Aplnra/b Sequentially Regulate Organ Left-Right Patterning via Distinct Mechanisms

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Abstract

The G protein-coupled receptor APJ/Aplnr has been widely reported to be involved in heart and vascular development and disease, but whether it contributes to organ left-right patterning is largely unknown. Here, we show that in zebrafish, ahlra/b coordinates organ LR patterning in an apela/apln ligand-dependent manner using distinct mechanisms at different stages. During gastrulation and early somitogenesis, ahlra/b loss of function results in heart and liver LR asymmetry defects, accompanied by disturbed KV/cilia morphogenesis and disrupted left-sided Nodal/spaw expression in the LPM. In this process, only ahlra loss of function results in KV/cilia morphogenesis defect. In addition, only apela works as the early endogenous ligand to regulate KV morphogenesis, which then contributes to left-sided Nodal/spaw expression and subsequent organ LR patterning. The ahlra-apela cascade regulates KV morphogenesis by enhancing the expression of foxj1a, but not fgf8 or dnh9, during KV development. At the late somite stage, both ahlra and ahlrb contribute to the expression of lft1 in the trunk midline but do not regulate KV formation, and this role is possibly mediated by both endogenous ligands, apela and ahlra. In conclusion, our study is the first to identify a role for ahlra/b and their endogenous ligands apela/apln in LR patterning, and it clarifies the distinct roles of ahlra-apela and ahlra/b-apela/apln in orchestrating organ LR patterning.

Key words: ahlra/b, apela/apln, left-right patterning, spaw, midline

Introduction

G protein-coupled receptor (GPCR) APJ, being close to the angiotensin II (Ang II) receptor, was identified as an orphan GPCR[1]. It remained an orphan receptor until a 36-amino acid peptide apelin was discovered [2]. In cardiac development and disease, the function of apelin-APJ has been studied in many cases [3-6]. In apelin and APJ knockout (KO) mice, the sarcomeres of cardiomyocytes are impaired in isolated ventricular myocytes [3]. Apelin/APJ also shows a role in the sustainability and amplification of the cardiac response to stress [5] and in essential hypertension (EHT) [6]. Apelin-APJ plays a role in the
Cripto signaling pathway in mammalian cardiac myogenesis by extracellular signal-regulated kinase/p70S6 kinase [7]. In vascular diseases, the upregulation of apelin in the atherosclerosis of human coronary artery suggested apelin-APJ signaling contributes to coronary vasospasm [8], while conflicting evidence in KO studies has shown antagonistic and inducing roles of apelin-APJ signaling in atherosclerotic formation [9, 10]. All these data indicate that the role of apelin/APJ and its mechanism of action in mammals need to be clarified.

The zebrafish homologs of APJ, aplnra/b and their endogenous ligands apela/apln were identified recently [11-14]. Aplnra/b is involved in regulating gastrulation cell movement [14, 15] and heart [13, 16-18] and vasculature development [19-21], which motivated the study for aplnra/b and its ligands apela/apln in disease and embryonic development. In the zebrafish grinch (grn) mutant, aplnrb loss of function leads to a reduced myocardial progenitor cells (MPCs) via cell-autonomous way [13, 18]. Aplnra/b directly modulates Nodal/TGFβ signaling to determine heart progenitor cells in a another cell-non-autonomous fashion during gastrulation [16], as well as regulating progenitor movement through a G-protein signaling-independent manner in later stages [17]. Detailed analyses showed that overexpression of apln, though not loss of function, phenocopied the heart development defect of the grinch (grn)/or apela mutant [12, 13, 18]. These findings suggested that apln, apela and their receptor aplnra/b might play different roles in heart development, as well as the possibility that another receptor may exist. The ligand Apela but not the receptor Aplnr is expressed in human embryonic stem cells [12], suggesting the distinct roles of apln, apela and receptor aplnra/b in heart or other organ development. All these reports have shown the association between aplnr and the underlying complicated mechanisms during heart development. However, the role of aplnr in cardiovascular development is still not clear.

Left-right (LR) patterning is a fundamental process in early development, and most of its mechanisms are conserved in the animal kingdom [22,23]. In zebrafish, left-sided Nodal/Spaw in the lateral plate mesoderm (LPM) [24] is initiated and amplified by the Node flow in Kupffer’s vesicle (KV), which sequentially regulates organ LR patterning [25-29]. In addition to the central role of KV/cilia (or Node/cilia in mouse) in initiating asymmetric Nodal/Spaw, pegasus, nek8 and atp1a1a.1 regulate left-sided Nodal/Spaw expression pattern in a KV/cilia-independent manner [30-32], suggesting that the procedure of initiating and maintaining asymmetric Nodal/Spaw is intricate.

In zebrafish, aplnra/b are zygotic genes and are expressed after blastula stage [11, 18]. At the early gastrulation stage, aplnra loss of function leads to gastrulation movement defect [15] and heart progenitor decreasing [12, 13, 18]. More recently, apela was discovered to work as the ligand for aplnra/b to guide vascular precursor migrate to the midline [33]. During gastrulation, aplnra but not aplnrb is expressed in the cells near dorsal forerunner cells (DFCs) and in DFCs (Fig. S1), the progenitors of KV. At the somite stage, aplnra/b were expressed in the cells near the midline (Fig. S1 and [33]). Since the roles of DFCs/KV and the midline in LR asymmetry patterning are widely reported [24, 26, 28, 29], we hypothesized that aplnra/b might play a crucial role in LR asymmetry patterning. Here, we found that aplnra/b were involved in organ LR patterning via the ligands apela/apln at different developmental stages.

Results

The complementary roles of aplnra/b in organ LR asymmetry patterning

The aplnra/b expression pattern and the critical role in gastrulation cell movement [11, 14] led us to hypothesize aplnra/b play vital roles in LR patterning. To examine this hypothesis, we synthesized the antisense morpholino oligos for zebrafish aplnra/b (MOATG, Gene tools) to block the translation of aplnra/b [13, 18]. Since aplnrb loss of function leads to heart progenitors disappearing or decreasing greatly [13, 18] (Fig. S2 C), which inhibits the analysis of heart LR patterning in aplnrb morphants, we first examined the heart LR patterning in aplnra morphants (Fig. 1 B-D) and defective in looping in aplnra morphants (Fig. 1 B-D, O), but no distinct neural epithelium laterality was observed (Fig. 1 Q). At 72 hours post fertilization (hpf), the embryos displayed abnormal morphology, and the liver laterality was also disturbed in aplnra morphants (Fig. 1 F-G, P), displaying right-sided (Fig. 1 G, P) and both-sided liver (Fig. 1 F, P). Since apela and aplnrb are involved in regulating heart progenitor development in a redundant way [13, 16], we explored whether aplnrb also contributed to organ LR patterning. The experiments indicated that although aplnrbMO injection (500 µM) resulted in the heart disappearing, deformed embryos ([13, 18] and Fig. S2 C) and head asymmetry (Fig. 1 M, Q), which prevented an analysis of heart laterality, titrating the concentration of aplnrbMO to 100 µM gave rise to 18.2% and 17.5% of embryos displaying heart and
liver laterality defects, respectively (Fig. 1 O, P), without clearly deformed embryos (Fig. S2B). Furthermore, coinjecting *aplnra*MO (400 µM) and *aplnrb*MO (100 µM) resulted in 40.2% of embryos displaying a liver laterality defect (Fig. 1 H-J, P). This ratio was higher than that in *aplnra* morphants (Fig. 1 P, 27.3%). These data indicated the redundancy of *aplnra/b* in organ LR patterning. To confirm the specific role of *aplnra* in organ LR patterning, we tried to rescue the organ LR patterning defect by coinjecting *aplnra* MO and *aplnra* mRNA together into the embryos. After titrating the concentration of *aplnra* mRNA, we found that *aplnra* mRNA injection (20 ng/µl) partially restored the LR defect in *aplnra* morphants (Fig. 1 O, P).

The CRISPR/Cas9 system has been broadly used in gene editing in zebrafish [34-36]. The high editing efficiency gave us the chance to analyze the role of the *aplnra* gene in Founder(Go) zebrafish embryos [35, 37]. To further confirm the specific role of *aplnra* in LR patterning, we designed and synthesized 4 guide RNAs for *aplnra* in vitro and then coinjected them with Cas9 protein into the cytoplasm at the one-cell stage to edit the genome of *aplnra* gene. The results demonstrated that, when the *aplnra* gene was edited (Fig. S3), the heart and liver LR defect phenotype in these embryos was also observed (Fig. 1 P and Fig. S3), which further confirmed the role of *aplnra* in organ LR asymmetry patterning.

All the data above suggest the specific role of *aplnra* in LR patterning, *aplnra* and *aplnrb* regulate organ LR asymmetry patterning in a redundant way.

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**Figure 1. Organ left-right lateral defect in embryos treated with different ways.** (A-D, O) The pattern of heart displayed in controls and *aplnra/b* morphants. Compared with controls (A, 98.6%, n=72), embryos injected with *aplnra* MO displayed normal-loop (B, 62.5%, n=112; p<0.01), reversed-loop (C, 17.8%, n=112; p<0.01) and no loop (D, 19.6%, n=112; p<0.01). (O, column 1 to 4) The cartogram of heart LR defect for embryos treated with different MOs or mRNA. Only more than 18.2% of *aplnrb* morphants displayed heart LR defect (O, column 4, n=115; p<0.05). *aplnra* mRNA injection (O, column 3, 26.8%, n=71; p<0.05) partially rescued the heart LR defect in *aplnra* morphants (O, column 2, 37.5%, n=112). (E-J, P) While 97.3% of control embryos showed left-sided expression of *cp* (E, n=112), 18.2% (F, n=112; p<0.01), 9.1% (G, n=112, p<0.05) and 10.8% (H, n=112, p<0.05) of *aplnra* morphants showed both sided and right-sided expression of *cp*, or disappear (H-J, P). Compared with that in *aplnra* morphants, *aplnra* mRNA partially rescued liver LR defect (24%, n=121, p<0.05). In *aplnra/b* double morphants, the expression of *cp* was greatly downregulated (100%, n=195; p<0.001), and more embryos displayed liver LR defect (H-J, P, 51.3%, n=195; p<0.05) than that in *aplnra* morphants (P, 38.1%, n=176). In embryos injected with *aplnra* sgRNAs and Cas9 protein, 15.8% and 6.4% of them displayed right sided and both sided/middle liver (P, n=233, p<0.05). (K-N, Q). *otx5* was expressed in the middle telencephalon. All the embryos injected with Cont MO displayed the expression of *otx5* in the middle telencephalon (K, 100%, n=32, and Q, column 1). While 61.7% of embryos injected with *aplnrb* MO showed both-sided *otx5* in telencephalon (M, Q, n=34), only 5.5% of *aplnra* morphants displayed both-sided *otx5* (Q, 37). For observation, heart, ventral view; *cp*, *spaw* and *otx5*, dorsal view.
Asymmetric Nodal/spaw in the LPM is disturbed when aplnra/b is downregulated

The crucial role of Nodal/spaw in LR patterning was shown in previous studies [21-23]. Downregulation of Nodal/spaw leads to organ laterality defect [38]. To reveal how aplnra/b regulates organ laterality, first we examined the left-sided Nodal/spaw in embryos injected with aplnraMO. The left-sided Nodal/spaw was disturbed in aplnra morphants, displaying both-sided, right-sided and disappeared spaw expression patterns (Fig. 2 A). Since apnra and apnrb regulate liver laterality in a redundant way (Fig. 1 P), we continued to evaluate whether left-sided Nodal/spaw was affected in embryos injected with apnrbMO or apnraMO+apnrbMO. In most embryos injected with apnrbMO or apnra+bMO, the left-sided Nodal/spaw disappeared (Fig. 2 B, and Fig. S4). Furthermore, we sequentially checked lefty2, the downstream gene of spaw in apnra morphants, and found that the left-sided lefty2 expression pattern in the heart field was substantially downregulated (Fig. 2 C, D), which was consistent with that of spaw in apnra morphants. The substantial downregulation of lefty2 in the heart field coincided with the role of apnra in heart progenitor development, i.e., apnra loss of function substantially downregulates heart progenitor development [12, 16]. The data above suggest that the left-sided Nodal/spaw or lefty2 were not only disturbed but also downregulated in apnra morphants (Fig. 2; Fig. S4). To determine when the downregulation of Nodal signaling was initiated, we examined the expression of lefty1 and other Nodal-related genes at the gastrulation stage. The experiments indicated that the Nodal-related gene sox32, mdr2 and mxtx2 were downregulated at 70% epiboly (Fig. S4). These data demonstrate that, from the late gastrulation stage, Nodal-related genes started to be downregulated. At the somite stage, in addition to the downregulation of Nodal signaling, the left-sided Nodal/spaw and the downstream gene lefty2 were also randomized. These data suggest apnra/b signaling contributes to organ LR patterning via the Nodal/spaw signaling pathway.

Figure 2. Expression of left-sided Nodal signaling in LPM. (A. a1-a4, B) Expression of left-sided spaw in embryos. In control embryos, near all the embryos expressed left-sided spaw (A. a1, 91.4%, n=93). While in apnra and apnrb morphants, left-sided spaw expression pattern was changed (A. a2-a4, B), 13.6% (p>0.05), 7.8% and 47.6% (p<0.001) of apnra morphants expressed right-sided, both-sided spaw or spaw disappeared (B, middle-left column, n=103). 10.2% (p<0.05) and 6.1% (p>0.05) of apnrb morphants showed right-sided and both sided spaw (B, middle-right column, n=98), majority of apnrb morphants showed no staining for spaw (B, middle-right column, n=98, p<0.001). (C. c1-c4, D) The expression of left-sided lefty2 in the heart progenitor field. In the control embryos, lefty2 was expressed in the left side of heart progenitor field (C.c1, 94.2%, n=52). Lefty2 was down-regulated in apnra morphants (C. c2-c4), the left-sided expression pattern was also perturbed (C. c2-c4, D), with 9.0%, 6.3% and 22.5% of embryos showing right-sided, both-sided lefty2 and lefty2 disappeared, respectively (C. c2-c4, D, middle column, n=74). The lefty2 expression pattern in apnrb morphants (D, right column, n=93) was similar to that in apnra morphants (D, middle column, n=74), but more embryos had no staining for lefty2 in apnrb morphants (D, right column, n=93) than in apnra morphants (D, middle column, n=74). All the embryos were observed from dorsal view. L, Left; R, Right.
**Aplnra but not aplnrb dominantly regulates KV formation and ciliogenesis**

Previous studies and our current research indicated that aplnra but not aplnrb is expressed in the DFCs at the gastrulation stage ([11, 18] and Fig. S1). In zebrafish, DFCs will form the KV at the early somite stage, and defective KV morphogenesis or defective ciliogenesis (or functional defect) will give rise to disturbed left-sided Nodal/spaw and subsequent organ LR defect [24, 26, 27]. To determine whether aplnra/b regulated KV morphogenesis or ciliogenesis, we analyzed the KV/cilia development in aplnra or aplnrb morphants. Compared with control morphants, at the 10-12 somite stage, the KV was smaller in a majority of aplnra morphants (Fig. 3 A-D) while not in aplnrb morphants (Fig. 3 D). Further, we examined the cilia development and found that aplnra morphants displayed a slightly shorter cilia than control morphants (Fig. 3 F, I). The cilia numbers were also decreased in aplnra morphants (Fig. 3 F, H). In aplnrb morphants, no distinct difference in cilia length or cilia number was observed when compared with control morphants (Fig. 3 G, H, I). To assess the specific role of aplnra in KV formation and ciliogenesis, the aplnra mRNA was used to rescue the KV morphogenesis and ciliogenesis defects in aplnra morphants. The results showed that the KV morphogenesis and ciliogenesis defects in aplnra morphants were partially rescued by coinjecting with aplnra mRNA (Fig. 3 D, H, I). Since aplnra expression was also found in DFCs, we studied whether aplnra loss of function in DFCs would lead to KV morphogenesis defect. Indeed, injecting aplnra MO at 256-512 cell to specifically downregulate the function of aplnra resulted in a KV morphogenesis defect (Fig. S5). In addition, DFC-specific downregulation of aplnra resulted in randomized spaw and the sequential organ LR defect (Fig. S5). These data show that aplnra but not aplnrb dominantly regulates KV formation and ciliogenesis during the early somite stage.

![Figure 3. KV morphogenesis and Ciliogenesis in treated embryos.](image)

(A-D) In control, 91.3% of embryos showed normal KV (A, n=181), but 35.6% (p<0.001), 54.2% (p<0.001) and 10.2% (p<0.001) of embryos injected with aplnra MO showed normal KV, smaller KV and no KV, respectively (B, C and D, column 2, n=175); this kind of KV phenotype also can be found in aplnra+b morphants (D, column 4, n=213, p<0.001) or apela morphants (D, column 7, n=208, p<0.001), embryos injected with apela mRNA (D, column 8, n=153, p<0.001) or embryos injected with apln mRNA (D, column 6, n=135, p<0.001) . In aplnrb or apln morphants, no distinct phenotype about KV was discovered (D, column 3, n=147 and column 5, n=193). Injection of aplnra mRNA partially restored the KV phenotype in aplnra morphants (D, column 9, n=116, p<0.05). (E-I) In the KV, compared with that in control morphants (E and H, n=25), the cilia number is decreased in aplnra morphants (F and H, n=28, p<0.01), difference was also found about the length of cilia (F, I, n=12, p<0.05). In aplnrb morphants, only mild difference about cilia number and cilia length was observed (G, H, I, n=9, p>0.05). Co-injection of aplnra mRNA with aplnra MO in the embryos resulted that the number (G, H, n=26, p<0.05) or length of cilia (G, I, n=14, p<0.05) was closed to that in control. *, p<0.05; **, p<0.01; NS, not significant.
**Foxj1a is downregulated in aplnra morphants**

In zebrafish and mouse, the Node/KV plays an important role in initiating asymmetric Nodal/spaw in LPM [3, 29, 39-41]. At the gastrulation stage, the DFCs move together to the tailbud and then form the KV at the early somite stage [42, 43]. During this process, decreasing the DFC number or disturbing the critical signaling pathways, such as FGF or Wnt signaling, leads to deformed KV morphogenesis or a ciliogenesis defect [28, 44]. To determine how KV morphogenesis and ciliogenesis were disturbed in *apltnra* morphants, we checked whether the primordial cells of KV in *apltnra* morphants were intact at the late gastrulation stage. In zebrafish, sox17 is generally used as the marker of DFCs. First we examined the expression of sox17 under loss of function. *sox17* showed three kinds of expression pattern in *apltnra* morphants, 1) similar to that of control (Fig. 4 B), 2) scattered expression (Fig. 4 C) and 3) decreased expression (Fig. 4 D). By measuring the area of *sox17* expression, we confirmed that the area of *sox17* expression was slightly smaller than that in controls (Fig. 4 E e2, red group). However, the downregulation of *sox17* in DFCs was not found in *aplnrb* morphants (Fig. 4 E e3, pink group).

Fgf8, dnah9 and foxj1a are involved in KV formation and ciliogenesis [20, 28, 33, 39, 45]. *dnah9* also lies downstream of FGFR signaling to regulate ciliogenesis [39]. To study how *apltnra* regulates the development of KV and cilia, we examined the expression of fgf8 and its downstream genes *erm, dnah9* and *foxj1a*. At the 2-somite stage, in situ and Q-PCR experiments showed that there was no distinct difference in fgf8 (Fig. S6 A, C) or *dnah9* expression (Fig. S6 D, E) between *apltnra* morphants and control embryos. Interestingly *erm*, the downstream gene of fgf8, was upregulated in *apltnra* morphants (Fig. S6 B, C). Finally, we found that *foxj1a* was greatly downregulated (Fig. 4 F-H), which was also confirmed by analyzing the area of *foxj1a* expression (Fig. 4 I) and Q-PCR (Fig. 4 J). To examine whether *foxj1a* mediates the role of *apltnra* in KV/cilia development, we performed rescue experiments. The results indicated that transient expression of *foxj1a* by injecting *foxj1a* mRNA partially rescued the KV phenotype and the heart LR patterning defect in *apltnra* morphants. In *apltnra* morphants, 54.2% of embryos displayed smaller KV (Fig. S 7 B), while 78.6% of embryos coinjected with *apltnra*MO and *foxj1a* mRNA displayed slightly larger KV than that in embryos injected with *apltnra*MO (Fig. S7 C). For heart LR patterning, only 22.1% of embryos coinjected with *apltnra*MO and *foxj1a* mRNA displayed the LR patterning defect (Fig. S 7 E, F). This ratio was lower than that in *apltnra* morphants (Fig. 1 O, column 2, 37.5%). All these data demonstrate that *foxj1a* but not *fgf8* was downregulated in *apltnra* morphants and that *foxj1a* possibly at least partially mediated the role of *apltnra* to regulate KV morphogenesis and the subsequent organ LR patterning.

Our early data in this study showed that injection of *aplnrb*MO enhanced the organ LR patterning defect in *apltnra* morphants (Fig. 1 P, column 5). Although *aplnrb* loss of function did not lead to distinct defective KV and ciliogenesis (Fig. 3), we could not exclude the possibility that *aplnrb* was partially involved in helping *apltnra* to regulate the expression of cilia-related genes. Here, we examined this possibility, and the result indicated that no distinct difference in the expression of cilia-related genes was observed between *apltnrb* morphants and control morphants (Fig. 4 E I; Fig. S6). We also did not find a significant difference in the expression of cilia-related genes between *apltnra* morphants and *apltnra+b* morphants (Fig. 4 E E and Fig. S6). This result further confirmed that *aplnrb* contributes to organ LR patterning in a KV/cilia-independent way.

**Apela and apln regulate organ LR asymmetry**

The first identified endogenous ligand of *apltnrb* was apelin(*apln*) [18], the late ligand. More recently, *apela* was identified to work as the early endogenous ligand of *apltnrb* in heart development [12]. To evaluate whether *apela/apln* work as the ligands of *apltnrb* during LR patterning, we examined the expression patterns of *apela/apln* from gastrulation to somitogenesis. The results demonstrated that, starting from the bud stage, *apln* was expressed in the midline (Fig. S8 Aa3-a8) and heart progenitors (Fig. S8 Aa7, left arrow; a8, down arrow), while it was not expressed in the KV epithelium (Fig. S8 Aa6, the down black arrow). *Apela* was expressed ubiquitously at 75% epiboly (Fig. S8 Bb1), in the midline and presomite mesoderm (PSM) at the bud stage (Fig. S8 Bb2-3), in the midline and KV epithelium at the 4-somite stage (Fig. S8 Bb4-b5, white arrow), and in the midline and heart progenitors at the 15-somite stage (Fig. S8 Bb6-b8, arrow). These expression data demonstrate that the expression patterns of *apln/apela* are different at early developmental stages. If *apln/apela* work as the ligands of *apltnrb* during LR patterning, they might play different roles during this process. To evaluate this hypothesis, we continued to analyze the organ laterality in *apela* morphants and *apltn* morphants. In *apela* morphants, the majority of embryos had no heart or a very small heart (Fig. S9), consistent with a previous report [12]. This phenotype blocked the analysis of heart LR in *apela* morphants. At day 3, in situ staining for cp demonstrated that liver laterality was perturbed in *apela* morphants (Fig. 5
Aa2-a3), similar to the phenotype in aplnra morphants (Fig. 1 G,P). In addition, the liver LR patterning defect was also observed in Tg(fabp10:GFP) transgenic embryos injected with apela MO (Fig. 5 Bb2-b3). To confirm the general role of apela in LR patterning, otx5 was used to examine the head laterality(46, 47). We found otx5 was expressed in both sides of the head in major of apela morphants (Fig. 5 C), phenocopying aplnrb morphants (Fig. 1 M, Q). In apln morphants, the heart progenitors were not decreased, but the heart (Fig. 5 D, H and Fig. S10 A) and liver (Fig. 5 E,G and Fig. S10 B) LR patterning were disturbed. To further confirm the specific roles of apela/apln in organ LR patterning, we also used the CRISPR/Cas9 method to confirm the general role of apela/apln in LR patterning. In a previous report, as the ligand of aplnra/b, apela or apln gain of function led to decreased heart progenitor cell number [18], Here, apela or apln gain of function also resulted in a liver LR patterning defect (Fig. 5 F, G; and Fig. S10), implying apela/apln and the receptors aplnra/b are in the same cascade in LR patterning.

Given that apela/apln and aplnra/b work in the same cascade in LR patterning, simultaneously downregulating aplnra/b and their ligands apela/apln will result in a stronger LR defect phenotype than that in aplnra/b morphants or apela/apln morphants. Indeed, the liver LR asymmetry defect in apela morphants was enhanced by coinjecting with aplnra/b MO or aplnrb MO (Fig. 5 G column 6 and column 7), further confirming that apela/apln and the receptors aplnra/b were in the same cascade in LR patterning. Since apela was expressed from the early stage and apln was expressed in the midline from the late gastrulation stage [12, 18, 21] (and Fig. S8 Aa3-a8), it seems that the ligands apela and apln sequentially couple with aplnra/b to regulate LR patterning at different stages.

Figure 4. Expression of sox17 and foxj1a. (A-E) Expression of sox17 in DFCs at 90% epiboly. In control embryos, 89.5% of embryos showed normal expression (A, n=88). In aplnra morphants, three kinds of expression pattern were discovered: mild decreased (B, 52.4%, n=89, p<0.001), scattered (C, 21.3%, n=89, p<0.001) and decreased (D, 19.6%, n=89, p<0.01). The area of sox17 expression was measured and in control embryos, the average level was 3.68x10^3 uM² (E, e1 group, n=19), the area of sox17 expression was 2.55x10^3 uM² (E, e2 group, n=22, p<0.05). Sox17 expression areas were also measured for aplnrb morphants, aplnra/b morphants, apela morphants, embryos injected with apln mRNA and embryos injected with apln mRNA, respectively (E, e3-e7 group). The average areas were 3.63x10^3 uM² (E, e3 group, aplnrb morphants, n=15, p<0.05), 2.53x10^3 uM² (E, e7 group, aplnra/b morphants, n=15), 3.28x10^3 uM² (E, e4 group, apela morphants, n=15, p<0.05), 2.46x10^3 uM² (E, e5 group, apln mRNA, n=15, p<0.01) and 2.64x10^3 uM² (E, e6 group, apela mRNA, n=15, p<0.001); these data indicated sox17 expression was down regulated in all these treated embryos, except for aplnrb morphants. (F-J) Analysis of foxj1a expression. Foxj1a was expressed in DFCs at 90% epiboly in control (F) and treated embryos (G, H). Compared with control embryos (F, 80.5%, n=82), foxj1a was greatly down regulated in aplnra/b morphants (H, 68.9%, n=87, p<0.001) as well as in aplnra/b morphants (I, 65.7%, n=76, p<0.001), but not in aplnrb morphants (I, 18.9%, n=74, p>0.05). Foxj1a expression area in control embryos, aplnra/b morphants, and aplnra/b morphants were average 3.75 x10^3 uM², 2.56 x10^3 uM², 3.57 x10^3 uM² and 2.39 x10^3 uM² respectively (J). Compared with that in control embryos, q-PCR experiment showed the quantity of foxj1a expression in aplnra/b morphants and aplnra/b morphants were down-regulated with 0.48 folds and 0.46 folds respectively, while the expression of foxj1a in aplnrb morphants was not affected (J). *, p<0.05; **, p<0.01; ***, p<0.001; NS, not significant.
Apela loss of function depresses left-sided Nodal/spaw in the LPM in a KV-dependent way

Our current data showed that apela/apln worked as the ligands of aplnra/b to regulate organ LR patterning, but how they regulated LR patterning was unknown. The different expression patterns between apela and apln gave rise to the possibility that the mechanisms by which apela/apln regulate LR patterning are different. To evaluate this possibility, we examined the KV development and the expression pattern of Nodal/spaw. In apela morphants, the KV morphogenesis was affected, most of embryos displayed smaller KV (Fig. 6 Aa1-a3, B), similar to the phenotype in aplnra morphants (Fig. 3 B-D). In contrast, the defective KV formation was not found in apln morphants (Fig. 6 B). This result implied the possibility that apela but not apln works as the ligand of aplnra to regulate KV development and the downstream left-sided spaw in the LPM. Indeed, further experiments showed that left-sided spaw (Fig. 6 C.c2-c5, D) and lft2 (Fig. 6 E.e2-e4, F) were substantially downregulated, and the left-sided expression pattern of both of them was disturbed in apela morphants (Fig. 6 C,E), similar to that in aplnra morphants (Fig. 2). In apln morphants, although the left-sided expression patterns of spaw and lft2 were also disturbed (Fig. 6 D, F), neither of them was substantially downregulated.

Since apela was found to be expressed in KV epithelium, we analyzed whether apela loss of function in DFCs resulted in LR patterning defects. As shown in Fig. S5, the KV development and left-sided spaw expression were all disturbed, but spaw was not downregulated in embryos with apela loss of function in DFCs (Fig. S5 Aa4 and Dd4). Heart and liver LR patterning defect were also observed in these embryos (Fig. S5 Bb4 and Cc4). These data further demonstrate that apela is involved in LR patterning via a KV/cilia-dependent cascade.

Figure 5. Organ LR asymmetry defect in embryos with aplnr ligands loss or gain of function. (A, G) Majority of control embryos expressed left-sided liver marker cp in day 3 (A. a1, G. column 1, 90.2%, n=51). Liver LR asymmetry was disturbed in apela MO injected embryos (A. a2-a3, G. column 2, 30.6%, n=121, p<0.01) and apela mRNA injected embryos (G. column 3, 34.9%, n=103, p<0.01). In transgenic line Tg(fabp10:GFP), the liver LR defect was also observed, displayed left-sided and right-sided liver in 76.5% and 23.5% of apela morphants, respectively (B. b2 and b3, n=51), but the GFP in liver region started to express in day 5 (B. b2 and b3). (C) Head marker otx5 was expressed in the middle telencephalon (C. c1, H. column 1, 100%, n=32), but many of apela morphants (C. c2, H. column 2, 65.4%, n=26) and aplnrb morphants (H. column 3 61.7%, n=34) showed both-sided otx5 in telencephalon. (D, I) Apln loss of function resulted heart LR asymmetry defect (D. d2-d4, I), displayed reversed loop (D. d4, 24.7%, n=117, p<0.05), normal loop (D. d2, 58.9%, n=117, p<0.01) and linear heart (D. d3, 16.2%, n=117, p<0.05). (E. e1-e3, G) In apln morphants, liver development was not delayed (E), but 25.4% liver LR asymmetry defect was found (E. e2-e3, G. column 4, n=185, p<0.001). Apela mRNA or apln mRNA gain of function also resulted in liver LR asymmetry defect, 34.9% (p<0.01) and 37.2% (p<0.01) of embryos displayed right-sided or both-sided expression of cp (G. column 5, n=97). When compared with the liver LR defect in apela morphants (G. column 2, 30.6%, n=121), high ratio of embryos co-injected with apela MO and aplnra MO (G. column 6, 37.1%, n=70), or embryos co-injected with apela MO and aplnrb MO (G. column 7, 40.0%, n=91) showed liver LR asymmetry defect. In embryos injected with apela/apln sgRNAs with Cas9 protein, the livers also showed left right asymmetry defect (G. column 8 and 9). L, left; R, right; V, ventral view; D3, day 3; D5, day 5.
Since apinra regulated the expression of foxj1a and erm, if the ligand apela but not apin truly couples with apinra to regulate KV formation and ciliogenesis, the expression of foxj1a and erm in apela morphants should be similar to that in apinra morphants. Indeed, the expression of fgf8 (Fig. 7 A) and dnah9 (Fig. 7 C) was not affected in apela morphants, while erm (Fig. 7 B) and foxj1a (Fig. 7 D-F) were increased and decreased, respectively, similar to apinra morphants (Fig. 4 G-I). These data suggest that apela but not apin works as the ligand of apinra to regulate LR patterning in a KV-dependent way.

**Lft1 is downregulated in midline in both apela and apln morphants**

Our data above showed that apin was involved in regulating organ LR patterning and left-sided spaw and lft2, but this process was independent of KV morphogenesis and foxj1a expression. How does apin regulate organ LR patterning? Since midline defects lead to randomized expression of spaw and lft2 in a KV/cilia-independent manner [48], and apin is expressed in the midline from the late gastrulation stage to the somite stage [18, 21] (Fig. S8 Aa3-a8), so we hypothesized that apin might be involved in regulating midline formation or function. To evaluate this possibility, we examined whether apin loss of function affected midline formation. In apin morphants, no deformed midline was observed in the living embryos (data not shown), and no decreased expression of shh was discovered (Fig. 8 Aa3). We also did not find any distinct difference in the expression of shh among the apela morphants, apinra morphants, apinrb morphants and apinra+b double morphants (Fig. 8 Aa2-a6, shown by black arrow head). These data show that apin (including the early ligand apela) and their receptors are not involved in regulating the expression of shh, one of the critical genes in the midline to maintain the left-sided Nodal/spaw in LPM.

Since intact lft1 in the midline is vital to ensure left-sided spaw expression in the LPM and the later organ LR patterning, we examined whether the
expression of \( \text{lft1} \) was affected in \( \text{apln} \) morphants. At 20 SS, \( \text{lft1} \) was expressed in 4 domains in wild-type embryos, including the left telencephalon, left heart field, trunk midline and tail midline (Fig. 8 Bb1, shown by the black arrow head). In \( \text{apln} \) morphants, \( \text{lft1} \) in the trunk midline disappeared or decreased greatly in the majority of embryos (Fig. 8 Bb4-b6, class 3 to class 5; C, the second column). In the control morphants, the expression of \( \text{lft1} \) in the midline was intact (Fig. 8 Bb1, C, column 1). This result indicated that the expression of \( \text{lft1} \) in the trunk midline was decreased in \( \text{apln} \) morphants, implying that \( \text{lft1} \) in the midline mediates the effect of \( \text{apln} \) to regulate LR patterning.

Since \( \text{apela} \) is expressed in the midline and guides angioblasts to move to the midline [33], and its receptor \( \text{aplnra/b} \) is expressed close to that of \( \text{apln/apela} \), we supposed that \( \text{apela} \) and the receptors \( \text{aplnra/b} \) were also involved in regulating the expression of \( \text{lft1} \) in the trunk midline. Indeed, further experiments showed that the defective expression pattern of \( \text{lft1} \) was also found in \( \text{apela} \) morphants, \( \text{aplnra} \) morphants and \( \text{aplnrb} \) morphants (C, column 3 to 5). Nearly half of embryos showed greatly decreased \( \text{lft1} \) in the trunk midline in all the morphants. This result provided additional evidence that \( \text{apln} \) (and \( \text{apela} \)) works as the ligand of \( \text{aplnra/b} \) to regulate organ LR patterning at the somite stage. In summary, at the somite stage, \( \text{apln/apela-aplnra/b} \) are involved in the regulation of \( \text{lft1} \) in the trunk midline to maintain the midline function and the subsequent organ LR patterning.

**Discussion**

The complementary role of \( \text{aplnra} \) and \( \text{aplnrb} \) in organ LR patterning

In vertebrates, \( \text{aplnra} \) (\( \text{aplnra/b} \)) in zebrafish has multiple roles in organ development and diseases [5, 21, 39, 40, 49]. In mouse, \( \text{aplnra} \) regulates cardiac contractility [50], heart looping and vascular maturation [21]. In zebrafish, functions in regulating gastrulation cell movement [14], heart [16-18], angiogenesis [33] and lymphatic system development [19, 20] are also reported. While in regulating heart development, zebrafish \( \text{aplnra} \) and \( \text{aplnrb} \) play different roles via multiple mechanisms: In \( \text{aplnrb} \) mutants, most heart progenitors are absent [18], but in \( \text{aplnra} \) mutants or morphants, the heart progenitors are only decreased [12]. Similarly, the vasculogenesis defect in \( \text{aplnra} \) mutants is not as strong as that in \( \text{aplnrb} \) mutants, but knockdown of \( \text{aplnra} \) enhances the vasculogenesis defect in \( \text{aplnrb} \) mutants [33]. It is possible that the different roles of \( \text{aplnra/b} \) in heart development and angiogenesis come from the different expression patterns of \( \text{aplnra/b} \). In our current study, the data demonstrate the critical roles of \( \text{aplnra/b} \) in organ LR patterning (Fig. 1). During this process, \( \text{aplnra} \) and \( \text{aplnrb} \) have redundant roles during liver LR patterning (Fig. 1 H-J, P). Interestingly, the head laterality in \( \text{aplnrb} \) morphants was more severe than that in \( \text{aplnra} \) morphants (Fig. 1 K-Q), phenocopied that in \( \text{squint} \) or MZoep mutants [46]. This result was consistent with a more recent report that Nodal/TGFβ signaling is greatly downregulated in \( \text{aplnrb} \) mutants [16]. On the role of \( \text{aplnrb} \) in heart LR patterning, although \( \text{aplnrb} \) loss of function results in heart absence or heart size decrease in most of embryos (Fig. S2 Cc2-c4), we still believe the role of \( \text{aplnrb} \) in organ LR patterning is general for all organs, since the left-sided \( \text{spaw} \) was substantially downregulated or disturbed in \( \text{aplnrb} \) morphants (Fig. 2 B, Fig. S4 A, B).

By carefully analyzing the ratio of the heart and liver LR patterning defects in embryos injected with the same MOs, we found that the rates of the heart and liver LR defects were different (Fig. 1 and Fig. 5). Our early studies also found this kind of phenotype [38, 51]. Here, we wanted to know, in the embryos injected with \( \text{aplnra MO} \), whether the liver LR patterning was also defective in the embryos with a reversed heart loop. We sorted out the \( \text{Tg(cmIc2:GFP)} \) embryos with a reversed heart loop, incubated them to 5 days post fertilization, and then examined the liver LR patterning using **in situ** experiments (Fig. S12). The results demonstrated that, in the embryos with a reversed heart loop, 29.4% of embryos displayed right-sided liver (Fig. S12 Aa1 and a3), while in the embryos with a normal heart loop, 18.7% of embryos displayed left-sided liver (Fig. S12 Bb1 and b3). Clearly, this result indicates that the heart and liver LR defects do not always occur at the same time in the \( \text{aplnra} \) morphants. It also implies that the detailed mechanisms underlying heart and liver LR patterning are at least partially different.

One other interesting phenotype was that \( \text{aplnrb} \) morphants displayed liver and head laterality defects, but no KV morphogenesis defect. For this result, two kinds of data can explain how the organ LR patterning was affected in \( \text{aplnrb} \) morphants. First, although \( \text{aplnrb} \) loss of function did not lead to defective KV development, downregulated Nodal signaling at the gastrulation stage and the somitogenesis stage contributed to the organ LR patterning defect. This explanation is supported by our current data (Fig. 2 B, D and Fig. S4 A, B) and previous reports [16]. Second, at the somite stage, the downregulation of \( \text{lft1} \) in the midline also contributed to the organ LR patterning defect in \( \text{aplnrb} \) morphants (Fig. 8 B, C).
**Apnra regulates LR patterning in KV/cilia-dependent and -independent ways**

The mechanism by which organ LR laterality is established is complicated and needs more study [50, 52]. In zebrafish, two events are critical for LR asymmetric signaling initiating and maintenance: normal KV morphogenesis/ciliogenesis and an intact midline [47, 48, 53-56]. Our results first identify the dominant role of *apnra* in LR patterning (Fig. 1 A-H). In this mechanism, the left-sided *spaw* and *lft2* were perturbed (Fig. 2 A-D), which mediated the disturbed organ LR patterning in *apnra* morphants. Further data indicated that *foxi1a* in DFCs was downregulated in *apnra* morphants (Fig. 4 F-J), possibly mediating the role of *apnra* in regulating KV morphology and ciliogenesis (Fig. 3), the downstream left-sided *spaw*, *lft2* and the subsequent organ LR patterning (Fig. 2 A-D). Since *apnra* is expressed ubiquitously in the embryos from the dome stage to the late gastrulation stage [12, 14], and in the early somite stage it is also expressed near the midline [33], these data argue against the idea that *apnra* is involved in regulation of LR patterning in only a KV morphogenesis/ciliogenesis-dependent manner. Indeed, besides the perturbed left-sided *spaw* expression pattern in *apnra* morphants (Fig. 2 A), *spaw* was downregulated or disappeared in most *apnra* morphants (Fig. 2 B) meaning that *apnra* loss of function not only randomized the left-sided *spaw* in LPM but also depressed the expression of *spaw* in LPM. This result was consistent with the earlier report that Nodal and Nodal-related genes were downregulated in *apnra* mutants [16] (Fig. S4 C-F). In addition, our previous data showed that *spaw* loss of function resulted in randomized organ LR patterning [38], further supporting the hypothesis that *apnra* contributes to left-sided *spaw* expression and the sequential organ LR patterning via not only a KV/cilia cascade but also a KV/cilia-independent signaling pathway.

The expression of *apnra* in the midline at the somite stage encouraged us to investigate the role of *apnra* in midline development. There was no distinct morphogenesis defect in living *apnra* morphants, and the expression of *slh* in *apnra* morphants was intact (Fig. 8 Aa5), while the expression of *lft1* in trunk midline was substantially depressed (Fig. 8 B, C). These data suggest that *apnra*, besides contributing to KV and cilia development at an early stage, also regulates midline function by regulating the expression of *lft1* at the somite stage. In *apnra* morphants or mutant embryos, *lft1* was unaffected at the early gastrulation stage.
stage (50% epiboly) [37]. To assess when \( lft1 \) was downregulated in embryos with \( aplnra \) loss of function, we examined the expression of \( lft1 \) from the shield stage in \( aplnra \) morphants. The data indicated that \( lft1 \) was slightly downregulated from the shield stage, suggesting downregulation of \( lft1 \) in the midline at the somite stage comes from two separate time points: gastrulation and somitogenesis stage. In brief, \( aplnra \) contributes to organ LR patterning in KV/cilia-dependent and -independent ways.

**Apela but not apln works as the ligand to regulate KV formation and ciliogenesis**

Cardiovascular development defects have been reported in APJ KO mice, but not in Apelin KO mice [3, 21, 41]. Heart contractility defects and disturbed looping were also found in APJ KO mice, but these functional and morphgenic defects do not exist in Apelin KO mice, even though the Apelin KO mice obtained a heart contractility defect during aging and severe heart failure was observed in response to pressure overload [5]. These data from mice suggest the possibility that Apln regulates heart development in an apelin-independent manner. This possibility was also confirmed by data from zebrafish. In zebrafish, two endogenous ligands apela and apln were discovered [14, 18]. During heart development, \( aplnra/b \) contribute to heart progenitors specification and migration [13, 16, 18], while only loss of function for the ligand apela and not apln phenocopies \( aplnrb \) loss of function [16, 18]. Here, our data further show the specific role of apela but not apln in KV morphogenesis and ciliogenesis during LR patterning. During the gastrulation and early somite stages, apela loss of function resulted in KV development defect and disturbed ciliogenesis (Fig. 3 D, E, 6 A, B), which contributes to, at least partly, the downstream left-sided gene \( spaw \) expression defect (Fig. 6 C,D) and organ LR defect (Fig. 5 A-C). In this process, it is possible that the downregulated \( foxj1a \) dominantly contributes to the decreased KV progenitors and the smaller KV in apela morphants (Fig. 7 D-F). On the other hand, \( apln \) was mildly expressed from the late gastrulation stage, and downregulation of \( apln \) did not lead to abnormal KV morphogenesis (Fig. 3 D), heart or endoderm organ progenitor specification or outgrowth (Fig. 5 D, E and data not shown [18]). Of course, we cannot exclude the possibility that some unknown \( apln \) ligand is also involved in KV morphogenesis.

**Figure 8. Expression of midline related genes in different treated embryos.** (A. a1-a6) Shh expression at 24SS. Compared with that in control embryos (A. a1, 100%, n=31), no clearly decreased or increased expression of \( shh \) in midline was found in apela morphants (A. a2, 82.7%, n=29), \( aplnra/b \) double morphants (A. a4, 78.2%, n=23), \( aplnra \) morphants (A. a5, 85.1%, n=27) and \( aplnrb \) morphants (A. a6, 86.4%, n=22). In \( apln \) morphants, the expression of \( shh \) was slightly increased (A. a3, 67.9%, n=28). (B. b1-b6, C) Expression of \( lft1 \) at 20SS. In wild type embryos \( lft1 \) was expressed in 4 domains, including left telencephalon, left heart field, trunk midline and tail midline (B. b1, black arrow head, n=45), and in dorsal view, \( lft1 \) was found to be expressed in left telencephalon, left heart field, trunk midline (B. b2, black arrow head). In \( apln \) morphants, \( lft1 \) expression was found to be decreased with different phenotypes (B. b3-b6, n=85). Among all the \( apln \) morphants, \( lft1 \) in midline was disappeared or decreased greatly in more than half of embryos (B. b4-b6, class 3 to class 5; C, the second column, 64.7%, n=85, p<0.01). The expression pattern of \( lft1 \) in \( apln \) morphants was also found in apela morphants (C, column 3, 58.2%, n=79, p<0.01), \( aplnra \) morphants (C, column 4, 60%, n=75, p<0.01) and \( aplnrb \) morphants (C, column 5, 56.2%, n=89, p<0.01), near or more than half of embryos showed greatly decreased \( lft1 \) in the trunk midline.
Conclusion

Zebrafish G protein-coupled receptors aplnra/aplnrb were involved in organ LR patterning via an apela/apln ligand-dependent pathway. At the gastrulation and early somite stages, aplnra but not aplnrb was specifically involved in regulating KV/Cilia morphogenesis, coupled by the early ligand apela but not ligand apln. Thereafter, aplnra continued to regulate midline function by specifically regulating lft1 expression in the trunk midline, and this role also depended on the role of the ligands apela/apln. Although aplnrb was not involved in KV/cilia morphogenesis during the gastrulation stage, at the somite stage, aplnrb regulated lft1 expression in the trunk midline, left-sided spaw and lft2 expression in the LPM, and subsequent organ laterality in an apela/apln-dependent manner. Mechanistically, at the gastrulation and early somite stages, foxj1a potentially lies downstream of the Apela-Aplnra cascade to regulate KV morphogenesis and ciliogenesis. In summary, being coupled with the endogenous ligands apela and apln, aplnra sequentially orchestrates organ LR patterning in a KV/cilia-dependent and -independent manner at different stages.

Experimental procedures

Ethics statement

All the experimental protocols were approved by Chengdu Medical College (Sichuan, China) and QMRC, University of Edinburgh. Zebrafish were maintained in accordance with the Guidelines of Experimental Animal Welfare from Ministry of Science and Technology of People’s Republic of China (2006) and the Guidelines of Experimental Animal Welfare from Home office in UK.

Zebrafish

Zebrafish (Danio rerio) of the AB genetic background (wild type), Tg(fabp10:GFP) (57) and Tg(cmlc2:GFP) (58) lines were raised and maintained at 28.5 °C, and staged by hours post-fertilization (hpf) at 25-25 °C, and applied to knock down aplnra (aplnra MO, 5'-TGATTCCACAGTGGCCTCCATTG-3', 400 uM) (13); aplnrb (aplnrb MO, 5'-CAGAGAAGTTGTTTGTCATGTGCTC-3', 500uM, or 100uM for low concentration) (13, 18), apela (apela MO, 5'-TGGAGAAGTGGTTGTGTCATGTGCTC-3', 200uM) and apln (apln MO, 5'-AACAGCCGTCAGCCGACTTAC-3', 300 uM) (13), the plasmids used for synthesizing apela mRNA and apln mRNA were gifts from Ian C Scott lab. aplnra mRNA, aplnrb mRNA, apela mRNA and apln mRNA were synthesized in vitro using mMESSAGE mACHINE Kit (AM1340, Ambion). The concentration for mRNA injection was as the following: aplnra mRNA (for rescue experiment), 20ng/ul; aplnrb mRNA, 50ng/ul; apela mRNA, 50ng/ul; foxj1a mRNA, 10ng/ul. All the MOs or mRNAs were injected at 1-4 cell stage to downregulate the expression of target genes or to rescue/overexpress the target genes in whole embryos. For specific knockdown for the target genes (aplnra or apela) in DFCs, the MO was injected into the yolk at 256-512 cell stage.

Whole Mount In Situ Hybridization (WISH)

Whole Mount In Situ Hybridization (WISH) was performed according to the established methods (38) using the established antisense probe otx5, apela, aplnra, aplnrb, apln, spaw, sox17, lft1, lft2, shh, fgf8 and erm (12, 13, 25, 60). To prepare the antisense probe of cp, foxj1a, sox32, vmhc and dnh9, we applied the cDNA (prepared from day 3 embryos and 10 hpf embryos) to amplify the template by PCR, then synthesized the antisense probe according to the Kit manual. The embryos for in situ experiments were dechorionated and fixed in 4% PFA overnight at 4 °C, then washed with PBS (PBS-Tween, 0.1%), dehydrated with MeOH(100%) and stored in MeOH at −20 °C at least for 24 hours.

Antibody staining

The embryos were fixed in 4% PFA at 4 °C for overnight and then washed with PBST (5 minutes/times, 3 times) and 100% methanol 3 times, stored at -20 °C for at least overnight. Embryos were incubated with antibodies against alpha-tubulin (1:300; Sigma, 6793), then incubated at 4 °C overnight with Alexa fluorescent-conjugated secondary antibodies (1:1000; Invitrogen) which was diluted in the blocking solution. After washed with the PBST, embryos were preceded for mounting and imaging.

Quantitative PCR (Q-PCR)

The cDNA template synthesis was performed in vitro using RT kit (Fermantas). Quantitative PCRs were performed for fgf8, erm and foxj1a. The following primers were used: fgf8 (5-GAGGCCTATAACATGAGACTCATAC-3, 5-GAACCTGCATCTCCAAATGTGTC-3); erm (5-GTGAAGAAGACAGCTGAGATG-3, 5-GAGTCTCTGCTCTTGTCCACATG-3), foxj1a (5-CTATCGAGAGAAAGGAGAATTTG-3, 5-CTGCTGGCATGCTTATAGACGC-3), mxtx2 (5-CAGCACAATGCGATGCGACA-3, 5-GTCAGTTGAGCAGCACGTCG-3), ndnr2 (5-CTGCTACTGCCAGTGCTGCTG-3), sox32 (5-CTGCTACTGCCAGTGCTGCTG-3, 5-CTGCTACTGCCAGTGCTGCTG-3).
(5-CAGCATGTATCTCGACCGATG-3; 5-GTGTTGCGTCCTTG-3; 5-GAATAGGCCACCCGAGTCTG-3). Transcription of beta-actin (5-CATGGAGAGGAAATCGCTGCC-3, 5-GCTCAGGATACCTCTCTTGCTC-3) was used for normalization.

SgRNAs preparing and Cas9 protein co-injection

Four sgRNAs for aplnra, apln and apela were designed according to previous report (61). The individual sgRNA templates for each gene were prepared and then pooled to synthesize the sgRNAs in vitro (NEB #E2040S). The prepared sgRNAs were mixed with Cas9 protein (NEB, #M0646T) (The final concentration for sgRNAs and Cas9 protein were 4uM and 600ng/ul, respectively), and co-injected into the cytoplasm at the one cell stage. For one pool of embryos injected, part of the injected embryos were raised to 27 hpf for analyzing whether the heart cells were decreased greatly, and part of the sorted embryos were used to check genome editing efficiency using PCR. While the remaining embryos injected were raised to stages needed for LR asymmetric phenotype analysis. The sgRNAs targeted for each genes were as: aplnra sgRNA1: ACGGGAATGAGAGAGTAAGA, aplnra sgRNA2: TTTCCAAATGGACCAACGT, aplnra sgRNA3: CGCCGTTCCCGGAAACCG, aplnra sgRNA4: TGTGGCGCGCGAAATCAAAT; apln sgRNA1: CTGGGGAGAGGAGGGAAA, apln sgRNA2: GACTGGCAGGGAAACGGA, apln sgRNA3: CGCTGGTGATTGTGCTGG, apln sgRNA4: AGCGTGACGGCTGTTGCC; apela sgRNA1: AGCAGCAGATACAGCGGG, apela sgRNA2: CAGCAGCAGCAGATACAG, apela sgRNA3: AGACTCGACTCTCCCTAC, apela sgRNA4: GTGATGCTCAGGGTGGTT. The specific primers for checking genome editing efficiency were as the following: aplnra_F: 5_CTGCTCAAGAAGGACTCAAAGCC_3, aplnra_R: 5_GTGGACGATGGCGAGGTAG_3; apln_F: 5_CGCACTGAAGAAGCAAACAGTC_3, apln_R: 5_CATGCAGAAGTCGGCAAGTAATT_3; apela_F: 5_GGATTTCTACAGTCCGTAC_3, apela_R: 5_CTCGAATCGTTTGCCTCATG_3.

Microscope

To examine the KV morphogenesis, the heart and liver of the living embryos in transgenic lines, the embryos were laid in the proper holes made in the Agar plate (1.5% of Low Melting-point Agar (LMP, Sigma)) or directly laid on the Agar plate. In situ hybridized, immunostained embryos were mounted in the 100% glycerol for imaging. Images for living embryos and for in situ experiments were captured at room temperature using AxioVision4 software (Carl Zeiss, Inc.). Cilia images were captured by using LAS AF software (Leica), a x20/0.70 N.A. HC Plan Apochromat oil objective (Leica).

Abbreviations

hpf: hours post fertilization; dpf: days post-fertilization; WISH: whole-mount in situ hybridization; qPCR: quantitative PCR; LR patterning: left-right patterning; GPCP: G protein coupled receptor; MPCs: myocardial progenitor cells; DFCs: dorsal forerunner cells; KV: Kupffer's vesicle; LPM: lateral plate mesoderm; KO: Knock Out.

Supplementary Material

Supplementary figures. http://www.ijbs.com/v15p1225s1.pdf

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Author contributions statement

S.H, C.Z, Z.G designed the experiments. C.Z., Y.Z, M.L, ZG, and S.H performed the experiments, K.C, Y.W, M.Y, X.Z, H.Z, H.T, M. L, C. L, B. S, Y. F, S. L and X.Y discussed the results and commented on the manuscript, S.H, C.Z, Z.G, F.Y, Y.W wrote the manuscript.

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Competing Interests

The authors have declared that no competing interest exists.

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