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Multiple left-right asymmetry defects in *Shh*$^{-/-}$ mutant mice unveil a convergence of the Shh and retinoic acid pathways in the control of *Lefty-1*

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ABSTRACT **Asymmetric expression of *Sonic hedgehog* (*Shh*) in Hensen's node of the chicken embryo plays a key role in the genetic cascade that controls left-right asymmetry, but its involvement in left-right specification in other vertebrates remains unclear. We show that mouse embryos lacking *Shh* display a variety of laterality defects, including pulmonary left isomerism, alterations of heart looping, and randomization of axial turning. Expression of the left-specific gene *Lefty-1* is absent in *Shh*$^{-/-}$ embryos, suggesting that the observed laterality defects could be the result of the lack of *Lefty-1*. We also demonstrate that retinoic acid (RA) controls *Lefty-1* expression in a pathway downstream or parallel to *Shh*. Further, we provide evidence that RA controls left-right development across vertebrate species. Thus, the roles of *Shh* and RA in left-right specification indeed are conserved among vertebrates, and the *Shh* and RA pathways converge in the control of *Lefty-1*.**

A model of left-right determination involving a complex cascade of genetic interactions has emerged recently, based on results obtained from a variety of vertebrates (1). In the chicken embryo, for example, the transforming growth factor β (TGF-β) member activin βB, expressed on the right side of Hensen's node at stages 3-5+, restricts expression of *Sonic hedgehog* (*Shh*) to the left side of the node (2). Subsequently, *Shh* induces *Nodal* (another gene encoding a TGF-β factor) in the left lateral plate mesoderm (LPM; ref. 2). *Nodal*, in turn, induces the expression in the left LPM of the bicoid-type homeobox gene *Pitx2*, which encodes a transcription factor recently shown to mediate *situs* specific organ morphogenesis in different vertebrates (1, 3). Two related genes, *Lefty-1* and *Lefty-2*, also encode TGF-β factors that play important roles in left-right development. In the mouse, *Lefty-1* is predominantly expressed in the left prospective floor plate, whereas *Lefty-2* is expressed more strongly in the left LPM. In *Lefty-1*$^{-/-}$ mouse embryos, *Nodal*, *Lefty-2*, and *Pitx2* are ectopically expressed on the right side (4). It has been proposed that *Lefty-1* acts as (or induces) a midline barrier that prevents induction of *Nodal*, *Lefty-2*, and *Pitx2* on the right side.

Shh activity in the left side of the chicken node has been shown to be both necessary and sufficient to induce *Nodal* expression in the left LPM of the embryo (5). Interestingly, asymmetric expression of *Shh* has not been reported in mouse or *Xenopus*, and the original description of the phenotype of *Shh*$^{-/-}$ mutant embryos did not report laterality defects (6). This raised doubts about the existence of a *Shh*-dependent left-right pathway conserved among vertebrates.

Another important factor known to be involved in left-right determination in vertebrates is vitamin A. This vitamin plays numerous roles during embryogenesis (7), affecting cell fate, differentiation, growth, and axis formation. Animals convert ingested vitamin A to the active derivative retinoic acid (RA) in reactions catalyzed by enzymes such as retinaldehyde dehydrogenase-2 (Raldh2; refs. 8 and 9). There are other enzymes that synthesize RA but, at least in the mouse, Raldh2 appears to be the primary enzyme that catalyzes the conversion of vitamin A to RA (10, 11). Exogenous RA can induce laterality defects in mouse, hamster, and rat (12), and both RA excess and deficiency cause *situs inversus* of the heart in quail and chicken embryos (13, 14). These results indicate that RA is required for the normal specification of heart left-right asymmetry. However, the mechanism by which RA does so is not known.

Here we show that *Shh* is required for normal left-right development in the mouse, and that RA is required for proper left-right development in mouse, zebrafish, *Xenopus*, and chicken embryos. The *Shh* and RA pathways converge in the control of the transcription of the *Lefty-1* gene, and laterality defects caused by an excess or deficiency of *Shh* and RA are likely to be mediated by the effects of those factors on the expression of *Lefty-1*. Our results provide a molecular link between two pathways that play key roles in the establishment of left-right asymmetry in vertebrates.

MATERIALS AND METHODS

Mice. *Shh*$^{-/-}$ mice (6) were kindly provided by Phil Beachy, Johns Hopkins University, Baltimore. Mouse embryos at head fold stage were collected and cultured as described (15). All-trans RA (Sigma) and retinoid antagonist AGN 193109 (synthesized at Allergan; ref. 16) were added at 10^{-7} M to the culture medium. *In situ* hybridization was performed as described (17). To avoid cross-hybridization, 3′ specific untranslated probes were used for the mouse *Lefty-1* and *Lefty-2* and

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Abbreviations: E(*n*), embryonic day; LPM, lateral plate mesoderm; RA, retinoic acid; Raldh2, retinaldehyde dehydrogenase-2; Shh, Sonic hedgehog.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AF179483 for chicken *Lefty-1*, AF181680 for chicken *Raldh2*, and AF181681 for zebrafish *Pitx2*).

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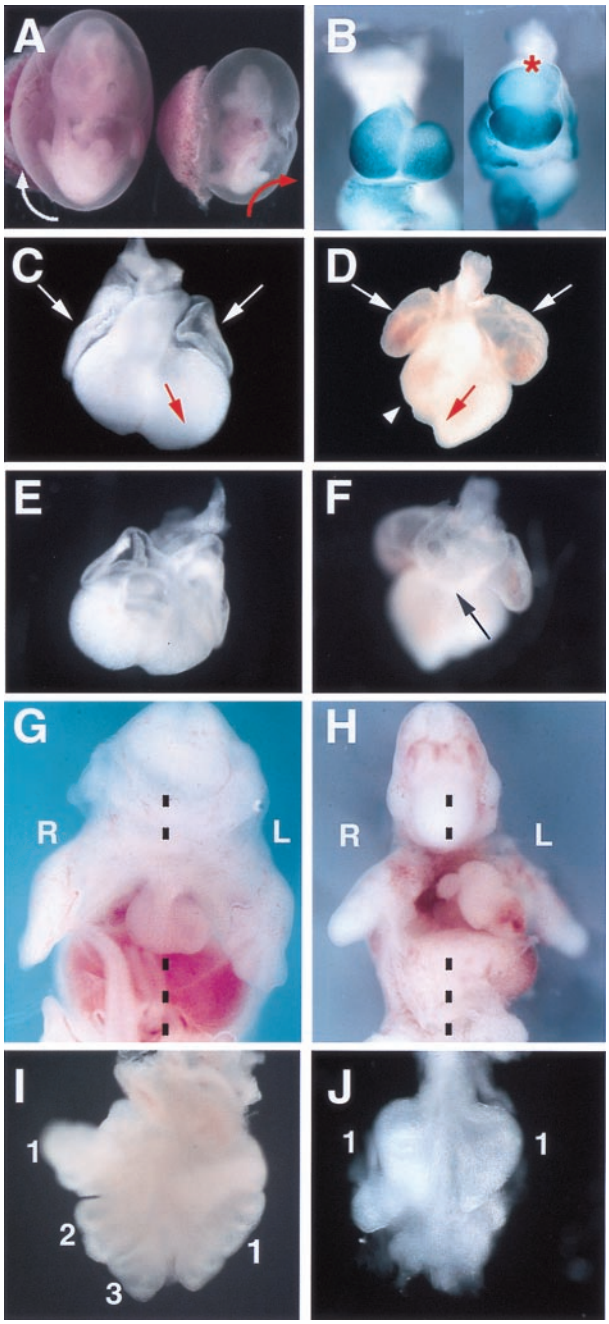

FIG. 1. *Shh*$^{-/-}$ embryos show multiple laterality defects. Shh$^{+/-}$ embryos (*C, E, G,* and *I*) are indistinguishable from wild-type ones and from now on will be referred to as such. (*A*) *Shh*$^{-/-}$ embryos (*Right*) display randomization of axial turning (red arrow indicates reversed turning of the tail, which points to the left side of the body in the mutant; compare with the *Shh*$^{+/-}$ littermate on the left, where the tail points to the right). (*B*, ventral views) Whole-mount *in situ* hybridization using a probe to detect the ventricular marker *MLC-2V* shows a dilated conotruncus (red asterisk) and failure to complete rotation in the *Shh*$^{-/-}$ heart (compare with the normal heart on the left). (*C* and *D*, ventral views) *Shh*$^{-/-}$ hearts display a severe reduction of the right ventricle (arrowhead in the mutant heart shown in *D*) and enlarged atriae (arrows in *C* and *D*). *Shh*$^{-/-}$ hearts display an enlarged apex that points toward the right side (red arrow in *D*, compare with the normal apex pointing toward the left side in the normal heart shown in *C*). (*E* and *F*, dorsal views) Wild-type (*E*) and *Shh*$^{-/-}$ (*F*) heart, where the *Shh*$^{-/-}$ heart has an abnormally enlarged inflow tract (arrow in *F*). (*G* and *H*) The heart of E12.5 wild-type mice normally is positioned in the midline (*G*). In *Shh* mutant mice, the heart is positioned toward the left side of the thoracic cavity (*H*). R, right; L, left. Broken lines indicate the axes of the embryos. (*I* and *J*) In contrast to the wild-type pattern (*I*), which is four lobes on the right and one on the left (the fourth right lobe is not visible in *I*), the lungs of *Shh*$^{-/-}$ mice display a single hypoplastic lobe on each side (*J*; only the portions marked with 1 are lung tissue).

for the chicken *Lefty-1* genes. The rest of the probes were produced as described (3, 18).

Retroviral Infections and Bead Implantation. Chicken embryos were grown *in vitro* as described (19, 20). *RCAS* retroviral stocks containing full-length chicken *Lefty-1* or *Raldh2* were produced and used as described (3). Beads were soaked in Shh protein, a blocking anti-Shh antibody (5), all-trans RA, or retinoid antagonist as described (5).

Cloning of Chicken *Lefty-1* and *Raldh2* and Zebrafish *Pitx2* Genes. Cloning of the chicken *Lefty-1* gene was as described (31). The *Raldh2* gene was isolated by screening a stage 22 chicken limb library with a PCR fragment kindly provided by Ramón Merino and Juan Hurlé (Universidad de Cantabria, Spain). The zebrafish *Pitx2* gene was isolated by screening a 24-hr embryonic library with a PCR fragment obtained from zebrafish cDNA. The chicken *Lefty-1*, *Raldh2*, and zebrafish *Pitx2* sequences have been deposited in Genbank under accession numbers AF179483, AF181680, and AF181681, respectively.

RESULTS

Multiple Laterality Defects in *Shh*$^{-/-}$ Embryos. We examined the phenotype of a total of 22 *Shh*$^{-/-}$ embryos ranging from embryonic day (E) 8.5 to E12.5. In *Shh*$^{-/-}$ embryos the tail points to the left side of the body, which indicates randomized turning of the body axis [Fig. 1*A* (*Right*) compare with the *Shh*$^{+/-}$ littermate (*Left*), where the tail points to the right side]. Also, as shown by *in situ* hybridization with a ventricular marker, *MLC-2V* (21), mutant E10.5 embryos display a dilated conotruncus and deficient cardiac rotation (Fig. 1*B*; red asterisk indicates the conotruncus in the mutant, on the right; compare with the *Shh*$^{+/-}$ heart on the left). Severe reduction of the right ventricle (arrowhead in Fig. 1*D*), a prolonged left ventricle, and an extended apex pointing toward the right (red arrow in Fig. 1*D*, compare with 1*C*) also are observed. Mutant E11 hearts display dilated inflow tract (arrow in Fig. 1*F*) and atrial defects that consist either in hypermorphic atria or a single unseptated common atria (compare with *Shh*$^{+/-}$ heart in Fig. 1*E*). Although we did not detect alterations in the direction of cardiac looping, *Shh*$^{-/-}$ hearts display an incomplete and delayed looping with impaired dorsal/right rotation. As a result, a failure in ventricular alignment occurs. In addition, in *Shh*$^{-/-}$ mutants the heart is positioned on the left side of the thorax (Fig. 1*H*), instead of displaying the normal midline placement (indicated by the dotted line in Fig. 1*G*; E12.5 embryos). In wild-type and *Shh*$^{+/-}$ mice the right lung has four lobes and the left lung has only one (Fig. 1*I*). *Shh*$^{-/-}$ embryos have bilaterally monolobed lungs that are severely hypoplastic, and branching morphogenesis is severely impaired (Fig. 1*J*; ref. 22).

We next analyzed the expression of the left-specific genes *Lefty-1*, *Lefty-2*, *Nodal*, and *Pitx2* in the *Shh*$^{-/-}$ embryos. *Lefty-1* expression is completely absent from its normal domains in the left prospective floor plate and left LPM in *Shh*$^{-/-}$ embryos (eight embryos of eight analyzed; Fig. 2*B*, compare with 2*A*). In contrast, in addition to their normal domains of expression in the left LPM, *Lefty-2* (6/14; Fig. 2 *C* and *D*), *Nodal* (8/11; Fig. 2 *E* and *F*), and *Pitx2* (8/9; Fig. 2 *G* and *H*) are all ectopically induced in the right LPM (red arrows in Fig. 2 *D*, *F*, and *H*; black arrows indicate normal expression in left LPM). Expression of these three genes was stronger in the left (normal domain) than in the right LPM (ectopic domain) of the *Shh*$^{-/-}$ embryos, and the ectopic patches of expression usually were restricted to the anterior part of the right LPM,

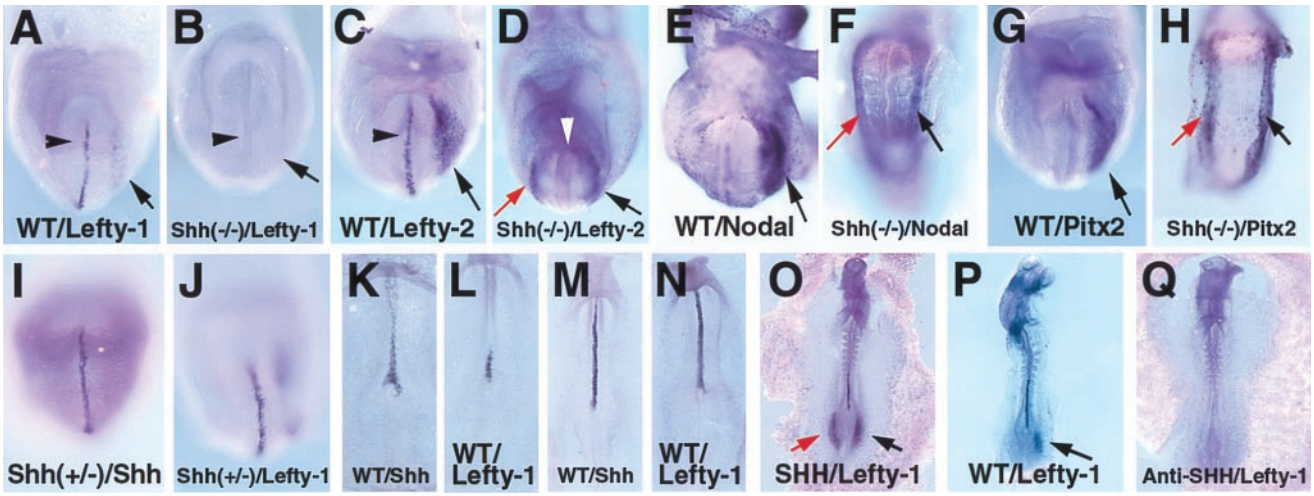

FIG. 2. *Shh* controls *Lefty-1* expression. All embryos are viewed from the ventral side (left side of the embryo corresponds to the reader's right). Phenotypes or experimental manipulations, followed by the probe used for *in situ*, are shown at the bottom of each panel, unless otherwise indicated. (*A-H*) Expression of *Lefty-1*, *Lefty-2*, *Nodal,* and *Pitx2* in E8–8.5 $Shh^{-/-}$ embryos. (*A* and *B*) *Lefty-1* normally is expressed on the left side of the presumptive floor plate (arrowhead in *A*) and weakly on the left LPM (arrow in *A*). In $Shh^{-/-}$ mice (*B*), expression of *Lefty-1* is not detected, neither in the midline (arrowhead) nor the left LPM (arrow). (*C* and *D*) *Lefty-2* also is expressed in the midline (arrowhead) and more strongly on the left LPM (arrow) of wild-type embryos (*C*). In $Shh^{-/-}$ embryos, *Lefty-2* expression in the midline is not detected (white arrowhead), whereas an ectopic induction on the right LPM (red arrow) is observed (*D*; black arrow shows normal expression in the left LPM). (*E and F*) *Nodal*, normally expressed on the left LPM (arrow in *E*), is ectopically induced on the right LPM of $Shh^{-/-}$ embryos (red arrow in *F*). (*G* and *H*) Similarly, *Pitx2*, normally expressed on the left LPM (arrow in *G*) is ectopically induced on the right LPM in $Shh^{-/-}$ embryos (red arrow in *H*). (*I-J*) In the mouse, *Shh* is expressed symmetrically in the head process at E7.5-E8.5 (*I*). *Lefty-1* transcripts are localized on the left side of the perspective floor plate (*J*). (*K* and *L*) In the chick, asymmetric expression of *Shh* on the left side of the node at stage 5+ coincides with the initial asymmetric expression of *Lefty-1*. (*M* and *N*) In subsequent stages (7 to 9), *Lefty-1* and *Shh* transcripts overlap in a symmetrical pattern in the notochord of the chicken embryo. (*O-Q*) Shh beads implanted on the right side of the chicken node at stage 6 induce an ectopic patch of *Lefty-1* expression that is observed on the posterior right LPM (red arrow in *O*; black arrow shows normal expression in the posterior left LPM). When beads soaked in a blocking anti-Shh antibody are implanted on the left side of a stage 6 chicken embryo, *Lefty-1* expression is neither detected in the midline nor in the posterior left LPM (*Q*, compare with the normal pattern in *P*). WT, wild type.

always appearing after the normal left LPM expression. Expression of *Lefty-2* transcripts was absent in the midline of the $Shh^{-/-}$ mutant embryos (white arrowhead in Fig. 2*D*, black arrowhead in Fig. 2*C* indicates normal *Lefty-2* expression in the midline).

Some of the morphological alterations observed in $Shh^{-/-}$ embryos, including pulmonary left isomerism, resemble those described in $Lefty\text{-}1^{-/-}$ mutant mice (4), where delayed bilateral expression of *Nodal*, *Lefty-2*, and *Pitx2* in the LPM also was reported. Thus, our results suggest a possible genetic interaction between the *Shh* and *Lefty-1* pathways.

Shh Controls *Lefty-1* Expression. *Shh* and *Lefty-1* genes are expressed in similar domains of the midline in mouse embryos (Fig. 2 *I* and *J*; expression in $Shh^{+/-}$ embryos is indistinguishable from the wild type). We also cloned the chicken homologue of the *Lefty-1* gene and analyzed its pattern of expression. At stage 5+ (the stage at which *Shh* becomes asymmetrically expressed in the left side of the chicken Hensen's node, ref. 2), *Lefty-1* transcripts also become asymmetric and overlap with *Shh* (Fig. 2 *K* and *L*). Afterward, and coinciding with the disappearance of the asymmetric expression of *Shh* in the node, *Lefty-1* transcripts become symmetrically expressed in the notochord, where they overlap with *Shh* (Fig. 2 *M* and *N*; ref. 2 and data not shown). At stage 10 chicken *Lefty-1* transcripts no longer are detected in the midline, but a patch of expression is detected in the posterior left LPM between stages 8 and 11.

We further explored the relationship between *Shh* and *Lefty-1* by performing experimental manipulations in chicken embryos. *In situ* hybridization 6–12 hr after implantation of beads soaked in Shh protein on the right side of the chicken node at stages 5–7 indicated that Shh is able to induce *Lefty-1*, as revealed by a ectopic patch of *Lefty-1* expression in the posterior right LPM (observed in 33% of the embryos; red arrow in Fig. 2*O*, compare with wild-type embryo in Fig. 2*P*). Furthermore, blocking *Shh* signaling by implanting beads soaked in a blocking anti-Shh antibody on the left side results in repression of *Lefty-1* expression in the midline (observed in 52% of the embryos; Fig. 2*Q*). *Shh* is unaffected in $Lefty\text{-}1^{-/-}$ mouse embryos (4), and we also have confirmed that *Lefty-1* misexpression in the chicken embryo does not affect *Shh* transcription (data not shown). Taken together, these results show that *Shh* is an upstream regulator of *Lefty-1* in mouse and chicken embryos.

Conserved Role of RA in Controlling Left-Right Development Among Vertebrates. We decided to study the effects of excess and deficiency of RA activity in four different vertebrates. As a reporter of alteration of gene expression we monitored the left-specific gene *Pitx2*, because it is induced by *Nodal* and normally is detected subsequent to *Nodal* expression in the left LPM of mouse, zebrafish, *Xenopus*, and chicken embryos (Fig. 3 *B*, *E*, *H*, and *L*; refs. 1 and 3). Furthermore, *Pitx2* misexpression alters heart *situs* and looping and alters the *situs* of other organs in mouse, *Xenopus*, and chicken (1, 3).

Down- or up-regulation of *Pitx2* is observed after RA antagonist (10^{-7} M) or RA (10^{-7} M) treatment, respectively, of mouse (4–6 hr at the head fold stage; Fig. 3 *A-C*), zebrafish (1 hr at 50% epiboly; Fig. 3 *D-F*), and *Xenopus* (1 hr at stage 8; Fig. 3 *G-J*) embryos. In the chick, application of a bead soaked in RA antagonist (10^{-7} M) to the left side of Hensen's node at stages 5–7 abolishes endogenous *Nodal* (data not shown) and *Pitx2* expression (Fig. 3*K*, compare with Fig. 3*L*) and results in randomization of heart looping (41% of treated embryos have *situs inversus*, Fig. 3*N*, compare with normal heart *situs* in Fig. 3*O*). Application of a bead soaked in RA (10^{-6} M) to the right side of the node induces ectopic expression of *Nodal* (data not shown; ref. 23) and *Pitx2* (red arrow in Fig. 3*M*, compare with normal expression in Fig. 3*L*). This results in 43% of treated embryos having *situs inversus* (Fig. 3*P*, compare with Fig. 3*O*). Our data, along with the

FIG. 3. RA alters *Lefty* and *Pitx2* gene expression. (*A–C*, ventral views) Treatment of mouse embryos for 4–6 hr with the retinoic antagonist AGN 193109 (10^{-7} M) down-regulates *Pitx2* expression on the left LPM (arrow in *A*; wild type in *B*). Treatment of mouse embryos with RA (10^{-7} M) for 4–6 hr at the head fold stage induces *Pitx2* expression on the right LPM (red arrow in *C*). (*D–M*) Treatment of zebrafish (*D–F*, dorsal views), *Xenopus* (*G–J*, lateral views), and chicken (*K–M*, dorsal views) with RA antagonist, or RA, down-regulates or induces *Pitx2* expression, respectively (black arrows indicate normal expression in the left LPM and red arrows indicate ectopic expression in the right LPM). (*N–P*) Chicken embryos that were allowed to develop until stage 12 after treatment with RA antagonist (*N*) or RA (*P*) display a randomization of heart looping. Here we show hearts looping to the left (compare with the wild-type heart that loops to the right in *O*). (*Q–V*, ventral views) Treatment of mouse embryos at the head fold stage with RA antagonist (10^{-7} M) for 6 hr results in down-regulation of *Lefty-1* and *Lefty-2* genes in both the midline and LPM (arrows in *Q*; normal expression in *R*). On the other hand, similar treatment with RA induces ectopic expression of *Lefty* genes (red arrow in *S*). In the chicken embryo, when a bead soaked in RA antagonist is implanted on the left side of the node at stage 6, *Lefty-1* expression is abolished (*T*; normal expression in *U*). On the contrary, RA beads implanted on the right side of the node of stage 6 chicken embryos induce *Lefty-1* expression on the posterior right LPM (red arrow in *V*; black arrow indicates normal expression on the posterior left LPM). In the dorsal views (*D–F* and *K–M*), the left side of the embryo corresponds to the reader's left). WT, wild type.

randomization of heart looping caused by vitamin A deficiency in quail embryos (14, 24), demonstrate that endogenous RA has an essential role in establishing normal heart *situs*.

RA Controls *Lefty-1* Expression. Treatment of mouse embryos at the head fold stage with RA antagonist (10^{-7} M for 4–6 hr) abolishes *Lefty-1* and *Lefty-2* transcription (34% of the embryos; Fig. 3*Q*, normal pattern in Fig. 3*R*). Conversely, treatment with RA (10^{-7} M) results in bilateral induction of both *Lefty-1* and *Lefty-2* transcripts (47% of the embryos; Fig. 3*S*; red arrow indicates right LPM). Similarly, beads soaked in RA antagonist (10^{-7} M) implanted to the left side of the chicken node at stages 5–7, repress *Lefty-1* expression (38% of the embryos; Fig. 3*T*, compare with Fig. 3*U*). Conversely, beads soaked in RA (10^{-6} M) induce *Lefty-1* expression when implanted to the right side of the node (35% of the embryos; red arrow in Fig. 3*V* indicates right LPM). Thus, RA, as described above for *Shh*, controls *Lefty-1* expression in chicken and mouse embryos. RA also regulates *Lefty-2* in the mouse embryo (a *Lefty-2* gene has not been isolated in chick).

Shh and RA Pathways Act in Parallel to Determine Left-Right Development. In the chicken embryo, the effects of RA on heart *situs* and *Pitx2* expression do not appear to be mediated by Shh, because neither RA nor RA antagonist affects *Shh* expression (refs. 23 and 25, and Fig. 4 *A–C*), suggesting that RA acts either downstream or in parallel to the Shh signal. *Shh* could influence the enzymes that synthesize RA, the levels and/or activity of RA receptors, or other components of the pathway such as the cellular retinoic acid-binding proteins. Because recently it has been shown that the enzyme Raldh2 is the primary enzyme that catalyzes the conversion of vitamin A to RA (11), we decided to investigate a possible regulation of Raldh2 by *Shh*. We cloned the chicken *Raldh2* gene and examined its pattern of expression, which is largely equivalent to that of its murine counterpart (10). During the early stages of gastrulation, *Raldh2* expression is widespread in the entire posterior part up to the node of the chicken embryo. Transcripts are excluded from the node and symmetrically distributed in the mesoderm that surrounds it (Fig. 4*D*). Later, *Raldh2* expression is detected in a variety of structures, in a pattern that coincides with the presence of high levels of RA (26). The bilaterally symmetrical pattern of *Raldh2* expression observed during gastrulation suggests that RA is synthesized at similar levels in both sides of the node. However, overexpression of *Raldh2* on the right side of the chicken node, using an *RCAS* retrovirus, ectopically activates the target gene *Pitx2* (36% of the infected embryos; red arrowhead in Fig. 4*E*, compare with Fig. 3*L*). This, similar to

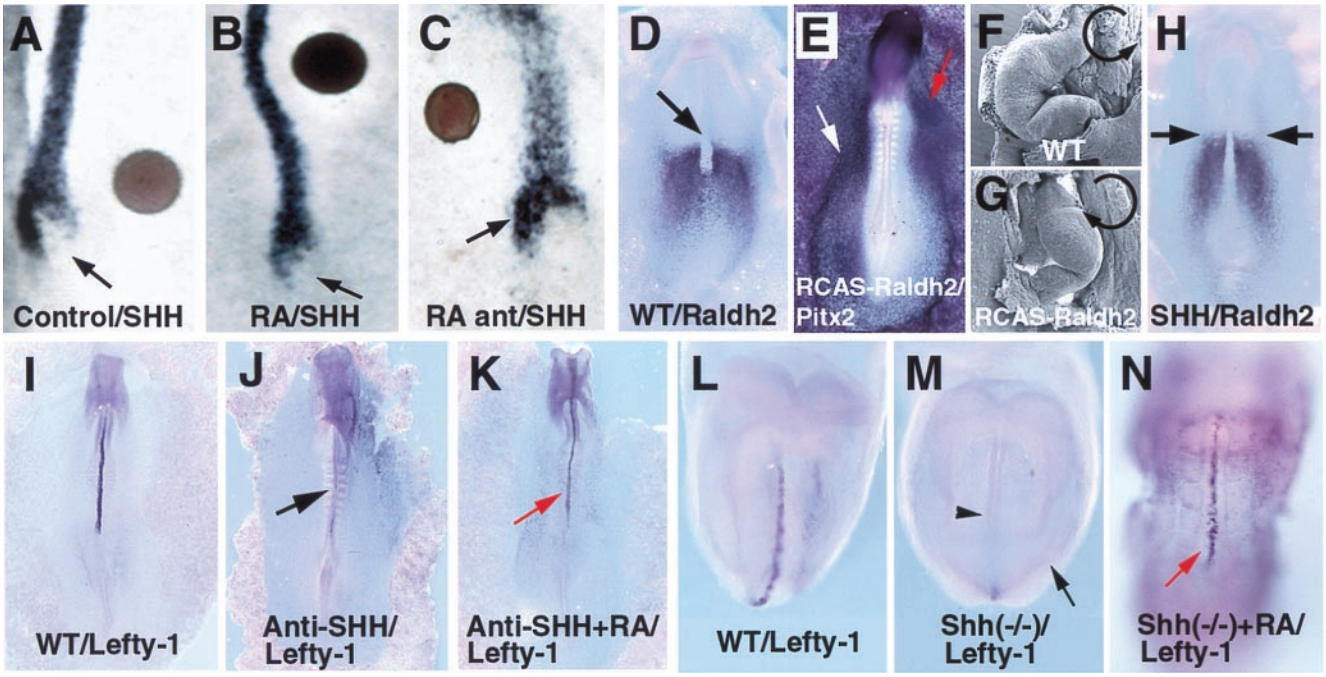

FIG. 4. Relationship between the *Shh* and RA pathways. (*A-C*, dorsal views) Beads soaked in RA (10^{-7} M, on the right side of the node at stage 4, *B*), or RA antagonist (10^{-7} M, on the left side of the node at stage 4, *C*) do not alter the normal left-sided expression of *Shh* in the node of the chicken embryos (*A*; control bead). Beads are the brown circles. (*D*, dorsal view) Expression pattern of *Raldh2* in stage 5+ chicken embryos. *Raldh2* transcripts are absent from the node (arrow) and are symmetrically detected on the left and right mesoderm adjacent to the node. (*E*) Dorsal view. Overexpression of *Raldh2* at stage 4, using a retroviral competent vector (*RCAS-Raldh2*), induces ectopic expression of *Pitx2* on the right LPM of chicken embryos (red arrow in *E*; white arrow indicates normal expression in the left LPM). (*F-G*) When embryos are allowed to develop until stage 12 after *Raldh2* misexpression, we observe a change in the direction of heart looping (in 30% of injected embryos, the heart loops to the left, *G;* compare with the normal looping to the right in *F*). Black semicircular arrows indicate direction of heart looping. (*H*, dorsal view) Application of Shh protein, either to the left or right side of the node at stages 4–5, does not alter the symmetrical expression of *Raldh2* (arrows). (*I-K*, ventral views) Beads soaked in anti-Shh antibody implanted at stage 4 on the left side of the node, down-regulate *Lefty-1* expression in the midline (arrow in *J*, compare with the normal pattern in *I*). *Lefty-1* expression can be rescued (red arrow in *K*) when a bead soaked in RA is implanted 3 hr after implantation of the anti-Shh bead (compare with normal pattern in *I*). (*L-N*, ventral views) *Lefty-1* is absent in both the midline (arrowhead) and the left LPM (arrow) of $Shh^{-/-}$ mouse embryos (*M*, compare with *L*). When $Shh^{-/-}$ mouse embryos are cultured in the presence of RA (10^{-7} M for 6 hr at the head fold stage), *Lefty-1* expression is restored (red arrow in *N*). WT, wild type.

the right-sided application of RA, results in alterations of heart looping (30% of the infected embryos, Fig. 4*G*, shows *situs inversus* of the heart, compare with normal *situs* in Fig. 4*F*). Clearly, a local increase in the amount of RA activity is able to perturb left-right development. We could not detect any change in the transcription levels of the *Raldh2* gene after ectopic application of Shh protein (Fig. 4*H*, compare with Fig. 4*D*). Thus, should there be a regulation of RA activity by *Shh*, this regulation does not appear to operate through the modulation of *Raldh2* transcription.

In chicken embryos, treatment with RA (10^{-6} M) is able to antagonize the repression of *Lefty-1* caused by exposure to a blocking anti-Shh antibody (33% of the treated embryos; Fig. 4 *I-K*). Similarly, application of RA (10^{-7} M for 4–6 hr at the head fold stage) to $Shh^{-/-}$ mouse embryos in culture is able to rescue the expression of *Lefty-1* (30% of the embryos; Fig. 4 *L-N*). During development, expression of *Shh* in the left side of the node could conceivably synergize with some component of the RA pathway. In this way, activation of *Lefty-1* would occur only on the left side of the embryo, even though RA also appears to be present at similar levels on both sides of the embryo (26).

DISCUSSION

We have shown that embryos lacking *Shh* display a variety of laterality defects. *Lefty-1* is absent in $Shh^{-/-}$ mutants, and *Nodal*, *Lefty-2*, and *Pitx2* are ectopically expressed on the right side of the LPM (our results and refs. 27 and 28). These results demonstrate that *Shh* indeed plays a key role in left-right specification in the mouse embryo. The fact that several alterations observed in $Shh^{-/-}$ mutants are reminiscent of the $Lefty\text{-}1^{-/-}$ phenotype suggests that the ectopic expression of *Nodal*, *Lefty-2*, and *Pitx2* observed in the right LPM of $Shh^{-/-}$ embryos is likely to be caused by the absence of *Lefty-1*. The patterns of expression of *Shh* and *Lefty-1* are similar in both mouse and chicken embryos, and *Shh* activity is both necessary and sufficient to induce *Lefty-1* expression in the chick. *Shh* transcription is unaffected in $Lefty\text{-}1^{-/-}$ mutant embryos (4). We conclude that, in the mouse, *Shh* is required (via its control of *Lefty-1*) to induce and/or maintain the midline barrier (4) that has been proposed to restrict expression of *Nodal*, *Lefty-2*, and *Pitx2* to the left side of the embryo. In this scenario, *Shh* would not be required for left-sided expression of these genes. Because it has been suggested that *Shh* acts as a right determinant in the mouse (28), these results contrast with its proposed role as a left determinant in the chick. A deeper understanding of left-right asymmetry pathways in mouse and chicken is required to decide to what extent the role of *Shh* is conserved among vertebrates.

In four different vertebrates, RA is absolutely required for the correct expression of left-specific genes, as revealed by the disappearance of *Pitx2* expression in embryos exposed to a highly specific RA antagonist. Conversely, exposure to RA results in ectopic expression of *Pitx2* in the right side of the embryos. Also, heart defects caused by diminished levels of RA resemble those resulting from reduction or elimination of *Shh*, identified here as a key regulator of *Lefty-1* expression. Treatment of embryos with a RA antagonist completely

abolishes *Lefty-1* expression in both chicken and mouse (and also *Lefty-2* in the mouse), whereas RA treatment results in ectopic expression of *Lefty-1* and *Lefty-2* in the right side of mouse embryos (and ectopic *Lefty-1* in the right LPM of chicken embryos). Dollé and collaborators (29) have reached similar conclusions concerning the effects of RA on left-right genes. These results suggest that the effects of RA and RA antagonists on left-right development are also likely to be mediated by changes in *Lefty-1*. *Lefty-2* has been previously shown to be a target of *Lefty-1* (4).

It is tempting to hypothesize about the existence of an asymmetric distribution of RA, its derivatives, or RA responsiveness needed for proper downstream gene expression and organ *situs*, but so far no component of the RA pathway has been shown to be preferentially localized on the left side of the vertebrate embryo (26). In the left-right cascade, manipulation of RA levels does not affect *Shh* transcription. The pathway does not seem to work the other way around either, because manipulation of *Shh* levels does not affect *Raldh2* transcription. Nevertheless, the RA pathway could still be regulated by *Shh*, but we have determined that it does not occur at the level of *Raldh2* transcription. The fact that RA is present in the embryo in regions where *Shh* is not transcribed (26) indicates that, if *Shh* regulates RA activity at all, this regulation is most likely to operate at the local level (in or near the node).

We have established that pharmacological levels of RA can overcome the lack of *Shh* activity and restore *Lefty-1* expression in both mouse and chicken embryos. We interpret that physiological levels of both *Shh* and RA are required to ensure proper organ placement along the left-right axis and normal transcription of *Lefty-1*. It is also conceivable that RA may influence the interpretation of the *Shh* signal at some level of the genetic cascade other than the transcriptional regulation of *Lefty-1*. For example, RA also induces *Lefty-2* in the mouse, and this also could be the case for the *Nodal* gene (30). Another possible target of RA regulation could be the "X" factor that has been proposed to mediate the *Shh*-dependent induction of *Nodal* in the left LPM (5). These possibilities remain to be explored.

In conclusion, our results demonstrate that, despite apparent differences between species, the roles of *Shh* and RA in the specification of left-right asymmetry during embryonic development are conserved among vertebrates, and that the *Shh* and RA pathways converge to ensure the proper activation of *Lefty-1* in the embryo.

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