Serotonin has early, cilia-independent roles in *Xenopus* left-right patterning

Laura N. Vandenberg1, Joan M. Lemire1 and Michael Levin1,*

**SUMMARY**

Consistent left-right (LR) patterning of the heart and viscera is a crucial part of normal embryogenesis. Because errors of laterality form a common class of birth defects, it is important to understand the molecular mechanisms and stage at which LR asymmetry is initiated. Frog embryos are a system uniquely suited to analysis of the mechanisms involved in orientation of the LR axis because of the many genetic and pharmacological tools available for use and the fate-map and accessibility of early blastomeres. Two major models exist for the origin of LR asymmetry and both implicate pre-nervous serotonergic signaling. In the first, the charged serotonin molecule is instructive for LR patterning; it is redistributed asymmetrically along the LR axis and signals intracellularly on the right side at cleavage stages. A second model suggests that serotonin is a permissive factor required to specify the dorsal region of the embryo containing chiral cilia that generate asymmetric fluid flow during neurulation, a much later process. We performed theory-neutral experiments designed to distinguish between these models. The results uniformly support a role for serotonin in the cleavage-stage embryo, long before the appearance of cilia, in ventral right blastomeres that do not contribute to the ciliated organ.

**INTRODUCTION**

Understanding how consistent left-right (LR) asymmetry of the body-plan is established is important for developmental biology and medicine. Individuals with LR patterning defects, including heterotaxia (the loss of concordance among the visceral organs), and isomerisms (loss of asymmetry), suffer grave medical consequences (Hackett, 2002; Peeters and Devriendt, 2006; Zhu et al., 2006). The frog *Xenopus laevis* has proven to be an excellent model for the study of LR asymmetry because of the vast number of developmental, molecular-genetic, physiological and pharmacological tools that are available for use in this organism. Specific benefits of this model system also include the large number of eggs available for study at the earliest of stages (which allowed the discovery of many very early asymmetry-causing mechanisms that have so far been too difficult to study in mice), and a well-defined fate-map that allows targeting of left- and right-side cells at will (which is not possible in zebrafish for example). Thus, the frog embryo is a uniquely powerful model in which to study the earliest events that link biophysical chirality to asymmetric gene expression and subsequent organ sites.

There are currently two competing paradigms of how the LR axis is established. One model proposes that symmetry is first broken during neurulation, when cilia localized to a node-like structure generate a chiral fluid flow that asymmetrically distributes a morphogen, or causes asymmetric bending of sensory cilia (reviewed by Basu and Brueckner, 2008; Hashimoto and Hamada, 2010). This model implies considerable evolutionary divergence among phyla. Because many different model systems (including amniotes such as the chick and pig) orient their asymmetry without the benefit of cilia (Levin and Palmer, 2007; Spéder et al., 2007; Gros et al., 2009), it is unclear which species is the best model for human disease-relevant symmetry breaking. The other model proposes that a highly conserved chiral intracellular cytoskeleton component drives asymmetric localization of ion transporters, thus establishing a biased voltage gradient that results in asymmetric localization of a charged molecule that is instructive for LR identity (reviewed by Aw and Levin, 2009; Vandenberg and Levin, 2010). This model is supported by molecular-genetic gain- and loss-of-function data that indicate that asymmetry is established during the first cell cleavages (Levin et al., 2002; Aw et al., 2008) and identify the neurotransmitter serotonin as the small molecule that is redistributed at cleavage stages to provide LR identity to blastomeres (Fukumoto et al., 2005a; Fukumoto et al., 2005b; Adams et al., 2006; Carneiro et al., 2011).

A recent study (Beyer et al., 2012) suggests a permissive role for serotonin in the specification of the gastrocoel roof plate (GRP; the *Xenopus* node) and cilia-dependent fluid flow. Thus, the two main LR asymmetry models now converge on a common molecule, the neurotransmitter serotonin. The question of timing, i.e. when serotonin actually acts during LR patterning, is crucial not only because it impacts the plausibility of the late origin versus early origin models of asymmetry but also because it identifies the embryonic stages that would be most sensitive to the serotonergic compounds in widespread medical use today (Shuey et al., 1992; Alwan et al., 2007; Noorlander et al., 2008; Sadler, 2011).

Here, we report the results of experiments that allowed us to resolve the role of both the timing and the location of serotonin in LR patterning of the frog embryo. These studies were designed to experimentally distinguish between two hypotheses: one that suggests a role for serotonin in right-sided ventral blastomeres...
Clinical issue
Normal human embryos exhibit asymmetric (sided) anatomy of the heart, visceral organs and brain. Disturbances in this process result in isomerism (lack of asymmetry), situs inversus (reversal of asymmetry) or heterotaxia (randomization of asymmetry among the organs). Because such errors lead to birth defects, understanding the molecular mechanisms that establish consistent and correct left-right (LR) asymmetry during embryogenesis is crucial. Work in chick and frog embryos has revealed a novel role for serotonin in LR patterning: dissecting the molecular mechanism and timing of this neurotransmitter’s involvement is important for assessing the impact of serotonergic pathway modulator drugs on fetal development.

Two main models for serotonin function during LR patterning exist. The EARLY model views serotonin as an instructive signal, because it is asymmetrically localized during early cleavage stages and initiates a cascade of asymmetric gene expression on the right side via interaction with epigenetic control machinery. The driving force for serotonin’s rightward movement is a LR voltage gradient established by very early intracellular (cytoskeletal) chirality present immediately after fertilization. By contrast, the LATE model proposes that serotonin is a permissive factor needed for the induction of ciliated tissue during neurulation stages (and holds that asymmetry is first generated by the vertical action of these cilia fairly late in development). A role for cilia in a symmetry-breaking (initial) mechanism requires that no prior asymmetrical signaling exists.

Results
The authors used theory-neutral experiments, designed to distinguish between two competing hypotheses, to determine both the timing (early versus late) and the location of serotonin’s role in LR patterning. A gain-of-function experiment, injecting ectopic serotonin into targeted blastomeres, revealed that excess serotonin on the left side randomizes asymmetric gene expression. A loss-of-function experiment, injecting a molecular construct that disrupts serotonin signaling, revealed that serotonin is required in the ventral right cells. A final experiment demonstrated that genes with asymmetric expression become expressed on the left side of the embryo even when ciliary flow is not present. Importantly, a meta-analysis of ciliary parameters in the literature revealed that the variability in cilia length and number, even in unperturbed animals, is such that many ciliary function mutants fail indistinguishably in the range of ‘normal’ ciliary measurements, revealing that quantification of cilia properties does not reliably distinguish between animals that have correct and incorrect LR patterning.

Implications and future directions
These results uniformly support the EARLY model. Moreover, the meta-analysis refutes the often-cited link between parameters of cilia function and LR asymmetry. Together with the recent discovery that, even in mouse, the early blastomeres are not functionally LR-equivalent, these data support the view that very early mechanisms of LR symmetry breaking should be considered, and that cilia-dependent signaling is unlikely to be a well-conserved initiating step. Important future directions include the investigation of early (pre-node) mechanisms of serotonergic signaling in the mouse, and a dissection of the epigenetic steps that lead from intracellular serotonin redistribution to the differential gene expression that drives asymmetric organ morphogenesis. A better understanding of instructive patterning roles for this important neurotransmitter will significantly impact our understanding of neuropharmacology and the etiology of a wide range of birth defects.

during cleavage stages (i.e. the EARLY model) (Levin and Palmer, 2007), and one that requires a role for serotonin late, in the left side (Vick et al., 2009) of the dorsally derived GRP cells (i.e. the LATE model). The results of these experiments support a role for serotonin during early cleavage stages and exclude a role for serotonin signaling in the GRP.

RESULTS
Ectopic serotonin randomizes the LR axis
The LATE model proposes that serotonin is localized throughout the embryo and acts as a competence factor for canonical Wnt signaling to specify the GRP (Beyer et al., 2012). By contrast, the EARLY model suggests that serotonin is an instructive signal asymmetrically localized to the right side of the early cleavage-stage embryo (Fukumoto et al., 2005b; Vandenberg and Levin, 2010). To determine the spatial requirement for functional serotonin signaling in LR patterning, we injected one blastomere of four-cell embryos with 15 or 30 ng of ectopic serotonin (plus lineage tracer to confirm targeting). We then scored animals for organ position (Fig. 1A), not pitx2 expression as in other studies (Beyer et al., 2012), because incidence of incorrect asymmetric gene expression strongly overestimates the effects of treatments on randomizing organ situs, making cilia-targeting treatments appear more penetrant than they are (supplementary material Fig. S1) (Vandenberg, 2012). Whereas the LATE model predicts that there should be no effect from ectopic serotonin on either side, the EARLY model predicts that excess serotonin signaling, especially on the left side, will randomize the LR axis by ectopic activation of intracellular receptors (Carneiro et al., 2011). Indeed, overabundance of serotonin introduced to any blastomere significantly affected organ situs, with the greatest effects observed when injected on the left side (Fig. 1B). These results are compatible with the need for differential serotonin signaling on the left and right sides, a result predicted by the EARLY model, and with data showing that endogenous serotonin is normally moved away from the left and towards the right side within the first few cell cleavages (Fukumoto et al., 2005b; Vandenberg and Levin, 2012). The results are not consistent with the LATE model’s view of serotonin as a competence factor whose presence above a baseline threshold simply allows ciliated cells to form.

Loss-of-function reagents targeting serotonin signaling implicate the ventral right blastomere in LR patterning
The LATE model suggests (Beyer et al., 2012) that loss-of-function serotonin reagents randomize asymmetry via direct effects on the flow-relevant cells of the GRP (i.e. only those on the dorsal, left side) (Vick et al., 2009). By contrast, the EARLY model predicts that injections of such reagents into the ventral right side precursors should be most effective because endogenous serotonin signaling occurs in these blastomeres. We injected embryos with miRNA encoding a dominant-negative Xenopus serotonin receptor 3A (Gunthorpe and Lummis, 2001) at the four-cell stage, specifically targeting blastomeres that contribute to flow at the GRP (dorsal left) or blastomeres that do not (ventral right). We observed that injections into the ventral right blastomere were significantly more effective in randomizing organ situs (Fig. 1C). Such asymmetry defects from loss-of-function serotonin reagents in cells that do not contribute to the GRP are incompatible with the cilia role hypothesized by the LATE model.

Lateral plate mesoderm explants indicate that Xnr-1 is induced independently of ciliary flow
One of the key questions in this field is how biophysical events regulate asymmetric transcription of key laterality genes such as the one coding for Xnr-1 protein. We isolated explants from the
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left lateral plate mesoderm of embryos at stage 13 (before ciliary flow), stage 18 (after ciliary flow) and stage 22; at all stages, these explants exclude the GRP and its associated cilia. We aged these explants to stage 23 and then examined the expression of Xnr-1 mRNA, which is normally asymmetrically expressed on the left side of the embryo. The LATE model predicts that explants isolated before ciliary flow begins will not express Xnr-1 because cilia-dependent signaling is required to turn on Xnr-1 expression in left-side cells. By contrast, recent data suggested that Xnr-1 is instead actively suppressed by the early presence of serotonin on the right (Carneiro et al., 2011); therefore the EARLY model predicts that left-side explants will express Xnr-1 at all stages regardless of when they were isolated (whether or not they participated in ciliary-dependent events). We observed strong Xnr-1 mRNA expression in explants isolated at all three stages (Fig. 1D,E). The patterns of expression were similar in shape and relative intensity to Xnr-1 mRNA expression patterns seen in whole embryos (Fig. 1D and data not shown). The subsequent expression of Xnr-1 in explants isolated at stage 13, prior to the onset of ciliary motion, directly refutes a requirement for ciliary flow in the induction of expression of Xnr-1.

To ensure that these results were not specific to Xnr-1 mRNA and applied to asymmetric gene expression in general, we repeated these experiments for two additional laterality genes, lefty and pitx2.
We found significant differences in reported values for cilia number, cilia length and flow rates in wild-type (untreated) embryos, even though we controlled for embryonic stage (Fig. 2). Most striking was the fact that some mutants exhibited ciliary parameter values solidly within the range of what was reported as ‘normal’ in other studies, meaning that there is no reliable way to associate any observed set of ciliary properties with a normal or abnormal laterality outcome. From this meta-analysis, we conclude that there is no support for the claim that ciliary parameters could functionally distinguish between correct and incorrect LR patterning.  
A similar analysis was attempted for the mouse literature. However, this analysis was hindered by the small number of studies that report means, s.e.m. or s.d., and sample sizes for wild-type populations. There are a few examples where large differences in reported measures suggest that ciliary parameters are not consistent in the mouse literature, similar to our findings for the zebrafish literature (Fig. 2). For example, although one study reports the number of node cilia in the range of 60-80 (Shinohara et al., 2012), another reports the number in the range of 130-210 (a large range in itself) (McGrath et al., 2003). In addition, two studies report nodal flow rates in wild-type embryos in the range of 2-5 μm/second (Tanaka et al., 2005; Shinohara et al., 2012), although others report flow in the range of 12-17 μm/second (Nonaka et al., 2002; Buceta et al., 2005); a final study reports flow in the range of

Quantitative meta-analysis reveals that ciliary parameters do not distinguish between normal and randomized embryos  
In order to quantitatively assess whether cilia are functionally responsible for correct LR patterning, we performed a meta-analysis of the cilia literature and collected reported values for cilia number, cilia length, and ciliary flow rate in normal and LR-compromised conditions. Only the zebrafish literature contained enough studies to perform an analysis. We found significant differences in reported values for cilia number, cilia length and flow rates in wild-type (untreated) embryos, even though we controlled for embryonic stage (Fig. 2). Most striking was the fact that some mutants exhibited ciliary parameter values solidly within the range of what was reported as ‘normal’ in other studies, meaning that there is no reliable way to associate any observed set of ciliary properties with a normal or abnormal laterality outcome. From this meta-analysis, we conclude that there is no support for the claim that ciliary parameters could functionally distinguish between correct and incorrect LR patterning.

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20-50 μm/second (Okada et al., 1999). We conclude that given the large divergence and overlap between measurements from normal controls and LR-impaired animals in the published literature, it is impossible to conclude what measured values for these ciliary functions correspond to normal versus abnormal states or consequences for asymmetry.

### DISCUSSION

We utilized *Xenopus laevis*, a popular model organism for the study of LR patterning, to distinguish between early and late roles of pre-nervous serotonin. These experiments were theory-neutral, allowing outcomes consistent with either or both major models, and distinguishing between two plausible roles for serotonin in LR patterning: an early instructive function whereby serotonin is asymmetrically localized to the right side of the embryo and actively suppresses *Xnr-1* mRNA (Fukumoto et al., 2003; Fukumoto et al., 2005b; Carneiro et al., 2011), or a late role whereby serotonin is a permissive factor required for the specification of the GRP which in turn is necessary for cilia-dependent fluid flow (Beyer et al., 2012) (Table 1). The results of all three experiments indicate that serotonin has an endogenous role on the right side, in ventral cells that do not contribute to the GRP, and that the asymmetric expression of *Xnr-1* mRNA is induced even when ciliary flow is absent. These data are consistent with previously published HPLC analyses, which indicate high levels of serotonin during early cleavage stages and low levels during gastrula and neurula stages (Fukumoto et al., 2005a). They are also consistent with molecular-genetic functional experiments implicating serotonin signaling during early cleavage stages on the ventral right side of the embryo (Fukumoto et al., 2003; Fukumoto et al., 2005a; Fukumoto et al., 2005b; Carneiro et al., 2011). Ventral right-side serotonin-dependent events are incompatible with the LATE model because only left-dorsal cells are relevant for ciliary flow (Vick et al., 2009). Taken together, these data uniformly support a role for serotonin in LR patterning in a GRP-independent manner.

Recent experiments directed at a late role for serotonin in LR patterning (Beyer et al., 2012) did not test early roles of maternal serotonin or serotonin receptor proteins because they relied on morpholinos. Maternal serotonin and serotonin receptor proteins known to be present in the cleavage-stage embryo (Fukumoto et al., 2005b) would not be targeted by these reagents. However, the report implicating serotonin in GRP specification suggested that inhibition of serotonin signaling causes serious patterning defects in treated embryos, including altered specification of the superficial mesoderm (Beyer et al., 2012), a phenotype we never observed with our reagents but one that could potentially contribute to non-specific laterality defects due to toxicity. We found that properly-titrated gain- and loss-of-function reagents cleanly affected LR patterning without producing such teratogenic side-effects. Indeed, this allowed us to score organ situs in perfectly formed tadpoles, instead of the indirect assay of asymmetric gene expression that is often used as a surrogate readout when significant other abnormalities manifest later in development and preclude assessment of actual anatomical asymmetry (Walentek et al., 2012).

Our results on early (pre-ciliary) roles of serotonergic signaling are consistent with many previous observations and functional data showing that embryos from a wide variety of phyla exhibit molecular and physiological asymmetries long before the appearance of (or without the presence of) cilia, and a number of mutant analyses have now dissected LR-relevant roles of ‘ciliary’ proteins from the functional roles of cilia themselves (reviewed by Vandenberg and Levin, 2010). Although cilia might have a role somewhere downstream in the LR pathway (Schweickert et al., 2007), they cannot be the initiator of LR asymmetry in *Xenopus* (Lobikin et al., 2012).

Although ciliary proteins have earlier intracellular roles (Qiu et al., 2005; Armakolas and Klar, 2007) that are inevitably abrogated in genetic experiments (and often interpreted as evidence of ciliary roles), many studies interpreted as supporting the LATE model use ciliary structure or function as the only analysis endpoint. However, our meta-analysis of the published zebrafish literature shows that ciliary parameters are highly variable, even among unperturbed embryos. Crucially, the values for the length and quantity of cilia in many ciliary mutants are within the range of normal variability found in controls (Fig. 2). Thus, it is not possible to make a quantitative link between cilia function and asymmetry outcome in the zebrafish system. Similar analyses of the mouse data are ongoing.

The relative plausibility of early rather than late origins of asymmetry is further impacted by questions about the timing of action of mechanisms such as serotonin signaling. Our data suggest that the primary role for serotonergic signaling in asymmetry is most likely to take place at very early stages. Furthermore, the early timing of LR symmetry breaking and orientation is indicated by recent findings that, even in mouse, the early blastomeres are structurally and functionally non-equivalent with respect to LR identity (Gardner, 2010; Jefferson and Williams, 2012) and that consistent asymmetries are present prior to the onset of ciliary flow in both *Xenopus* and mouse embryos (Kramer et al., 2002; Kramer and Yost, 2002; Bunney et al., 2003; Qiu et al., 2005; Ohkawara and Niehrs, 2011; Roberts et al., 2011; Sun et al., 2011; Lobikin et al., 2012). Some asymmetries, such as the right-sided bias of PKCγ-dependent phosphorylation of the proteoglycan syndecan-2, occur temporally between the earliest steps in the LR asymmetry pathway (ion flux and serotonin) and asymmetric gene expression (Kramer et al., 2002), and therefore might act as downstream mediators in the serotonin pathway.

It is now necessary to examine the role of serotonin in the early stages of the LR patterning process in numerous model systems.

### Table 1. Theory-neutral experiments: predictions and results

<table>
<thead>
<tr>
<th>Experiment</th>
<th>EARLY model prediction</th>
<th>LATE model prediction</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ectopic serotonin injected at four-cell stage</td>
<td>Greatest effects on left side</td>
<td>No effect, regardless of where injected</td>
<td>Consistent with EARLY model</td>
</tr>
<tr>
<td>Injected dominant-negative serotonin receptor</td>
<td>Greatest effects on ventral right</td>
<td>Effects only on dorsal left</td>
<td>Consistent with EARLY model</td>
</tr>
<tr>
<td>Left LPM explants (timing)</td>
<td>Explants express <em>Xnr-1</em>, <em>lefty</em> and <em>pitx2</em> regardless of when isolated</td>
<td>Explants express <em>Xnr-1</em>, <em>lefty</em> and <em>pitx2</em> only after onset of ciliary flow (stage 18)</td>
<td>Consistent with EARLY model</td>
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Although it is still possible that this neurotransmitter plays some additional permissive role in the later development of ciliary flow or GRP specification, serotonin first functions in LR patterning at early stages in spatiotemporal patterns not consistent with ciliary roles. Thus, serotonin signaling is one of the many mechanisms now known to underlie the events proximal to symmetry breaking shortly after fertilization.

METHODS

Xenopus husbandry

Xenopus laevis embryos were fertilized in vitro in 0.1× modified Marc’s Ringers (MMR) pH 7.8 according to standard protocols (Sive et al., 2000), housed at 14-18°C and staged according to published methods (Nieuwkoop and Faber, 1967). All experiments were approved by the Animal Care and Use Committee at Tufts University and were conducted according to the Guidelines for the Care and Use of Laboratory Animals.

Construction of dominant-negative serotonin receptor 3A construct

A Xenopus laevis serotonin receptor 3A cDNA was purchased from Open Biosystems (clone 4724653) and mutated as described (Gunthorpe and Lumbins, 2001), using a site-directed mutagenesis kit (Agilent). The insertion of proline and the point mutation of two other amino acids produced the sequence: 265-GPARLGFKVTTLGFLSTLLLILND.

Microinjections

For injections of molecular constructs, capped synthetic mRNAs encoding a dominant-negative serotonin receptor 3A and mRNAs encoding β-galactosidase, a lineage tracer, were dissolved in water. For injections of serotonin, stock solutions were prepared in distilled water and used immediately because serotonin has a very limited shelf-life in solution. For all injections, droplet size was calibrated to 5-6 nl of liquid using standard methods (50-150 millisecond pulses with borosilicate glass needles calibrated for a bubble pressure of 50-70 kPa in water). Embryos were placed in 3% ficol and reagents were injected into a single blastomere of four-cell embryos.

Scoring of LR phenotypes

At stage 45, Xenopus embryos were analyzed for situs of the heart, stomach and gall bladder according to published methods (Levin and Mercola, 1998). Heterotaxia was defined as the reversal in position of one or more organs. Only embryos with a normal dorsoanterior index (DAI=5) were scored. Embryos were then fixed, washed, and stained with X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) to verify targeting of injections to specific blastomeres. Percentage heterotaxia was calculated as the number of heterotaxic embryos divided by total embryos. A χ² test with Pearson correction for increased stringency was used to compare absolute counts of heterotaxic embryos. Groups were considered significantly different when P<0.01.

LPM explants

At the indicated stage, embryos were placed in 1× MMR and a sharp scalpel was used to remove the left lateral plate mesoderm (LPM) from the remainder of the embryo. These explants were maintained in 1× MMR for 1 hour to allow them to heal, and were then returned to 0.1× MMR. Uncut siblings were also placed in 1× MMR for the same period of time to allow careful staging of the explants. Explants and uncult siblings were aged to the appropriate stage for Xnr-1, lefty or pitx2 expression, fixed overnight, dehydrated and stored at −20°C for analysis.

In situ hybridization

Whole mount in situ hybridization was performed using standard protocols (Harland, 1991). In situ hybridization probes against mRNAs encoding Xnr-1 (Sampath et al., 1997), lefty (Meno et al., 1997) and pitx2 (Campione et al., 1999) were generated in vitro from linearized templates using DIG Labeling Mix (Roche, Branford, CT).

Analysis of ciliary parameters

Data was collected from 16 published zebrafish studies (Oishi et al., 2006; Shu et al., 2007; Okabe et al., 2008; Ferrante et al., 2009; Hatler et al., 2009; Lin and Xu, 2009; Neugebauer et al., 2009; Francescato et al., 2010; Gao et al., 2010; Lopes et al., 2010; Kim et al., 2011; Liu et al., 2011; Wang et al., 2011; Bighove et al., 2012; Caron et al., 2012; Chen et al., 2012) and analyzed using Graphpad version 5. Only studies examining cilia number, cilia length and ciliary flow in wild-type fish, and including means, s.e.m. or s.d., and sample sizes could be used for analysis. For cilia number and cilia length, analysis was limited to studies examining fish at the 10-somite stage. One-way ANOVA and, when appropriate, Bonferroni posthoc analyses were performed to determine whether there were significant differences between groups. Bartlett’s test for equality of variance was performed as well.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge Punita Koustubhan and Amber Currier for assistance with Xenopus husbandry, Claire Stevenson for assistance with molecular techniques, and members of the Levin laboratory for helpful discussions. The authors also thank Chris Wright for the Xnr-1 and Lefty probes, and H. Joseph Yost for the Pitx2 probe.

COMPETING INTERESTS

The authors declare that they do not have any competing or financial interests.

AUTHOR CONTRIBUTIONS

L.N.V. and M.L. conceived and designed the experiments. L.N.V. performed experiments. J.M.L. contributed reagents. L.N.V. and M.L. analyzed the data. L.N.V., J.M.L. and M.L. wrote and edited the manuscript.

FUNDING

This work was supported by an American Heart Association Established Investigator Grant [grant number 074088N to M.L.], and the National Institutes of Health [grant number R01-GM077425 to M.L., fellowship F32GM087107 to L.N.V.].

SUPPLEMENTARY MATERIAL

Supplementary material for this article is available at http://dmm.biologists.org/lookup/suppl?doi=10.1242/dmm.010256/-/DC1

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Fig. S1. Regression analysis of abnormal pitx2 expression and improper organ laterality indicates that asymmetric gene expression overestimates effects on LR patterning. To determine how predictive pitx2 expression is of asymmetric organ position, we analysed published *Xenopus* studies that measured both for treatments that affect LR patterning (Chen et al., 2004; Hatayama et al., 2011; Kramer and Yost, 2002; Levin et al., 2002; Maisonneuve et al., 2009; Mogi et al., 2003; Sakano et al., 2010; Schweickert et al., 2007; Vick et al., 2009; Yasuhiko et al., 2001) and performed a regression analysis. Similar to what has been reported previously for the relationship between asymmetric expression of Xnr-1 and organ position (Vandenberg, 2012), abnormal localization of pitx2 overestimates the effects of treatments on organ laterality. This is demonstrated by the regression line with a Y-intercept of 18.5, indicating that a significant amount of abnormal pitx2 expression would have little to no effect on asymmetric organ position. Additionally, we examined the effects of three treatments we have studied including vibrations of 7Hz and 15Hz from 1-cell to st. 19 (Vandenberg et al., 2011) and injections with serotonin in the dorsal left blastomere at the 4-cell stage. This regression analysis indicated a Y-intercept of 24.9, again suggesting that pitx2 overestimates the effects of treatments on organ laterality. These results indicate that pitx2 expression should not be used as an accurate substitute for examining the effects of treatments on organ situs, the LR patterning event that is most important for health outcomes.