Two Molecular Models of Initial Left-Right Asymmetry Generation

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Summary

Left-Right (LR) asymmetry is a fascinating problem in embryonic morphogenesis. Recently, a pathway of genes has been identified which is involved in LR patterning in vertebrates (1,2). Although, this work characterizes the interactions of several asymmetrically-expressed genes, it is still entirely unclear how such asymmetric expression is set up in the first place. There are two promising molecular candidates which may play a role in such a process: the motor protein *dynein*, and the gap junction protein *connexin-43* (*Cx43*). We present two models, significantly supported by previous findings, which hypothesize that (1) *dynein* asymmetrically localizes LR determinants in individual cells to establish cell-autonomous LR biasing, and (2) asymmetric activity of *Cx43* gap junctions within key cells sets up electric potentials in multi-cellular fields, thus establishing large-scale LR asymmetry.

Introduction

Symmetry in morphogenesis

Animal body plans occur in a wide variety of symmetries: spherical (volvox), radial (starfish), chiral (snails, ciliates), bilateral (drosophila) and pseudo-bilateral (man). Most vertebrates have a generally bilaterally-symmetrical body plan, but this symmetry is broken further into a pseudo-symmetry by the consistently asymmetric placement of various internal organs such as the heart, liver, spleen, and gut, or an asymmetric development of paired organs (such as brain hemispheres or lungs).

Symmetries are often broken in development. For example, the radial symmetry of the early chick blastoderm is broken into a bilateral symmetry by the appearance of Köhler's sickle and then the primitive streak (3). This is further broken into a pseudo-symmetry by the right-sided looping of the heart tube. In contrast, the early sea-urchin larva has bilateral (and then, pseudo-bilateral) symmetry. The adult, however, has a five-fold radial symmetry. Such axial patterning is the most fundamental process in embryogenesis because it lays a foundation and provides a context for all subsequent morphogenetic events.

Left-Right Asymmetry

Asymmetry along the left-right (LR) axis (defined as an invariant, among normal individuals, difference between the left and right sides of an animal's morphology) is fundamentally different from asymmetries in the other two axes. First, there is no feature of the macroscopic world which differentiates right from left. While gravity is a ubiquitous feature of the world which can be used to define the dorso-ventral axis, and any chosen direction of motion automatically picks out an anterior end (since that is the end which is best used for sensory and processing organs), there is no independent way to pick out the left (or right) direction.

Second, all normal members of a given species are asymmetrical in the same direction. However, animals with complete mirror reversal of internal organs are otherwise phenotypically unimpaired (4,5). Thus, while it is possible to come up with plausible evolutionary reasons for why organisms might be asymmetric in the first place (optimal packing of viscera, etc.), there is no obvious reason for why they should all be asymmetric to the same direction. It is, after all, easier to imagine a developmental mechanism for generating bilateral asymmetry (such as positive-feedback and amplification of stochastic biochemical differences) than for biasing it to a given direction. The left-right axis is thus unique, and especially interesting, among the three axes.

Many kinds of situs anomalies have been reported in the human teratology literature, associated with such syndromes as Kartagener's and Ivemark's (5). These include dextrocardia, *situs inversus* (a complete mirror-image reversal of the sidedness of asymmetrically positioned organs and asymmetric paired organs), heterotaxia (where each organ makes an independent decision as to its situs), and right or left isomerism (where the organism is completely symmetrical, leading to polysplenia or asplenia). Of these, only the complete (and rare) *situs inversus* is not associated with physiological difficulties. The rest, especially heterotaxia, often result in serious health problems for the patient. Laterality defects can arise in a single individual but are especially associated with twinning (4, 6, 7). These syndromes are paralleled to various degrees by mouse mutants such as: *iv* (8) which results in roughly 50% of the offspring being phenotypically *situs inversu*s, and *inv* (9) which have 100% of the offspring showing mirror image inversions of the internal organs.

The molecular mechanisms underlying antero-posterior and dorso-ventral asymmetry have been studied in detail (10). However, the basis for LR asymmetry is much less well understood. Neville (11) presents an extensive and fascinating survey of various asymmetries, including the well-known asymmetric organs such as the heart, as well as flatfish which consistently settle on and undergo eye

migration to one side, and even a species of parasite (the arthropod *Bopyrus*) which lives only on one side of prawn and shrimp. There has been little information, however, shedding light on the mechanisms which determine the sidedness of such asymmetries. Previously, information on the molecular basis of LR asymmetry centered around three lines of inquiry: the genetics of chirality in snails, a list of drugs which cause alterations in LR patterning, and several mammalian mutants which have phenotypes associated with LR asymmetry. Recently, a pathway of genes has been described which are asymmetrically expressed in the chick embryo and control the situs of the heart and other organs (1, 12).

The initial steps of LR determination remain unknown

LR patterning can be conceptually divided into three phases: (1) cell(s) in the very early embryo must ascertain their own right vs. left sides, presumably by a model like that involving a tethered chiral molecule (13), and (through lineage relationships, migration, and cell-cell inductive interactions) this cell-autonomous LR information is converted into asymmetrical multi-cellular domains of expression; (2) these asymmetrically-expressed genes regulate each other in sequential (and perhaps branched) pathways to establish and maintain asymmetric gene expression domains; and finally, (3) the various organ primordia read this information and determine their situs.

With the identification of a cascade of asymmetrically expressed genes which regulate each others' expression (1), a significant part of phase 2 has been uncovered. Likewise, it has become clear that this pathway controls the LR patterning of many aspects of laterality (12), and experiments are currently under-way to determine the mechanisms by which organs such as the heart respond to this information (Sylvia Pagan, personal communication). Thus, significant progress is on the horizon for phase 3. However, what is conspicuously missing are clues to the most interesting part of this problem: how the LR axis is oriented with respect to

the AP and DV axes in the first place, and how this orientation at the single-cell level is converted into asymmetry on the scale of the whole embryonic field.

The existence of isomerisms (where the body is symmetrical, consisting of either two normally-left sides or conversely, two right sides) and randomized asymmetry (i.e., 50% incidence of complete *situs inversus*) as two distinct genetic conditions suggests that normal LR asymmetry is accomplished in two dissociable steps: a random asymmetry is generated, which is then biased in the correct direction with respect to the other two axes (13). The pathway identified by Levin *et al*. most likely directs the second step, since right-sided misexpression of left-determining genes such as *Shh* or *nodal* results in heterotaxia (1,12), not true isomerism. Thus, there is still no molecular data on how (random) asymmetry is generated in the first place.

We would like to sketch out some ideas regarding two of the only promising molecular candidates for the primary steps in LR patterning, *dynein* and *connexin-43.* While the models described in this paper are quite speculative, they illustrate the types of mechanisms which are very likely to play a role in these early events. The *dynein* model is designed to show how a chiral molecule could differentiate L from R within a single cell. The *Cx43* model shows how, once a cell becomes LR asymmetric, this information could become transduced into multi-cellular domains of asymmetric gene expression. It should be noted that the two models are independent of each other and describe complementary phases in LR patterning.

A Specific chiral molecule model

Microtubule motors

The first model, based on the ideas of Brown and Wolpert (13), hypothesizes a cytoskeletal component, such as a centriole, which is chiral. It is oriented with respect to the AP and DV axes of the egg by means of other cytoskeletal filaments,

and serves as a nucleation center for filaments or microtubules which run along the LR axis. Consistent with this model, tubulin was identified as one of the proteins modified in *iv* homozygotes relative to w.t. mice (14). The head-tail attractive feature of microtubule assembly (15) ensures that the chiral nature of the nucleating center is passed on as a directionality of the LR tracks. Interestingly, the mouse egg has no centriole (one forms anew after several cell divisions), so that defects in the origin of chirality would show up as zygotic (as in the mouse LR mutants, such as *iv*); in contrast, the maternal mode of inheritance of chirality in snails (16) may be explained by the fact that the snail egg's cytoskeletal components are formed by the mother.

The next step results in a microtubule motor, such as dynein, riding the LR tracks carrying mRNA or protein determinants, which become transported to one side of the cell. These determinants could become localized with cell division (which is possible in molluscs or even frogs), or this process could happen anew in each cell during various phases (which is most likely in the chick and mouse) followed by the kind of process discussed below for generating domains of LR gene expression.

Evidence for a *Dynein* model

An excellent candidate for such a mechanism is *dynein* (17), a motor protein which serves to actively translocate sub-cellular cargo (18-20). There appear to be at least 13 axonemal (used in cilia and flagella), and 2 cytoplasmic (presumably involved in axonal transport, mitosis, etc.) *dynein* genes (reviewed in (21)).

Despite the involvement of dynein proteins in many disparate events (such as ciliary function, vesicle transport in axons, mitosis, etc.), it is clear that certain *dynein* genes have very specific expression. For example, Dhc64c is expressed only in ovaries, testes, and very early embryos in *Drosophila*; furthermore Dhc64c is asymmetrically (though not LR) localized in the *Drosophila* oocyte (22-23). Thus, it may be expected that specific lesions in one of the many *dynein*

genes can affect LR patterning without lethal effects on mitosis or organelle transport. There is compelling evidence that early AP and DV embryonic patterning is controlled in part by the cytoskeleton (24-25), and most importantly, disruption of the microtubule array in *Xenopus* by UV light causes 25% situs inversus (26).

Predictions of this model

Under this model, the heterotaxia phenotype could result from a broken dynein motor which is unable to perform localization of determinants. This would allow the factor to homogeneously accumulate in both halves of a cell, resulting in double-R or double-L (depending on the nature of the determinant) compartments. As shown by Levin *et al*. (12), this leads to independent randomization of organ situs. Interestingly, human patients with heterotaxia as part of Kartagener's syndrome do show defects in *dynein* (27-29).

The *inv* mutant could result from a nucleation center that is either the opposite enantiomer of one with the proper chirality, or simply becomes oriented incorrectly. The former possibility is much less likely (since the complete reversal of such a complex structure would require several coordinated mutations); it is most likely that whatever binding site is used to tether it with respect to the DV and AP axes is altered. This would result in embryos which are normal except for the consistently incorrect *situs* (as is observed in *inv* mice, where *nodal* is expressed always on the incorrect side only (2)).

The *iv* mutant may represent a nucleation structure that was not tethered at all. Thus, it would face in different directions (randomly) in different cells. Depending on stochastic events, this would result in a mosaic of domains of cells which were oriented properly, adjacent to cells which were not. This would be magnified by cell proliferation and lineage relationships and could thus easily account for the full spectrum of *nodal* expression patterns observed in the mutant mice (2), corresponding to normal situs, reversed situs, or double L or R sides.

An alternative explanation for why *dynein* defects are associated with laterality disturbances has been proposed: that cilia directly influence the situs of the gut (27-30). This is unlikely because it has been shown that several kind of asymmetries are present long before gut looping (1), and because some patients with heterotaxia do have normal cilia function (31-32). This may be a consequence of the fact that only cytoplasmic, not ciliary, *dynein* is important for this process.

Future directions

Several approaches can be taken to test the *dynein* model. Determining the expression of the various *dynein* genes in early embryos is crucial, to guide misexpression experiments and to begin to address the question of how a defect in a basic cell function protein can have such a subtle phenotype. The question of whether cytoplasmic *dynein* or ciliary function is important for LR patterning can be addressed by specifically disabling cilia motion, by mechanisms such as Ni^{++} (33), halothane (34), or vanadate (35), which inhibits cilia but not organelle motion. Finally, a direct test of the model can be made by overexpressing in *Xenopus* dominant negative versions of the appropriate *dynein*, consisting of the endogenous *dynein* cargo binding-domain alone, or fused to a kinesin motor domain (which moves in a direction opposite to that of *dynein* (36)). The resulting animals may be expected to exhibit LR phenotypes as the dominant negative *dynein* mis-localizes LR determinants.

How cell-autonomous LR information is transduced into cell fields

A condition known as isomerism is occasionally observed in human patients (5). This condition, sometimes called Ivemark syndrome, where the organism is bilaterally symmetric (polysplenia when two left sides are present, or asplenia when both are right sides (37)) is especially interesting because the known LR pathway provides no obvious clues as to how this might happen. For example, as shown by the *Shh* misexpression studies (12), producing bilateral *nodal* expres-

sion does not result in two morphologically identical left sides, but rather causes a heterotaxic phenotype where each organ decides its situs randomly. Isomerism is likely to shed clues on a mechanism quite different from that in which the genes and mutants discussed above are involved: the generation of LR asymmetry, as opposed to its biasing in an appropriate direction.

The relevance of *Cx43*

Connexin-43 (*Cx43*) is a member of a family of at least 12 distinct genes, whose proteins make up the intercellular channels of gap junctions (reviewed in (38)). These channels are composed of hexamers of connexins and provide conduits for the transfer of ions and other small molecules (such as 2nd messengers) between cells. Gap junctions have been implicated in normal embryonic development (39-40) as well as in tumor growth (41-42), since reduced communication between cells stimulates tumor promotion.

Most interestingly, it was found that human patients with isomerisms exhibit mutations in *Cx43* (43). This suggests the exciting possibility that gap junctions also play a role in LR patterning. This is also suggested by the observation that LR asymmetric transfer of dye takes place between blastomeres in the early embryo (44-45, Fig. 1A), consistent with gap junctions' providing an asymmetric partitioning of LR determinants. Moreover, transgenic mice that overproduce *Cx43* have situs anomalies (C. W. Lo, personal communication). Finally, expression of *cx43.4* is missing in zebrafish *ntl* mutant embryos (46). *Ntl* embryos lack an organized notochord and have randomized heart situs (47). Although this has been attributed to an active role of notochord in LR compartmentalization, it is equally possible that *cx43.4* is needed earlier for LR patterning. Gap junctions could potentially be involved in LR asymmetry by either providing an asymmetric path for the localization or distribution of a LR morphogen, or by generating an electric field which produces asymmetrical localization of charged molecules by electrophoresis.

The *Cx43* model

The idea that endogenous electric fields specify large-scale embryonic pattern is quite old (48-51); this has been suggested to involve galvanotaxic control of cell migration, electrophoresis of morphogens, electromagnetic cell-cell signaling, etc. The distribution of communication channels shown in Fig. 1B (45) suggests a model for LR asymmetry generation by gap junctions: that cells A and/or H are a battery, which generates a potential difference (due to asymmetric placement of ion pumps on the cell surface, established by a *dynein*-like mechanism). Since the other cells appear to be connected to each other by gap junctions, they represent an open circuit with respect to the current generated by the cells at A. Thus, charged LR determinants molecules would experience a net electromotive force and would tend to electrophorese to different halves of the embryo. A similar electrophoretic mechanism for directing the movement of maternal components has been characterized in egg-ovary systems (52-54); likewise, endogenous electric fields have also been shown to be involved in symmetry breaking in the *Fucus* embryo (55). The finding that placement of amphibian embryos in applied electric fields results in reversals of LR asymmetry (56) is also consistent with this model, which represents one possibility by which asymmetry at the level of the cell becomes transformed into asymmetric fields of gene expression.

The *Cx43* knock-out mouse (57) does not recapitulate exactly the human phenotype associated with the *Cx43* mutations (43): while a heart defect is observed, it is not a true situs abnormality but a problem with heart morphogenesis *per se*. This suggests that instead of simply a lack of *Cx43* gap junctions, a more subtle mutation in *Cx43* is responsible for isomerism (and specifically, that the w.t. asymmetry is not purely a result of open channels, but rather may be based on a specific pattern of cell communication restrictions). Britz-Cunningham *et al*. (43) find that 5 out of 6 of the (unrelated) patients had Ser364 mutated to a Pro, and suggest that this may interfere with serine phosphorylation which is known to affect *Cx43* function (58).

Future Experiments

Several directions need to be followed to elucidate the role of *Cx43* in LR asymmetry. First, detailed expression patterns of various connexins (not just 43) in frogs and mice need to be ascertained. Second, mutants such as the *Cx43*$^{-/-}$ transgenic (57) and the *Cx43* overexpression transgenic (mentioned in (43) as having randomized turning and heart looping) need to be examined for *nodal* expression (59). Third, whether or not the Ser364Pro mutation found in the human isomerism patients is directly responsible for their phenotype should be tested by producing a knock-in transgenic mouse (replacing the mouse's endogenous *Cx43* sequence with the Ser364Pro mutant); besides answering that question, the resulting mice may turn out to be a good medical model for human laterality defects and their treatment. Fourth, since it is unclear whether LR asymmetry may arise from the restriction of some morphogens due to closed gap junction channels between particular cells, or conversely, from specific cell-cell signaling resulting from open gap junctions, the exact nature of communication needed for correct asymmetry needs to be determined by functional studies. Finally, our model of electrophoretically-based asymmetry can be directly tested by short-circuiting this proposed current, as recently done by Hotary and Robinson (60). We are currently pursuing several of these approaches.

Conclusion

An understanding of these processes would have important implications for research in embryonic development, both in terms of basic questions of LR asymmetry, as well as a better appreciation of the role of endogenous electric fields (51,61-64) and the cytoskeleton in embryonic morphogenesis. The models described here represent specific, testable hypotheses which can serve to guide experiments directed toward that end.

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Figure Legends

Figure 1: Gap-junctional communication in the early *Xenopus* embryo (modified after Guthrie *et al*., 1988).

A 32-stage frog embryo, (future ventral side of animal pole is at top), with blastomeres labeled a-h. Arrows indicate open cell-cell communication, as determined by dye transfer experiments (Guthrie *et al*., 1988). This asymmetric pattern of gap-junctional communication suggests a model for the electrophoresis of LR determinants.