiotrophoblast cells, but contain abundant terminally-differentiated trophoblast giant cells instead. The absence of Cx31 channels seems to enhance the ability of chorionic trophoblast stem cells to differentiate as giant cells. This disturbance of the differentiation pathway leads to death of 60% of the embryos between days 10.5 and 13.5 pc. That some Cx31 deficient embryos do survive to term has been attributed to the fact that in some placentae, recovery of the normal structure occurs from day 9.5 pc onwards, correlated with induction of Cx43 expression in the spongiotrophoblast. Thus, while they may serve no specific function in blastocysts, both Cx31 and Cx43 are essential for early postimplantation morphogenesis. The same is true for Cx45 (29). Although preimplantation development is normal when this connexin is lacking, the embryos die *in utero* around day 10. Cx45 is strongly expressed in the allantoic mesenchyme as well as the mesodermal component of the yolk sac. The mutant embryos are not capable of developing yolk sac vessels and the ingrowth of the allantoic mesenchyme into the labyrinthine part of the placenta is impaired.

6. SUMMARY AND PERSPECTIVE

Despite more than two decades of research, there is still uncertainty about the role of gap junctional intercellular communication in preimplantation development. Gap junction assembly is a post-translationally regulated event associated with compaction, yet no one connexin that has been tested by genetic ablation has yet to exhibit an indispensible role before implantation. It remains possible that the co-expressed connexins play

redundant roles in preimplantation development, but do so by co-assembly into heteromeric channels such that an injected connexin-specific antibody can affect the assembly or function of the entire population of channels. A test of this model must await the interbreeding of single connexin knockout lines to produce preimplantation embryos lacking two or even three different connexins. It is clear, however, that individual connexins present in blastocysts have indispensible roles to play after implantation in the development of extraembryonic tissues.

It must be kept in mind that all of the analyses of preimplantation embryos after perturbation of gap junction assembly or function have been conducted *in vitro* where the factors affecting developmental success may be different from those *in vivo*. For example, preimplantation embryos develop in a hypoxic environment in the female reproductive tract (40), and may require gap junction channels to facilitate sharing a limited supply of energy substrates among blastomeres in this situation. Such a dependence on gap junctional coupling *in utero* would possibly go undetected when tested *in vitro*. Thus further experimentation may reveal hitherto unappreciated roles of gap junctional coupling in preimplantation development.

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