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The Role of Gap Junction Membrane Channels in Development

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In most developmental systems, gap junction-mediated cell–cell communication (GJC) can be detected from very early stages of embryogenesis. This usually results in the entire embryo becoming linked as a syncytium. However, as development progresses, GJC becomes restricted at discrete boundaries, leading to the subdivision of the embryo into communication compartment domains. Analysis of gap junction gene expression suggests that this functional subdivision of GJC may be mediated by the differential expression of the connexin gene family. The temporal-spatial pattern of connexin gene expression during mouse embryogenesis is highly suggestive of a role for gap junctions in inductive interactions, being regionally restricted in distinct developmentally significant domains. Using reverse genetic approaches to manipulate connexin gene function, direct evidence has been obtained for the connexin 43 (Cx43) gap junction gene playing a role in mammalian development. The challenges in the future are the identification of the target cell populations and the cell signaling processes in which Cx43-mediated cell–cell interactions are critically required in mammalian development. Our preliminary observations suggest that neural crest cells may be one such cell population.

KEY WORDS: Development; gap junctions; mouse embryos; *Drosophila*; connexin 43; transgenic mouse; neural tube defects; neural crest cells.

INTRODUCTION

A possible role for gap junctions in embryogenesis and development has been proposed since early studies showing the presence of low-resistance junctions in a variety of nonexcitable cells and tissues (see for example, Furshpan *et al.*, 1968; Sheridan, 1966, 1968; Ito and Loewenstein, 1969; Bennett and Trinkaus, 1970). This possibility seems particularly tantalizing given the permeability properties of gap junction channels. Thus, gap junctions may provide the pathway through which calcium, cAMP, inositol triphosphate, or other second messengers may pass from cell to cell. Moreover, gap junctional communication (GJC) may provide the context in which gradients may be generated in a community of cells (Michalke, 1977). This is particularly tantalizing given the wealth of evidence indicating the importance of gradients in patterning and morphogenesis. However, the precise role(s) of gap junctions in development remains unresolved. Much of the experimental evidence has been indirect, although recent studies employing reverse genetic approaches for manipulating gap junction gene function have provided more definitive insights into this very important problem.

DEVELOPMENTAL RESTRICTION IN GAP JUNCTIONAL COMMUNICATION

Some of the first evidence that gap junctions may be important in development comes from the analysis of the pattern of cell–cell communication in developing embryos and tissues. These studies showed that in most embryos, GJC is established within the first few cell cleavages, and results in the entire embryo becom-

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ing interconnected as a "syncytium" (Ito and Hori, 1966; Ito and Loewenstein, 1969; Bennett and Trinkaus, 1970; Lo and Gilula, 1979a; Guthrie, 1984; Serras *et al.*, 1989). This can be demonstrated by using microelectrode impalements to monitor the cell-to-cell movement of ions (ionic coupling) or low-molecular fluorescent tracers such as Lucifer yellow or carboxyfluorescein (dye coupling). Although ionic coupling, once established, frequently persists, even between cells of different differentiated states or with differing developmental potentials, dye coupling is frequently restricted as development progresses. Of particular note is the fact that GJC usually is segregated into multicellular units known as "communication compartments" (Lo and Gilula, 1979b). Such compartments often coincide with developmentally significant domains. Below we briefly summarize our observations of GJC in the mouse embryo and in the *Drosophila* system.

MOUSE EMBRYOS

An example of the progressive restriction in coupling which accompanies embryogenesis can be seen in the developing mouse embryo. In the mouse embryo, ionic and dye coupling is detected from the late eight cell stage (Lo and Gilula, 1979a), a time in development when all the blastomeres of the embryo are still uncommitted (as indicated by their totipotency). With implantation at the blastocyst stage, coupling begins to break down. At this stage of development (equivalent embryonic day 6.5; E6.5), the embryo has segregated into two distinct cell lineages, the inner cell mass (ICM) which will give rise to the yolk sac tissues and the embryo proper, and the outer cells which will differentiate into the trophectodermal derivatives of the embryonic placenta. In such embryos, dye coupling is lost between the ICM and the trophectoderm, although ionic coupling continues to link these two cell populations together (Lo and Gilula, 1979b). However, by the egg cylinder stage of development (E7.5), ionic coupling also ceases, such that the embryo proper and the trophectodermal tissues of the placenta constitute "global" communication compartments that are completely isolated from one another. This segregation in GJC may be of particular importance to the development of the embryo proper, as the placenta makes intimate cell–cell contact, including gap junctional contacts, with the maternal tissues. Hence the complete loss of GJC between the embryo and the placental tissues may allow the segregation of metabolite pools such that signaling processes important to embryogenesis may proceed unperturbed.

Of further note is the fact that cells within each of the two global communication compartments are subdivided into smaller communication compartment domains. In contrast to the two global compartments, these communication compartments, though not dye coupled to one another, show the persistence of a low level of ionic coupling across the compartment border. Thus, in the developing placenta, the extra-embryonic tissues were segregated into a number of separate communication compartment domains, each of which remained ionically coupled to the others (Kalimi and Lo, 1989), while in the embryo proper, each embryonic germ layer is comprised of a separate communication compartment, with ionic but not dye coupling continuing to link all three germ layers together (Fig. 1). In addition, within each embryonic germ layer, cells are further segregated into a number of additional communication compartments. Most striking was the finding of compartments comprised of boxlike domains in the ectoderm/mesoderm layer (Kalimi and Lo, 1988). Multiple impalements into the same embryo suggested the presence of a tandem array of such boxlike compartments in the egg cylinder stage embryo. As this was observed at the late gastrulation stage of development, that is, before the appearance of metameric structures, we suggest that such boxlike communication compartments may be the analogs of somites or neuromeres.

DROSOPHILA

Analysis of GJC in *Drosophila* has also revealed the segregation of cells into communication compartment domains in conjunction with the patterning of development. Thus in the *Drosophila* larval epidermis, dye coupling is restricted at the boundaries of body segments (Ruangvoravat and Lo, 1992). This segmental restriction in GJC has also been demonstrated for a number of other insects including *Calliphora* and *Oncopeltus* (Warner and Lawrence, 1982; Blennerhasset and Caveney, 1984). The possible developmental significance of these communication restrictions is suggested by the fact that each body segment is a unit within which patterning is regulated. This is recognizable externally via the deposition of distinct cuticular structures by the underlying epidermis of each body segment (Lawrence, 1966; Stumpf, 1966; for a review, see Locke, 1967). We further observed that in each

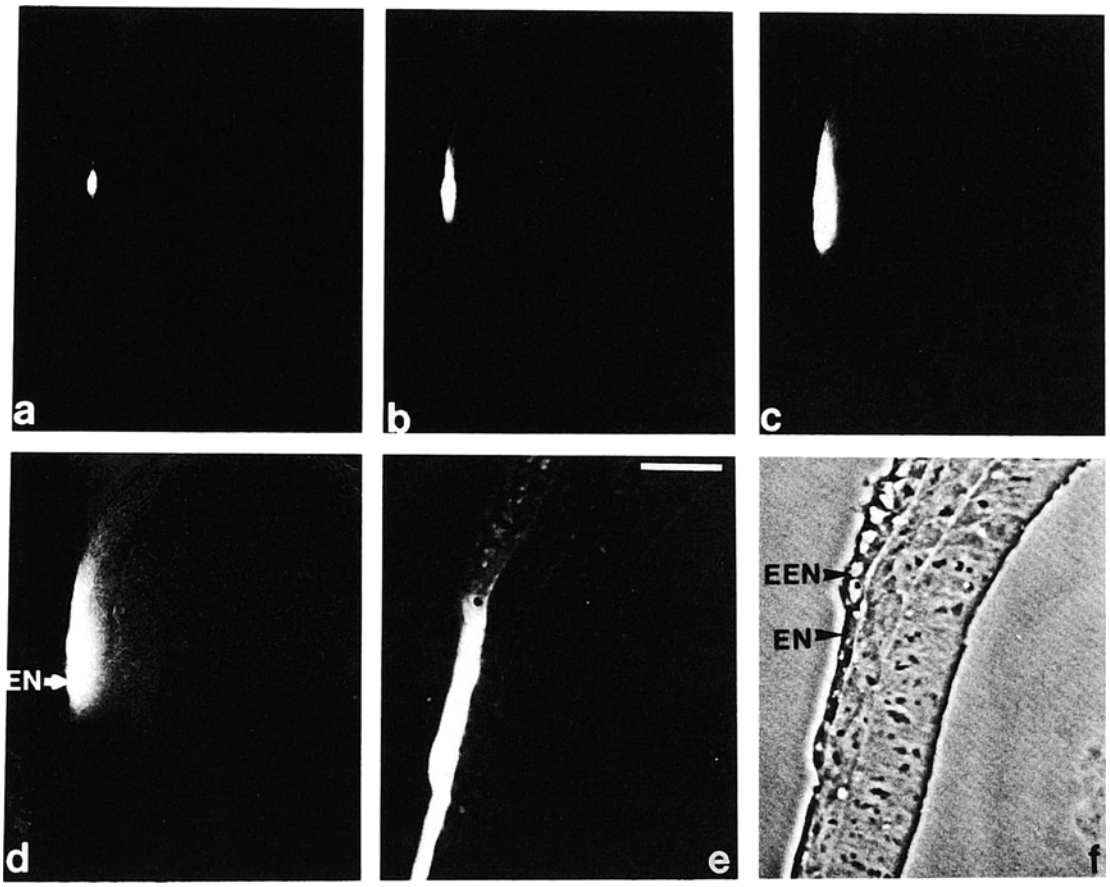

Fig. 1. Gap junctional communication restrictions in the mouse embryo. A gastrulation stage mouse embryo (E7.5) was examined for dye coupling. A microelectrode was inserted into a visceral endodermal (EN) cell bordering the extra-embryonic endoderm (EEN). The Lucifer yellow was injected into the impaled cell, and over a period of time (a = 30 s, b = 2 min, and c = 6 min), the fluorescent tracer spread in a highly asymmetric manner from the impalement site. This pattern of spread would indicate the presence of a restriction in gap junctional communication between the EN and EEN. Thick section histology of the same embryo in (e,f) confirmed that the injected dye is localized in the endodermal cell layer, and not in the extra-embryonic endoderm. Note that sections shown in (e,f) are inverted in orientation relative to the dye injection images. Reprinted with permission from Kalimi and Lo (1989).

Drosophila larval segment, the epidermis is actually subdivided into a dorsal, ventral, and two lateral communication compartments (Ruangvoravat and Lo, 1992). It is interesting to note that the two lateral compartments are reminiscent of the domains of *wingless* expression—a gene required for patterning of segmentation. Of further note is the fact that dye injection into the larval epidermis revealed dye transfer not only between the epidermal cells, but also from the epidermal cells into the overlying cuticle, a pattern of cell connectivity which would suggest a direct role for GJC and communication compartments in regulating the segmental patterning of cuticle deposition (Ruangvoravat and Lo, 1992).

Dye coupling studies in the *Drosophila* wing imaginal disk also showed the segregation of cells into communication compartment domains. Cells of the wing disk epithelium normally give rise to cuticular structures of the adult mesothoracic segment (Weir and Lo, 1982, 1984). Restrictions in dye coupling were observed in the wing disk epithelium, in particular along boundaries that coincided with those of lineage compartment borders (Fig. 2; Weir and Lo, 1982, 1984). Lineage compartments are multicellular units within which some of the genes that regulate pattern formation appear to act (Crick and Lawrence, 1975; Garcia-Bellido, 1975). Thus, dye coupling is restricted at a boundary which bisects the disk epithelium along its long axis, a position identical to that of the anterior/posterior (A/P) lineage compartment border. It is interesting to note that in the wing disk of *engrailed* mutants, this A/P communication restriction boundary is maintained in the absence of the A/P lineage

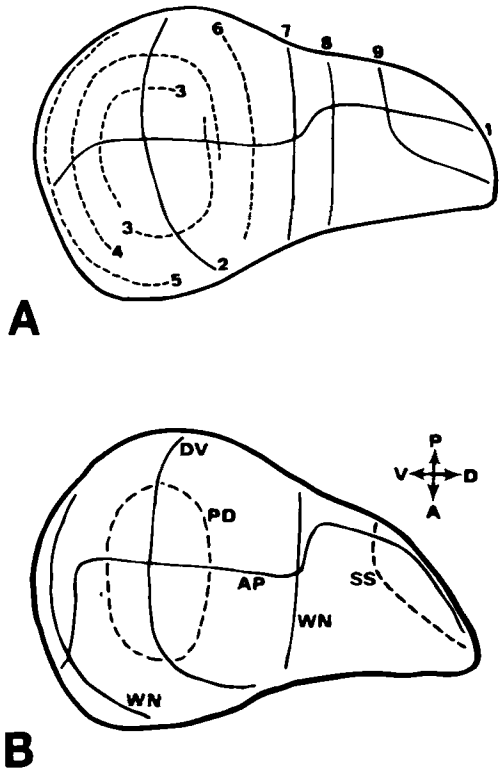

Fig. 2. Communication restrictions and lineage compartment boundaries in the *Drosophila* wing imaginal disk. The communication restriction boundaries observed in dye coupling studies (A) show a close correlation with that of lineage compartment boundaries (B). Reprinted with permission from Weir and Lo (1984). AP = anterior/posterior, DV = dorsal/ventral, PD = proximal/distal, SS = scutum/scutellum versus postscutum/postscutellum, WN = wing/notum.

compartment border (Weir and Lo, 1985). Given that positional information is likely undisturbed in *engrailed* mutants (only its interpretation, as these mutants exhibit position-appropriate differentiation leading to mirror symmetric duplication), these observations would suggest that communication compartments rather than lineage compartments are the functional units responsible for the patterning of the wing disk epithelium.

CONNEXIN GENE EXPRESSION AND DEVELOPMENTAL RESTRICTION IN GJC

Although it is still not known how gap junctional communication restrictions are established, one possibility is that they are generated as a result of the differential expression of connexin genes encoding gap junctions with different permeability and gating properties (see article by Veenstra in this issue). Thus gap junctions encoded by some connexin genes exhibit selective dye and ionic permeabilities (Steinberg *et al.*, 1994; Brisette *et al.*, 1994; Veenstra *et al.*, 1994). For example, gap junctions formed by Cx45 exhibited dye coupling with dichorofluorescein but not the commonly used fluorescent tracer, 6-carboxyfluorescein (Veenstra *et al.*, 1994). Moreover, among the connexin isoforms, only some can interact to form heterotypic gap junctions (Werner *et al.*, 1989; Bruzzone *et al.*, 1993; Tomasetto *et al.*, 1993; Moreno *et al.*, 1995; Ghosh *et al.*, 1995). Thus cells expressing Cx40 cannot communicate with Cx43-expressing cells (Bruzzone *et al.*, 1993). In light of this complexity, the differential expression of connexin genes could be a mechanism by which GJC is spatially regulated, such that communication compartments and restrictions in GJC are established.

In mouse embryos, connexin 43 (Cx43) is one of the first gap junction genes to be expressed, coming on at the 4–8 cell stage, just prior to the onset of gap junction-mediated dye and ionic coupling (Nishi *et al.*, 1991; Valdimarsson *et al.*, 1991). As development progresses, Cx43 expression becomes restricted to the embryo proper, with none found in the trophectodermal lineage (Ruangvoravat and Lo, 1992; Pauken and Lo, 1995). In contrast, expression of another gap junction gene, connexin 26 (Cx26), is elicited and restricted to the trophectodermal lineage in the early post-implantation embryo (Pauken and Lo, 1995). This differential temporal-spatial expression of Cx26 and Cx43 coincides with the segregation of GJC into two global communication compartments. It is of further interest to note that Cx26 expression is actually found in only a subset of cells in the developing placenta (Pauken and Lo, 1995), an expression pattern reminiscent of the segregation of the placenta into several communication compartment domains (Kalimi and Lo, 1989). These observations suggest that the differential expression of connexin genes may play a role in the functional segregation of GJC in the early mouse embryo.

GAP JUNCTIONS AND CELL SIGNALING IN DEVELOPMENT

The restriction of GJC and the formation of communication compartments during embryogenesis and development may help to establish communities of cells within which cell signaling processes may be organized, relayed, and perhaps even amplified. The

persistence of ionic coupling between compartments may provide the low level of cell–cell communication needed to coordinate the activities of separate communities. It is interesting to note that within the context of this hypothesis, Cx43 expression in the mouse embryo is regionalized in a manner indicative of a role in various inductive tissue interactions. Thus, Cx43 exhibits an asymmetric or restricted distribution in the metanephric kidney, in the nasal placodes, the infundibulum of the diencephalon, or the forming otic and optic vesicles (Fig. 3A) (Ruangvoravat and Lo, 1992; Yancey *et al.*, 1992). Particularly striking is Cx43 expression in the developing central nervous system (CNS), where it is localized in domains that are similar to those of various members of the *wnt* gene family. A band of Cx43 expression is observed at the midbrain/hindbrain junction, a distribution identical to that of *wnt-1*, the vertebrate homologue of *wingless* (Fig. 3B–D) (Wilkinson *et al.*, 1987; Ruangvoravat and Lo, 1992). Since *wnt* genes encode short-range diffusible factors that regulate patterning of the CNS, it is tantalizing to speculate that gap junctions may play a role in CNS development by helping to relay cell signaling cascades triggered by *wnt* and other short-range signal-

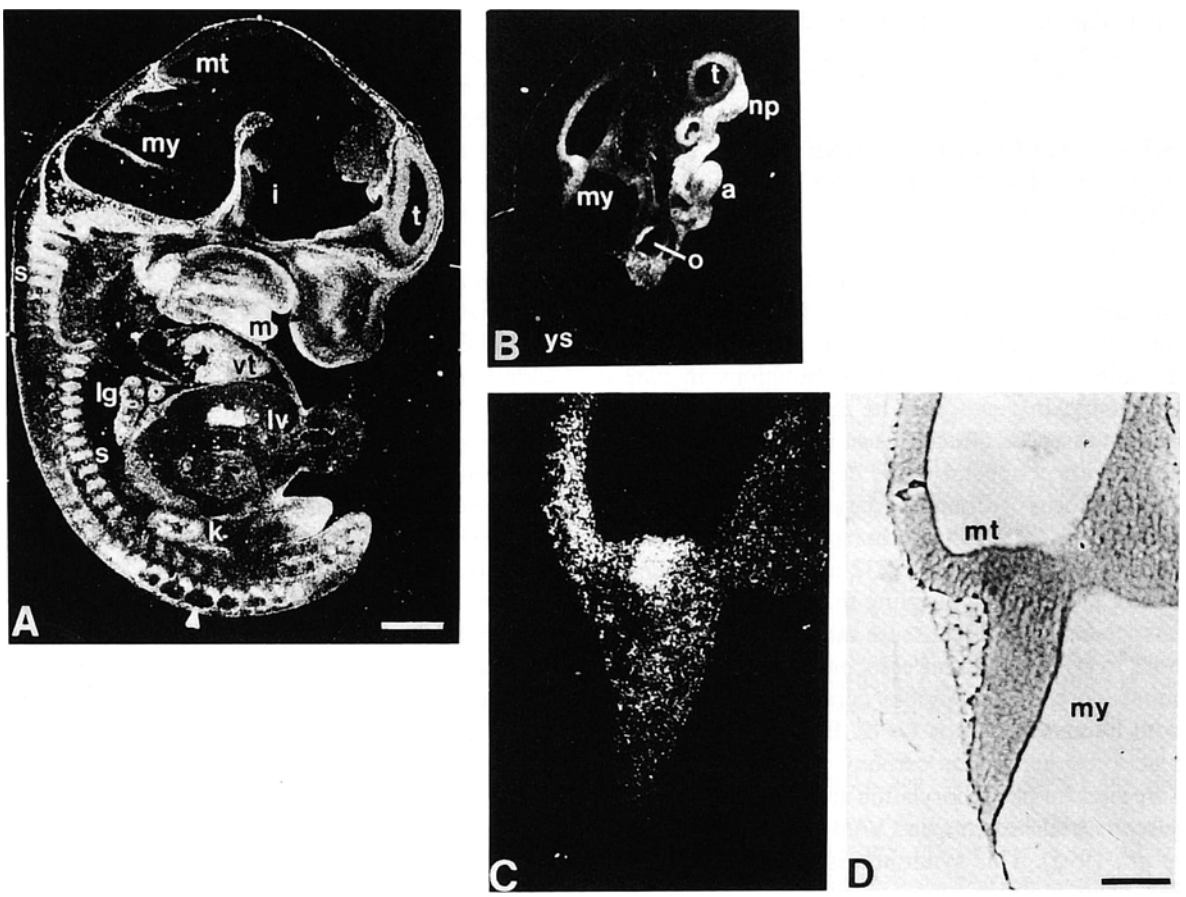

Fig. 3. Expression of Cx43 transcripts in the mouse embryo. *In situ* hybridization analysis of mouse embryos revealed Cx43 transcript expression in many developmentally significant domains. (A) Darkfield image of a sagittal section of an E12.5 embryo. Note the abundance of Cx43 expression in many regions of the embryo where inductive interactions play an important role in development, such as in the condensing mesenchyme of the metanephric kidney (k), or in the neural crest derived ectomesenchyme of the facial/manibular (m) regions of the head. Also, note strong Cx43 expression in the migrating sclerotomal (s) masses that will give rise to the vertebral elements of the axial skeleton. (B) An oblique sagittal section of an embryo at E10.5 show abundant Cx43 transcript expression in dorsal aspects of the developing brain. Note the high level of transcript expression in the nasal placode and also the branchial arches. The latter is comprised mostly of ectomesenchymal cells of neural crest origin. (C,D) Higher magnification of the same image shown in (B), detailing Cx43 transcript distribution in the midbrain/hindbrain. Note the stripe of Cx43 expression in the metencephalon (mt). a = branchial arches, i = infundibulum, k = kidney, l = lung, lv = liver, mt = metencephalon, my = myelencephalon, m = mandible, n = nasal placode, o = otic vesicle, s = scleratome, t = telencephalon, vt = ventricle, ys = yolk sac. Reprinted with permission from Ruangvoravat and Lo (1992).

ing molecules. This close correlation between the expression pattern of *wnt* genes and that of Cx43 is particularly intriguing, given that ectopic expression of *wnt-1* in *Xenopus* embryos brings about axial duplication in conjunction with changes in the pattern of GJC (Olson *et al.*, 1991; Olson and Moon, 1992). High levels of Cx43 are expressed in neural crest and sclerotomal cells (Ruangvoravat and Lo, 1992) (see Fig. 3A,B). As both of these are migratory cells, it is interesting to consider whether gap junction-mediated cell–cell signaling also may play an important role in guiding the migration and development of motile cell populations. This is a novel concept which may be of particular importance in considering the possible role of Cx43 in mouse development (see below).

GENETIC AND REVERSE GENETIC ANALYSIS OF GAP JUNCTIONS IN DEVELOPMENT

To determine the role of gap junctions in development, availability of mutants with the disruption of gap junction gene function would be invaluable. In fact, recent studies have indicated that mutations in two different connexin genes may be responsible for specific human diseases. Mutations in the Cx32 gene are associated with peripheral nerve degeneration seen in the X-linked form of Charcot–Marie–Tooth syndrome (Bergohoffen *et al.*, 1993; Bruzzone *et al.*, 1994). Although the precise role of Cx32 in nerve demyelination is not known, it is puzzling that the Cx32 mutations associated with this disease range from true null mutations to point mutations associated with conservative amino acid changes. Another connexin gene associated with human disease is Cx43. Point mutations in amino acid residues at the carboxy terminus of Cx43 which are sites for phosphorylation are found in patients with visceroatrial heterotaxia (VAH) (Britz-Cunningham *et al.*, 1995). This syndrome is characterized by complex heart malformations in addition to visceral organ defects, all of which appear to arise from fundamental perturbations in left/right patterning. As expected, these mutations are not nulls, although they exhibit aberrant regulation by protein kinases. These observations suggest that the Cx43 gap junctions may play a significant role in left/right patterning and that their precise regulation is of critical importance in development. Further evidence consistent with a role for Cx43 gap junctions in development comes from the analysis of knockout and transgenic mice.

Using the embryonic stem cell method of gene disruptions, Cx43 null mutant mice have been generated by the laboratories of Kidder and Rossant (Reaume *et al.*, 1995). Such mice develop to term, but die shortly after birth. As such null mutant mice exhibit the swelling and blockage of the right ventricular outflow tract, death likely is the result of a failure in pulmonary gas exchange. Other developmental abnormalities are also observed in the null mutant mice, such as defects in gonadogenesis and a thinning of the skin (Kidder, personal communication). Interestingly, developmental defects have been observed with incomplete penetrance in animals that are heterozygous for the Cx43 null allele, such as female sterility associated with dysgenic ovaries, deficiencies in the formation of cranial and dorsal root ganglia and nerves, and cranial neural tube defects (Sullivan and Lo, unpublished observations). These observations together with the findings in the VAH patients would suggest that it is not merely whether Cx43 gap junctions are present, but also of importance to mammalian development, is the precise level and regulation of Cx43-mediated GJC. Further evidence consistent with this possibility are our recent transgenic experiments examining the developmental effects of up-or down-regulating Cx43-mediated GJC. When wildtype Cx43 is expressed via the CMV promoter, which directs expression predominantly in neural crest derivatives (Koedood *et al.*, 1995), we observed cranial neural tube defects, deficiencies in the formation of cranial and dorsal root ganglia, and also the perturbation of heart morphogenesis (Ewart *et al.*, 1995; Ewart and Lo, unpublished observations). A similar constellation of defects was observed in transgenic mice in which Cx43 function was down regulated via the expression of the dominant negative Cx43/lacZ fusion protein (Sullivan and Lo, 1995; Sullivan *et al.*, 1995). As expression in both sets of transgenic animals is predominantly restricted to the dorsal neural tube and neural crest cells, it is likely that these developmental defects arise from the perturbation of neural crest cells. With regard to this possibility, it should be noted that the heart defects seen in the Cx43 knockout mouse and our transgenic mice all center around the conotruncal region of the heart, an area where neural crest cells are required for normal heart morphogenesis (for review see Kirby, 1993). This would suggest that Cx43 gap junctions may play an important role in the behavior of neural crest cells. It should be noted that given the possibility for functional redundancy among the connexin gene family members, observations from the

knockout mouse cannot exclude the role of Cx43 gap junctions in other aspects of mammalian embryogenesis and development.

PERSPECTIVES

That gap junctions may play a role in development is an attractive possibility, given that these membrane channels can mediate the transmission of second messengers and cell signaling molecules that may be important in development. In this regard, the finding that cells in the embryo are subdivided into communication compartment domains is of particular interest, as in principle they could serve as the functional units for propagating inductive interactions or orchestrating community effects. Recent analysis using reverse genetic approaches to perturb gap junction function in transgenic and knockout mice have provided evidence indicating a role for gap junctions in various aspects of mammalian development. The phenotypes exhibited by these transgenic and knockout mice suggest that neural crest cells may be affected by the perturbation of Cx43, a surprising finding given the migratory nature of this cell population. These and other studies suggest that in considering the role of gap junctions in development, it is not merely whether gap junctions are present or absent, but their precise regulation and expression levels may be of great importance.

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