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Calcium signaling: A common thread in vertebrate left-right axis development

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Introduction

The internal organs of vertebrates exhibit extensive asymmetry with respect to the left-right axis, both in terms of their relative positions and morphology. Evidence accumulated over the past decade has delineated a conserved molecular pathway involving the TGF β superfamily member Nodal. In all species analyzed, left-biased *nodal* expression in the lateral plate mesoderm (LPM) is required for the proper upregulation of downstream genes including *lefty1/2* and the transcription factor *pitx2* in the left-LPM. Abnormal expression of these genes in the LPM is associated with abnormal positioning of the internal organs later in development (for review see (Hamada et al., 2002). While this pathway has been studied extensively, mechanisms by which LR symmetry is broken are still being explored. In this review, we will briefly discuss the divergent mechanisms establishing the LR axis in multiple vertebrate model organisms and then focus on the roles of calcium (Ca²⁺) in LR patterning of the chick and mouse and the recent findings of the involvement of Ca²⁺ signaling at multiple decision points in LR patterning in the zebrafish.

Initial break of LR symmetry

Frog and Chick

In the *Xenopus*, asymmetric distributions of serotonin, the chaperon protein 14-3-3E, and the ion pump H⁺,K⁺-ATPase are noted as early as the first few cell divisions (Bunney et al., 2003; Fukumoto et al., 2005; Adams et al., 2006). Disrupting such asymmetric gene activity by forced expression or by chemical inhibitors results in the randomization of *nodal* expression and the placement of the internal organs, indicating that asymmetric gene expression is a very early step in frog LR patterning. While asymmetric localization of H⁺,K⁺-ATPase has not been noted in chick embryos, blocking the activity of H⁺,K⁺-ATPase with chemical inhibitors is sufficient to perturb the left-biased *shh* expression around Hensen's node, which precedes asymmetric *nodal* expression in the chick, and cause randomization of the internal organs (Levin et al., 2002; Raya et al., 2004). Furthermore, gap junctions are also required at very early steps of LR determination in chick and *Xenopus* (Levin and Mercola, 1998; Levin and Mercola, 1999). Disrupting gap junctions results in a loss of asymmetric *shh* in chick and heterotaxic organ situs in *Xenopus*. Given the physiological role of H⁺,K⁺-ATPase in pumping protons out of the cell to generate a negative membrane potential, it has been hypothesized that in chick and *Xenopus*, H⁺,K⁺-ATPase might maintain an asymmetric cellular voltage potential, allowing charged, low molecular weight signaling molecules to travel through gap junctions and accumulate preferentially on one side of the embryo. Alternatively, these two molecules may be

functioning in parallel to influence the secretion and transport of LR determinants (Oviedo and Levin, 2007).

Mouse

The first noticeable LR asymmetry in the mouse is a transient cilia-driven leftward fluid flow in the node. This cilia-driven flow precedes and is required for the establishment of asymmetric nodal signaling. Mice deficient in ciliary motor proteins such as KIF3 (a plus-end microtubule motor) and Left/right dynein (Lrd, a minus-end microtubule motor) do not have notable nodal flow and display a randomization of the internal organs (Nonaka et al., 1998; Okada et al., 1999; Takeda et al., 1999; McGrath et al., 2003). Similarly, organ situs is perturbed in mice deficient for *Inversin*, another ciliary protein required for generating nodal flow (Okada et al., 1999; McGrath et al., 2003). Furthermore, artificially supplied leftward flow can direct the development of normal situs in *lrd* and *inversin* mutant mice (Nonaka et al., 2002; Watanabe et al., 2003), indicating that nodal flow is the critical component of LR patterning disrupted in these mutants.

Two models have been proposed to explain how nodal flow directs LR asymmetry. Both of these models place a left-sided elevation in intracellular calcium levels just downstream of nodal flow in the process of LR axis determination. Using immunohistochemistry and the *lrd*-GFP knock-in mouse, McGrath et al. showed that there are at least two types of cilia in the node of the mouse: the more centrally located motile cilia that express both *Lrd* and *Pkd2* and the more peripherally located *Pkd2* positive but *Lrd* negative non-motile cilia which may function as mechanosensors (McGrath et al., 2003). *Pkd2* is a Ca^{2+} -permeable channel that has been shown to localize to the primary cilium of kidney cells. In kidney cells, *Pkd2* senses fluid flow and causes an increase in intracellular calcium levels (Nauli et al., 2003). The two cilia model proposes that sensory cilia on the left periphery of the node respond to fluid flow leading to an accumulation of intracellular calcium on the left side of the node (Tabin and Vogan, 2003).

An alternative model is based on the discovery of small membrane-sheathed objects, termed nodal vesicular parcels (NVP), that travel across the mouse node in a leftward direction (Tanaka et al., 2005). Chemical inhibition of Fgf receptors showed that Fgf signaling is required for the production of NVPs and asymmetric calcium signaling on the left side of the node, but is dispensable for the generation of leftward nodal flow. Sonic hedgehog (Shh) and retinoic acid (RA) are present in the NVPs and NVP production and asymmetric calcium signaling defects induced by the inhibition of Fgf signaling can be restored by exogenous application of Shh protein or RA, suggesting that FGF-dependent surface accumulation of morphogens may be essential for NVP production (Tanaka et al., 2005). How the fusion of Shh and RA-containing NVPs to the left edge of the node might trigger an increase in intracellular calcium levels is not currently known.

Zebrafish

Blocking H^+, K^+ -ATPase activity in zebrafish results in LR defects indicating that like the chick and *Xenopus*, zebrafish requires H^+, K^+ -ATPase activity at an early step in LR patterning (Kawakami et al., 2005). The exact mechanisms by which early ion flux influences LR determination in zebrafish are not yet known.

In zebrafish, like the mouse, cilia-driven fluid flow is required for establishing the LR axis. Kupffer's vesicle (KV), a transient embryonic organ positioned at the posterior end of the notochord, is the fish equivalent of the mouse node as far as LR patterning is concerned (Amack and Yost, 2004; Bisgrove et al., 2005; Essner et al., 2005; Kramer-Zucker et al., 2005). Disturbing the morphogenesis of KV by knocking down two T-box genes, *ntl* and

spt, results in randomized placement of the internal organs (Amack et al., 2007). Like the mouse node, the rotation of KV cilia generates fluid flow in the zebrafish KV. Loss of function of intraflagellar transport proteins such as IFT88/Polaris and IFT57/Hippi inhibits ciliogenesis, resulting in a loss of directional fluid flow in KV and randomization of organ placement in zebrafish (Bisgrove et al., 2005; Kramer-Zucker et al., 2005; Kreiling et al., 2007). Also in parallel to the mouse node, a left-sided elevation of intracellular calcium levels has been detected in tissues surrounding the zebrafish KV (Sarmah et al., 2005), indicating that the early steps in LR patterning in mouse and zebrafish may in fact be identical or highly similar.

Calcium signaling in LR patterning

Calcium is an important signaling molecule regulating various biological processes. Accumulating evidence suggests that calcium is involved in LR patterning in all of the major vertebrate model organisms (mouse, chick, *Xenopus*, and zebrafish). However, like the mechanisms by which symmetry is broken, calcium's role in LR patterning does not appear to be strictly conserved among vertebrates.

Mechanisms for generating asymmetric intracellular Ca^{2+} signaling around the node

As discussed earlier, elevated intracellular calcium levels are present at the left edge of the mouse node prior to the onset of asymmetric gene expression (Figure 1). The two cilia hypothesis proposes that nodal flow triggers calcium release via mechanosensory Pkd2-expressing cilia (Tabin and Vogan, 2003). This hypothesis is supported by data showing that in embryos lacking Pkd2, calcium levels are not elevated on either side of the node (McGrath et al., 2003). However, the finding that blocking NVP formation by inhibiting Fgf signaling prevents elevated calcium levels around the node without affecting nodal flow (Tanaka et al., 2005) suggests that the mechanosensory model alone may not be able to explain how calcium levels are specifically elevated on the left-side. Whether one hypothesis can supersede the other or these mechanisms function in parallel warrants further investigation. It will be important to determine if NVPs are produced and transported normally by nodal flow in *pkd2* mutants and if Fgf-inhibited embryos have the Pkd2-expressing sensory cilia population. The impact of loss of Pkd2 on nodal/KV cilia motility has not yet been analyzed directly. However, given the findings that pronephric cilia motility is not affected in zebrafish *pkd2* morphants and mutants (Bisgrove et al., 2005; Obara et al., 2006; Schottenfeld et al., 2007; Sullivan-Brown et al., 2008), it is not likely that *pkd2* is required for the motility of nodal/KV cilia. It is possible that Pkd2 expressing cilia may serve as sensors for NVPs since cilia are known to be required for Hedgehog signaling and Shh is present in the NVPs (Tanaka et al., 2005). However, despite the presence of Shh and retinoic acid in NVPs, no evidence for asymmetric Shh or retinoic acid signaling around the node has been detected yet (Tabin, 2006).

Observation of the perinodal calcium phenotype in *lrd* (*iv*) mutants, which have non-motile nodal cilia, has yielded puzzling yet important information relevant to both the two cilia and NVP models. McGrath et al. show that elevation of calcium levels is absent in most *lrd* mutants (McGrath et al., 2003), whereas Tanaka et al. observe a bilateral elevation of calcium in most *lrd* mutants (Tanaka et al., 2005). Bilateral elevation of calcium in *lrd* mutants would be consistent with the NVP model, in which morphogen containing parcels are generated on both sides of the node and fracture near their point of origin in the absence of flow. On the other hand, a loss of calcium elevation would be more in line with the mechanosensory model, in which Pkd2 containing cilia can not be synchronously triggered to release calcium without nodal flow. Further studies will be required to resolve the discrepancies between the two-cilia and NVP models and to formulate a new model explaining how exactly nodal flow generates a left-sided increase in calcium.

Like the mouse, a transient elevation of intracellular calcium levels has been observed on the left side of the KV and zebrafish requires Pkd2 for proper asymmetric gene expression and organ laterality. These data suggest that in zebrafish, like the mouse, Pkd2 expressing cilia may be performing a sensory role, detecting either flow or morphogens transported by that flow, and translating this laterality information into an asymmetric calcium signal. It is not yet known whether zebrafish possess NVPs or non-motile Pkd2-expressing sensory cilia, but it will be interesting to determine if zebrafish and other vertebrates share these mechanisms for triggering asymmetric calcium signaling on the left side of the node.

Asymmetric extracellular Ca^{2+} signaling around the chick node

In the chick, elevated *extracellular* calcium levels have been detected on the left side of Hensen's node, and a model has been developed to explain how these elevated calcium levels could influence LR asymmetric gene expression (Raya et al., 2004). In this model, increased extracellular calcium on the left side is thought to potentiate Notch signaling, resulting in an increase in the expression level of the Notch ligand *delta-like 1* (*dll1*) on the left (Figure 1). Symmetric *dll1* expression and a loss of left-sided *nodal* were observed in embryos treated with the calcium chelator BAPTA, showing that asymmetric calcium signaling is essential for LR asymmetric gene expression. Raya et al. also use a cell culture model to show that by increasing the concentration of calcium in the culture medium, the response of a Notch activity reporter to ligand can be enhanced. Overexpression of a dominant-negative form of *dll1* in the chick perinodal region led to a loss of left-sided *nodal* expression, placing Notch signaling upstream of Nodal signaling in the cascade of LR asymmetric gene expression. It will be interesting to determine if there is any relationship between the asymmetric extracellular calcium detected in the chick and the asymmetries in intracellular calcium seen in the mouse and zebrafish. For example, elevation of intracellular and extracellular calcium levels may go hand in hand in all three organisms. Interestingly, a requirement for Notch signaling in LR development has also been reported in the zebrafish and mouse models (Krebs et al., 2003; Raya et al., 2003; Gourrionc et al., 2007), but has not yet been connected to intracellular or extracellular calcium levels.

Ca^{2+} signaling in zebrafish LR patterning

Recent work in the zebrafish model has uncovered additional roles for calcium in LR patterning that are upstream of nodal flow: regulation of KV morphogenesis and KV cilia function. (Figure 1). Imaging the intracellular calcium levels of zebrafish embryos revealed that transient releases of calcium occur in the region of the shield and dorsal forerunner cells (DFC, precursors of KV) (Schneider et al., 2008). Two independent studies have shown that treatment with thapsigargin, a Ca^{2+} ATPase inhibitor that prevents the pumping of Ca^{2+} back into the endoplasmic reticulum, disrupts KV formation and randomizes asymmetric gene expression and organ laterality (Kreiling et al., 2008; Schneider et al., 2008). However, mechanisms by which thapsigargin treatment causes LR defects remain to be clarified. It has been shown that thapsigargin causes a transient increase in cytoplasmic Ca^{2+} levels, but will ultimately lead to the depletion Ca^{2+} from the ER (Thastrup et al., 1990; Thastrup et al., 1994). An increase in cytosolic Ca^{2+} was detected in those embryos treated with a low concentration of thapsigargin (0.5 μM for 2 hours) (Kreiling et al., 2008) whereas a repression of Ca^{2+} flux was noted in those embryos treated with a high concentration pulse of thapsigargin (2.5 μM for 10–20 minutes) (Schneider et al., 2008). Whether this discrepancy is due to the different methodologies used for measuring cytosolic Ca^{2+} levels or the differences in the dosage and timing of treatments requires careful investigation. However, the findings that embryos treated with other agents that suppress Ca^{2+} release causes phenotypes similar to embryos treated with a high concentration of thapsigargin indicates that inhibiting DFC regional Ca^{2+} flux interferes with LR patterning by disrupting DFC migration and coalescence and suppressing KV formation (Schneider et al., 2008).

In addition to regulating KV formation, cytosolic Ca^{2+} levels in DFC/KV also modulate cilia motility. NCX4a (also known as *slc8a4a*) and Na,K-ATPase $\alpha 2$ (also known as *atp1a2a*) are two genes required for calcium homeostasis in DFC and KV (Shu et al., 2007). Knocking down NCX4a and Na⁺,K⁺-ATPase $\alpha 2$ in DFC/KV disrupts nodal signaling and randomizes organ laterality, indicating a role for NCX4a and Na⁺,K⁺-ATPase $\alpha 2$ in LR patterning. The overall morphology of KV and the number and length of KV cilia appear normal in NCX4a and Na⁺,K⁺-ATPase $\alpha 2$ morphants (morpholino injected embryos). However, cilia within the KV of these embryos are immotile as demonstrated by a lack of KV fluid flow and direct observation using high-speed video imaging. NCX function is one of the primary mechanisms by which calcium is extruded from cells, and the Na⁺ gradient established by Na⁺,K⁺-ATPase helps to drive calcium extrusion via NCX (Reviewed in (Blaustein and Lederer, 1999)). In blastula stage NCX4a and Na⁺,K⁺-ATPase $\alpha 2$ morphants, intracellular calcium levels are globally elevated. Interestingly, increasing calcium signaling by injecting a constitutively active form of Calcium/calmodulin-dependent protein kinase II (CaMKII) also led to defects in organ laterality, and decreasing CaMKII activity with the chemical inhibitor KN-62 was able to rescue the defects in organ laterality and KV flow of NCX4a and Na⁺,K⁺-ATPase $\alpha 2$ morphants (Shu et al., 2007). Furthermore, inhibition of CaMKII was also able to suppress the LR defects in embryos treated with A23187, a calcium ionophore that allows calcium in the extracellular space to enter the cell, increasing its intracellular levels. Thus, the elevation of cytosolic Ca^{2+} levels inhibits KV cilia motility by a CaMKII mediated pathway.

More evidence for the role of calcium in KV cilia motility comes from the knockdown of *Ipk1*, a kinase that converts inositol 1,3,4,5,6-pentakisphosphate (IP_5) to inositol hexakisphosphate (IP_6) (Sarmah et al., 2007). The intracellular messenger IP_6 has been previously linked to calcium signaling in numerous studies. Knockdown of *ipk1* (also known as *ippk*) in zebrafish reduces Ca^{2+} flux in tissues around the KV and, like knockdown of NCX4a and Na⁺,K⁺-ATPase $\alpha 2$, results in a loss of KV cilia motility and LR developmental defects (Sarmah et al., 2005; Sarmah et al., 2007). The KV cilia in *ipk1* morphants are also shorter than in wildtype, implicating calcium signaling in the regulation of KV cilia biogenesis as well (Sarmah et al., 2007). Further studies will be required to determine if the KV ciliary defects in *ipk1* morphants are a result of aberrant calcium signaling or another pathway influenced by IP_6 .

Remaining Questions

While the mechanisms by which symmetry is initially broken appear to be divergent among vertebrates, recent findings suggest that directional fluid flow in a ciliated “node-like” structure might be a common feature of the early steps of vertebrate LR axis formation. Nodal or KV flow has been noted in numerous fish and mammalian species (Okada et al., 2005). Recently, a cilia-driven leftward fluid flow was also noted in the gastrocoel roof plate in *Xenopus* (Schweickert et al., 2007). Blocking the cilia-driven nodal flow resulted in laterality defects, indicating that directed fluid flow precedes and is required for asymmetric *nodal* expression in *Xenopus* as it is in fish and mammals. The *Lrd* protein has been reported to be located in the cilia of Hensen’s node (Essner et al., 2002), suggesting that cilia-driven flow may also be present in Hensen’s node. It will be interesting to investigate whether these cilia drive directional fluid flow and influence LR determination in the chick embryo as they do in all the other major vertebrate model organisms.

How is leftward fluid flow established in the node? In the mouse, the nodal cilia are tilted posteriorly, and fluid dynamic modeling confirmed that rotation of posteriorly tilted cilia is capable of generating a net leftward flow (Nonaka et al., 2005). In the zebrafish, cilia are distributed densely on the dorso-anterior surface and lightly on the ventral surface of KV

(Kreiling et al., 2007) and in *Xenopus*, cilia in the gastrocoel roof plate are polarized to the posterior pole of cells (Schweickert et al., 2007). Thus, it is clear that cilia are not randomly distributed in the node or its equivalent structures. Molecular mechanisms that establish the pattern of cilia distribution in the node are worth studying in the near future. Furthermore, once asymmetric intracellular calcium levels have been established on the left side of the mouse node and zebrafish KV, they must somehow be converted into asymmetric gene expression. The specific pathways influenced by calcium that are relevant to LR patterning should be a focus of further investigation.

Studies in the zebrafish have revealed multiple roles for Ca^{2+} in the formation and function of KV cilia. Interestingly, *Xenopus* embryos treated with the calcium ionophore A23187 also have defects in organ laterality (Toyoizumi et al., 1997). In light of the finding that *Xenopus* has a structure analogous to the zebrafish KV and mouse node, Ca^{2+} may play a role in regulating the motility of its cilia as well. Another interesting finding from the zebrafish studies is that the repression and elevation of intracellular Ca^{2+} levels influence LR patterning by different cellular mechanisms. Inhibiting calcium release interferes with DFC migration and KV formation whereas elevating intracellular calcium levels inhibits KV cilia motility without affecting KV morphogenesis or KV cilia biogenesis. How Ca^{2+} flux directs KV morphogenesis is currently not known. An accumulation of β -catenin was detected in the nuclei of thapsigargin treated zebrafish and *Xenopus* embryos, implying an antagonistic relationship between Ca^{2+} and the Wnt/ β -catenin pathway during KV morphogenesis (Schneider et al., 2008). However, the causative relationship between Ca^{2+} and Wnt signaling in guiding DFC migration needs further investigation. Furthermore, the mechanism by which calcium regulates nodal cilia motility also requires further investigation. Calcium levels have been proposed to modulate dynein-regulated microtubule sliding, and thereby affect the waveform of *Clamydomonas* flagella and the motility of sea urchin sperm flagella (Brokaw, 1979; Gibbons and Gibbons, 1980; Nakano et al., 2003; Wargo et al., 2004). Since CaMKII is known to be an important component of calcium signaling in zebrafish LR development, it is possible that CaMKII directly or indirectly regulates dynein activity in KV cilia. Alternatively, proper calcium homeostasis and signaling might be required for the biogenesis of ultrastructurally normal motile KV cilia.

Studies in the past decade have delineated a conserved molecular cascade involving nodal signaling that functions to transmit LR axis information globally. However, very little is known about how individual organs interpret and respond to these signals. In the zebrafish model, mutants have been identified that have defects in laterality of one organ but not the others. For example, normal cardiac laterality, but a low penetrance of visceral organ laterality defects, has been reported in *heart and mind*, a mutant deficient for Na,K-ATPase $\alpha 1\text{B1}$ (also known as *atp1a1*) (Shu et al., 2003; Ellertsdottir et al., 2006) and *lost-a-fin*, a zebrafish mutant in the type I BMP receptor, *Alk8*, has cardiac looping defects but normal visceral laterality (Chen et al., 1997). Consistently, BMP4 signaling is required for cardiac, but not visceral laterality (Chocron et al., 2007). These findings suggest that the heart and the visceral organs respond to global LR signals independently. It has been noted that asymmetric BMP4 expression in the newly fused heart is required for proper placement and morphogenesis of the developing zebrafish heart (Chen et al., 1997). In an elegant cell tracing study, Smith et al. further demonstrate that BMP signaling regulates the direction of cardiac progenitor cell migration and thereby directs cardiac laterality (Smith et al., 2008), providing a mechanism by which one organ's laterality is specified. How the sodium pump directs visceral organ-specific laterality is worth pursuing and genetic and molecular dissection facilitated by the zebrafish model is likely to assist the investigation of mechanisms utilized by each organ to interpret global LR signals.

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Fig. 1.

Model for the roles of calcium in LR patterning. Proposed role of calcium in the mouse, zebrafish and chick are illustrated. Cilia-driven fluid flow has been proposed to generate asymmetric calcium fluxes on the left edge of the mouse node by triggering pkd-2 expressing mechanosensory cilia or by transporting morphogen-laden NVPs leftward. Calcium functions at different steps in zebrafish left-right axis patterning. Calcium fluxes in dorsal forerunner cells (DFC) prevent excessive nuclear localization of β -catenin and are necessary for their normal migration and coalescence into the Kupffer's vesicle (KV). Within KV, intracellular calcium levels, regulated by NCX4a and Na,K-ATPase $\alpha 2$, modulate CaMKII signaling to permit normal cilia beating. Asymmetric calcium fluxes have also been detected on the left side of KV. In the chick, asymmetric extracellular calcium is required for inducing Dll1 expression on the left side of Hensen's node and Nodal signaling in the LPM.