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Topical Review

Isolation and Community: A Review of the Role of Gap-Junctional Communication in Embryonic Patterning

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Summary. Gap junctions are specialized channels formed between the membranes of two adjacent cells. They permit the direct passage of small molecules from the cytosol of one cell to that of its neighbor, and thus form a system of cell-cell communication that exists alongside familiar secretion/receptor signaling. Gap junction states can be regulated at many levels by factors such as membrane voltage, pH, phosphorylation state, and biochemical signals. Because of the rich potential for regulation of junctional conductance, and directional and molecular gating (specificity), gap junctional communication (GJC) plays a crucial role in many aspects of normal tissue physiology, as well as in tumor progression. However, arguably the most exciting role for GJC is in the regulation of information flow that takes place during embryonic development. This review summarizes the current knowledge of how GJC controls various aspects of embryonic morphogenesis in both vertebrate and invertebrate systems. Modern molecular embryology approaches have complemented biophysical and ultrastructural data, and we are beginning to unravel the patterning roles of GJC in embryonic events such as the patterning of the embryonic left-right axis, as well as the morphogenesis of the heart and limb. Proteins from the *Connexin* (*Cx*) gene family, as well as innexins and ductin, are now beginning to be understood as the basis for GJC underlying important embryonic patterning events.

Keywords: Gap junction — Embryo — Development — Morphogenesis — Connexin

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Introduction

The mechanisms that allow an embryo to reliably self-assemble the complex morphology, physiology, and behavior appropriate to its species, represent one of the most fundamental and fascinating areas of research in modern science. Pattern is acquired simultaneously on three orthogonal axes, and on several logs of scale, from the molecular (e.g., cytoskeletal elements) to the organismic (graded antero-posterior (AP), dorso-ventral (DV), and left-right (LR) axial specification).

Though some animals possess a stereotyped (mosaic) development, most embryos show impressive abilities to regulate in the face of environmental or genetic perturbations. The events underlying this generation of form require a complex web of information flow between cell and tissue subsystems during development. With the advent of multi-cellularity, the cell membrane has become a key nexus for regulatory information flow. Receptor-mediated signal exchange via secreted messenger molecules has been studied extensively. However, another system of signaling exists: the direct cell-cell exchange of small molecules through gap junctions.

The cell biology of gap junctions has been described in a number of excellent recent reviews (Goodenough, Goliger & Paul, 1996; Falk, 2000); the reader is also referred to the classic (Loewenstein, 1981) for a historical and detailed perspective of some of the definitive studies on gap junction properties. Briefly, early findings that voltage shifts were able to propagate to adjacent cells (Weidmann, 1952; Furshpan & Potter, 1959) were augmented by the pivotal discovery that fluorescein can also be transferred (Kanno & Loewenstein, 1964; Loewenstein & Kanno, 1964). The model was proposed that the cell-cell junction channel was a pair of tightly-joined proteins spanning the gap between adjacent cells (Loewenstein,

Table 1. Roles of GJC in biological control processes not directly relevant to embryogenesis

Process or context	References
Cardiac conduction	Jalife, Morley & Vaidya, 1999; Jongsma & Wilders, 2000
Hearing and auditory function	Rabionet, Gasparini & Estivill, 2000
Aging and senescence	Xie, Huang & Hu, 1992
Neuronal function, pathfinding, and glial signaling	Bruzzone & Giaume, 1999; Naus et al., 1999; Rozental, Giaume & Spray, 2000
Eye and lens physiology	White & Bruzzone, 2000
Immune system function	Saez et al., 2000
Bone and tooth development	Donahue, 1998; Donahue, 2000; Lecanda et al., 2000
Neural tube defects	Ewartetal., 1997
Hematopoiesis	Montecino-Rodriguez, Leathers & Dorshkind, 2000
Myogenesis	Alien & Warner, 1991; Constantin & Cronier, 2000

1974), and a number of studies subsequently used permeant molecules of various sizes and other characteristics as tracers to probe the size of the gap junctional channel (Simpson, Rose & Loewenstein, 1977; Flagg-Newton, Simpson & Loewenstein, 1979).

It is now thought that the gap junction channel is formed by the assembly and docking of hexamers of proteins from the connexin family (one hexamer in each of two adjacent cell membranes). The junctional permeability between cells can be regulated at the mRNA level (by the transcription of connexin genes), but is also subject to a number of post-translational regulatory events, including connexin subcellular localization/targeting (Evans et al., 1999), degradation/recycling (Saffitz, Laing & Yamada, 2000), and gating of existing junctions (Peracchia & Wang, 1997).

The open intercellular channels are generally permeable to molecules of less than 1,000 MW (Loewenstein, 1981), but the exact permeability is a function of (1) precisely which connexin family members form the junction (White & Bruzzone, 1996), (2) the charge and size of the permeant molecule (Landesman, Goodenough & Paul, 2000; Nicholson et al., 2000), (3) the pH of cytoplasmic and intercellular space (Morley et al., 1997), (4) V_{membrane} of the cells (Brink, 2000), (5) the phosphorylation state of the connexin protein subunits (Lampe & Lau, 2000), and (6) the activities of a number of chemical gating molecules (Granot & Dekel, 1998). Thus, functional gap-junctional communication (GJC) is dependent on the existence of compatible hemichannels on the cells' surfaces, the permeability of the hemichannels to the substance, and the open status of the gap junction.

This extremely versatile system for communication allows for rapid synchronization among cells in a tissue and the passage of signals, both of which can be regulated at many levels. Thus, it is a perfect conduit for information flow during development, which depends on the ability of cells and tissues to communicate. The converse, however, is also paramount—embryos contain independent compartments, which must remain isolated for proper morphology to result.

Gap junctions have been studied for years using biophysical methods (such as electron microscopy and X-ray diffraction), but some of the most exciting information on gap junctions has come more recently, from the use of molecular embryology techniques to probe the role of GJC in physiological processes (Goodenough & Musil, 1993; Lo, 1999). A number of human syndromes have been identified as mutations in connexin genes (Maestrini et al., 1999), and transgenic mice are beginning to allow the molecular dissection of GJC function in these contexts (Krutovskikh & Yamasaki, 2000). For example, GJC is now known to be a general mechanism for achieving rapid syncitial communication within a tissue. Contexts include the spread of electric waves in cardiac tissue (Kimura et al., 1995; Severs, 1999) and the brain (Budd & Lipton, 1998), and the spread of signals through gland cells to synchronize hormonal action and secretion (Meda, 1996). GJC is now known to be involved in a host of phenomena not fundamentally related to embryonic morphogenesis, and for reasons of brevity, a partial list (and citations to relevant reviews) is given in Table 1.

One of the most tantalizing roles for GJC is in the processes that distinguish normal tissue from tumor cells (Loewenstein & Rose, 1992; Yamasaki et al., 1995; Krutovskikh & Yamasaki, 1997; Li & Herlyn, 2000; Omori et al., 2001). It has been shown that normal tissue possesses a much higher degree of GJC than tumor tissue, and a loss of GJC accompanies early steps in neoplastic transformation (Pitts, Finbow & Kam, 1988). Moreover, a neoplastic phenotype can be induced in cell culture by ectopic closing of gap junctions using pharmacological agents or dominant-negative constructs (Omori & Yamasaki, 1998). Most interestingly, neoplastic characteristics can be suppressed by ectopic induction of GJC in tumor tissue (Mehta et al., 1991; Rose, Mehta & Loewenstein, 1993; Hellmann et al., 1999). These data are consistent with a role of GJC in mediating the information flow that is necessary for coordinated tissue activity and morphogenesis, which is lost in tumors.

In light of the many aspects of cell physiology and behavior that are regulated by GJC, it is not surprising that they play a key role in several aspects of embryogenesis (Lo, 1996; Warner, 1999). This review will be focussed on several examples of patterning roles of GJC during development. Because of space limitations, this can not be exhaustive; likewise, a number of important advances in contexts where gap junctions play a "housekeeping" or physiological-integrity role will not be covered. One example is the role of GJC in supporting the proper function of the placenta (Winterhager, Kaufmann & Gruemmer, 2000; Cronier et al., 2001). Another example is the requirement for GJC in the hatching of frog embryos (Levin & Mercola, 2000). Though hatching is certainly a normal part of embryonic development in *Xenopus*, the role of connexins in this process is likely to be limited to the dependence of hatching-enzyme secretion on GJC, and is thus not a true morphogenetic event. Of course, this distinction is somewhat arbitrary and the boundary between morphological patterning and physiological roles will shift as we gain a better understanding of GJC and pattern formation mechanisms in general. For example, future research may well show that the GJC that is required between oocytes and follicle or nurse cells in both vertebrate and invertebrate systems (Bohrmann & Haas-Assenbaum, 1993; Goodenough, Simon & Paul, 1999; Juneja et al., 1999) plays not only a nutritive and house-keeping role but also participates in the construction of a pre-pattern of morphogenetic material laid down in the egg as it matures.

GJC in Embryonic Tissues and Connexin Expression

A number of labs have sought to characterize GJC in embryogenesis. A basic question concerns which gap junction mRNA is present in a given tissue. Three different connexin mRNAs were described in *Xenopus* embryos (Gimlich, Kumar & Gilula, 1988, 1990); in situ hybridization showed that *Xenopus* Cx32 is expressed in the pronephros and the hatching gland (Levin & Mercola, 2000). Many more studies described the expression of connexin genes in the embryonic (Nishi, Kumar & Gilula, 1991) and extra-embryonic (Kalimi & Lo, 1989) tissues of the mammalian embryo; Cx43, for example, is expressed in discrete domains in the brain, neural tube, prevertebra, and limb (Ruangvoravat & Lo, 1992).

Because of the many levels of regulation to which gap junctions are subject, the presence of connexin mRNA in a tissue is no guarantee of the existence of gap junction complexes or of functional cell-cell connectivity. Thus, some studies have addressed the localization of connexin protein by immunohistochemistry. For example, in the rabbit, Cx43 and Cx32 are expressed in the blastoderm at streak stages (Liptau & Viebahn, 1999).

The rich regulation to which mature gap junctions are subject make it necessary to ascertain functional GJC in addition to mRNA and protein localization data. Direct dye coupling assays have demonstrated compartments of communication in a number of embryonic systems, including a very early observation in the squid embryo (Potter, Furshpan & Lennox, 1966), the detection of ionic coupling among cells of the *Triturus* (Ito & Loewenstein, 1969), and an investigation of electrotonic GJC in the chick blastoderm (Sheridan, 1966). It has also been shown that the wing imaginal disk in *Drosophila* is subdivided into a number of communication compartments during differentiation (Weir & Lo, 1984).

Complex patterns of restriction of GJC among cells of the cleavage and implantation stages of the mouse embryo have also been observed (Lo & Gilula, 1979a,b). At 7.5 days, the mouse embryo was found to be subdivided into at least nine GJC compartments with respect to Lucifer Yellow (LY) transfer, but only two domains with respect to ionic coupling (Kalimi & Lo, 1989). These results clearly underscore the need to explore GJC with a variety of different probes to truly understand what paths are available for endogenous small molecules in the embryo.

The *Xenopus* embryo features early stereotypical division of the egg into large blastomeres, which are predictably oriented with respect to the three spatial axes. LY has been used to analyze junctional coupling in the early frog embryo (Guthrie, 1984; Guthrie, Turin & Warner, 1988). These studies identified linkages between geometric embryonic axes and modulation of GJC: different degrees of coupling were observed for cells along the dorso-ventral axis, and the data also hinted at unidirectional transfer along the left-right axis (LR).

Thus, GJC is a natural candidate for restricting the flow of determinants between embryonic compartments. Significantly, the ability of GJC to partition molecules among embryonic destinations based on characteristics such as charge has now been demonstrated in vivo in the fish embryo. In the loach (*Misgurnus fossilis*), LY, fluorescein, and DAPI showed consistent differences in their ability to transfer between tissues during mesoderm induction and patterning (Bozhkova & Rozanova, 2000). The detection of connexin mRNA expression and patterned junctional transfer in many tissues and stages of embryos in most species evaluated has led to the idea that GJC plays a causal role in pattern formation.

Early Functional Studies

A number of early functional experiments indicated that endogenous patterns of embryonic GJC have important morphogenetic roles. Introduction of antibodies raised to specific portions of connexin pro-

teins in mouse embryos resulted in developmental defects (Becker et al., 1995). In *Xenopus*, microinjection of antibodies has been reported to disrupt axial patterning (Warner, Guthrie & Giluia, 1984); the treated embryos contained differentiated mesodermal derivatives such as notochord and muscle tissue and Warner et al. argued that GJC has a role in pattern formation per se, rather than in the induction of specific cell types. In the mouse zygote, cells injected with an anti-connexin antibody became extruded, consistent with an early role in the maintenance of compaction (Lee, Gilula & Warner, 1987; Becker, Leclerc-David & Warner, 1992). Interestingly, the *Cx43* homozygous null mutant mice reach implantation stages normally (De Sousa et al., 1997); this may be due to contributions from other connexin family members coexpressed in the same cells. This result illustrates a theme that turns up in many contexts of GJC research (such as limb and LR patterning), and underscores the need for exhaustive analysis of GJC in tissues during genetic deletion experiments.

The development of a dominant-negative connexin protein (''243H7'') by fusing Cx32 and Cx43 sequences was a key advance in this area. Misexpression of this construct in *Xenopus* embryos completely blocked channel conductances, and resulted in a loss of adhesion and delamination of the progeny of the injected cell (Paul et al., 1995). Because of the ability of this construct to exert a dominant-negative effect on gap junctions composed of multiple connexins, and the existence of Cx37, which is unaffected (and can thus serve as a rescue control), 3243H7 and other dominant-negative constructs (Sullivan & Lo, 1995; Duflot-Dancer, Mesnil & Yamasaki, 1997) will emerge as important tools to address the problem of compensation of levels of individual connexin family members by other connexins. Transgenic mice expressing these dominant-negative constructs in specific tissues and at early stages should prove especially informative.

GJC and Heart Morphogenesis

The patterning of the heart during development requires a number of orchestrated processes including the early delimitation of the heart field, the formation of a heart tube and eventual bending of the tube along the LR axis (Harvey, 1999; Mercola, 1999). The cells that participate in these events express a variety of genetic markers as they migrate and differentiate into tissue identities appropriate to their location within the heart and their physiological role.

Several members of the connexin family are expressed in the mammalian heart, including Cx37 (Haefliger et al., 2000), Cx43 (Dasgupta et al., 1999), and Cx45 (Alcolea et al., 1999). A variety of Connexin mutant mice have implicated GJC in the morphogenesis of the heart. Unlike the effects of GJC on the laterality of the heart tube bending (discussed below), these observations represent true defects in cardiac morphogenesis. For example, $Cx45^{-/-}$ mice exhibit an endocardial cushion defect which appears to result from an impairment of the epithelial-mesenchymal transformation of the cardiac endothelium (Kumai et al., 2000). Cx43 has also been specifically implicated in heart morphogenesis (Ya et al., 1998), based on reports that Cx43 knockout mice possess hearts with a blockage of the right ventricular outflow tract (Ewart et al., 1997).

Consistent with the ability of connexins to form heterotypic and heteromeric gap junctions, and to compensate for each other's function in the context of a genetic lesion, compound mouse knockouts sometimes exhibit additional phenotypes not seen in either of the single-null mice. For example, $Cx40^{+/-}$/$Cx43^{+/-}$ display severe and lethal cardiac malformations including abnormal atrioventricular connection, ventricular septal defects, and premature closure of the ductus arteriosus (Kirchhoff et al., 2000). In these mice, Cx43 haploinsufficiency aggravates the cardiac defects resulting from a genetic deletion of Cx40 (however, the reverse is not the case).

A number of the cardiac phenotypes resulting from Cx mutations, knockouts, or misexpression have been attributed to effects on neural crest cells (Lo, Waldo & Kirby, 1999). These migratory cells contribute to and are crucial for the formation of the embryonic heart (Creazzo et al., 1998; Farrell et al., 1999; Hong, 2001). Neural crest cell properties are dependent on appropriate GJC during migration and differentiation (Huang et al., 1998a). Neural crest cells appear to express Cx43, and indeed, the mammalian neural crest cells appear to possess functional GJC (Lo et al., 1997; Waldo, Lo & Kirby, 1999), an unusual property for a migratory cell. It has now been shown directly that the rate at which neural crest cells will migrate in an in vitro system correlates with the level of Cx43 expression (Huang et al., 1998a). Moreover, their survival (in vitro) has been shown to depend on GJC (Bannerman et al., 2000). Transgenic mice, in which the Cx43 has been replaced by a Cx43/β-galactosidase fusion (a dominant-negative construct), exhibit defects in right ventricular outflow tract morphogenesis as well as inhibition of GJC in cardiac neural crest cells (Sullivan et al., 1998). Interestingly, overexpression of Cx43 also results in conotruncal and right ventricle defects (Huang et al., 1998b), as well as cranial neural tube defects (Ewart et al., 1997).

The malformations resulting from over- as well as under-expression of Cx43 underscores another theme that is also seen in the studies of GJC in LR asymmetry: that certain morphogenetic events depend on junctional isolation as well as on communi-

cation between cells. The nature of the inappropriate intracellular signaling between tissues that disrupts LR and cardiac morphogenesis upon introduction of exogenous connexin proteins remains a very important line for future inquiry.

GJC in the Patterning of the Vertebrate Limb

Vertebrate limb development has proved to be a very fertile context within which to understand control of morphogenesis, since it involves tightly orchestrated programs of directed cell migration, differentiation, and patterning of the nervous system, musculature, and bone (Pizette & Niswander, 2001; Schaller et al., 2001). A variety of signaling molecules such as proteins from the FGF (fibroblast growth factor), Hedgehog, and BMP families determine the position of the limb field within the flank, guide cell contributions from the somitic mesoderm and neural tube, set the polarity of the limb with respect to the AP, DV, and medio-lateral axes, and control elongation of the limb bud. These early patterning events lead to the proper positioning of skeletal elements as cells form precartilagenous condensations and subsequently differentiate into chondrocytes allowing the cartilage template to ossify into the bony skeleton. Interestingly, GJC serves an important role in the early events that determine the morphology of the embryonic limb.

Using the scrape-loading/dye transfer technique, it was shown that chick limbs exhibit a gradient of GJC along the AP axis (Coelho & Kosher, 1991). Highest GJC is observed in cells adjacent to the zone of polarizing activity, and no GJC is present at the opposite end of the limb bud. Mesenchymal tissues in the middle of the limb have an intermediate level of dye coupling. This gradient is dependent on signaling from the apical ectodermal ridge (AER), an important signaling center (Meyer et al., 1997), since they disappear when the AER is surgically removed (Makarenkova et al., 1997). At later stages, micromass cultures of chondrogenic precursor cells display high levels of GJC, suggesting that GJC may be present in the cells undergoing cartilage differentiation in the maturing limb.

Cx43 mRNA is found in the AER; using antibodies to examine the localization of Cx43 and Cx32 in the mouse limb (Laird et al., 1992), it was determined that Cx43 protein is also present at the AER. Cx32 was strongly detected only in the mesenchyme, but also became localized to the ectoderm by 14.5 days of development. Direct LY transfer indicated that the AER cells are coupled to non-ridge ectoderm, but never showed transfer to mesenchyme. Mesenchyme cells were also coupled among themselves. These data suggest distinct developmental signaling compartments in the limb; indeed, use of probes other than LY (which may have different GJC properties based on size and charge) may eventually delineate even finer subdivisions (such as between AER and non-ridge ectoderm). Since unidirectional junctions are thought to form between Cx32 and Cx43 (Robinson et al., 1993; Xin & Bloomfield, 1997), this arrangement of connexin expression may underlie directed signaling between patterning centers in the limb.

Recently, it has become possible to add functional experiments to the descriptive data, and thus directly test for the importance of GJC in limb development. Application of anti-gap junction antibodies (Alien, Tickle & Warner, 1990) or antisense oligonucleotides specific for Cx43 mRNA (Makarenkova & Patel, 1999) to chick limb buds *in ovo* leads to severe defects in limb patterning, including truncation, fragmentation, or splitting of the limb bud. The ectopic lobes induced by the splitting do not express *Shh* or *BMP-2*, suggesting that reduction of Cx43 protein does not induce novel signaling centers such as the zone of polarizing activity (ZPA). However, Cx43 protein knockdown does result in discontinuities and downregulation of FGF genes in the limb. These results are consistent with a feedback model between FGF signaling and Cx43 function. In culture, application of FGF-4 protein substantially improves GJC between posterior but not anterior mesenchyme cells (Makarenkova et al., 1997). Interestingly, proteins of the FGF family are known to regulate Cx43 expression in other contexts, including in the embryonic brain (Nadarajah et al., 1998; Reuss, Dermietzel & Unsicker, 1998). Analogously, Wnt family members are upstream of Cx43 expression in the contexts of the limb (Meyer et al., 1997) and the cleavage-stage frog embryo (Olson, Christian & Moon, 1991), suggesting that regulation of connexin expression by a specific signaling molecule forms a cassette that can be used in many developmental contexts.

A particularly interesting observation was made by Meyer et al. (1997): the expression of *Cx43* in the AER of mouse limbs is asymmetric between the left and right limbs. For example, at E9.5 in the CDI strain mouse, significantly more right forelimbs show *Cx43* signal than left limbs. This appears to be due to an asymmetry in the timing of initiation of *Cx43* expression, but is specific for this gene since it is not observed for other genes such as *FGF-8*. This finding is reversed in the hind limbs, and is altered in other genetic backgrounds, suggesting the presence of genetic modifiers. GJC now is known to have an important role in left-right asymmetry (*see* below). Meyer et al. examined this asymmetry in the *iv* mouse (a dynein mutant in which 50% of the offspring exhibit reversal of organ situs, (Supp et al., 1997)), and found the effect not to be reversed (although the sample size was fairly small). These are extremely

interesting data, in light of other findings suggesting that, though apparently symmetrical, the limbs bear a special relationship with body asymmetry (Schreiner et al., 1993). Using the asynchrony of *Cx43* expression as a molecular marker of laterality, much could be learned if other LR mutants were examined (such as the *inv* mouse (Yokoyama et al., 1993), which exhibits almost total reversal of body *situs*). Other species such as chicks and zebrafish should also be examined, to determine whether the limb asymmetry is conserved.

Interestingly, though a slowing of the ossification of limb bones has been reported (Lecanda et al., 2000), connexin mutant mice have not demonstrated the sort of severe limb defect that would be predicted from the chick data described above (Houghton et al., 1999). Houghton et al. (1999) analyzed compound mutants in Cx43 and Cx32 to test for the possibility of compensation, but no real limb defects were observed. Targeted limb expression of a dominant-negative connexin construct is needed to determine whether GJC is maintained by another member of the connexin family in the limb, or whether GJC represents a basic mechanism of limb bud morphogenesis that differs between avian and mammalian species.

GJC and Left-Right Asymmetry

The body plan of vertebrates (and many invertebrate species) is based on a bilaterally symmetrical structure; however, the visceral organs and brain display marked and consistent asymmetries in their location or geometry with respect to the embryonic midline. Left-Right asymmetry is a fascinating example of large-scale embryonic patterning and raises many deep theoretical issues linking molecular stereochemistry with multicellular pattern control (Levin & Mercola, 1998a; Burdine & Schier, 2000). Because no macroscopic force distinguishes right from left, a powerful paradigm has been proposed to leverage large-scale asymmetry from the chirality of sub-cellular components (Brown & Wolpert, 1990; Brown, McCarthy & Wolpert, 1991). In this class of models, some molecule or organelle with a fixed chirality is oriented with respect to the antero-posterior and dorso-ventral axes, and its chiral nature is thus able to nucleate asymmetric processes. Thus, the first developmental event that distinguishes left from right would take place on a subcellular scale. However, it is now known that a pathway of multicellular fields of asymmetric gene expression directs the laterality of asymmetric organs (Levin, 1998; Yost, 2001). A mechanism must then exist to transduce subcellular signals to cell fields.

Based on published dye transfer studies, which indicated strong dorso-ventral asymmetries in cleavage-stage *Xenopus* embryos and hinted at a LR bias in transfer of tracers (Guthrie, 1984; Guthrie et al., 1988; Guthrie & Gilula, 1989; Olson et al., 1991), Levin and Mercola hypothesized that a circumferential path of GJC around a zone of isolation could be the mechanism by which asymmetry at the level of a cell can be transduced into embryowide asymmetry of gene expression. Specifically, we proposed that small molecule morphogens are initially randomly distributed, but traverse the circumferential GJC path in a chiral fashion (one-way), accumulating in a gradient on one side, which could then induce gene expression in conventional ways. Capitalizing on the complementary strengths of the chick and *Xenopus* embryonic systems, we showed that such a mechanism is indeed very likely to be an obligate event upstream of asymmetric gene expression (Levin and Mercola, 1998b; Levin and Mercola, 1999).

We tested junctional paths available to small molecules in early *Xenopus* embryos. Using injections of a system of a small junctionally-permeable fluorescent dye together with a large molecule, junctionally-impermeable dye (to mark the injected cell and rule out false positives due to cytoplasmic bridges and incomplete cell cleavage), we showed that there is, indeed, an asymmetry, with a zone of isolation across the ventral midline and good junctional coupling on the dorsal side. This asymmetry in ability to transfer dye was shown to be modified by chemical agents that are known to regulate junction permeability.

Using pharmacological agents that target gap junctions and affect permeability in known ways, we showed that a global closure of junctional transfer, as well as a global induction of GJC in embryos results in heterotaxia. The independent randomization of the *situs* of the heart, stomach, and gallbladder is a specific effect of the disruption of patterns of GJC and occurred in the absence of other defects (including normal dorso-anterior development). By showing that global opening or closure of gap junctions both destabilize laterality, we demonstrated that endogenous dorso-ventral differences in GJC are necessary for correct LR patterning.

The drug studies allowed a dissection of the timing: using the GJC drugs applied at different stages during embryonic development, we showed that the most sensitive developmental period was between stages 5 and 12 (gastrulation). Consistent with such an early role of GJC in LR patterning, we showed that manipulation of GJC states randomizes the asymmetric gene expression of *xNR-1* (a left-sided marker) as well as the *situs* of organs. Thus, we concluded that GJC was upstream of asymmetric gene expression in *Xenopus*. Importantly, the question of which connexin provides the LR-relevant GJC in early frog embryos is still open.

To specifically test the model that the ventral junctional isolation, together with the dorsal GJC,

are both crucial to correct LR morphogenesis, we made use of mRNA constructs developed and generously provided by Daniel Goodenough and David Paul. We showed that misexpression of constitutively active connexins across the ventral zone of isolation, as well as misexpression of a dominant-negative connexin on the dorsal side, both specifically induced heterotaxia. The opposite placement of either construct had no effect on laterality, consistent with the model. Crucially, examination of the distribution of a lineage tracer coinjected with the construct mRNA demonstrated that organs, whose *situs* had been reversed, originated from cells that most frequently *did not* receive any of the construct mRNA. Together with the early time period discovered to be sensitive to GJC-targeting drugs, we interpreted this to mean that GJC was not directly affecting the individual morphogenesis of asymmetric organs (a late step in LR patterning), but rather that the GJC dorso-ventral asymmetry was an early and fundamental system by which the embryo patterns the LR axis.

GJC has also been shown to be involved in LR patterning in chick embryogenesis. A particularly elegant set of experiments showed that Hensen's node, an important patterning center in the chick, does not generate its own LR orientation, but rather derives it from surrounding tissue (Psychoyos & Stern, 1996; Pagan-Westphal & Tabin, 1998). Pagan-Westphal et al. suggested that the tissue immediately adjacent to the node contains the crucial LR information. Because our data in the frog embryo implicated large-scale, embryo-wide information exchange as an early step in LR patterning, we began to characterize the spatial extent of tissue that is necessary for patterning asymmetric gene expression in the chick node.

Previous work on asymmetric gene expression in chicks has led to the paradigm of distinct left and right compartments that are separated by a midline barrier and carry on independent cascades of repression and induction of asymmetric genes (Levin, 1997). Cultured chick embryos exhibit the normal left-sided expression of *Shh* and *Nodal*. Surprisingly, we found that when distant lateral left-side tissue is cut away (far away from the primitive streak and node) at stage 3 (early node formation), ectopic right-sided *Nodal* expression often results (Levin & Mercola, 1999). Because this effect involves an ectopic gene expression, rather than the lack of it, it represents a specific LR defect, not a general death of the embryo due to tissue loss. Conversely, removal of the distant right-lateral tissue often results in the lack of normal *Nodal* expression on the left side. The same effect is observed on the expression of the early asymmetric gene *Shh*. These data argued for the importance of distant lateral regions to early LR patterning upstream of asymmetric gene expression, and suggested that, contrary to the compartment paradigm, signaling between cells at the distant halves of the early blastoderm is required for proper LR asymmetry in the node.

A natural candidate for such long-range communication is GJC, and we directly tested its role in chick LR development. The circumferential GJC model can be easily extended to the chick embryo (which has a very different mode of gastrulation). We proposed a radial movement of small-molecule morphogens through the plane of the early blastoderm, allowing them to accumulate on one side of the nascent streak (midline), if the net movement was biased (clockwise or counterclockwise). Circumferential, unidirectional sorting of a small LR morphogen through gap junctional paths at stage 2–3 would lead to the formation of a concentration gradient across the primitive streak, which could then induce asymmetric gene expression in conventional ways. The accumulation at the streak in later stages of this step would be consistent with the signaling role of the tissue immediately adjacent to the node found by Pagan et al. at stage 4. Moreover, the large-scale embryo-wide path taken by the morphogens would predict the dependence on distant tissues which we found in the ablation experiments.

To test this model, we cultured embryos that received single slits in the blastoderm far away from the primitive streak. Our model predicted that this would destabilize LR asymmetry. Consistent with the prediction of the long-range path model, disruption of the radial contiguity of the blastoderm leads to a randomization of *Shh* and *Nodal* expression. To test whether the circumferential path, which is necessary for correct expression of asymmetric genes, is dependent on GJC, we used Lindane — an agent that closes gap junctions. Exposure of stage 2–3 embryos to Lindane specifically destabilizes expression of asymmetric markers. This result is consistent with a dependence of the long-range, circumferential transfer on functional GJC.

We then examined a variety of Connexin family members' expression; our model predicted that since functional GJC paths have to result from the expression of a connexin, a radial expression of some connexin would be found at the relevant time period during development (early streak formation). We found that *Cx43* has a circumferential pattern around the primitive streak, which specifically excludes this mRNA (and thus is equivalent to the zone of isolation). Interestingly, *Cx43* expression is also asymmetric in Hensen's node at later stages (st. 5). To test whether the function of *Cx43* is specifically involved in LR asymmetry, we cultured early embryos in the presence of anti-*Cx43* oligonucleotides. Incubation with these oligos (but not with control oligos) significantly reduced the presence of *Cx43* mRNA, and randomized asymmetry of *Shh* and *Nodal*. The same

result on sided gene expression was observed by culturing embryos in the presence of functionally blocking anti-Cx43 antibodies.

Thus, a very similar picture emerged of the role of GJC in LR patterning in two embryonic systems. In both chicks and frogs, a circumferential large-scale pattern of GJC exists around a zone of isolation. The contiguity of this path is crucial for normal LR expression of asymmetric genes; interruption of this path by surgical, pharmacological, or molecular-biological methods specifically causes heterotaxia. Conversely, it is seen that the zone of isolation is likewise crucial for LR patterning. Introducing GJC through this zone randomizes gene expression, probably because it can no longer serve as a barrier for accumulation of LR morphogens on either side of the midline. The main features of this system appear to be the same in birds and amphibians; however, a couple of differences have been found. Firstly, due to the different modes of gastrulation, the circumferential path has to be overlaid on the two embryos with a 90° rotation: it is perpendicular to the DV axis in chicks, but to the animal-vegetal axis in *Xenopus*. Secondly, the zone of isolation appears to be maintained at the mRNA level in chick (based on the exclusion of *Cx43* from the streak). In contrast, *Xenopus* appears to rely on a protein-level mechanism to exclude junctional transfer from the zone of isolation, because drug agents that open existing gap junctions are effective in inducing GJC on the ventral side in *Xenopus* embryos.

The Ser365Pro Mutation in *Connexin-43*

Despite the strong evidence that GJC is centrally involved in LR patterning, none of the reported connexin knockout mice have true laterality defects (Reaume et al., 1995; Ewart et al., 1997). This is probably due to the fact that functional junctions can be formed from the proteins of coexpressed connexin family members, which can substitute for one another in a tissue when a single family member is targeted by genetic knockout. The generation and analysis of a transgenic mouse misexpressing a dominant-negative connexin construct at early streak stages would be necessary to determine whether connexins play a role in LR patterning in rodent embryos. Interestingly, a controversial patterning role has been suggested for a mutation in human Cx43. Mutation of serine^{364} to proline in a putative regulatory domain of Cx43 has been reported to occur in several unrelated human patients with heterotaxia (Britz-Cunningham et al., 1995), although several other studies did not find such mutations in a differently-selected patient sample (Casey & Ballabio, 1995; Gebbia, Towbin & Casey, 1996; Debrus et al., 1997). For example, Ivemark syndrome (which induces a type of isomerism) does not seem to be associated with this mutation of Cx43 (Chen et al., 2000).

We directly tested whether such a mutation causes laterality disturbances by injecting mRNA for the human Cx43^{Ser364Pro} generated by site-directed mutagenesis (Britz-Cunningham et al., 1995) into *Xenopus* embryos at the 4- or 8-cell stages (Levin & Mercola, 1998b). We found that both dorsal and ventral misexpression induces heterotaxia. This suggests that Cx43^{Ser364Pro} is able to function as a connexin, albeit not as well as the wild-type form. Thus, the mutant protein is both a mild hypomorph and a potent antimorph: on the dorsal side it functions as a weak dominant negative and induces heterotaxia by inhibiting the normal GJC, while on the ventral side its partial activity as a functional junction would induce heterotaxia by penetrating the zone of isolation (consistent with the injections described above). Our data indicate that mutations in *Cx43* can induce heterotaxia; aberrations in LR patterning can surely arise from mutations in many different genes and it remains to be determined precisely which subset of human laterality patients possess this mutation in Cx43.

Differential GJC along Dorso-Ventral Axis in *Xenopus*

The difference in GJC between the dorsal and ventral side of early embryos in wholemount has been described by a number of labs (Guthrie, 1984; Guthrie et al., 1988; Guthrie & Gilula, 1989; Nagajski et al., 1989; Olson et al., 1991; Olson & Moon, 1992; Krufka et al., 1998; Levin & Mercola, 1998b; Brizuela, Wessely & DeRobertis, 2001). However, based on analysis of sectioned embryos, it was recently argued that this difference does not exist. In a re-examination of the DV differences in the early frog embryo, Landesman et al. found no GJC using LY as a probe, and identical dorsal and ventral GJC using neurobiotin (a smaller molecule). Landesman et al. hypothesized that previous reports of DV differences in GJC were artifacts caused by lens-flare effects from adjacent blastomeres, and by pigmentation differences between dorsal and ventral *Xenopus* cells (Landesman et al., 2000). They suggest analysis of sections as opposed to wholemount embryos as a means of overcoming these potential problems.

It is probably unlikely that pigment differences can account for the observed DV differences because the same observation has been described in albino embryos (Brizuela et al., 2001) and on the ventral (pigmented) side when Cx26 is misexpressed there (Levin & Mercola, 1998b). Likewise, lens flare effects are unlikely to explain the observed differences because a number of labs have reported changes in endogenous GJC (induction on the ventral side or

abolition on the dorsal side) caused by the application of heptanol (Levin & Mercola, 1998b; Brizuela et al., 2001) or Wnt constructs (Olson et al., 1991, Olson & Moon, 1992). If analysis of embryos in wholemount inhibited the accurate assessment of GJC by fluorescent tracer methods (i.e., produced positives which were all false), one would not expect specific treatments that target GJC to alter the observed degree of dye transfer. Changes in scored instances of GJC that are caused by drugs or constructs suggest that the dye transfer observed represents a real phenomenon.

However, the reason for the failure of Landesman et al. to detect LY transfer in sections is an important issue and remains to be resolved by future studies on two fronts. Firstly, it has to be demonstrated that the sectioning analysis methods used by Landesman et al. are able to detect a genuine instance of junctional transfer, since they reported no transfer of LY in any region of the early embryo. This could easily be done by injecting embryos with an expression construct of Cx26 at the 1-cell stage (to introduce the ubiquitous expression of a connexin that lacks regulatory elements responsible for junctional gating). The embryos could be tested by LY dye transfer at the 8-cell stage, and sectioned. Such embryos should provide unmistakable instances of junctional transfer. This is important to ensure that loss of LY signal during fixation, embedding, or sectioning (or another factor) is not responsible for the lack of detection of GJC. Secondly, the converse is equally important. The labs that work with GJC in wholemount embryos should be able to demonstrate junctional transfer by sectioning their embryos. A disparity in the results of such experiments could be due to technical differences in the methods used (for example, Levin et al. used a Potassium salt of LY, while Landesman et al. utilized a Lithium salt, which is known to have developmental effects in frog embryos (Hedgepeth et al., 1997)).

Another important finding of Landesman et al. concerns the ubiquitous GJC observed for the neurobiotin tracer, in contrast to the restricted LY transfer. Such differences in GJC reflect the chemical selectivity of junctional paths, which are likely to be a crucial aspect of their regulatory function in LR asymmetry and other morphogenetic effects. This area of research requires a detailed study of the paths in early embryos available to molecules of different charges and sizes.

Invertebrate GJC

Although no evidence for connexin genes has been found in invertebrate systems, there are a number of proteins that provide GJC between cells during invertebrate embryogenesis (Phelan & Starich, 2001). Hydra, a freshwater coelenterate, is known by ultrastructural methods to contain gap junctions (Wood & Kuda, 1980), though the epithelial cells appear to exhibit no electrotonic or dye coupling (de Laat, Tertoolen & Grimmelikhuijzen, 1980). These junctions have been proposed to serve as the conduit for positional information during regeneration (Wakeford, 1979) because they form between graft and host tissue during inhibition of head regeneration by grafted head tissue. Indeed, application of antibodies made against a liver gap junction protein eliminates GJC in hydra cells and perturbs the ability of grafted tissue to inhibit regeneration (Fraser et al., 1987). Thus, the regenerating hydra offers a tractable context within which to identify the endogenous small molecules that traverse gap junctions during pattern formation. In the sea urchin (Yazaki, Dale & Tosti, 1999), vegetal pole blastomeres exhibit electrical communication; 1-octanol inhibited this GJC and induced defects in formation of the archenteron, suggesting that perhaps GJC is involved in a basic gastrulation process in urchin embryos.

More recently, molecular and genetic approaches have focussed on understanding the basis of GJC in invertebrates. Genes from the family now known as Innexins (formerly called OPUS) comprise a set of important developmental proteins that show no sequence homology to connexins but have the same topology, including four transmembrane domains. For example, the *Passover* and *Shaking-B* transcripts in *Drosophila* have dynamic expression patterns in the embryonic mesoderm and pupal nervous system and are required for electrical synapse formation between giant-fiber neurons (Crompton et al., 1995; Zhang et al., 1999). Though the molecular basis underlying GJC in imaginal discs of the fruit fly has not been identified, a mutation of the *dco* locus leads to a reduction of GJC and a severe overgrowth in the discs (Jursnich et al., 1990), suggesting that perhaps GJC is the mediator of size-limit control in this context.

In *C. elegans*, a mutant screen for suppression of hypersensitive response to the GJC blocker halothane identified the gene *unc-7*, mutations in which result in an uncoordinated phenotype due to the formation of ectopic electrical junctions between a subset of neurons; likewise, the worm mutation *eat-5* impairs the electrical and dye coupling of specific muscles in the pharynx (Starich et al., 1996). In *Drosophila* as well as the worm, GJC is necessary for the development of potassium currents, which forms a necessary part of normal development of the muscle (Todman et al., 1999), and, indeed, functional GJC is necessary for myoblast fusion in some vertebrates (Mege et al., 1994).

Although the ability of innexins to form functional gap junction channels has been controversial, it has recently been demonstrated directly for a number of innexins (Phelan et al., 1998; Landesman et al.,

1999; Stebbings et al., 2000). To date, genetic mutants in *Drosophila* and *C. elegans* have implicated innexins in the development of muscle and neuronal cell types. Identification of roles of GJC in other aspects of invertebrate patterning awaits the characterization of other GJC genes that are known to be present, based on genomic and PCR studies (Curtin, Zhang & Wyman, 1999). Interestingly, innexin homologs have recently been identified in mammals (Panchin et al., 2000), although no expression or functional data is available for these sequences proposed to be called "pannexins".

In general, the issue of what genes underlie GJC in invertebrate and vertebrate animals is crucial to evolutionary developmental biology. For example, an innexin is expressed in the grasshopper embryonic limb (Ganfornina et al., 1999); because of the important role of GJC in vertebrate limb patterning, a demonstration of the importance of this innexin's activity in grasshopper limb morphogenesis would be an important element in the discussion on the evolutionary relationships between vertebrate and invertebrate limbs (Shubin, Tabin & Carroll, 1997; Capdevila & Johnson, 2000).

The functional (but not sequence) relationship between innexins and connexins highlights another crucial question: why, if innexins are the basis of GJC in lower animals, would a whole new family of gap junctional proteins have arisen in vertebrates (Finbow, 1997)? Interestingly, one putative element of GJC is highly evolutionarily conserved: ductin, which, like connexins, has four transmembrane domains, exists in plants and most metazoan animals and has approximately 85% identity between insects and human.

Ductin

Data indicate that another substratum for GJC may exist alongside the Connexin family. Ductin is a critical 16 kDa proteolipid component of H^+ pumps (V-ATPases), which generates large electrochemical gradients at the expense of ATP when it is expressed in the plasma or vesicle membrane (Harvey & Nelson, 1992). Ductin has been suggested to oligomerize to form gap junctions between cells (Finbow et al., 1987; Finbow & Pitts, 1998). Inhibition of ductin function by exposure to drugs that specifically target this proteolipid (such as DCCD), through introduction of functionally blocking antibodies (Serras, Buultjens & Finbow, 1988; Bohrmann, 1993), or through misexpression of dominant-negative constructs (Saito et al., 1998), leads to a drastic reduction in GJC in mammalian and insect systems (Finbow et al., 1992, 1993). Since the ductin dominant-negative construct causes mis-localization of Cx43 (Saito et al., 1998), there may well be interactions between connexin and ductin proteins. The ductin basis of GJC is only now beginning to be understood and there are many unanswered questions about its function and its relationship to connexins (Bruzzone & Goodenough, 1995; Finbow, Harrison & Jones, 1995).

Consistent with its involvement in two key aspects of cell biology (GJC and V-ATPases), ductin has been implicated in carcinogenesis (Ohta et al., 1996; Saito et al., 1998), viral infection (Faccini et al., 1996; Ashrafi et al., 2000), and neurotransmitter release (Israel & Dunant, 1999). Its role as a GJC molecule is controversial (Finbow & Pitts, 1993; Bruzzone & Goodenough, 1995); for example, it has been suggested that modulation of ductin activity affects GJC indirectly, through changes in V-ATPase activity, which leads to altered pH (to which connexin-based gap junctions are known to be sensitive). Though a full discussion of whether ductin is truly a component of gap junctions is outside of the scope of this review, evidence based on ductin sequence, tertiary structure in the membrane, and localization in gap junction preparations, together with the finding that inhibiting ductin function abolishes GJC, make ductin an interesting candidate for an endogenous gap junction component (Finbow & Pitts, 1993).

Ductin is known to be crucial for embryonic development in a number of species. It is present in both plasma and vesicle membranes in *Drosophila* ovarian follicles, midgut, salivary gland, nervous system, and muscles (Bohrmann & Braun, 1999; Bohrmann & Bonafede, 2000). Gastrulation in *Drosophila* may be dependent on ductin function, since microinjection of anti-ductin antibodies abolishes dye tracer coupling in embryonic cells and induces specific developmental defects in the region of injection (Bohrmann & Lammel, 1998).

While much existing data on ductin have come from cultured cells or invertebrate systems (John et al., 1997; Bohrmann & Lammel, 1998), ductin is clearly important in the development of mammals: mice homozygous for the deletion of ductin die very early (around day 4) in development (Inoue et al., 1999; Sun-Wada et al., 2000). This precludes the study of complex embryonic phenotypes in simple ductin knockout mice. Thus, understanding the role of ductin within mammalian experimental embryonic systems will require the generation of conditional knockouts. It is likely that more rapid progress will be achieved by the characterization of ductin function in other model systems such as *Xenopus* and zebrafish. An unequivocal demonstration that ductin is either necessary or sufficient for junctional transfer would open the door to a number of novel possibilities in the cell biology of ion flux, and provide new questions in the evolution of cell-cell communication mechanisms.

Future Prospects

The characterization of GJC in embryonic patterning is currently at a very exciting stage; molecular tools now exist to probe every aspect of this type of intercellular information exchange in contexts where it is known to be important. Important future research areas include the characterization of factors that set up patterns of differential GJC in various embryonic tissues (at the transcriptional level, as well as at the level of controlling GJC states, such as by endogenous patterns of pH and voltage gradients), and the mapping of paths that exist in and between different tissues to molecules of various charges and sizes. Particularly interesting is the possible role of chemically-rectifying (unidirectional) gap junctions in embryogenesis (Robinson et al., 1993; Xin & Bloomfield, 1997).

Another important set of questions concerns the nature of the small molecules which traverse gap junctions. Many molecules that can pass through junctions (such as LY, Ca^{++}, $InsP_3$ Acetylcholine, ATP, etc.) have been identified, but in specific cases such as LR patterning and limb morphogenesis, it is not known what small molecules carry information through GJC. Of course, it is also crucial to characterize the genetic targets downstream of this junctional flow. Connexins may also have signaling effects through direct binding to other proteins—the yeast two-hybrid system has been utilized (Jin, Lau & Martyn, 2000) to search for connexin-interacting proteins, although no embryologically relevant molecules have yet been characterized.

The question of why mouse connexin knockouts often have fairly limited phenotypic consequences is important, and may best be approached by the analysis of transgenic mice misexpressing connexin mutants, such as dominant-negative constructs, as well as connexins which have altered voltage- and pH-gating properties (Verselis et al., 1997). Simple knockouts will still be informative, for example in probing the role of innexin-like proteins recently cloned from vertebrates.

The zebrafish will likely emerge as an excellent embryonic system for studying GJC since the embryos are transparent, allowing easy dye-based analysis of GJC paths in vivo as well as use of more sophisticated pH- and voltage-sensitive dyes for studying the spatial distribution of factors that control junctional gating. Expression of Cx43.4 has already been followed in zebrafish using a GFP fusion construct (Essner et al., 1996). The amenability of the zebrafish to genetic screens may also be used to analyze mutants in junctional transfer, by designing lines containing fluorescent small molecules.

There are many parallels to be drawn between embryonic morphogenesis and the processes of neoplastic growth. It is anticipated that the study of GJC in development will have important implications for the understanding of cancer; conversely, progress on GJC involvement in tumor growth will advance our appreciation for how GJC controls the events which shape the embryo. The involvement of GJC in embryonic development and cancer emphasizes the central importance of gap junctions in growth and pattern control.

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