A gap junction connexin is required in the vertebrate left–right organizer

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Abstract

Early patterning of vertebrate embryos involves the generation of asymmetric signals across the left–right (L–R) axis that position and are required for the proper function of internal organs. This patterning is directed by a conserved nodal/lefty signaling cascade on the left side of the embryo, thought to be asymmetrically directed by ciliary beating that generates a leftward fluid flow in the mammalian node and in Kupffer’s vesicle (KV), the related structure in zebrafish. Following morpholino knockdown of Cx43.4, asymmetric gene expression and global organ distribution are randomized, consistent with the expression of Cx43.4 in KV. Randomization is recapitulated in mosaic embryos in which Cx43.4 is depleted preferentially in KV cells, showing that Cx43.4 is specifically required in KV for proper L–R axis formation. The mechanistic basis for the laterality anomalies in Cx43.4-deficient embryos is a primary morphogenesis defect during lumen formation in KV. Additionally, the role of Cx43.4 appears to be conserved given that its ortholog, human Cx45, is able to functionally compensate for zebrafish Cx43.4 during L–R patterning. This is the first report linking connexin function in the ciliated, node-like cells of KV with normal L–R axis development.

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Introduction

While vertebrates typically exhibit external symmetry across the left–right (L–R) axis, the distribution of internal viscera and the functions of particular organs are strikingly asymmetric. Internal L–R asymmetry is a tightly regulated patterning process such that all normal individuals within a species display a precise morphological and functional arrangement. The failure to establish the L–R axis during early embryogenesis leads to heterotaxy, which is associated with organ laterality defects, including congenital heart defects and spleen anomalies (Bisgrove et al., 2003; Peeters and Devriendt, 2006).

Much progress has been made toward understanding the mechanisms that lead to proper asymmetric patterning, including the identification of a conserved, left-sided TGF-β signaling cascade (Meno et al., 1996). Propagation and direction of this signaling cascade requires the action of motile monocilia within the node in mouse (Nonaka et al., 1998) and Kupffer’s vesicle (KV) in zebrafish (Essner et al., 2005; Kramer-Zucker et al., 2005). These monocilia rotate to produce a leftward flow in the node and a counterclockwise movement of extracellular fluid in KV. Additionally, the presence of motile monocilia in the related organizing structure of Xenopus suggests that a cilia-based mechanism for L–R development is conserved among some vertebrate species (Basu and Brueckner, 2008; Essner et al., 2002; Schweickert et al., 2007).

Zebrafish have served as a useful model for understanding L–R patterning as they provide a number of advantages for pursuing the role of nodal cilia. In particular, the ability to easily access, manipulate, and monitor the ciliated cells of KV in live embryos has resulted in a model outlining the distinct steps in the development of this structure (Amack et al., 2007). KV is a transient structure that arises from the dorsal forerunner cells (DFCs). DFCs are a population of mesenchymal cells that migrate ahead of the shield (zebrafish organizer) during epiboly and eventually involute to reside within the developing tail bud (Cooper and D’Amico, 1996). At this site, they differentiate into the ciliated epithelial cells that line the lumen of KV. Surgical disruption of KV showed that a critical developmental window exists from 3 to 7 somites when ciliary function and nodal flow are required for normal L–R patterning (Essner et al., 2005). In the present study, we demonstrate that a connexin (Cx) protein is required for the DFCs to develop into KV and undergo morphogenesis into a fully functional epithelial lumen capable of generating an asymmetric signal.

Recent mouse knockouts of Cxs, the protein subunits of vertebrate gap junctions (GJs), have clearly demonstrated that Cx-based intercellular communication is required for normal organ function and, in some cases, for proper development (Wei et al., 2004). For example, Cx45 null embryos die at embryonic day 10 (Kruger et al., 2000; Kumai et al., 2000), and Cx43 knockouts exhibit defects in heart development (Reaume et al., 1995). However, the mechanisms by
which Cx proteins might regulate early events in embryonic development and patterning remain largely unexamined.

Since Cx proteins are required for development, we theorized that GJ channels play an early role in embryonic patterning. Specifically, GJ channels connecting the cytoplasmas of adjacent cells could function through the direct, cell–cell transfer of small signaling molecules, such as ions, cAMP, and IP3 in to regulate the migration, differentiation, and physiology of cells during L–R patterning (Wei et al., 2004). For example, inositol phosphates are required for L–R patterning in zebrafish (Sarmah et al., 2005), which could necessitate Cx-based GJs as a mechanism for the transfer of this signaling molecule between the cells adjacent to KV. In addition, previous reports have implicated Cx43-based GJ communication in L–R patterning of Xenopus (Levin and Mercola, 1998), chick (Levin and Mercola, 1999), and rabbit embryos (Feistel and Blum, 2008). However, the role of Cx43 in L–R patterning may not be conserved. Cx43 knockout mice do not show laterality defects and it is unclear whether human patients with laterality defects also have Cx43 point mutations (Britz-Cunningham et al., 1995; Debrus et al., 1997).

Here, we show that morpholino (MO) depletion of Cx43.4 randomizes L–R patterning. These defects can be rescued by coinjection of MOs and Cx43.4 mRNA, showing that the L–R phenotype is a specific consequence of Cx43.4 knockdown. Consistent with Cx43.4 localization in KV cells, targeted knockdown of Cx43.4 within KV cells leads to randomization. This indicates that Cx43.4 function within KV cells is necessary for L–R patterning; KV cells were correctly specified following Cx43.4 depletion, given that cilia in the morphants were comparable to those in controls. However, following MO knockdown, Cx43.4 morphant KVs failed to inflate and morphogenetic defects were apparent. Taken together, these results indicate that the laterality defects associated with Cx43.4 depletion are a result of defects during the morphogenesis of KV, the L–R organizer in zebrafish.

Materials and methods

Morpholino injections

The following morpholinos (MOs) were obtained from GeneTools (Eugene, OR) and were injected into 1- to 4-cell stage embryos as described (Nasevicius and Ekker, 2000):

Cx43.4 MO: 5′-TCAGAAGTACACCGTCTCAGTC-3′
Cx43.4 mismatched MO: 5′-TCAGAAGTACACCGTCTCAGAC-3′
Cx44.2 MO: 5′-AACTGTCAGAAGCTCCCAACTAC-3′
Cx43 MO: 5′-AGGGAGTTCTAGCTGGAAAGAAGTA-3′
ntl-MO, 5′-GACCTGAGCCGACATTTCCGAT-3′

Fluorescein-labeled MOs of the same sequences listed above were injected at the mid-blastula stage to knock down expression specifically in the dorsal forerunner cells (DFCs) according to published methods (Amack and Yost, 2004). Embryos were screened by fluorescence microscopy at the shield stage for fluorescence in the DFCs, which was lacking in other embryonic cells.

mRNA rescue

The Cx43.4 coding sequence was cloned using the following 5′ primer 5′-ATGAGTTGAAGCTTCTCAAGCGGTTTGG-3′. This fragment was generated with silent mutations (shown in lower case letters) to prevent recognition by start codon-targeting MOs and cloned into the vector, pT3S. A human Cx45 plasmid was provided by E. Beyer and V. Berthoud (Kanter et al., 1994) (University of Chicago). Full-length, capped mRNA was synthesized using Ambion’s mMessage Machine (Austin, TX) transcription kit. Cx43.4 or human Cx45 mRNA was injected into one-cell embryos at doses ranging from 20 to 200 pg/embryo. For MO-mRNA coinjection experiments, a dose of 60 or 100 pg/embryo was used.

In situ hybridization

Whole-mount in situ hybridization was performed as described (Essner et al., 2005). Cx43.4 probes were used as before (Essner et al., 1996). The Cx44.2 antisense probe was synthesized from the full length c44.2 coding sequence. Antisense RNA probes used to analyze L–R patterning events were cmlc2 (Yelon et al., 1999), spaw (Long et al., 2003), lefty1 (Bisgrove et al., 1999), and lefty2 (Bisgrove et al., 1999). The midline was analyzed with ntl antisense probes.

Whole-mount immunohistochemistry

Cilia in KV were labeled with anti-acetylated tubulin (Sigma T7451) as described (Essner et al., 2002). Cx43.4 rabbit polyclonal antibodies (Desplantez et al., 2003), kindly provided by D. Gros, University of Marseilles, were used at 10 µg/ml. To stain nuclei, Hoechst dye (Molecular Probes, 33342) was added to the wash solution at a concentration of 10 µg/ml. Embryos were mounted in Prolong Antifade (Molecular Probes) and z-series images were collected using either a Nikon C1si confocal microscope or a Zeiss fit with an Apotome and analyzed using ImageJ software (NIH, Bethesda, MD).

Nodal flow analysis

Fluorescent latex beads (Polysciences, Inc.), 0.5 µm in diameter, were diluted in PBS, injected into KVs of 8–12 somite stage embryos, and visualized as described (Essner et al., 2005). Movies were taken with a Sony HandiCam digital camera and analyzed with QuickTime software.

Results

Cx43.4 knockdown randomizes left–right asymmetry

We sought to determine the role of Cx43.4 in embryonic patterning given that it is highly expressed during gastrulation and somite-forming stages in zebrafish (Essner et al., 1996). Interestingly, when antisense MO methods (Nasevicius and Ekker, 2000) were utilized to knock down the level of Cx43.4, morphant embryos displayed heart laterality defects that indicated a L–R patterning defect. Normally, the heart tube loops from the left to the right side of the embryo and can be visualized by in situ hybridization using a cardiac myosin light chain (cmlc2) probe (Fig. 1A). However, upon MO depletion of Cx43.4, heart looping was abnormal in 47% of embryos (notably, 39% reversed and 8% midline; Figs. 1B and C), compared with only 7% aberrant looping in embryos injected with a mismatched control MO (Fig. 1C, p < 0.0003). The effects of Cx43.4 knockdown on heart looping were not significantly different from the theoretical maximum of 50% reversal upon randomization (χ2 (1, N = 64) = 0.360, p > 0.5). Knockdown of a closely related Cx family member, Cx44.2, however, produced insignificant effects on heart looping (Fig. 1C, p = 0.2), suggesting that Cx43.4 plays a more significant role in L–R specification despite the localization of c44.2 transcripts to KV (Supplementary Fig. 1). Therefore, the remaining studies focused on the role of Cx43.4, and not Cx44.2, during L–R development.

To determine whether Cx43.4 is required for global embryonic L–R patterning processes versus a more limited function during later heart morphogenesis, other known asymmetric organ systems were examined. Ninety percent of control embryos (n = 103) had normal positioning of the gall bladder on the right and pancreas on the left at 6 days postfertilization (Fig. 1D). In contrast, the developing guts of 44% (n = 104) of the morphant embryos displayed reversals of the...
development. As previously reported (Essner et al., 1996), Cx43.4 is expressed in and around KV transcripts were identified in KV cells of 10-somite stage embryos. Colabeling with polyclonal Cx43.4 antibodies (Desplan et al., 2003) showed distinct puncta within the ciliated area of KV and throughout the surrounding tail tissue (Fig. 2A). Notably, Cx43.4 puncta were not co-localized with cilia or Hoechst-labeled nuclei and were often found between nuclei, suggesting that the Cx43.4-positive plaques are localized to regions of cell–cell apposition. Also, there is no apparent L–R asymmetry of Cx43.4 protein expression in the ciliated KV cells. Overall, the expression of Cx43.4 in KV cells during the developmental stage when KV cilia are actively producing nodal flow is consistent with a requirement for Cx43.4 during L–R development.

We also analyzed the effects of MO injection on Cx43.4 localization. Upon injection of Cx43.4 MOs, the punctate staining in KV and elsewhere throughout the embryo was abolished (Fig. 2B), demonstrating extensive MO knockdown efficacy. Overall, Cx43.4-depleted embryos were grossly normal at 24 hours postfertilization (hpf) (data not shown) and high survival rates were observed, averaging 86% (after normalizing uninjected controls to 100%). This suggests that the observed disruption of L–R patterning in Cx43.4 morphants was not a secondary defect given the normal patterning of other embryonic axes and likely represents a direct requirement for Cx43.4.

Fig. 1. Cx43.4 is specifically required for L–R patterning. Dorsal views of 30 hpf embryos processed for in situ hybridization with a cardiac myosin light chain probe show normal looping of the heart tube towards the right in controls (A) and aberrant looping to the left in Cx43.4 morphants (B). (C) Quantification of heart looping in four independent knockdown experiments is represented as means ± SEM. The dotted line in all graphs shows the theoretical maximum value for randomization. (D) Ventral view of autofluorescence in the gut of a 5 dpf larva, specifically the gall bladder (arrowhead) and pancreas (arrow); compare with (E) the Cx43.4 morphant where these organs are reversed.

gallbladder and/or pancreas (Fig. 1E). These data, along with the analysis of asymmetric central nervous system (CNS) patterning below, indicate that the laterality of multiple organ systems is affected in Cx43.4-deficient embryos and suggest that Cx43.4 is required during early establishment of the L–R axis throughout the embryo.

Cx43.4 is expressed in and around KV

To explore further the role of Cx43.4 in asymmetric patterning, we next asked if its localization supports a function for Cx43.4 during L–R development. As previously reported (Essner et al., 1996), Cx43.4 was expressed throughout the somite stage embryo with higher expression observed posteriorly and in the notochord. Here, Cx43.4 transcripts were identified in KV cells of 10-somite stage embryos (Supplementary Fig. 2). Interestingly, the DFCs do not display expression of cx43.4 during gastrulation (Essner et al., 1996), suggesting a later role for Cx43.4 during KV development. Protein localization was also analyzed to determine whether it coincided with mRNA expression. For this analysis, KV was identified using antibodies for acetylated tubulin to label cilia in the tail region of 10-somite stage embryos. Colabeling with polyclonal Cx43.4 antibodies (Desplan et al., 2003) showed distinct puncta within the ciliated area of KV and throughout the surrounding tail tissue (Fig. 2A). Notably, Cx43.4 puncta were not co-localized with cilia or Hoechst-labeled nuclei and were often found between nuclei, suggesting that the Cx43.4-positive plaques are localized to regions of cell–cell apposition. Also, there is no apparent L–R asymmetry of Cx43.4 protein expression in the ciliated KV cells. Overall, the expression of Cx43.4 in KV cells during the developmental stage when KV cilia are actively producing nodal flow is consistent with a requirement for Cx43.4 during L–R development.

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Cx43.4 is specifically required for L–R patterning

To rule out off-target effects of Cx43.4 MO injection, the specificity of the morphant L–R phenotype was verified by a rescue approach. Cx43.4 MOs were coinjected with in vitro transcribed, MO-insensitive cx43.4 mRNAs at the one-cell stage. In these experiments, injection of MOs alone resulted in 42% of the embryos (n = 178) displaying aberrant heart looping at 30 hpf, while Cx43.4 mRNA injected alone had no effect. Coinjection of MOs and 100 pg of rescuing mRNA significantly reduced the percentage of embryos with aberrant heart looping from 42% to 27% (n = 148, p < 0.04) (Fig. 2C). These results indicate that the effects of Cx43.4 morpholino injection on L–R development are specifically related to Cx43.4 depletion and are not a side effect of morpholino injection.

Cx43.4 functions in KV during L–R patterning

Given the widespread distribution of Cx43.4 puncta observed by immunofluorescence, it is possible that Cx43.4 is not required in KV itself. Cx43.4 could influence L–R patterning through its expression in the lateral mesoderm and/or notochord. To address whether Cx43.4 expression is required specifically in KV cells, mosaic animals were produced by an experimental method that is unique to zebrafish. Because the DFCs maintain cytoplasmic bridges to the yolk between the 256- and 1000-cell stages while other blastomeres do not, MO injection into the yolk at this stage knocks down expression in a KV cell-specific manner (Amack and Yost, 2004). After DFC-targeted knockdown of Cx43.4 (Supplementary Fig. 3), defects in heart looping were seen in 34% of embryos (Fig. 2D), which is quantitatively comparable to previously reported defects in DFC-targeted knockdowns of a ciliary dynein, ldr1 (Essner et al., 2005) and the transcription factor, ntl (Amack and Yost, 2004). In our hands, DFC-targeted ntl depletion produced comparable alterations in heart looping to knockdown of Cx43.4 (Fig. 2D) and served as a positive control. This mosaic analysis provides strong evidence of a tissue-autonomous requirement for Cx43.4 in KV during establishment of the L–R axis.
Given previous reports on the role of Cx43 during L–R patterning in chick and Xenopus, we also asked whether zebrafish Cx43 was required specifically in KV cells for L–R axis development. Consistent with the lack of cx43 expression in KV cells (data not shown), abnormal looping in DFC-targeted Cx43 morphant embryos was not significantly different than in uninjected control embryos (Fig. 2D, *p* > 0.4). This suggests that Cx43 is not required within the cells of KV for normal L–R development and serves as a control for the specificity of Cx43.4 function in KV.

**Asymmetric gene expression patterns are consistent with a role for Cx43.4 function in KV**

To determine whether the KV tissue-autonomous role of Cx43.4 influenced downstream signaling events, we analyzed the expression of genes in an evolutionarily conserved, left-sided TGF-β cascade known to direct asymmetric organ development. One of the first asymmetrically expressed genes, spaw, is activated in the left-lateral mesoderm adjacent to KV at 10–12 somites (Long et al., 2003). Left-sided spaw expression is dependent on an intact KV with normal nodal flow. Initiation of spaw expression in 12-somite stage Cx43.4 morphants was predominantly bilateral (66%, *n* = 35; Figs. 3A–E), while 82% of control embryos expressed spaw on the left side. These data suggest that cells lateral to KV in Cx43.4-deficient embryos are capable of receiving a signal and activating spaw expression, however, the directionality of activation is impaired.

The embryonic midline, comprising the notochord and floor plate of the CNS, is required to restrict asymmetric gene expression to the left or right sides of the embryo. Disruption of the midline or of repressive signaling from this midline barrier results in bilateral expression of genes that are normally expressed asymmetrically (Danos and Yost, 1996; Meno et al., 1998), similar to the spaw expression pattern in 12-somite stage Cx43.4-deficient embryos. Since Cx43.4 is expressed in the notochord, we next examined the signaling barrier function of the midline in Cx43.4-deficient embryos to determine if it was intact. No apparent differences in the expression of ntl in the notochord domain were detected when comparing controls (Figs. 4A and C) and Cx43.4 morphants (Figs. 4B and D) at 10 or 20 somites, suggesting that the midline was continuous. Additionally, at 20 somites, spaw expression was randomized in morphants (Fig. 3F), rather than being expressed bilaterally as observed at 12 somites (Fig. 3E). Resolution of early bilateral spaw expression to randomization at 20 somites provides further evidence, of a functional nature, that the midline barrier is intact in Cx43.4-deficient embryos and is capable of restricting gene expression to one side of the embryo. Taken together, the spaw expression data at two developmental time points suggest that Cx43.4 morphants do not have defects in midline barrier function, but KV function is altered.

We next analyzed the asymmetric expression of genes that are downstream of spaw signaling, lefty1 is normally expressed in the left, dorsal diencephalon (Fig. 3G) and can be used as a marker for asymmetry in the CNS at the 20-somite stage. Upon Cx43.4 knockdown, lefty1 expression was randomized in the left in only 22% of embryos, while all of the control embryos that had activated lefty1 displayed normal, left-sided expression (Figs. 3H and I). Importantly, right-sided or bilateral expression patterns were never seen in controls, whereas 33% of Cx43.4 morphant embryos had reversed, right-sided lefty1 expression.

Cx43.4 knockdown also resulted in randomization of lefty2 (Figs. 3J–L), a gene normally expressed in the left side of the developing heart field at 20 somites. Notably, bilateral lefty2 expression was observed only in rare cases (4 out of 64). Overall, the low percentage

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**Fig. 2.** Cx43.4 is expressed in a spatiotemporal manner to be competent for KV function. Confocal fluorescence imaging (A) shows Cx43.4 labeling (red) in puncta that are distinct from the nuclei and cilia (green) of KV cells (arrow). Note also significant Cx43.4 labeling in the surrounding tail bud tissue. Grid-like confocal imaging (B) demonstrates Cx43.4 MO efficacy by the lack of staining (red), punctate or otherwise, in knockdown embryos. Cilia (green) of KV are labeled with tubulin antibodies and marked with an arrow as in (A). (C) Defects in heart laterality are partially rescued by coinjection of cx43.4 mRNA, while 82% of control embryos expressed lefty1, lefty2, nodal, and spaw expression in 12-somite stage Cx43.4 morphant embryos was not significantly different than in uninjected control embryos (Fig. 2D, *p* = 0.04). (D) DFC-targeted injections of Cx43.4 MOs have a significant heart reversal phenotype. (C, D) Data are averages of three independent experiments and are represented as means ± SEM.
of Cx43.4 knockdown embryos with bilateral spaw, lefty1, or lefty2 expression at 20 somites is consistent with maintenance of midline integrity. Together, these data provide strong evidence that Cx43.4 functions specifically in KV to help direct asymmetric gene expression rather than in establishment or maintenance of a midline barrier function.

Fig. 3. Molecular markers support a role for Cx43.4 in KV function. In situ hybridizations are used to probe for expression of genes in the L–R signaling cascade. (A–D) Twelve-somite Cx43.4 morphant embryos probed for spaw expression (arrowheads, throughout) show left, right, bilateral, and absent expression, respectively. spaw expression data are quantified (E) at 12 somites and (F) at 20 somites. Twenty-somite embryos probed with lefty1 show (G) normal expression in wild-type and (H) reversed expression in a DFC-targeted Cx43.4 knockdown embryo. (I) lefty1 data are summarized. (J) Twenty-somite embryos probed with lefty2 show a (K) reversal in the developing heart field of the Cx43.4 morphant. (L) Quantification of lefty2 expression was compiled from three independent experiments. Graphs in (E, F, I, L) represent means ± SEM from three independent experiments.

Cx43.4 is required for KV ciliary development but not KV cell specification

Since mosaic analysis following DFC-targeted MO injections showed that Cx43.4 is required in KV cells and gene expression data indicated a role for Cx43.4 in KV function, we next asked whether
Cx43.4 is required for KV cell specification, differentiation, morphogenesis, or function. Based on immunofluorescence, KV cilia in 10-somite control embryos were notably well-developed and the distribution of cilia encompassed a circular area (Fig. 5A). In Cx43.4 morphant embryos, although ciliated cells had developed in a distinct region of the tail, they occupied a smaller and more irregularly shaped area (Fig. 5B). These results are similar to previously published ciliary characteristics in ntl mutant and morphant embryos (Amack et al., 2007) and suggest that Cx43.4 is similarly required for complete KV morphogenesis.

To quantify the differences between control and morphant cilia, additional analyses were performed using ImageJ software. Morphant cilia, although similar in number compared to controls (Fig. 5C), were significantly shorter than the average wild-type cilium (Fig. 5D). The analysis of nodal cilia showed that KV cells are specified and have begun a differentiation program in order to acquire this specialized cell fate. Additionally, since no significant differences were seen in the number of cilia, KV cell progenitors (DFCs) likely migrated to the correct position in the embryo and were then specified correctly.

Since KV cilia in Cx43.4 morphant embryos were shorter compared to controls, we asked whether Cx43.4 was required for ciliary motility and production of a net leftward fluid flow. Fluorescently labeled beads were injected into inflated KVs of live 10-somite stage embryos and video microscopy was used to track their movement. In wild-type embryos, as previously reported (Essner et al., 2005; Kramer-Zucker et al., 2005), the beads circulated in a counterclockwise direction, driven by motile monocilia (data not shown). Interestingly, while attempting to inject beads into KVs of Cx43.4-deficient embryos, we found that 94% of the knockdown embryos (n = 38) did not possess a luminal KV space observable by DIC microscopy (Figs. 6A–C). In contrast, only 3% of mismatched control MO injected embryos lacked an inflated KV. To determine whether the Cx43.4 MO injection produced a developmental delay, the posterior region of Cx43.4 morphants was observed until 16 somites with no recovery of lumen inflation observed. Thus, a fundamental part of the Cx43.4 knockdown phenotype is a lack of KV inflation. Our interpretation is that without an inflated luminal space, the cilia in KV are likely unable to generate a net leftward flow, thus rendering KV incapable of directing normal asymmetric development.

**Human Cx45 can functionally compensate for zebrafish Cx43.4 in L–R development**

Finally, we addressed whether the role of Cx43.4 in KV cells during L–R patterning is conserved. The expression pattern of zebrafish Cx43.4 resembles that of mammalian Cx45 during somitogenesis (Kruger et al., 2000; Kumai et al., 2000), and the two Cxs are highly related by sequence and phylogeny (Cruciani and Mikalsen, 2006; Eastman et al., 2006). Additionally, physiological analyses of Cx43.4 channel properties (Barrio et al., 1997; Desplantez et al., 2003) provide evidence for substantial functional conservation. Therefore, we asked whether human Cx45 could functionally compensate for Cx43.4 during L–R patterning.

Control experiments showed that when 80 pg of in vitro synthesized human cx45 mRNA was injected into single-cell embryos, there were no significant L–R heart looping aberrations (Fig. 7). As in earlier experiments, more than 40% of Cx43.4 morphants displayed aberrant heart looping. However, when cx45 mRNA was co-injected with Cx43.4 targeting MOs, the percentage of embryos with aberrant heart looping was significantly reduced to only 25% (Fig. 7). This level of rescue is comparable to that observed after injection of rescuing cx45 mRNA (Fig. 2D), suggesting that human Cx45 can effectively compensate for Cx43.4 during L–R patterning. Additionally, we note that Cx45 null mice have specific defects in positioning of the atrium and ventricle of the heart (Kumai et al., 2000), which are reminiscent of a laterality defect. These observations are consistent with the idea that Cx45 orthologs have a conserved role in L–R patterning. Asymmetry defects have not been described for other
organ systems in Cx45 null mice. However, these embryos die shortly after gastrulation, and the analyses to date have focused on the cardiovascular system, given the observed defects in cardiac function in these knockout mice.

**Discussion**

**A role for Cx43.4 in development**

It is well known that gap junctions equip neighboring cells for communication by providing cell-to-cell channels that allow small molecules to pass directly between cells. As a result, these pathways have been envisioned as a means of signaling and coordinating cells during various developmental processes. Particularly relevant to this study, it has been theorized that GJ communication is critical for normal patterning during early embryogenesis. For example, future dorsal cells can be distinguished from their ventral counterparts in 32- and 64-cell Xenopus embryos by a difference in GJ communication (Olson et al., 1991; Warner et al., 1984), and beta-catenin, a key player in early embryonic development, is the endogenous regulator of the increase in communication in dorsal cells (Krufta et al., 1998). Moreover, previous studies have implicated GJs during L–R patterning in both chick and Xenopus embryos (Levin and Mercola, 1998; Levin and Mercola, 1999). However, we have only a limited understanding of Cx involvement during early embryonic development in terms of spatiotemporal requirements (Chuang et al., 2007; Guthrie et al., 1988; Levin and Mercola, 1998). Additionally, the extent of mechanistic conservation of Cx function across species is not yet clear.

Here, we sought to capitalize on the experimental advantages of the zebrafish embryo, in order to evaluate the role of a Cx that is highly expressed in early embryos, suggestive of a role in patterning. We show that Cx43.4, which is expressed in cells of the zebrafish L–R organizer, and elsewhere in the embryo, is specifically required for L–R patterning. The high level of penetrance leading to nearly 50% randomization after MO knockdown underscores the significance of
this requirement. Importantly, DFC-targeted MO injection allowed for
the analysis of mosaic embryos and the first observation that Cx
function is required within a specific tissue, KV, during L–R
axis development. Although KV cells are still specified in Cx43.4-deficient
embryos and develop cilia, a functional lumen fails to develop. This
indicates that Cx43.4 is required for KV morphogenesis – at a specific
place and time within the embryo—during early L–R patterning.
Additionally, it appears that this function could be conserved in
vertebrate development, as human Cx45 was sufficient to rescue the
L–R defects associated with zebrafish Cx43.4 depletion. Consistent with
this, knockout of Cx45 in mice produces heart morphogenesis
anomalies indicative of looping defects (Kumai et al., 2000).

Other potential roles of connexin proteins during embryonic patterning

We stress that the present findings do not exclude the possibility
that Cx proteins play other roles, as well, during embryonic
patterning. In fact, it is likely that this is the case.

In order for KV to develop and function, the KV precursor cells,
DFCs, must migrate to the correct position in the embryo during
gastrulation and then undergo differentiation. This subpopulation
of cells migrates together, and it is possible that Cx proteins other than
Cx43.4 are required for DFC migration, similar to the involvement of
Cx43 during neural crest cell migration (Lo et al., 1999). Neural crest
cells migrate in streams away from the neural tube during
development and give rise to many tissues, including certain cells
within the nervous system and heart. Neural crest cells have been
shown to express Cx43 and be well-coupled, even during migration.
In the absence of Cx43, neural crest cell migration is altered (Huang et
al., 1998). In the present experiments, embryos depleted of Cx43.4
displayed a similar number of KV cilia (presumably one per cell) as
control embryos, suggesting that DFC migration and early KV cell
specification are not dependent on Cx43.4, but another Cx could be
required.

Yet another potential Cx role in L–R patterning comes from the
observation that the cilia on the epithelial cells of KV beat in a
coordinated fashion. In airway epithelia, the coordinated beating of
cilia is also required, specifically for the efficient clearance of mucus.
In these epithelial cells, the coordination is thought to be dependent
on GJ communication (Boitano et al., 1992). Coordinated ciliary
beating likely relies on elevated Ca2+ within cells that is generated by
mechanical stimulation (e.g., by means of the normal flow of mucus
across the epithelium) and propagated to neighboring cells via GJs. A
similar model could be envisioned in the ciliated KV epithelium,
where GJ communication could coordinate ciliary beating in order
to create a functional leftward fluid flow, without which KV would be
rendered incapable of directing L–R asymmetry. The data presented
here do not eliminate a possible role for Cx43.4 in coordinating KV
ciliary beating. However, in Cx43.4 morphant embryos, it is likely that
any effects on ciliary motility would be masked due to the uninflated
KV structure, which would render fluid flow ineffective regardless of
ciliary function.

Finally, once the cells derived from DFCs have differentiated
and nodal flow has been established in KV, a left-sided signaling wave is
observed in the cells adjacent to KV. That is, some type of signal
originating in KV cells needs to ultimately reach lateral mesodermal
cells adjacent to KV and subsequently activate the Nodal signaling
cascade. Cx proteins could also be required for the spread of this
signal. In fact, this communication question ranks as one of the most
fundamental in terms of understanding the process of L–R patterning,
not only in zebrafish, but in higher vertebrates as well.

Possible insight comes from previous studies in the chick and Xe-
nopus, which suggested that Cx43 could influence L–R development
(Levin and Mercola, 1998, 1999). This evidence supports a model
where GJs, comprised of Cx43, function in a population of embryonic
cells to establish an electrical gradient that would help distinguish the
future left side from the right (Levin, 2005). Support came from
experiments using dominant negative constructs and less specific
drug treatments. The model proposed that a L–R determinant like
serotonin (Fukumoto et al., 2005) could segregrate along the electrical
gradient and would then influence asymmetric gene expression
depending on its local concentration on a given side of the embryo.
Thus, Cx43 could function in conjunction with KV in a manner that is
distinct from the role of Cx43.4 within the L–R organizer. (Note that
these are not closely related connexins, despite the similar
terminology.)

Thus, although we have gained important insights into L–R
patterning as a result of the present analysis of Cx43.4, we have
much to learn about the role of Cxs in development, including roles in
embryonic patterning.

Cx43.4 is required for KV morphogenesis

What then is known about KV development and how might a Cx
function in this process? First, it is clear that for the motile KV cilia to
function optimally and produce a robust, leftward fluid flow, they
must operate within a fully inflated luminal space. Recent studies
have begun to examine the various processes that lead to KV
development (Amack et al., 2007), although the precise genes involved
in this morphogenetic process and the mechanistic details
remain unclear.

The formation of cilia is an early step in KV development that
precedes lumen expansion (Amack et al., 2007). In Cx43.4-deficient
embryos, KV cells are specified and cilia are present. However, we
observed defects in the length of KV cilia, suggesting that Cx43.4 is
required for this early phase of KV development. Interestingly, recent
work has implicated fibroblast growth factor (FGF) signaling in the
regulation of cilia length (Neugebauer et al., 2009). Moreover,
membrane permeability through the activation of Cx43 and Cx45
hemichannels (channels located on the plasma membrane but not
docked as in a GJ) is specifically increased upon FGF signaling
(Schalper et al., 2008), and FGF can stimulate ATP release via Cx43
hemichannels (De Vyust et al., 2007). These observations suggest
that, in a similar way, Cx43.4 and FGF signaling may function
together in KV to regulate cilia length, a necessary step for the
production of fluid flow. Alternatively, the defects in KV cilia length
could be consistent with a requirement for Cx43.4 during KV cell
development and may represent a secondary effect of inflation
deficiencies in KV.

After the acquisition of cilia, KV cells display expanded apical
membrane surfaces as the lumen of KV begins to inflate (Amack et al.,
2007). The transcription factor, ntl, is known to be required for KV
lumen formation (Amack and Yost, 2004; Essner et al., 2005), and the
defects associated with ntl depletion are strikingly similar to the
results described here for Cx43.4. Furthermore, we have previously
shown that ntl mutant embryos have reduced levels of Cx43.4
expression (Essner et al., 1996). These observations suggest that
Cx43.4 is one of the transcriptional targets of ntl, possibly even a key
player with an impact on downstream events, during the KV
morphogenesis program. Future experiments will be directed at
understanding the precise function of Cx43.4 during KV morphogen-
esis with the goal of identifying the critical point at which KV lumen
formation fails in the absence of Cx43.4.

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Appendix A. Supplementary data


References


