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Avian models and the study of invariant asymmetry: how the chicken and the egg taught us to tell right from left

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ABSTRACT While the external vertebrate body plan appears bilaterally symmetrical with respect to anterior-posterior and dorsal-ventral axes, the internal organs are arranged with a striking and invariant left-right asymmetry. This laterality is important for normal body function, as alterations manifest as numerous human birth defect syndromes. The left-right axis is set up very early during embryogenesis by an initial and still poorly understood break in bilateral symmetry, followed by a cascade of molecular events that was discovered 20 years ago in the chick embryo model. This gene regulatory network leads to activation of the *pitx2* gene on the left side of the embryo which ultimately establishes asymmetric organogenesis of the heart, gut, brain, and other organs. In this review, we highlight the crucial contributions of the avian model to the discovery of the differential transcriptional cascades operating on the Left and Right sides, as well as to the physiological events operating upstream of asymmetric gene expression. The chick was not only instrumental in the discovery of mechanisms behind left-right patterning, but stands poised to facilitate inroads into the most fundamental aspects that link asymmetry to the rest of evolutionary developmental biology.

KEY WORDS: *left-right, laterality, chirality, embryogenesis, chick, sonic hedgehog, nodal, bioelectricity, gap junction*

Introduction

One of the most immediately obvious features of the vertebrate body plan is its overall bilateral symmetry: most animals are constructed of two halves which are mirror images of each other, joined at a central midplane. This architecture is established extremely early as a fundamental feature of embryogenesis. However, subsequent development reveals a fascinating and progressive departure from left-right symmetry: numerous structures develop with a marked and consistent difference in their shape or placement with respect to the midplane. Perhaps the most remarkable aspect of animal anatomy is that organs such as the heart, gut, liver, brain, etc. are not only asymmetric, but are *consistently* so. All normal individuals have these organs on their correct respective side, exhibiting not simply asymmetry but a very strong bias of sidedness relative to the anterior-posterior and dorso-ventral axes.

The fundamental puzzle of left-right (LR) asymmetry

In fact, biological systems including bacteria (Mendelson and Keener, 1982), slime molds (Dimonte *et al.*, 2016), plants (Hashimoto, 2002), and individual vertebrate cells in culture (Chen *et al.*, 2012, Xu *et al.*, 2007) can all tell left from right. Here, we discuss the mechanisms that enable animal embryos to create consistently asymmetric internal organs, focusing on the contributions of avian models to this question. Minor deviations from perfect symmetry (e.g., subtle random differences in the lengths of the legs for example) are called fluctuating asymmetry (arising from developmental noise), and are not discussed here. True left-right asymmetry is a phenomenon that is far more difficult to understand. In 3D space, an organism breaks symmetry once to set up the head-tail axis, and places the sense organs at the end that will encounter things first, as the animal moves forwards. Together with the AP axis, an early embryo also acquires a dorso-ventral axis. As long as AP and DV axes are orthogonal, any choice of planes will do. But, having fixed the AP and DV axes, there is no longer any choice about the LR axis – the L and R directions are fixed. How does an embryo determine which direction is which, when, macroscopically, nothing distinguishes left from right? One can appreciate the problem by visualizing the difficulty of explaining to someone, over a telephone

Abbreviations used in this paper: HH, Hamburger-Hamilton developmental stage; LR, left-right.

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connection, which one is meant by "Left hand" (without reference to an already shared chiral object). Thus, the consistent establishment of invariant left-right asymmetry in all normal vertebrate embryos is a truly profound problem.

Steps to achieving LR asymmetry

Correct LR patterning requires several elements (Fig. 1). First, symmetry breaking – the L and R sides must be made different in some way. Then, the asymmetry has to be correctly oriented (so that the L and R sides are not merely different, but the L features are always on the left side of the organism). This information has to be propagated from the cellular level to large cell fields. Work in several (non-avian) species has revealed that these processes involve an amplification of cellular chirality (knowing which direction is L or R) into multicellular asymmetry (position along the L/R axis). Finally, this information needs to be interpreted by organ primordia to allow them to perform asymmetric morphogenesis. The flat blastoderm of the chick and amenability to surgical and molecular-genetic techniques has enabled it to play an instrumental role in revealing 1) the molecular genetics of asymmetric gene expression comprising the middle part of the pathway, 2) key elements of the upstream amplification process, and 3) the biophysics involved in heart and gut asymmetric morphogenesis.

The importance of LR asymmetry

LR asymmetry is a difficult puzzle; it is no coincidence that the first molecular inroads into unraveling this aspect of development came much later than insights into the AP and DV axes. Consistent asymmetry raises a host of questions in cell and evolutionary biology: why are we asymmetric at all? And even if asymmetric, why always in the same direction and not randomly oriented? Interestingly, the lessons learned about LR patterning (discussed below) are of value not only in explaining asymmetry: as it turns out, the mechanisms of LR asymmetry are a paradigm case of multi-scale biological integration. The study of asymmetry reveals to us how living systems can convert molecular-level information into large-scale anatomy.

Aside from these fundamental issues, LR asymmetry has a more practical side: clinical relevance to a number of human syndromes – primary defects of laterality, as well as other syndromes with lateralized presentations in the human patient. There are several ways for asymmetry to go awry: mirror image reversal, loss of asymmetry or randomization of individual organs' sidedness.

Human primary laterality syndromes

From the outside, adult human bodies generally look symmetrical between left and right, despite minor random variations between individuals. From the inside, however, numerous organs are positioned with a strong bias towards one side. This is the case for unpaired organs: most of the heart, spleen, pancreas and stomach are located on the left side, while most of the liver and gall bladder are found on the right side (Fig. 2). These organs are first formed symmetrically, at the embryo midline, and then undergo complex morphogenesis that positions them on one side. Some major blood vessels, such as aortas, are initiated symmetrically, then undergo by a regression phase on one side. Similarly, many paired organs also display asymmetrical development or looping, such as brain hemispheres, lungs, and gut. This left-right organization, which is the most commonly found in humans, is called *situs solitus*. Laterality disorders have a global incidence of 1/8-15,000 in humans (Orphanet website; Catana and Apostu, 2017). Full left-right reversal of internal organ organization, called *situs inversus* (or *situs inversus totalis),* has a rare incidence of

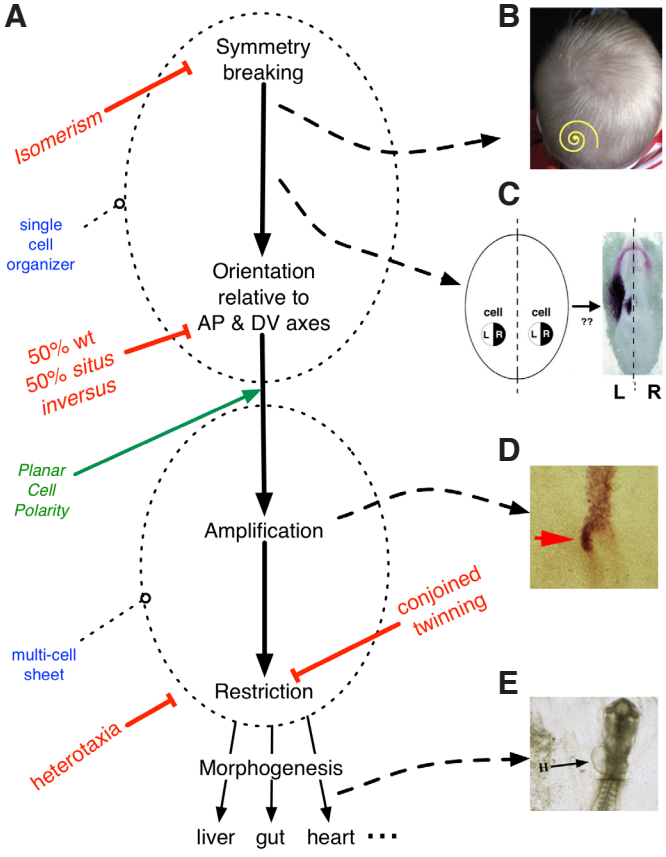


Fig. 1. Conceptual phases of left-right (LR) patterning and their subsequent readouts. (A) *LR symmetry breaking requires that a midline be established, and one side be made different from the other. This difference needs to be consistently oriented within the population. The information needs to be amplified and transmitted to multiple organ systems during organogenesis; midline structures must restrict side-specific signals from crossing over. Lack of coordination results in heterotaxia, where individual organs make independent decisions, resulting in a spectrum of random placement within an affected cohort. Red arrows indicate phenotypes arising from disruption of each step.* **(B)** *Hair whorls reveal the presence of chirality, a fundamental early step of asymmetry not requiring a midline, and its link to planar polarization of many later tissues such as skin and hair (Aw and Levin, 2009). In monozygotic twins, such hair whorls are mirror images (Golbin* et al., *1993, Klar, 2003), revealing a link to symmetry-breaking events occurring during the earliest cell cleavages.* **(C)** *During the orientation and amplification phases occurring after the embryonic midline has been established, cells must convert intracellular knowledge of direction along the LR axis or chirality (the same in all cells) into position relative to the midline (different in L vs. R cells), here illustrated by the expression of Nodal in lateral plate cells only on the left side of the chick embryo (dark purple is Nodal, light pink is Nkx2.5, taken from cover of Levin* et al. *(1995).* **(D)** *The amplification of primary events results in the asymmetric expression of genes like* Sonic hedgehog, *shown here on the left side of Hensen's node (taken from Levin* et al., *1995).* **(E)** *Asymmetric cues feed into morphogenesis of organs such as the heart tube (taken from Levin* et al., *1995). Figure modified after (Vandenberg and Levin, 2009).*

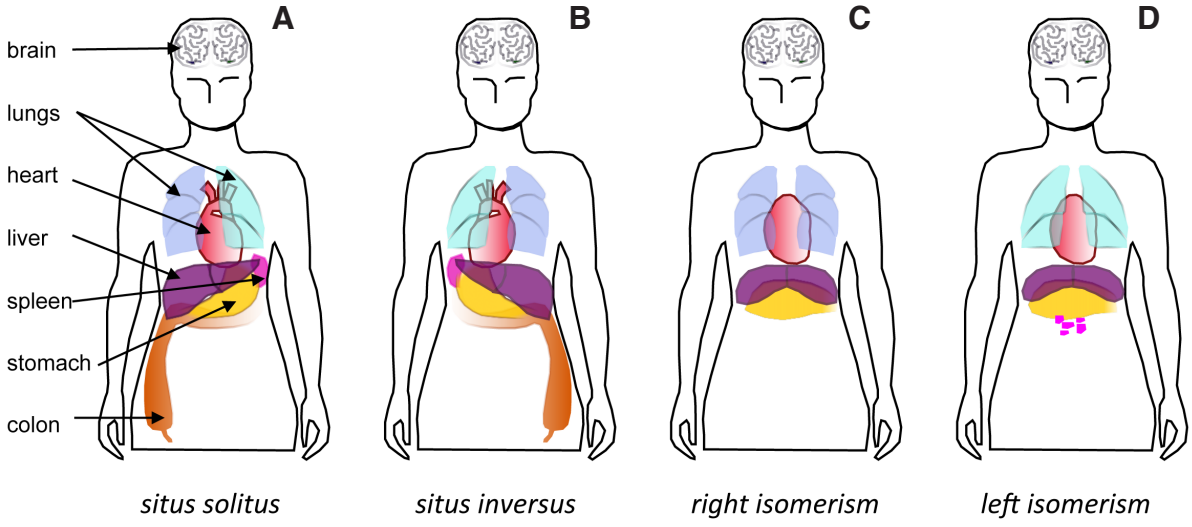


Fig. 2. Laterality in humans: *situs solitus* and some *situs* alterations. *Many organs are arranged asymmetrically along the LR axis within the human body cavity. The normal arrangement* **(A)**, *called* situs solitus, *includes the left lung with two lobes whereas the right lung has three, the heart pointing to the left side, the stomach and spleen on left while the liver is mostly on the right body side, as well as gut coiling in counterclockwise direction. Complete inversion of this arrangement* **(B)**, *called* situs inversus totalis, *usually does not cause physiological malfunctions. In contrast, the mirror duplication of the right* **(C)** *or left-***(D)** *sided organs, named* isomerism, *cause severe pathological conditions. Right isomerism, also known as asplenia syndrome, exhibits duplication of the right heart chamber, of the right lung, and of the liver, loss of spleen, abnormal positioning of the stomach. Left isomerism results in polysplenia syndrome, with multiple non-functional small spleen-like structures, often associated with gastrointestinal abnormal rotation and cardiac anomalies. Interestingly, the defects in these internal organs organization do not seem correlated to brain laterality modifications. The organs are color-coded for clarity and the schemes are simplified to show only the main features, as a large spectrum of anomalies is observed in patients.*

1/8-25,000, which is likely underestimated since most patients are asymptomatic (Casey, 1998, Peeters and Devriendt, 2006). Other laterality disorders, grouped under the name of *heterotaxy (*from the Greek *"other arrangement")* or *situs ambiguus,* cause severe medical condition, including organ malformations and functional disorders. Heterotaxy includes *left* or *right isomerism,* when the left-sided or right-sided organs respectively, are mirrored on the opposite side, and all other configurations of random relative positioning of internal organs. These defects in laterality, resulting in aberrant relationships between organs, cause complex congenital heart disease in 80% of the patients with heterotaxy (such as transposition of the great arteries, various ventricular defects, atrioventricular septum malformations, defective pulmonary venous connections) (Peeters and Devriendt, 2006, Ramsdell, 2005). The patients with heterotaxy represent 3% of total congenital heart defects (CHD) in human. In contrast to 80% of CHD in patients with heterotaxy, only 3-9% patients with *situs inversus* present CHD, which is still higher incidence that in the normal *situs solitus* situation (CHD in 0.6%), but means that total reversal of organ positioning results in a relatively normal physiological situation (Peeters and Devriendt, 2006).

In the brain, the anatomical signs of laterality are more subtle, and mainly found in areas involved in language processing (Duboc *et al.*, 2015). Interestingly, brain laterality seems independent from visceral organ arrangement, as patients with *situs inversus* have the same language lateralization as *situs solitus* individuals (Kennedy *et al.*, 1999).

Interestingly, laterality is a feature of other body systems that do not display overt anatomical asymmetry. A number of syndromes are present unilaterally in paired structures like the limbs (Smith *et al.*, 1979), face, or hips (Delaney and Boyd, 2007, Paulozzi and Lary, 1999). Even cancer incidence (Robichaux *et al.*, 2015, Sandson *et al.*, 1992; Wilting and Hagedorn, 2011) and immune response (Dane *et al.*, 2001; Meador *et al.*, 2004) exhibit invariant asymmetries, revealing that left-right patterning is embedded in body form and function far deeper than just the positioning of asymmetric organs.

Human syndromes with disorders of lateralization

Human laterality defects are found in diverse forms: they include familial or sporadic forms, with isolated or syndromic phenotypes. The genetic inheritance can involve autosomal recessive or X-linked recessive, or autosomal dominant transmission (Casey, 1998). About 230 genes and genetic phenotypes have been associated to left-right asymmetry anomalies (*45 OMIM entries for heterotaxy and 186 OMIM entries for situs inversus, June 2017).*

Situs inversus is well-defined phenotype, with autosomal recessive transmission. Numerous human phenotypes of *situs inversus* are associated with primary cilia dysfunction. Described in 1933, Kartagener Syndrome (OMIM # 244400) associates *situs inversus* to primary cilia dyskinesis (PCD). It is belongs to the familial Immotile Cilia Syndromes (ICS), with dyskinesic/immotile primary cilia and flagella caused by defective dynein arm (Mitchison *et al.*, 2012). ICS patients present malfunction of upper and lower airways and infertility (Casey, 1998, Fretzayas and Moustaki, 2016). Half of the ICS patients harbor *situs inversus*, while *situs ambiguus* is rare (6.7%; Kennedy *et al.*, 2007), suggesting a randomized lateralization in ICS. Other familial *situs inversus,* not associated to ICS, have sometimes been reported, also with autosomal recessive transmission (Chib *et al.*, 1977, Cockayne, 1938, Corcos *et al.*, 1989, Kosaki and Casey, 1998, Mital *et al.*, 1974).

Heterotaxia encompasses a broad spectrum of clinical fea-

tures, and it is unclear if isolated cardiac malformations, or other isolated visceral organ abnormalities, relate to laterality defects. The "classical" *situs ambiguus* associates cardiovascular defects to visceral organ malformations. Familial cases of *situs ambiguus* have been described in children born from consanguineous parents. Heterotaxy genetic transmission follows either autosomal dominant, or autosomal recessive, or X-linked modalities (Kosaki and Casey, 1998, Zhu *et al.*, 2006). However, most described cases of heterotaxia are likely sporadic, supporting that Mendelian inheritance is not the rule. A recent study suggests, however, after closer phenotypic examination of unaffected individuals in the pedigree, that 10% of infants with heterotaxy could belong to a family with congenital cardiac defects (Zhu *et al.*, 2006). The identification of the genes associated to *situs ambiguus* will further aid analyzing the familial history for cases of apparently isolated heterotaxy.

Early steps: asymmetry research prior to molecular work in the chick

It is somewhat remarkable, given that this is a major body axis, that the total sum knowledge about asymmetry prior to 1995 can be summarized as follows. Some such as Neville (Neville, 1976) had cataloged the numerous consistent asymmetries throughout the animal kingdom, at the level of anatomy and histology; examples (most of which did not turn into molecular model species in the LR field) include beetles that consistently fold one wing under the other, crustaceans with specialized right and left fore-limbs, flatfish that consistently settle on and undergo eye migration to one side, and parasites that lives only on one side of host prawn and shrimp. It was however known from developmental work in sea urchin and frog that the LR axis was probably specified after the anterior-posterior (AP) and dorso-ventral (DV) axes, and is determined with respect to them (McCain and McClay, 1994); early surgical work in chick had identified gastrulation as a sensitive period for affecting heart laterality (Hoyle *et al.*, 1992) – a finding backed up by early rodent studies (Fujinaga and Baden, 1991a). In amphibian (Yost, 1990) and rodent (Fujinaga and Baden, 1991b, Fujinaga *et al.*, 1990) embryonic models, drug experiments had identified a variety of compounds that caused LR inversions, randomizations, or unilateral defects (reviewed in Levin, 1997), but mechanisms were unclear.

Genetic approaches had provided interesting data. Selection attempts for LR asymmetries in *Drosophila*, in hopes of generating a genetically-tractable mutant, had failed (Tuinstra *et al.*, 1990). However work on molluscs, which undergo chiral spiral cleavage, implicated an unknown cytoplasmic determinant (Freeman and Lundelius, 1982, Murray and Clarke, 1966). Several mammalian mutants were known, which displayed either defects in basic LR patterning or phenotypes that differentially affect the left or right sides of the body (reviewed in Levin, 1997). For example, *iv* (Hummel and Chapman, 1959) produces racemic offspring (50% being phenotypically *situs inversus*), while *inv* (Yokoyama *et al.*, 1993) mice have ~85% of the offspring showing mirror image inversions of the internal organs. Mutants such as *legless* (Schreiner *et al.*, 1993, Singh *et al.*, 1991) exhibit limb phenotypes that are more pronounced on one side of the body. Ironically, it was the chick - a model system amenable to classical embryology but not genetics - that provided the first clues to the molecular basis of invariant LR asymmetry.

Classical data

Many classical studies have explored L/R patterning prior to the molecular identification of early players of the L/R asymmetry cascade in the late 1990's. In chick embryos, a series of experiments have identified that gastrulation is a critical timing period for L/R axis setting, and that the node (Hensen's node) is a key structure. To study sidedness of heart looping, which orients to the right-hand side in most embryos, Wolpert and colleagues performed delicate heterotopic grafts of left or right precardiac mesoderm during chick gastrulation (Hoyle *et al.*, 1992). In chick, gastrulation takes place during primitive streak elongation, from stage 3+ to stage 4, according to Hamburger and Hamilton (HH) staging table (Hamburger and Hamilton, 1951). Later on, gastrulation proceeds posteriorly, along the regressing primitive streak, while neurulation starts anteriorly (Gilbert, 2006). Hoyle *et al.,* thus demonstrated that embryos with bilateral right-sided precardiac mesoderm, taken from stage 4-5HH donor embryos, develop a high proportion of left-sided (inverted) hearts. Moreover, this effect decreases when stage 6HH precardiac mesoderm is grafted. This striking result matched the critical period identified for mammalian (rat embryos) L/R axis setup, with treatment with an adrenergic agonist (Fujinaga and Baden, 1991b) (see below). Furthermore, Hensen's node displays a clear morphological L/R asymmetry, very transiently between stage 5HH and 8HH (subtle asymmetries are even seen at earlier stage 4HH), suggesting that node development could be the influenced by early laterality regulators, and that the node could play a role in L/R axis formation (Cooke, 1995, Dathe *et al.*, 2002). Node rotation experiments in chick gastrulas, at either stage 4HH or stage 5HH, demonstrated that only a stage 5HH node influenced embryo laterality (Pagan-Westphal and Tabin, 1998). When the node was rotated at stage 4HH, the L/R axis was reset to the normal orientation respective to the embryo, not to the rotated node. This indicated that the node acquires knowledge of the L/R axis at stage 5HH, and that the node can subsequently influence laterality of the embryo (Pagan-Westphal and Tabin, 1998). It also indicated upstream regulation by the surrounding tissues, influencing node L/R patterning prior to stage 5HH. However, these experiments did not indicate the nature or properties of the mechanism nvolved in L/R axis determination.

Avian asymmetries

The embryonic chick turned out to be pivotal in unraveling these steps. Prior to considering the molecular work, it is helpful to sketch the anatomical and functional asymmetries in this model system. During gastrulation, the organizing center at the tip of the primitive streak, Hensen's node, already exhibits a consistent asymmetry: its right lip is much more prominent than the left and contains a cell condensation connected with the head process (Cooke, 1995, Dathe *et al.*, 2002). Another macroscopic aspect of whole-embryo asymmetry is the process of "turning": the wild-type embryo rotates to its right and lies on its side. There is some correlation between the turning and subsequent heart situs (Waddington, 1937), but these processes are dissociable and can occur in different directions if LR patterning signals are randomized (Hoyle *et al.*, 1992, Levin *et al.*, 1997).

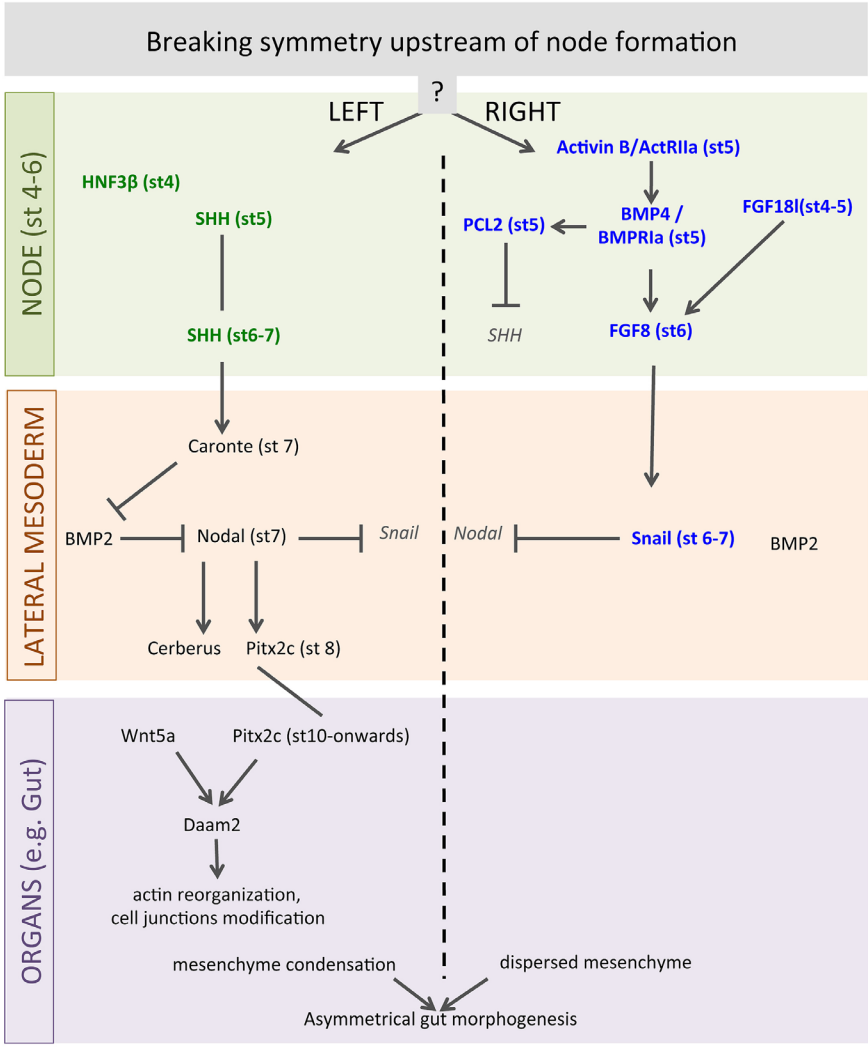
Organogenesis is highly asymmetric; the most obvious example of this is the heart tube, which bends and folds in a stereotypical

pattern that subsequently sets up the canonical example of asymmetry – the vertebrate heart. This process involves differential properties of cells on the right and left side of the precardiac fields and heart tube itself, including proliferation and traction forces, mediated by cytoskelon and retinoic acid signaling (Latacha *et al.*, 2005, Linask *et al.*, 2003, Linask *et al.*, 2002, Manner, 2004, Simard *et al.*, 2006, Stalsberg, 1969, Tsuda *et al.*, 1996, Yue *et al.*, 2004, Zamir *et al.*, 2003, Zile *et al.*, 2000). Likewise, the profound asymmetry of the viscera involve asymmetric morphogenesis and rotation of the gut tube, now known to be driven by differential extracellular matrix and cell adhesion properties, as well as physical forces transmitted from the mesentery (Branford *et al.*, 2000, Davis *et al.*, 2008, Kurpios *et al.*, 2008, Welsh *et al.*, 2013, Yuan and Schoenwolf, 2000).

In addition to these, most obvious anatomical aspects of asymmetry arising in singular (midline) structures, the chick offers a model for asymmetric development of paired organs. Interestingly, only the left ovary and oviduct persists in normal avian embryos. Useful strains of chickens exist, such as the PNO/DO line, in which the right oviduct persists (Hoshino *et al.*, 2005, Ishimaru *et al.*, 2008, Wakamatsu *et al.*, 2000), providing an opportunity to study the role of programmed cell death and remodeling in the sculpting of asymmetry. The lateralization of paired structures extends to the anatomy and function of the brain, including connections to the eyes. Chick brain hemispheres have consistently distinct responses to, and provide differential processing of, stimuli mediated by light and even magnetoreception (Rogers, 2008, Rogers *et al.*, 2008, Vallortigara *et al.*, 2001, Vallortigara *et al.*, 1996). This is thought to derive from different light exposure of the left and right eye, driven by the invariant rotation of the embryo which points one eye upward and one down into the darker portion of the egg.

The molecular age

A breakthrough in the understanding of the molecular basis of left-right asymmetry determination occurred some 20 years ago, with the observation of asymmetrical gene expressions in chick Hensen's node. A series of studies comprising gain and loss of function for various signaling molecules, as well as combined manipulations for phenotypical rescues, has allowed deciphering a left-right asymmetry gene regulatory network (LR-GRN) that can be subdivided into three main steps in chronological order, over a short period of development: establishing stable initial gene asymmetries in the node at the end of gastrulation, relay the asymmetry information to the adjacent mesoderm, propagate and control L/R asymmetry during organogenesis. As this LR-GRN involves a complex cascade of genetic activities, those three steps are depicted separately in Fig. 3.



Molecular asymmetries pattern left from right in the node, between stages 4HH and 6HH

As mentioned above, initial asymmetry in Hensen's node morphology can be detected as early as the stage of fully extended primitive streak (stage 4HH), suggesting that breaking of bilateral symmetry has already taken place at that early stage (Dathe *et al.*, 2002). From 1995 onwards, there was a period of active discovery of asymmetrical gene expressions in the node. Early *Hnf3b* expression was found transiently enhanced on the left side of the stage 4HH node. While symmetrically expressed until stage 4HH, at stage 5HH (nascent notochord stage), *sonic hedgehog* (*shh*) was detected on the left side of the node and maintained this expression pattern until stage 7HH (3-somites stage) (Levin *et al.*, 1995). In contrast, *activin receptor IIa* was expressed on the right side of the primitive streak just prior to stage 4HH, then on the right side of the node at stage 5HH (Levin *et al.*, 1995). At stage 5HH, *shh* and

Fig. 3. The molecular cascade of asymmetrically-expressed genes. *The temporal sequence of the regulatory genetic interactions starting in the node is described as three main steps: molecular events within the node, transmission of laterality information to the adjacent lateral mesoderm, and control of organogenesis, with gut morphogenesis chosen as an example. Details on gene activation and function of each factor are described in the text. The initial symmetry breaking events acting upstream of this LR-GRN are described in Figure 5 and text.*

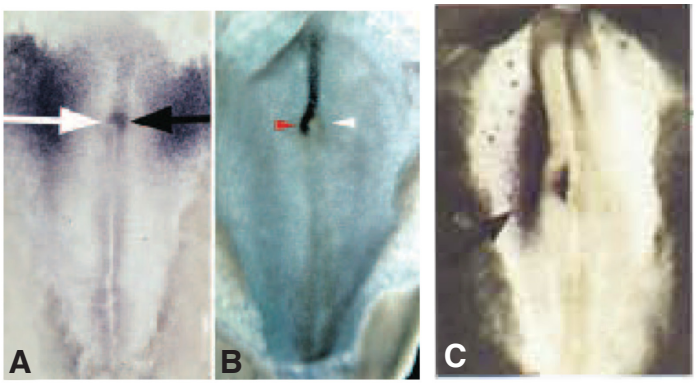
activin receptor IIa asymmetrical gene expression were found in the superficial layer of the node, which both contribute later to the notochord (Levin *et al.*, 1995). The initial left-sided expression of *shh* is further stabilized by inhibiting signals on the right side of the node, since, for example, blocking right-sided BMP signaling at stage 5HH results in bilateral *shh* expression at stage 6/7HH (Monsoro-Burq and Le Douarin, 2001). Indeed, on the right side of the node, a molecular cascade including early Activin signals triggers BMP signaling, which in turn activates FGF signals and blocks right-sided activation of *shh* (Monsoro-Burq and Le Douarin, 2000, Monsoro-Burq and Le Douarin, 2001). In addition, early expression of *fgf18* (from stage 4 to 5+HH) is also involved in the right-sided expression of *fgf4* and *fgf8* at stage 6HH (Boettger *et al.*, 1999, Ohuchi *et al.*, 2000, Shamim and Mason, 1999). Finally, polycomb-like PCL2 transcriptional repressor could mediate the repressive action of these right-sided signals on *shh* expression (Wang *et al.*, 2004). Hence, molecular asymmetries are initiated and stabilized by coordinated and antagonistic signaling cascades on each side of the node (Fig. 3).

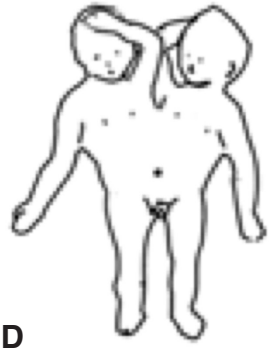
Left-sided information is transmitted to the lateral mesoderm around stage 7-8HH

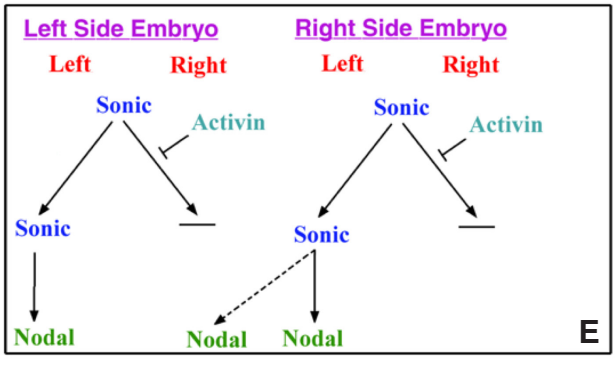
At the first somite stages, several markers identify left from right in the mesoderm lying adjacent to the node: on the left, *caronte, nodal* (chick nodal-related 1, *cNR-1*) and *pitx2* appear around stage 7HH, while *snai1 (*chick-related snail, *cSnR)* marks the mesoderm on the right (Isaac *et al.*, 1997, Levin *et al.*, 1995, Rodriguez Esteban *et al.*, 1999, St Amand *et al.*, 1998, Yokouchi *et al.*, 1999, Yoshioka *et al.*, 1998). SHH, on the left of the node, activates *caronte* (a member of DAN-Cerberus BMP antagonists family), in the lateral plate mesoderm. Caronte antagonizes BMP signaling asymmetrically, resulting in *nodal* activation on the left (Rodriguez Esteban *et al.*, 1999, Yokouchi *et al.*, 1999). In turn, Nodal and Lefty-1 trigger *cerberus* and *pitx2* expression (Levin *et al.*, 1995, Pagan-Westphal and Tabin, 1998, Yoshioka *et al.*, 1998, Zhu *et al.*, 1999). On the right side of the embryo, Activin and FGF8 signals activate *snai1* in the mesoderm while SHH and nodal repress it (Boettger *et al.*, 1999, Isaac *et al.*, 1997, Patel *et al.*, 1999). Snai1 further prevents ectopic activation of *pitx2* on the right side of the embryo (Patel *et al.*, 1999). All these experimental manipulations result in heart *situs* during later organogenesis (Boettger *et al.*, 1999, Isaac *et al.*, 1997, Levin *et al.*, 1995, Rodriguez Esteban *et al.*, 1999). Together, this series of studies highlights the transmission of left information from the node to the mesoderm (Fig. 3), by *nodal* and *pitx2* activation, active repression of the left-sided gene expressions on the right side. In addition, elegant experiments in early mouse embryos have shown that Lefty-2 prevents the diffusion of Nodal signals towards the right side of the embryo (Meno *et al.*, 2001). Furthermore, among other less well-explored cellular parameters, cell-cell adhesion via N-cadherin may influence L/R patterning by regulating *pitx2* and *snai1* expression (Garcia-Castro *et al.*, 2000).

Pitx2 from the mesoderm controls asymmetrical organogenesis

A landmark study, using several vertebrate model organisms, has demonstrated the pivotal role of Pitx2 in the transmission of L/R information during organogenesis and embryonic rotation. In addition to its expression in the left-side lateral mesoderm around stage 8-9HH, *pitx2* is also expressed on the left side of the heart from stage 10HH. During organogenesis, *pitx2* is found asymmetrically expressed in the digestive tract and in branchial arches. Misexpression of Pitx2 in chick and frog embryos resulted in heart and embryo turning side defects (Ryan *et al.*, 1998). This study thus placed Pitx2 function as central in the LR-GRN (Fig. 3). Pitx2 is a bicoid-like homeodomain transcription factor, which seems to be active via its N-terminal part (isoform Pitx2c; Simard *et al.*, 2009). Mutations in human Pitx2 have been related to CHD in patients (Yuan *et al.*, 2013). However, its transcriptional targets involved in organ laterality control were poorly known, until the discovery of asymmetrical WNT signaling in the dorsal mesentery, responsible for asymmetric midgut looping (Welsh *et al.*, 2013). Daam2, regulated by Pitx2 and asymmetrical Wnt signaling in the dorsal mesentery, interacts







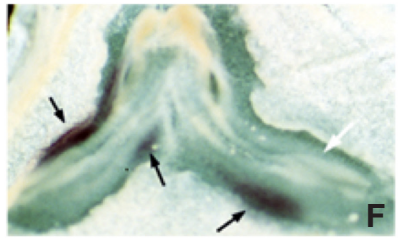


Fig. 4. Conjoined twins: laterality defects explained by the left-right (LR) pathway. *The asymmetric gene cascade, including Activin Receptor 2B* **(A)**, *Sonic hedgehog* **(B)**, *and Nodal* **(C)** *helped explain the previously mysterious observation that one of two laterally-conjoined twins (schematized in* **D***) would exhibit laterality disturbances. The model based on ectopic diffusion of asymmetric gene products such as Sonic hedgehog onto the right side of one of the embryos from its neighbor matched human data (Levin* et al.*, 1996) and was directly tested in chick twins, correctly predicting the ectopic induction of Nodal on the right side of the left twin* **(F)**.

with the cell junctions and cell cytoskeleton, thus affecting cell adhesion and behavior (Welsh *et al.*, 2013). This study highlights how asymmetrical gene expression, controlled by Pitx2, results in asymmetrical organogenesis.

In parallel, specific mechanisms buffer the LR-GRN cascade action, during symmetrical organ formation: during somitogenesis, retinoic acid signaling is required for the formation of bilaterally symmetrical somites (Vermot and Pourquie, 2005).

Conjoined twins – explaining human birth defects

The chick system also enabled a good example of the use of basic developmental genetics to illuminate problems in human medicine. It had long been noticed that inversions of various organs often occur in the context of human conjoined twins (Aird, 1959, Cuniff *et al.*, 1988, Torgersen, 1950). Classical studies also observed spontaneous and experimentally-induced twins in frog, armadillo, and fish embryos: frequent laterality defects affect one of the conjoined twins usually the one positioned on the right side (Morrill, 1919, Newman, 1916, Peeters and Devriendt, 2006, Schwind, 1934, Spemann and Falkenberg, 1919). An explanation for this fact was unknown, despite the fact that almost a century ago, the association between twinning and asymmetry had not escaped the notice of the giants of developmental biology (Mangold, 1921, Spemann and Falkenberg, 1919). These observations suggested that the left twin has an influence on the right-hand side twin, possibly via the action of long-range diffusible molecules. This observation has triggered early works using various pharmacological agents, from simple chemicals such as cadmium to complex agonists/antagonists (see below). An understanding of the molecular determinants of L and R identity provided a ready explanation for what is happening when two embryonic fields are conjoined (Fig. 4): the left-specific secreted signaling molecules (such as Nodal) can leak over and affect the right side of the adjacent twin. The predictions of this model were validated against chick experiments using induced twins, and the geometric requirements for mutual arrangement of the primary axes (that could allow side-by-side leak-over of signals) provided an explanation for why certain classes of human twins exhibit laterality disturbances and others do not (Levin *et al.*, 1996).

Upstream of asymmetric gene expression

Transcription can't tell left from right: events upstream of asymmetric gene expression

While experiments in the chick helped identify a cascade of left- and right-specific transcripts that ultimately dictated the sidedness of the internal organs, this left a major puzzle. Whatever turns out to be the very first asymmetrically-expressed gene, what makes *that* transcript only be expressed on its correct side? The work of chasing each asymmetric gene upstream to see what induces it cannot go on forever; because transcription alone cannot tell left from right, some other aspect of physics or physiology has to occur upstream of, and instruct, the first asymmetric gene in the LR pathway cascade (Fig. 5).

In 1999, the chick model facilitated the identification of the first such component. The distinct, mutually-repressive programs on the left and right sides of the embryo suggested that the L and R sides

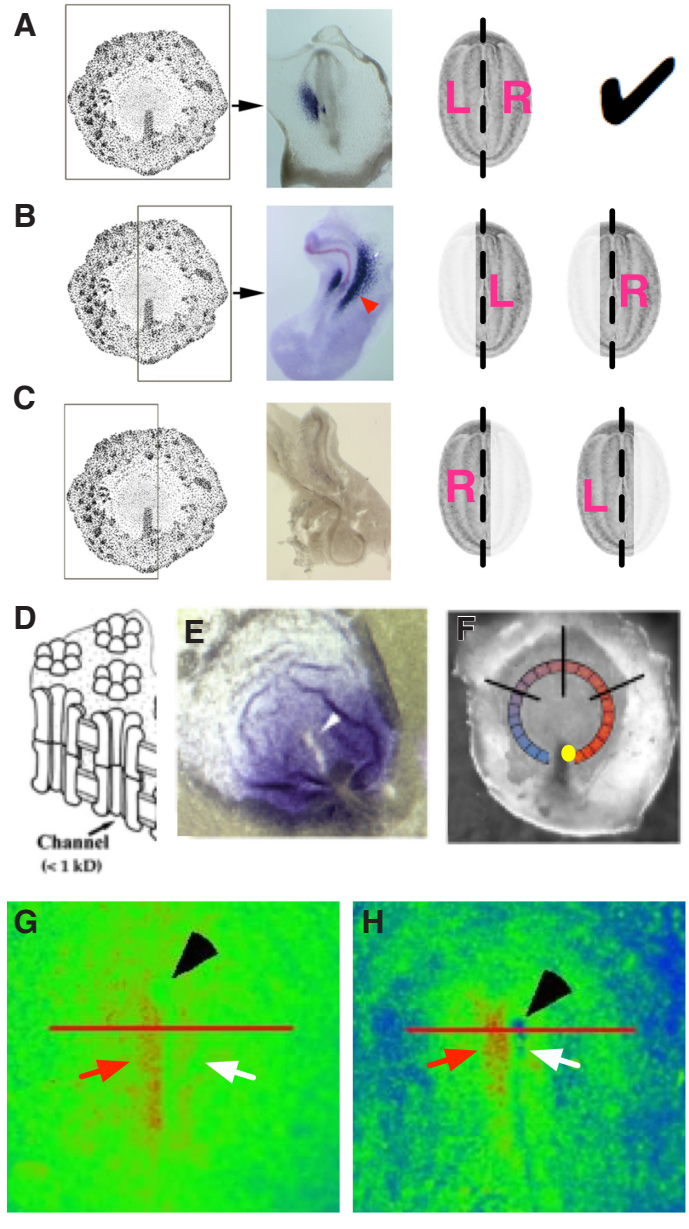


Fig. 5. Physiology: mechanisms upstream of asymmetric gene expression. *Upstream of asymmetric gene expression lies a system of physiological signals by means of which the L and R sides coordinate their identity.* **(A)** *In a normal cultured chick embryo, the two sides develop correct identity, shown here via the left-sided expression (purple stain) of Nodal.* **(B)** *When only the right side is cultured (left lateral tissues removed), its identity is randomized, often exhibiting inappropriate expression of Nodal.* **(C)** *Likewise, when the left side is cultured alone, its identity is randomized, often exhibiting a failure to turn on Nodal. Subsequent work (Levin and Mercola, 1999) revealed that a system of gap junctional channels* **(D)** *is encoded by genes like Connexin 43, expressed in a circumferential pattern around the nascent streak* **(E)** *(white arrowhead reveals zone of isolation in the streak). Interference with the circumferential path, either by knockdown of Cx43 or single slits in the blastoderm interfering with the long-range current path, randomize asymmetry, suggesting a model in which some LR morphogen is transported intracellularly across the blastoderm through the circumferential gap junctional path (Levin and Nascone, 1997). Such movement requires motive force, which could be provided by the observed voltage gradient (Levin* et al.*, 2002) created by differential resting potentials generated at the L and R sides of the st. 2* **(G)** *and st. 3-4* **(H)** *primitive streak (red stain indicates depolarized cells, revealed by voltage-sensitive fluorescent dye; black arrowhead indicates developing Hensen's node).*

are independent compartments, each running its own side-specific gene-regulatory network. But is it possible that prior to that, the L and R sides actually need to communicate to decide their identities, before turning on appropriate asymmetric genes? The flat architecture of the chick blastoderm, together with its amenability to explant culture, allowed testing of this hypothesis (Levin and Mercola, 1999). It was found that when the early-streak embryo is cultured with the right lateral portion of the blastoderm removed, the left side failed turn on Nodal in a significant number of the animals. Even more strikingly (proving that this was not simply a damaged embryo that failed to turn on genes appropriately), the right side – which normally does not express the left-side marker Nodal, would often express Nodal robustly if the left tissue was removed. Importantly, the removed tissue was quite lateral, well away from the primitive streak, ruling out interference with the midline as a possible cause. It was thus found that the L and R sides do not know their identity *a priori*, but rely on each other's presence to decide L/R identity. How?

Gap junctions – electrical synapses that mediate long-range LR coordination

How might the L and R side tissues communicate across the whole blastoderm? One way for cross-tissue communication is through paths of cells connected by gap junctions (GJs) – electrical synapses directly connecting cells' interiors via an aqueous pore that is permeable to small molecules (Mathews and Levin, 2017). Work in the frog model showed that gap junctional paths around the early cleavage embryo were essential for normal laterality; the data supported a circumferential model whereby connectivity all along the dorsal side of a 32-cell embryo, and isolation across the ventral midline, were both required for normal asymmetry (Levin and Mercola, 1998). Despite the fact that the chick embryo at the relevant stages had a much different architecture than the frog, and tens of thousands of cells instead of 32, experiments suggested a similar topology. Connexin43, a gap junction protein, was found to be expressed throughout the blastoderm at the early streak stage, *except* in the primitive streak, as predicted by the model of circumferential connectivity around a region whose L and R sides are physiologically distinct (the significance of the consistently asymmetric expression of Cx43 in the much later Hensen's node remains unknown). Antisense oligonucleotides targeting Cx43 revealed that it was required for the correct sidedness of expression of *Sonic hedgehog* (Levin and Mercola, 1999), placing the gap junction-mediated signals upstream of the early asymmetric gene cascade. Thus, the chick embryo appeared to be using precisely the same "circumferential pattern around a midline zone of isolation" geometry as was found in frog, although projected onto a much different embryonic bodyplan. In this paradigm, the LR identity originates outside of Hensen's node, and instructs it laterally; this was confirmed by a number of elegant transplantation experiments (Pagan-Westphal and Tabin, 1998, Psychoyos and Stern, 1996, Yuan and Schoenwolf, 1998).

Ion channels and neurotransmitters: propagating LR information

The embryo appears to require a circumferential path of physiologically-coupled cells upstream of the asymmetric gene cascade. Why? One hypothesis was that some small molecule signal(s) had to be shuttled from one side of the embryo to the other, enriching it in a spatial gradient as a kind of LR morphogen. Subsequent work addressed two questions: the nature of at least one left-right morphogen, and the transport mechanism.

A circumferential path of electrical connectivity around a zone of isolation is highly reminiscent of a simple circuit consisting of a conductor around a battery: a voltage gradient across the zone of isolation would exert an electrophoretic force across the gap junctionally-coupled ectoderm, forcing the unidirectional movement of specific charged molecules that could penetrate selective gap junctions. This simple model (which was motivated by prior work in developmental bioelectricity showing transport of biological molecules by endogenous electric fields (Poo, 1981, Woodruff and Telfer, 1980)) was tested simultaneously in chick and frog (Levin *et al.*, 2002). Several ion translocators were found to be specifically expressed in the zone of junctional isolation (in the chick streak); these included the V-ATPase proton pump, the H,K-ATPase proton/potassium exchanger, and two K^+ channels (Kir6.1 and Kir4.1) (Adams *et al.*, 2006, Aw *et al.*, 2008, Aw *et al.*, 2010, Gros *et al.*, 2009, Levin *et al.*, 2002). The chick model enabled the first example of *molecular* developmental bioelectricity work, as the use of a voltage-sensitive fluorescent dye revealed an endogenous bioelectric gradient, with the left side of the streak showing an H,K-ATPase-dependent depolarization of resting potentials. While the chick had been a popular model for classical bioelectric work using electrodes, implicating endogenous ionic signaling in dorso-ventral (Jaffe and Stern, 1979, Stern, 1982) and limb/intestinal (Hotary and Robinson, 1990, Hotary and Robinson, 1992) patterning, it was the context of left-right asymmetry in the chick that established the molecular approaches to developmental bioelectricity which did not rely on laborious electrophysiological measurements of individual cells but revealed at once the whole electric landscape of a patterning system. These early chick results kicked off a plethora of subsequent work in developmental bioelectricity (Levin, 2017, Ozkucur *et al.*, 2010).

Having identified a likely source of motive force for net-unidirectional movement of charged molecules, the next question became the molecular identity of such signals. The ideal candidate would be small (to fit through GJs, it has to be < 1kD), significantly charged (to respond to the electrophoretic force), and known to be able to penetrate through GJs and to signal intracellularly. A candidate approach, biased toward molecules with a well-developed pharmacology and genetics, suggested the neurotransmitter serotonin. Using the numerous known drug blockers, as well as misexpression of serotonin receptors and other machinery, in both frog and chick embryos, it was found that the serotonin transporter and serotonin receptors R3, R4, and an intracellular binding protein were crucial components of LR patterning upstream of *Sonic hedgehog* left-sided expression (Fukumoto *et al.*, 2005a, Fukumoto *et al.*, 2005b). These data are consistent with the electrophoretic model, and have been simulated computationally to provide a quantitative picture of events upstream of asymmetric gene expression (Esser *et al.*, 2006, Zhang and Levin, 2009b), although it is entirely possible that additional small molecule morphogens remain to be identified.

Serotonin movement was subsequently shown in the frog embryo to be dependent upon the V-ATPase function (Adams *et al.*, 2006), and collapsing the voltage gradient across the zone of isolation disrupted the whole process and led to LR randomization of asymmetric genes and organ situs. Follow-up work in *Xenopus* also identified the intracellular receptor (the transcription factor Mad3) and showed that it binds Histone Deacetylase 1 to repress

the Nodal transcriptional element on the right side. In retrospect, the early work in the chick led to perhaps the densest, best-understood (at the molecular-biological and quantitative levels) example of developmental bioelectricity, in which early biophysical events drive a spatialized second-messenger cascade that controls a gene regulatory network upstream of axial organ patterning of a major body axis. Moreover, the early discoveries on serotonergic signaling in pre-neural contexts in the chick have now fanned out to spur discoveries far beyond left-right asymmetry (Rea *et al.*, 2013, Vandenberg *et al.*, 2014, Vandenberg *et al.*, 2012), including the bioelectric induction of conversion to melanoma (Blackiston *et al.*, 2015, Lobikin *et al.*, 2015), craniofacial patterning (Sullivan and Levin, 2016), and control of innervation of transplants (Blackiston *et al.*, 2015). Together, a picture emerges in which decisions about large-scale properties are made via the control of neurotransmitter movement by electric activity – precisely as in the brain; this simple observation, initially made in chick, has presaged much work in the field of bioelectricity that echoed the same theme of deep conservation (Bates, 2015, Levin, 2017).

The origin of asymmetry: avian embryos and early biophysical events in LR patterning

Despite the drawback that avian embryos are generally not available for study until the blastoderm stages (hiding initial cleavage stages inside the maternal organism), avian models have nevertheless given important clues even about the earliest steps of LR patterning: midline determination and symmetry breaking.

Symmetry can be broken at a local level by clock-wise vs. counter-clockwise or L-hand vs. R-hand microscopic structures. However, in order to convert this information into a sense of position along the LR axis (important for cells to know whether to drive, for example, left-specific depolarization and gene expression), the embryo must establish a midline. When does this first take place? Conventional belief for amniotes is that the primary axis arises at the events that determine the location of the primitive streak at one point on the radial circumference of the early blastoderm (Khaner and Eyal-Giladi, 1989). However, a set of papers in avian models such as chick and finch suggest that we may be missing an important piece of LR-relevant biology. The study of gynandromorphs (animals that are half male and half female, due to chromosomal aberrations) reveals remarkably precise division of these characteristics along the midplane of the animal (Fig. 6). This is true not only in birds (Agate *et al.*, 2003, Clinton *et al.*, 2011, Lillie, 1931, Peer and Motz, 2014, Zhao *et al.*, 2010), but many other species across a very broad range of taxa (reviewed in Aw and Levin, 2008; Ma, 2013). Even humans, in cases of hermaphroditism (Mittwoch, 2000, 2001, 2008) and other syndromes (Happle *et al.*, 1995; Konig *et al.*, 2000), reveal a precise midline demarcation that is not consistent with an origin in cells that are random with respect to the midline. Precise left/right differences in such traits suggest that the separation into L and R halves occurred extremely early, allowing events like chromosomal nondisjunction post-fertilization to affect all of the descendants of one blastomere that gave rise to an entire body half. It is thus possible that, while the primitive streak can be artificially initiated anywhere (Bachvarova *et al.*, 1998, Shah *et al.*, 1997), the endogenous events of development in amniotes establish the L and R sides as early as do species like frog, where the first cell division usually sets the embryonic midline and thus defines the LR axis (Klein, 1987, Marrari *et al.*, 2004, Masho, 1990).

There is broad consensus, consistent with Brown and Wolpert's definitive and prescient analysis (Brown and Wolpert, 1990), that LR asymmetry is broken and oriented via biophysical events involving a chiral structure. There is however considerable controversy regarding the nature of that structure and the conservation of this set of mechanisms across body-plans. One model suggests extracellular cilia, operating during neurulation to set up an extracellular vortical flow (Basu and Brueckner, 2008, McGrath *et al.*, 2003), as the origin of asymmetry. Another model, driven initially by studies in *Xenopus*, first by Yost (Yost, 1991) and then others (Levin *et al.*, 2002, Lobikin *et al.*, 2012, McDowell *et al.*, 2016b, Qiu *et al.*, 2005), suggests the origin of asymmetry within chiral structures of the intracellular cytoskeleton – an interaction of microtubule organizing centers and cortical actin. The relative merits of the two models are discussed in detail in (Vandenberg *et al.*, 2013,

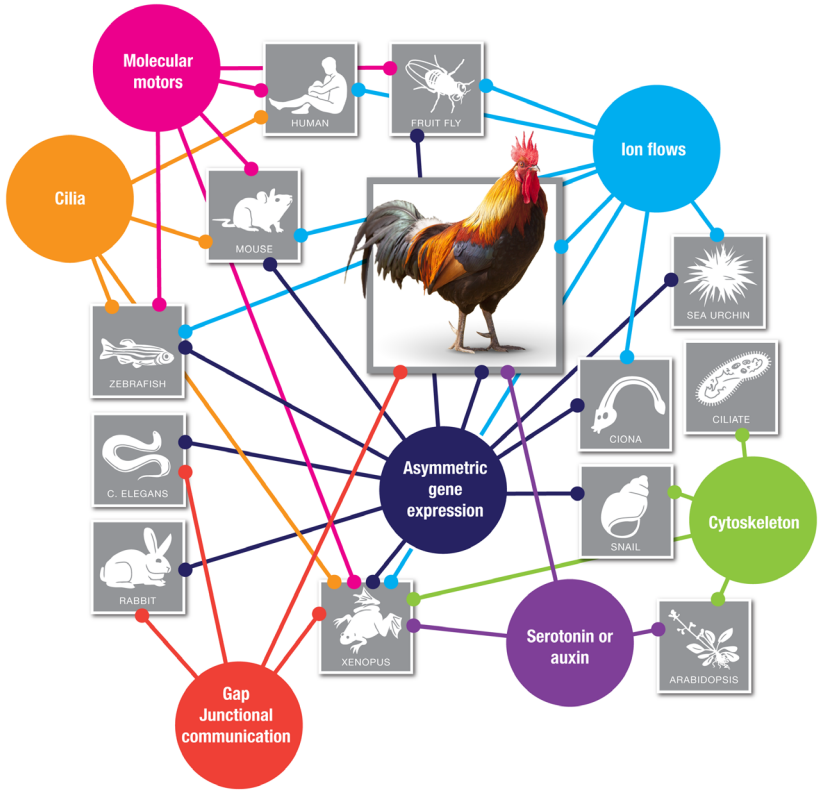


Fig. 6. Conservation of early left-right (LR) mechanisms across phyla. *This schematic shows the major classes of mechanisms involved in left-right patterning, and the model species in which they have been shown to operate. Details and references to each are given in (Levin and Palmer, 2007, Vandenberg and Levin, 2009). The chick model has been instrumental in the discovery of several of these, most notably the asymmetric gene cascade and upstream physiological signals like ion flows and gap junctional communication. Despite the significant differences in bodyplan architecture across phyla, many LR mechanisms are broadly conserved and utilized to pattern the left-right axis of the body.*

Vandenberg and Levin, 2009, Vandenberg and Levin, 2010b, Vandenberg and Levin, 2013). Because they utilize exactly the same molecular players (since almost all the same proteins function in ciliary motion as in intracellular transport), genetic experiments targeting these proteins, especially in mice where early stages are hard to analyze, usually do not distinguish between them. However, these two models make numerous different predictions (Vandenberg and Levin, 2013), and imply opposite conclusions about the evolutionary aspects of asymmetry. Because numerous phyla do not have cilia at the relevant stages but successfully establish asymmetry (*Drosophila*, *C. elegans, Arabidopsis*, etc.), the ciliary model is forced to suggest a highly divergent origin for asymmetry. Indeed, early studies suggested that this cannot even be conserved in amniotes, as the chick node does not have the required ciliated structure and hydrodynamic flow (Manner, 2001). More recent work showed that neither the chick (Bangs *et al.*, 2011, Manner, 2001) nor the pig (Gros *et al.*, 2009) depend on cilia for their asymmetry. In contrast, other mechanisms operating in chick appear conserved to much more disparate model systems (Fig. 7), and new data are revealing conservation of ion translocators in mouse (Miyachi, 2017) and human patients (Fakhro *et al.*, 2011), beyond the well-known examples in *C. elegans*, sea urchins, zebrafish, etc. (Vandenberg *et al.*, 2013, Vandenberg and Levin, 2013).

From data in a range of model species, it is clear that numerous aspects of development, including maternal protein localization (Adams *et al.*, 2006, Aw *et al.*, 2008, Lobikin *et al.*, 2012, Qiu *et al.*, 2005), transcription (Vandenberg *et al.*, 2014), Wnt signaling (Ohkawara and Niehrs, 2011), and localization of small signaling molecules (Onjiko *et al.*, 2016) are already consistently asymmetric long before cilia appear even in animals that have them; most embryos can tell their Left from their Right at very early stages. Thus, the search for the origins of asymmetry has been extended

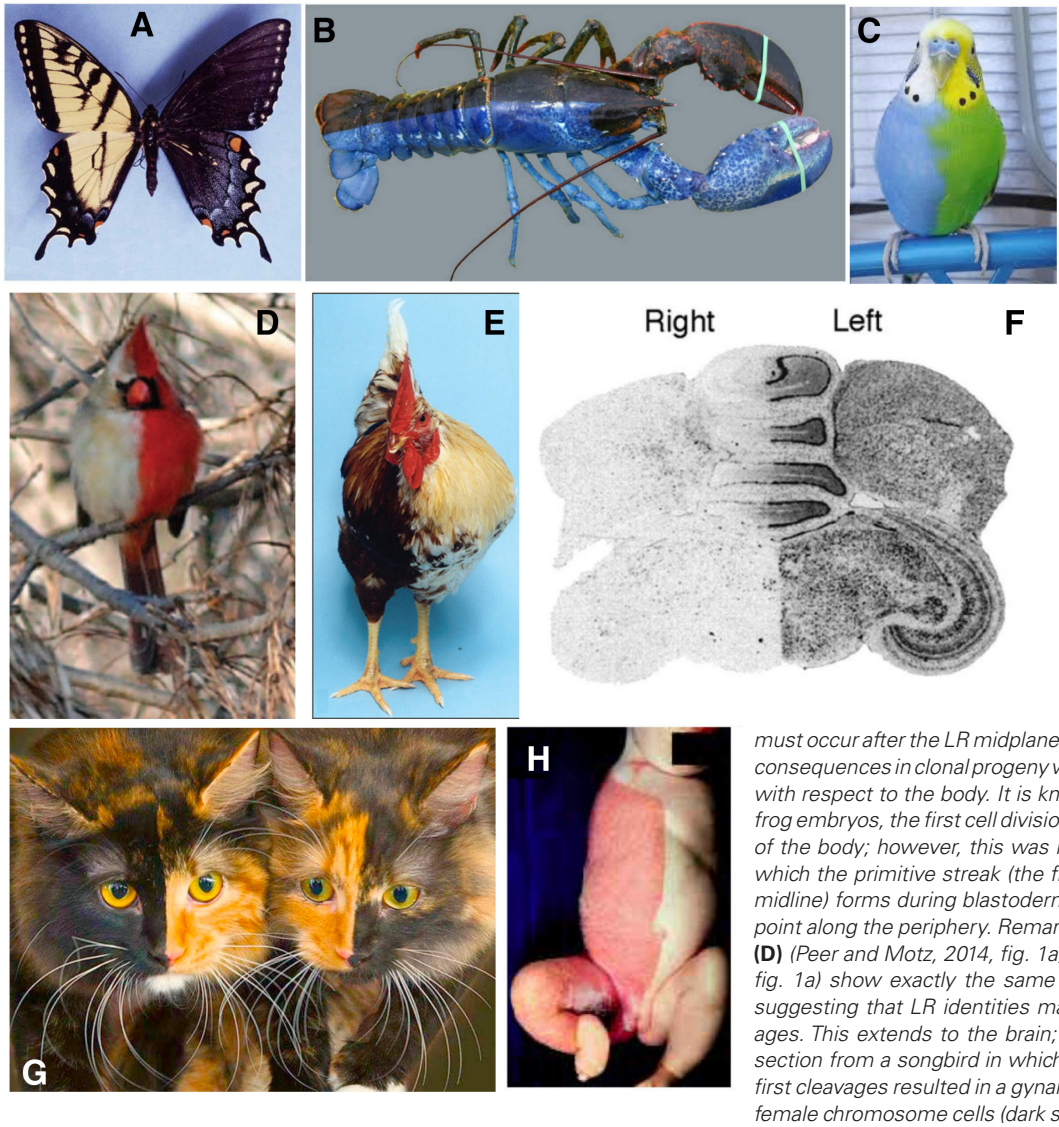


Fig. 7. Origin of asymmetry: conceptual issues for future progress. *One of the major conceptual issues is the establishment of the midline, and the early events that allow the LR axis to be consistently oriented with respect to the other 2 axes so that cells can determine their position with respect to the midline. While chick embryos do not facilitate studies of the earliest events after fertilization, avian data has provided important data suggesting that the embryonic midline might be established as early as during early cleavages (see (Aw and Levin, 2008) for more discussion). Numerous species, such as butterflies* **(A)** *and crustacea* **(B)** *exhibit gynandromorphy (male and female tissues in the same body) split precisely down the middle. Genetic events that lead to such states (such as loss of a chromosome during cell division) must occur after the LR midplane has been established, otherwise their consequences in clonal progeny would be spread in random orientations with respect to the body. It is known that in many organisms, such as frog embryos, the first cell division usually establishes the L and R sides of the body; however, this was not thought to be true of amniotes in which the primitive streak (the first anatomical sign of an established midline) forms during blastoderm stages and could be initiated at any point along the periphery. Remarkably, bird gynandromorphs, cardinals* **(D)** *(Peer and Motz, 2014, fig. 1a) and roosters* **(E)** *(Clinton* et al.*, 2011, fig. 1a) show exactly the same precisely-bilateral difference in cells, suggesting that LR identities may be set as early the first few cleavages. This extends to the brain;* **(F)** *an* in situ *hybridization of a brain section from a songbird in which chromosomal aberrations during the first cleavages resulted in a gynandromorphy. The division between the female chromosome cells (dark signal) and the male chromosome cells (no signal) is precisely down the anatomical midline of the brain, suggesting that the embryonic midline is determined long prior to streak development in bird in development. Taken from Fig. 6 of (Agate* et al.*, 2003), copyright held by National Academy of Sciences. Even mammals, such as cats* **(G)** *and human embryos exhibiting CHILD syndrome* **(H)** *(Happle* et al.*, 1995, Konig* et al.*, 2000) show the same midline sharp demarcation, indicating that there is still much to be learned about the earliest events of development and their contributions to subsequent LR patterning.*

far upstream of neurulation (Oviedo and Levin, 2007, Trulioff *et al.*, 2015); the chick is thus confirming a broad molecular conservation and not an outlier with respect to its non-reliance upon cilia. Thus, in this aspect, the chick model appears to be a much better conduit to understanding of amniote (and fundamental aspects of) asymmetry than mouse (Vandenberg, 2012), which has a very unique (cylinder-like) embryonic architecture. The embryonic chick offers a much more prototypical flat architecture and a conservation of early physiological events with a number of other species that suggest broad and deep principles by which evolution adapted the same components to impose asymmetry on radically different bodyplans.

Conclusion

Overview

The left-right (L/R) axis is defined as perpendicular to the two other main embryonic axes, anterior-posterior (A/P) and dorsal-ventral (D/V) axes. However, an intriguing feature of L/R asymmetry, is that there is no obvious reason why almost all normal individuals share the same left-to-right organization (*situs solitus)* rather than a population with equal proportion of *situs solitus* and *inversus*, since both are physiologically functional. The formation of A/P and D/V axes define an embryo with apparent bilateral symmetry. The lack of physical/visible difference between the left side and the right side of an embryo is a striking difference with A/P or D/V axes, which are set respective to well identified parameters such as the asymmetry of the oocyte or external cues (e.g. sperm entry point). This observation suggests the existence of an active and well-regulated developmental mechanism for breaking the apparent bilateral symmetry, rather than a pre-existing bias towards one side. Such a mechanism would initiate then amplify a subcellular-scale asymmetrical parameter, for example the chiral properties of actin (Danilchik *et al.*, 2006), tubulin (Lobikin *et al.*, 2012), or even DNA itself (Klar, 2008, Sauer and Klar, 2012).

What we learned from the chick

The chick embryo was instrumental in the discovery of the first molecular pathways establishing LR patterning: the asymmetric gene cascade during gastrulation. It has also facilitated the discovery of numerous other components, some still not well-understood, including tight junctions (Aw *et al.*, 2010, Collins *et al.*, 2015, Simard *et al.*, 2005, Simard *et al.*, 2006), asymmetric cell migration (Gros *et al.*, 2009), programmed cell death (Kelly *et al.*, 2002), and a set of bioelectric and neurotransmitter pathways upstream of the first known asymmetric genes (Fukumoto *et al.*, 2005a, Fukumoto *et al.*, 2005b, Levin *et al.*, 2002). Its flat architecture makes it uniquely amenable to the study of physiological, genetic, and biomechanical aspects of asymmetry, from single cell movement to asymmetric organogenesis. Its limitation is the internal development at cleavage stages, but advances in culture methods and increasingly-available transgenic birds (Mozdziak and Petitte, 2004, Nishijima and Iijima, 2013) continue to improve the usability of this classical model.

Future prospects: a central place for avian models in the next frontiers of this field

A number of aspects can be envisioned for the vibrant future of the chick model system in the field of LR asymmetry research. Issues of midline determination (events at cleavage stages, or mechanisms by which the primitive streak can accurately bisect itself) are paramount. The LR patterning of embryos derived from repositioned and ectopic primitive streaks needs to be investigated, and a chick model of early twin:twin LR instruction, as has been studied in the frog (Vandenberg and Levin, 2010a, Vandenberg and Levin, 2012), would be highly informative for understanding the relationship between the mechanisms establishing the AP, DV, and LR axes. More topologies for twin orientation are possible in chick than frog (and especially easy in duck embryos, (Lutz, 1949)), suggesting numerous interesting experiments that exploit the blastoderm as an arena for exploring physiological and transcriptional signaling under different spatial orientations of circuit components including the zone of isolation, gap junctional field, and Hensen's node. A requirement for planar polarity has been identified in the chick (Zhang and Levin, 2009a), which is likely revealing a fundamental linkage between LR asymmetry and general mechanisms that propagate subcellular molecular information into order on the large scale in multiple tissues (Aw and Levin, 2009).

The asymmetric gene cascade most well-characterized in the chick has been identified in numerous other models – it is highly conserved, and shown by functional experiments to be instructive for LR positioning of the organs. Interestingly however, recent data in the frog model (McDowell *et al.*, 2016a, McDowell *et al.*, 2016b) revealed that in a given cohort of animals, induced errors in upstream asymmetric genes' expression patterns are progressively reduced over developmental time, not maintained or amplified as would be expected from a linear model where each sided gene fully determines the sidedness of downstream target genes. This instead suggests the existence of parallel, reparative mechanisms that can partially compensate and reduce errors even when key elements of the main LR pathway re perturbed. Testing this property in the chick embryo, which offers by far the richest set of interacting components is a clear next step. If confirmed within the well-studied LR-GRN of the chick, this would significantly strengthen the role of LR patterning as not merely a self-contained aspect of development, but a prototypical example of living systems' robust regenerative capabilities. Likewise, important but poorly-understood links between organ asymmetry and clinically-relevant phenomena such as cancer, immune system function, and CNS lateralization await deep investigation in the highly tractable avian embryonic models. Thus, as clearly revealed by its history, the chick model offers the creative scientist boundless opportunities for investigation into some of the most fascinating areas of interdisciplinary, multiscale biology of growth and form.

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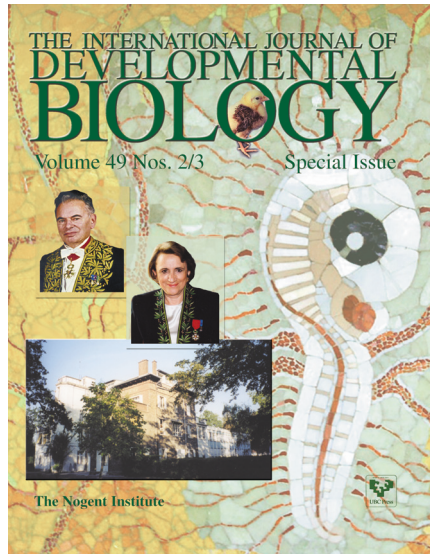

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