Cardiac looping and the vertebrate left-right axis: antagonism of left-sided Vg1 activity by a right-sided ALK2-dependent BMP pathway

Ann F. Ramsdell and H. Joseph Yost*

Huntsman Cancer Institute, Center for Children, Department of Oncological Sciences, University of Utah, Salt Lake City, UT 84112-5550, USA

*Author for correspondence (e-mail: joseph.yost@hci.utah.edu)

Accepted 9 September; published on WWW 9 November 1999

SUMMARY

The rightward looping of the primary heart tube is dependent upon upstream patterning events that establish the vertebrate left-right axis. In Xenopus, a left-sided Vg1 signaling pathway has been implicated in instructing cells to adopt a ‘left-sided identity’; however, it is not known whether ‘right-sided identity’ is acquired by a default pathway or by antagonism of Vg1 signaling. Here, we propose that an antagonistic, BMP/ALK2/Smad-mediated signaling pathway is active on the right side of the Xenopus embryo. Truncated ALK2 receptor expression on the right side of the blastula elicits heart reversals and altered nodal expression. Consistent with these findings, constitutively active ALK2 (CA-ALK2) receptor expression on the left side of the blastula also elicits heart reversals and altered nodal expression. Coexpression of CA-ALK2 with mature Vg1 ligand results in predominantly left-sided nodal expression patterns and normal heart looping, demonstrating that the ALK2 pathway can ‘rescue’ left-right reversals that otherwise occur following right-sided misexpression of mature Vg1 ligand alone. Results with chimeric precursor proteins indicate that the mature domain of BMP ligands can mimic the ability of the ALK2 signaling pathway to antagonize the Vg1 pathway. Consistent with the observed antagonism between BMP and Vg1 ligands, left-sided ectopic expression of Xolloid results in heart reversals. Moreover, ectopic expression of Smad1 or Smad7 identified two downstream modulators of the BMP/ALK2 signaling pathway that also can regulate cardiac orientation. Collectively, these results define a BMP/ALK2-mediated pathway on the right side of the Xenopus embryo and, moreover, suggest that left-right patterning preceding cardiac morphogenesis involves the activation of two distinct and antagonistic, left- and right-sided TGFβ-related signaling pathways.

Key words: ALK2, BMP, Cardiac morphogenesis, Left-right asymmetry, Smad, TGFβ, Vg1, Xolloid

INTRODUCTION

A critical and highly conserved event in vertebrate embryonic heart development is the rightward looping of the primary heart tube. As a result of looping morphogenesis, initially non-adjacent regions of the heart tube are brought into proximity with respect to one another and with the great vessels, thereby facilitating development of the inflow and outflow tracts of the heart (reviewed by Ramsdell and Yost, 1999). As observed in individuals affected with laterality disorders such as heterotaxia or isolated dextrocardia, failure of the heart tube to undergo a specific, rightward looping is frequently associated with complex cardiovascular malformations including, but not limited to, atrioventricular septal defects, ventricular septal defects, transposition (or corrected transposition) of the great arteries and anomalous venous pulmonary return (reviewed by Bowers et al., 1996). Thus, with respect to overall cardiac morphogenesis, the mechanism by which the heart is instructed to break its morphological symmetry and to undergo directional looping morphogenesis is fundamental to the establishment of a properly ordered and seamlessly connected cardiopulmonary system.

Significant progress has been made in identification of genes that are involved in vertebrate left-right patterning. Strikingly, the majority of genes thus far identified are expressed prior to organogenesis, and sources of left-right patterning signals include the organizer/node, the notochord and floorplate, the lateral plate mesoderm, as well as gap-junctional communication in the early gastrula (reviewed by Ramsdell and Yost, 1998, 1999). Based on expression patterns and/or inductive activities, it has become apparent that cardiac left-right patterning requires both left- and right-sided signaling pathways that function well before the formation of the primary heart tube. For example, in the chick embryo, a sonic hedgehog pathway is activated to the left of Hensen’s node (Levin et al., 1995, 1997), and an opposing pathway mediated by activin (Levin et al., 1995, 1997) and FGF8 (Boettger et al., 1999) is operative to the right of the node. Ectopic misactivation of either pathway on the contralateral side of the embryo results in inappropriate expression of downstream laterality genes by the lateral plate mesoderm, as well as reversed heart looping (Boettger et al., 1999; Isaac et al., 1997; Levin et al., 1995, 1997). Moreover, gene expression within or
near the node itself is perturbed, such that repression of left-sided signals follows ectopic expression of right-sided signals (and vice versa). Opposing activities of left- and right-sided signaling pathways have similarly been implicated for regulated, asymmetric expression of other left-right patterning genes expressed in the lateral plate mesoderm and the cardiac fields (Campione et al., 1999; Logan et al., 1998; Lohr et al., 1998; Meno et al., 1998; Piedra et al., 1998; Ryan et al., 1998; Smith et al., 1997; Yoshioka et al., 1998). Thus, current evidence suggests that cardiac left-right asymmetry results from an integration of early acting, left- and right-sided signaling pathways that have antagonistic activities in left-right development.

Despite progress in elucidating many of the molecular mechanisms underlying vertebrate left-right patterning, very little is known about the processes that are involved in first establishing left-right asymmetry in the embryo. Studies in chick and Xenopus indicate that molecular left-right asymmetry exists well before the formation of midline and lateral plate mesodermal tissues. In the chick, left-right asymmetry of the node is inductively patterned by signals emitted by tissues extraneous (and probably lateral) to the node (Pagan-Westphal and Tabin, 1998). In Xenopus, the only left-right patterning activities thus far implicated upstream of asymmetric nodal expression in the lateral plate mesoderm are gap-junctional communication (Levin and Mercola, 1998b) and Vg1 signaling (Hyatt et al., 1996; Hyatt and Yost, 1998).

Non-midline cell lineages that carry left-right identity and are capable of inducing left-right specification of the organizer/node have been termed the ‘left-right coordinator’ (LRC) (Hyatt and Yost, 1998). Examination of a variety of classes of signaling molecules suggests that LRC activity is mediated by left lateral vegetal cells that express Vg1, a member of the TGFβ superfamily of secreted polypeptide factors (Hyatt et al., 1996; Hyatt and Yost, 1998). Targeted misexpression of mature Vg1 ligand in right, but not left, lateral vegetal cells of the 16-cell stage Xenopus blastula results in morphological and molecular inversion of the left-right axis, as indicated by reversals of the heart and viscera, as well as inverted expression of downstream laterality genes (e.g. nodal) (Hyatt et al., 1996; Hyatt and Yost, 1998). Consistent with these findings, ectopic expression of a truncated, dominant-negative receptor that blocks Vg1 activity (Kessler and Melton, 1995) elicits left-right reversals when targeted to left lateral vegetal cells (Hyatt and Yost, 1998), and ectopic expression of mature Vg1 ligand can ‘rescue’ reversed heart looping that otherwise occurs in instances of conjoined twinning (Hyatt and Yost, 1998). Together, these results indicate a requirement for a left-sided signaling pathway in generating early molecular asymmetry in the vertebrate embryo and, moreover, implicate Vg1 in this process.

The mechanisms by which cells acquire right-sided identities are unknown. One possibility is that an early, right-sided pathway functions to prevent right-sided cell lineages from adopting a left-sided identity. To determine whether such a right-sided pathway exists, we have focused specifically on the BMP signaling pathway. BMPs were chosen for study as potential antagonists to Vg1 signaling for several reasons. First, maternally expressed BMP ligands are present in the early Xenopus blastula (Hemmatti-Briyianlou and Thomsen, 1995), and it has been shown that ectopically expressed BMP ligands can reverse the direction of heart looping in Xenopus when targeted to cell lineages that contribute to the LRC (Hyatt and Yost, 1998). Second, BMP signaling, including pathway-restricted Smads, is capable of attenuating Vg1/activin signaling in the context of dorsoventral patterning assays (Candia et al., 1997). Third, in the Xenopus blastula, coexpressed BMP and Vg1 chimeric proteins can inhibit reversed heart looping that otherwise occurs following expression of mature Vg1 alone (Hyatt and Yost, 1998).

Starting with use of dominant-negative forms of type I activin-like kinase (ALK) receptors (ALK2, ALK3, ALK4), we identified a potential role for a right-sided ALK2 pathway in left-right development in Xenopus. Ectopic activation of ALK2 signaling on the left side of the Xenopus embryo results in reversed heart looping and altered nodal expression, suggesting that ALK2 signaling might antagonize endogenous Vg1 activity. As a direct test of this possibility, expression of components of the ALK2 pathway, including a constitutively active ALK2 receptor or mature BMP ligands, was found to attenuate the ability of ectopic mature Vg1 to elicit reversed heart looping and right-sided nodal expression. Consistent with these results, targeted expression of Smad 1 and Smad 7, two downstream signal transducers of the ALK2 pathway, also elicit heart reversals. Given these findings, we propose that two antagonistic, TGFβ-related signaling pathways – Vg1 on the left and BMP/ALK2 on the right – function on opposite sides of the embryo to generate molecular left-right asymmetry required for formation of the vertebrate left-right axis.

**MATERIALS AND METHODS**

**Microinjection assays**

Eggs were obtained from PMSG- and hGC-injected adult, pigmented *Xenopus laevis* females, fertilized and dejellied with 2.5% cysteine. GppG-capped RNA was synthesized with the Ambion Message Machine kit following the manufacturer’s specifications. RNA was injected into left or right lateral vegetal cells of 16-cell stage embryos according to the methods and cell lineage nomenclature described in Hyatt and Yost (1998), in which left and right sides are identified as L and R, and cells are numbered from 1 to 4, dorsal to ventral. Dorsal and ventral sides of the embryo were identified on the basis of pigmentation patterns, and only embryos with unambiguous pigmentation were used for microinjection. The concentrations of RNA used were as follows: 250 pg A-BMP4, 250 pg AVg, 250 pg BMP2, 500 pg BMP4, 1 ng tALK2, 1 ng tALK3, 500 pg tALK4, 100-250 pg CA-ALK2, 400-500 pg CA-ALK3, 500 pg CA-ALK4, 2 ng Smad1, 0.5-2 ng Smad 7, 2 ng Xolloid. Injected embryos were allowed to recover in 5% Ficoll at 16°C for a minimum of 1 hour and then transferred to 1/3× MMR for culture at room temperature. After 5 days of development, embryos were anesthetized with 0.01% benzocaine and scored for directionality of heart looping. The percentages of heart reversals for control and experimental groups were tested for statistical significance by the χ²-squared test. Embryos exhibiting reduced dorso-anterior development were not scored for heart orientation, and non-injected sibling embryos were maintained for each experimental group to monitor background frequency of spontaneous heart reversals. It has been shown previously that injection of vehicle or control RNA (Hyatt et al., 1996) does not elicit cardiac reversals above background rates, which typically range between 0 and 8%, depending on the batch of embryos used (e.g. see also Hyatt and Yost, 1998; Lohr et al., 1997, 1998).

For some experiments, day 5-7 embryos were prepared for photography by whole-mount immunofluorescence staining with a
monoclonal troponin T antibody (CT3; Developmental Studies Hybridoma Bank, University of Iowa) to enhance visualization of cardiac and non-cardiac muscle tissues. Embryos were fixed in 3% paraformaldehyde, dehydrated in an ethanol series, serially rehydrated into PBS, blocked with 10% normal goat serum (Sigma), incubated with undiluted monoclonal CT3 supernatant, followed by extensive washes in 3% BSA-PBS, incubation with fluorescein-conjugated goat anti-mouse IgG (1 µg/ml; Sigma) and repeated washes in 3% BSA-PBS.

**Analysis of nodal expression**

Control and experimental embryos were collected at stages 24-26 and processed for in situ hybridization using digoxigenin-labeled antisense RNA probes as described (Lohr et al., 1997). Sibling embryos from control and experimental groups were maintained in culture for 5 days to monitor heart reversal rates for each group.

**RESULTS**

**Effects of truncated ALK receptors on heart looping and nodal expression**

Three type I ALK receptors have been identified in Xenopus: ALK2 (Chen et al., 1997; Suzuki et al., 1997b), ALK3 (Graff et al., 1994) and ALK4 (Chang et al., 1997). To determine whether a right-sided signaling pathway functions in early left-right patterning, RNA encoding dominant-negative forms of these ALK receptors was introduced into right-sided cell lineages of the 16-cell Xenopus blastula and hearts of injected embryos were scored at day 5 of development. tALK2 (Chen et al., 1997), tALK3 (Graff et al., 1994) and tALK4 (Chang et al., 1997) each encode truncated receptors, which lack an intracellular kinase domain and which inhibit BMP signaling in dorsoventral patterning assays. Because Vg1 signaling activity in vivo is thought to be maximal in the L2 and L3 cell lineages (Hyatt and Yost, 1998), the corresponding contralateral cell lineages, R2 and R3, were targeted for truncated receptor expression.

Cardiac reversal rates were low in embryos injected on the right side with either tALK3 (6%, \( n=89 \)) or tALK4 (8%, \( n=159 \)), suggesting that neither the ALK3 nor ALK4 pathways are required for left-right development. As a control for RNA expression and activity, tALK3 was targeted to ventral vegetal cell lineages, which resulted in dorsIALIZED embryos (not shown), consistent with its reported ability to disrupt dorsoventral patterning (Graff et al., 1994). In contrast to tALK3 and tALK4, the cardiac reversal rate in tALK2-injected embryos ranged from approximately 12% to as high as 50% (Table 1), depending on whether embryos with defects in addition to heart reversals were scored for heart orientation. For single lineage injections, the majority of embryos exhibited normal morphology, with two exceptions. First, reversed heart looping occurred in 12% and 17% of embryos injected in the R2 and R3 lineages, respectively. Second, the orientation of gut coiling appeared ambiguous in embryos showing reversed heart looping. A minority (18%) of embryos injected in the R3 lineage also showed other defects in addition to a 50% reversal rate of heart orientation, including deletion of anterior somites, bending of the axis and split tails. The non-cardiac defects observed in the R3 group and others described below did not appear to result from reduced dorsoanterior development, as indicated by normal head formation, but rather appear to be related to localized reduction of ventral mesodermal tissues (e.g. see Fig. 2). Because disturbances in dorsoanterior development have been shown previously to result in altered left-right development (Danos and Yost, 1995), embryos showing decreased dorsoanterior development (as assessed by DAI index; Kao and Elinson, 1988) in this group and in others described below were not scored for heart orientation. To determine whether higher rates of heart reversals could be obtained by targeting tALK2 expression to more than one cell lineage, the R2 and R3 lineages were coinjected. However, this resulted in embryonic lethality in 37% of the injected embryos. Of the surviving embryos, 15% exhibited reversed heart looping, and a minority (19%) exhibited 48% heart reversals in addition to mesodermal defects, as observed in the single R3 lineage injections. The defects other than reversed heart looping observed following the single and dual lineage injections are consistent with observations made in ALK2 null mice, which show severe disruption of mesoderm formation (Gu et al., 1999).

To determine whether the effects of tALK2 RNA on left-right development of the heart occur upstream of lateral plate mesoderm specification (Danos and Yost, 1996; Lohr et al., 1997; 1998), injected embryos were evaluated for nodal expression. The asymmetric, left-sided expression of nodal in the lateral plate mesoderm is highly conserved in all vertebrates thus far examined (Collignon et al., 1996; Levin et al., 1995, 1997; Lohr et al., 1997; Pagan-Westphal and Tabin, 1998).

**Table 1. Injection of tALK2 in right cell lineages alters cardiac orientation**

<table>
<thead>
<tr>
<th>Lineage</th>
<th>Normal hearts</th>
<th>Reversed hearts</th>
<th>% reversed hearts</th>
<th>Embryos with other defects</th>
</tr>
</thead>
<tbody>
<tr>
<td>R2</td>
<td>81</td>
<td>11</td>
<td>12±3</td>
<td>0</td>
</tr>
<tr>
<td>R2 control</td>
<td>71</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>R3</td>
<td>172</td>
<td>36</td>
<td>17±3</td>
<td>23</td>
</tr>
<tr>
<td>R3 control</td>
<td>157</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>R2+R3*</td>
<td>100</td>
<td>17</td>
<td>15±2</td>
<td>14</td>
</tr>
<tr>
<td>R2+R3 control</td>
<td>188</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Right cell lineages were injected with 1 ng of tALK2 RNA or 2 ng total for dual-lineage injections. The number of embryos and percentage (± s.e.m.) of heart reversals are shown for otherwise normal embryos. For embryos showing defects in addition to heart reversals (as described in the text), the number and percentage of heart reversals also are shown.

Results obtained from \( \chi^2 \)-squared analysis of embryos showing only laterality defects indicate that the percentage of heart reversals observed in experimental groups is significantly different from controls. \( \chi^2=9.10, P<0.001; \chi^2=31.97, P<0.001; \chi^2=28.93, P<0.001 \). *Significant mortality (37%) prior to gastrulation was observed in this group.**
Rebagliati et al., (1998b; Sampath et al., 1997), and serves as a molecular marker of left-right asymmetry that precedes formation of the primary heart tube and that is subject to regulation by earlier left-right patterning events in *Xenopus*, including Vg1 signaling (Hyatt et al., 1996; Hyatt and Yost, 1998). In contrast to control embryos (Fig. 1A), only 48% of tALK2-injected embryos showed normal, left-sided nodal expression (total n=40). A significant percentage (48%) of tALK2 injected embryos exhibited bilateral nodal expression, and for nearly all the embryos in this group, staining appeared much more intense in the left lateral plate mesoderm, compared to the very small area of ectopic expression faintly detected the right lateral plate mesoderm (data not shown). Absent expression was observed in 2% of embryos and right-sided only expression was also observed in 2% of injected embryos. Thus, the effects of tALK2 expression on heart looping are preceded by subtle alterations of nodal expression in the lateral plate mesoderm, suggesting that tALK2 can interfere with patterning processes that normally occur upstream to nodal expression and cardiac looping morphogenesis.

Ectopic activation of the ALK2 pathway elicits heart reversals and altered nodal expression

Because of the low frequency of heart reversals observed following expression of tALK2 RNA, we further tested the role of ALK2 signaling in left-right patterning with constitutively active forms of ALK receptors. CA-ALK2 (Suzuki et al., 1997b), CA-ALK3 (Candia et al., 1997) and CA-ALK4 (Chang et al., 1997) each contain a Gln-to-Asp mutation in the intracellular GS domain, which permits constitutive kinase activity in a ligand-independent manner. When targeted to L2 or L3 cell lineages, CA-ALK2 expression results in reversed heart looping (Table 2). As with tALK2 injected embryos, embryos injected with CA-ALK2 RNA were separated into two groups prior to scoring for the direction of heart orientation (Fig. 2). The first group appeared grossly normal, with the exception of reversed heart looping, which consistently occurred in approximately one-third of the embryos in this group (Fig. 3B, Table 2). As observed for tALK2-injected embryos, the orientation of gut coiling was ambiguous (Fig. 3B). The second group contained other notable defects in addition to reversed heart looping, including split tails, bending of the heads toward the axis, posterior truncation of axis, and deletion of 2-3 anterior somites on the injected side of the embryos (Fig. 2A, Table 2). The non-cardiac defects observed in CA-ALK2-injected embryos (Fig. 2A) are similar to those observed in some embryos injected with tALK2, and are consistent with the observed decreased ventral mesoderm formation following ubiquitous CA-ALK2 expression in the one-cell *Xenopus* embryo (Armes and Smith, 1997). Despite the presence of mesodermal defects in the latter group, it should be emphasized that dorsoanterior development appeared normal for most embryos; embryos that showed reduced DAI indexes were excluded from heart analyses (Fig. 2A). When CA-ALK2 was targeted to R2 or R3 cell lineages, high frequencies of heart looping were not observed, although mesodermal defects still occurred (Figs 2B, 3A, Table 2). Thus, the high rates of heart reversals elicited by CA-ALK2 occurred only when expression was targeted to left cell lineages and did not appear to be causally

### Table 2. Injection of CA-ALK2 in left, but not right, cell lineages alters cardiac orientation

<table>
<thead>
<tr>
<th>Lineage</th>
<th>Normal embryos</th>
<th>Embryos with other defects</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal hearts</td>
<td>Reversed hearts</td>
</tr>
<tr>
<td>L2</td>
<td>25</td>
<td>12</td>
</tr>
<tr>
<td>R2</td>
<td>55</td>
<td>0</td>
</tr>
<tr>
<td>Control</td>
<td>140</td>
<td>0</td>
</tr>
<tr>
<td>L3</td>
<td>36</td>
<td>19</td>
</tr>
<tr>
<td>R3</td>
<td>45</td>
<td>0</td>
</tr>
<tr>
<td>Control</td>
<td>111</td>
<td>0</td>
</tr>
</tbody>
</table>

Left or right cell lineages were injected with 100-250 pg of CA-ALK2 RNA. The number of embryos and percentage (± s.e.m.) of heart reversals are shown for otherwise normal embryos. For embryos with defects in addition to heart reversals (as shown in Fig. 2), the number and percentage of heart reversals are also shown.

Results obtained from χ<sup>2</sup>-squared analysis of embryos showing only laterality defects indicate that the percentage of heart reversals observed in experimental groups is significantly different from controls, and that the percentage of heart reversals resulting from left-sided injections is significantly different from right-sided injections: *χ<sup>2</sup>=48.71, P<0.001; *χ<sup>2</sup>=20.51, P<0.001; *χ<sup>2</sup>=43.30, P<0.001; *χ<sup>2</sup>=19.19, P<0.001. For embryos showing other defects in addition to heart reversals, the percentage of heart reversals resulting from left-sided injections is significantly different from right-sided injections: *χ<sup>2</sup>=12.26, P<0.001; *χ<sup>2</sup>=12.67, P<0.001.
ALK2 signaling antagonizes Vg1 activity

To test whether ALK2 signaling prevents cells from responding to Vg1, CA-ALK2 was coexpressed with AVg in the R3 cell lineage. AVg is an activin-Vg1 chimera that yields high levels of secreted, mature Vg1 via an activin prodomain containing the activin βB cleavage site (Kessler and Melton, 1995). When AVg RNA alone is targeted to the R3 lineage, heart reversals are observed in a majority (62%) of injected embryos (Table 3). In contrast, CA-ALK2 expression by the R3 lineage does not result in heart reversals, although mesodermal defects as described above are observed (Table 3, Fig. 3B). When coexpressed, CA-ALK2 significantly decreases the frequency of heart reversals that occurs following AVg expression alone (62% versus 16%; Table 3, Fig. 3C). Moreover, the mesodermal defects that occur following CA-ALK2 expression alone are greatly reduced by coexpression with AVg (52% versus 6%; Table 3). These results demonstrate

Fig. 3. Cardiac orientation of embryos injected with CA-ALK2 RNA. Embryos were injected with CA-ALK2 RNA in right or left cell lineages, and day 5-7 tadpoles were stained by immunofluorescence with monoclonal antibody CT3 to demonstrate heart and gut orientation. The orientation of embryos shown is a ventral view, with anterior at the top. CA-ALK2 expression in right cell lineages results in normal, rightward heart looping and ambiguous gut coiling (A). In contrast, CA-ALK2 expression in left cell lineages results in reversed, leftward heart looping and ambiguous gut coiling (B). Coinjection of AVg RNA with CA-ALK2 in left cell lineages results in normal, rightward heart looping and normal gut coiling (C). Arrowheads point to the conotruncus (outlet) of the heart.
that ALK2 and Vg1-mediated signaling activities are antagonistic, suggesting that ALK2 signaling might normally temper the response of right-sided cell lineages to Vg1 that is secreted from cells of the LRC.

In addition to heart looping, nodal expression in the lateral plate mesoderm of embryos coinjected with CA-ALK2 and AVg was examined. When expressed alone in right cell lineages, AVg expression results in a majority of embryos that exhibit predominantly right-sided nodal expression, with nearly all embryos showing reversed heart looping (Hyatt and Yost, 1998). Coinjection of CA-ALK2 and AVg greatly alters this expression pattern (total n=40). A high percentage (64%) showed bilateral staining, and in half of this group, staining appeared to be either equivalent or enhanced in the left lateral plate mesoderm, compared to staining in the right lateral plate mesoderm. An additional 18% showed normal, left-only expression (Fig. 1C), 15% showed an absence of expression, and 5% showed right-only expression. Thus, the overall trend of expression in coinjected embryos indicates a predominance of left-sided nodal expression (82% of embryos). Together, these results demonstrate that CA-ALK2 expression prevents the inversion of nodal expression to the right side that occurs following expression of AVg alone.

**BMP attenuates Vg1 activity through its mature ligand domain**

Similar to CA-ALK2, BMP2 or BMP4 expression in the L2 or L3 lineages results in stochastic heart reversals in *Xenopus* (Hyatt and Yost, 1998). Moreover, BMP4 can reduce the frequency of heart reversals elicited by a chimeric form of BMP-Vg1 (BVg1) if coexpressed in the R3 vegetal cell lineage (Hyatt and Yost, 1998). Although these results implicate BMPs as the ligands which activate ALK2 and other signaling pathways, it is possible that mechanisms other than signal transduction could account for this antagonistic activity. Given the multiple functions ascribed to prodomains of TGFβ-related ligands (Brunner et al., 1989, 1992; Constam and Robertson, 1999; Gentry and Nash, 1990; Gray and Mason, 1990; Wilson et al., 1993), and the presence of BMP prodomains in each protein, the previously reported attenuation of BVg1 activity by BMP may have resulted from artifactual interaction of the two ligands (e.g. heterodimerization) or from altered ligand secretion and/or stability. We therefore have examined whether mature BMP ligands are capable of inhibiting activity associated with mature Vg1 by using AVg in place of BVg1. *Xenopus* blastulae were injected with BMP2, BMP4 and AVg, either alone or in combination, at the 16-cell stage and allowed to develop until hearts could be scored for looping orientation. Expression of AVg, but not BMP4, in the R3 cell lineage elicits a high frequency of cardiac reversals (55% versus 4%; Table 4). However, coexpression of BMP4 substantially reduces the frequency of heart reversals obtained with AVg alone (55% versus 12%; Table 4), similar to the antagonism observed following coexpression of CA-ALK2 and AVg (Table 3). To test further whether antagonism between BMP and mature Vg1 activity is independent of prodomains, RNA injections were repeated with BMP2, which is approximately 90% identical with BMP4 in the mature ligand domain, but is much more divergent in the prodomain. Similar to BMP4 or CA-ALK2, coexpressed BMP2 was found to substantially reduce the frequencies of heart reversals that occur following expression of AVg alone (45% versus 10%; Table 4).

It was next tested whether heart reversals obtained following misexpression of BMPs on the left side of the blastula could occur following expression of mature BMP ligand derived from an activin-BMP4 (A-BMP4) chimera (Suzuki et al., 1997b). This chimera was chosen because wild-type activin alone does not elicit heart reversals when targeted to left-cell lineages in *Xenopus* (Hyatt and Yost, 1998). A-BMP4 elicits stochastic heart looping when targeted to the L3 cell lineage (55% reversals, N=109), similar to the frequency of reversed heart looping observed with left-sided misexpression of wild-type BMP2 or BMP4 (Hyatt and Yost, 1998). Cardiac orientation was normal in embryos injected on the right side (0% reversals, n=57) and in control embryos (3% reversals, n=117). In addition, ectopic expression of *Xolloid*, encoding a protease that yields active BMP ligands from BMP-chordin complexes (Piccolo et al., 1997), was targeted to the L3 lineage. In contrast to the rate of heart reversals in control embryos (2%, n=82), 24% (n=106) of injected embryos showed reversed heart looping. Thus, these results and those of the coinjections described above eliminate the possibility that BMPs interfere with Vg1 activity via a mechanism that requires native BMP and/or Vg1 prodomains, and suggest that BMP antagonism of Vg1 activity results from activation of receptor-mediated intracellular signaling.

**Table 4. Coinjection of BMP and AVg in right cell lineages**

<table>
<thead>
<tr>
<th>RNA</th>
<th>n</th>
<th>Total % cardiac reversals</th>
</tr>
</thead>
<tbody>
<tr>
<td>AVg</td>
<td>164</td>
<td>55±4.6&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>BMP4</td>
<td>133</td>
<td>4</td>
</tr>
<tr>
<td>AVg+BMP4</td>
<td>122</td>
<td>12±4&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Control</td>
<td>118</td>
<td>0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>AVg</td>
<td>115</td>
<td>45±5&lt;sup&gt;c,d&lt;/sup&gt;</td>
</tr>
<tr>
<td>BMP2</td>
<td>40</td>
<td>18±6</td>
</tr>
<tr>
<td>AVg+BMP2</td>
<td>90</td>
<td>10±5&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Control</td>
<td>93</td>
<td>0&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

The right R3 lineage was injected with 250 pg AVg, 500 pg BMP4 or 1-2 ng BMP2 RNA individually or in the combinations indicated.

The number and percentage (± s.e.m.) of control or experimental embryos with heart reversals are shown.

Results obtained from <sup>c</sup>-squared analysis indicate that the percentage of heart reversals observed in AVg-injected embryos is significantly different from control<sup>a,c</sup> or AVg+BMP coinjected embryos<sup>b,d</sup>:<sup>c</sup>x=95.11, P<0.001;<sup>b</sup> x=54.60, P<0.001;<sup>d</sup> x=56.07, P<0.001;<sup>a</sup> x=29.96, P<0.001.

**Downstream components of ALK2/BMP signal transduction elicit reversed heart looping**

Because ectopic BMP ligands or activated ALK2 receptor alter left-right development, downstream components of the BMP/ALK2 signal transduction pathway were tested for their ability to elicit reversed heart looping. Smad1 is a cytoplasmic protein that binds to the intracellular kinase domain of activated type I BMP receptors (including ALK2; Macias-Silva et al., 1998), but not to activin or TGFβ receptors (reviewed by Cho and Blitz, 1998; Heldin et al., 1997; Whitman, 1998). After phosphorylation by activated type I BMP receptors, Smad1 associates with other cytoplasmic proteins to form a transcriptional regulatory
complex which translocates to the nucleus to activate expression of BMP target genes. Similar to ectopic expression of CA-ALK2 or BMP ligands, ectopic expression of Smad1 RNA was found to elicit heart reversals in 28% or 32% of embryos when targeted to the L2 or L3 lineages, respectively, of the Xenopus blastula (Table 5), concurring with the idea that BMPs antagonize Vg1 signaling activity via a mechanism of signal transduction.

To determine whether BMP intracellular signaling in right cell lineages is required for normal left-right development, an inhibitor of TGFβ-related signaling, Smad7 (initially termed Smad8; Nakayama et al., 1998), was used. Similar to Smad1, Smad7 is a substrate for activated ALK2 receptors (Souchelnytskyi et al., 1998); however, unlike Smad1, Smad7 remains associated with activated type I receptors to inhibit binding of positive-acting Smads, such as Smad1 (reviewed by Cho and Blitz, 1998; Heldin et al., 1997; Whitman, 1998). The specificity of Smad7 also is broader than that of Smad1 in that low level expression of Smad7 inhibits BMP signaling and much higher levels inhibit activin/TGFβ-signaling (Casellas and Brivanlou, 1998). Because a role for activin signaling in left-right axis formation in Xenopus has been eliminated (Hyatt and Yost, 1998; Lohr et al., 1997, 1998; Pagan-Westphal and Tabin, 1998; Rebagliati et al., 1998a; Sampath et al., 1997), but also that in instances of bilateral expression, the cardiac primordia are capable of interpreting and responding to relative, quantitative left-right differences in nodal expression (Heymer et al., 1997; Lohr et al., 1997). In Xenopus, left lateral vegetal cells emit signal(s) that initiate the formation and orientation of the left-right axis relative to the dorsoventral and anteroposterior axes (Hyatt and Yost, 1998). These left-sided cell lineages have been termed the ‘left-right coordinator’ (LRC) and evidence suggests that Vg1 expression by cells of the LRC establishes the left-right axis (Hyatt et al., 1996; Hyatt and Yost, 1998). Because ectopic expression experiments indicate that mature Vg1 confers a left ‘identity’ to cells (e.g. nodal expression is elicited), it is unclear how right cell lineages are instructed to adopt a right ‘identity’. It is possible that cells of the LRC also emit signals that diffuse to the right side of the blastula to suppress left identity in right-sided cells, the latter of which acquire right identity by default. Alternatively, a second signal, expressed by right cell lineages, might act locally to antagonize a response to Vg1 signaling emanating from the LRC. The results presented here support the latter model of an active signaling pathway on the right side that antagonizes left-sided signaling in establishment of the left-right axis in the Xenopus blastula (Fig. 4).

Ectopic activation of ALK2 signaling in cells that contribute to the LRC results in stochastic heart reversals and aberrant nodal expression patterns, indicating that the effects of ALK2 signaling on laterality occur before the specification of lateral plate mesoderm. Specifically, nodal expression is bilateral, right-sided only, or absent in half of injected embryos, which is consistent with the observed rate of cardiac reversals. The ability of ALK2 signaling to override heart reversals that otherwise occur with AVg expression alone, as well as the ability of ALK2 signaling to interfere with the

### Table 5. Injection of Smad1 in left cell lineages and Smad7 in right cell lineages alters cardiac orientation

<table>
<thead>
<tr>
<th>RNA (cell lineage)</th>
<th>Normal hearts</th>
<th>Reversed hearts</th>
<th>% reversed hearts</th>
<th>Normal hearts</th>
<th>Reversed hearts</th>
<th>% reversed hearts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smad1 (L3)</td>
<td>46</td>
<td>18</td>
<td>28±6</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>L3 control</td>
<td>59</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Smad1 (L2)</td>
<td>23</td>
<td>9</td>
<td>32±8</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>L2 control</td>
<td>33</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Smad7 (R3)</td>
<td>15</td>
<td>10</td>
<td>40±10</td>
<td>9</td>
<td>10</td>
<td>53</td>
</tr>
<tr>
<td>R3 control</td>
<td>37</td>
<td>1</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Smad7 (R2)</td>
<td>18</td>
<td>1</td>
<td>5±5</td>
<td>31</td>
<td>25</td>
<td>45</td>
</tr>
<tr>
<td>R2 control</td>
<td>72</td>
<td>2</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Left cell lineages were injected with 2 ng of Smad1 and right cell lineages were injected with 0.5-2 ng Smad7 RNA.

The number of embryos and percentage (± s.e.m.) of heart reversals are shown for otherwise normal embryos and for embryos showing defects in addition to heart reversals as described in the text.

Results obtained from χ²-squared analysis of embryos showing only laterality defects indicate that the percentage of heart reversals observed in experimental groups is significantly different from controls, except for Smad7 injections in the R2 lineage. *χ²=19.44, P<0.001; bχ²=10.77, P<0.001; cχ²=14.61, P<0.001.

### DISCUSSION

The asymmetric expression of nodal and the orientation of cardiac looping are ultimately driven by upstream patterning processes that establish and maintain molecular asymmetry from the initially symmetric vertebrate body plan (reviewed by Ramsdell and Yost, 1998, 1999). It has been suggested that not only is nodal required for normal heart looping (Collignon et al., 1996; Levin et al., 1995, 1997; Lohr et al., 1997, 1998; Pagan-Westphal and Tabin, 1998; Rebagliati et al., 1998a; Sampath et al., 1997), but also that in instances of bilateral expression, the cardiac primordia are capable of interpreting and responding to relative, quantitative left-right differences in nodal expression (Heymer et al., 1997; Lohr et al., 1997). In Xenopus, left lateral vegetal cells emit signal(s) that initiate the formation and orientation of the left-right axis relative to the dorsoventral and anteroposterior axes (Hyatt and Yost, 1998). These left-sided cell lineages have been termed the ‘left-right coordinator’ (LRC) and evidence suggests that Vg1 expression by cells of the LRC establishes the left-right axis (Hyatt et al., 1996; Hyatt and Yost, 1998). Because ectopic expression experiments indicate that mature Vg1 confers a left ‘identity’ to cells (e.g. nodal expression is elicited), it is unclear how right cell lineages are instructed to adopt a right ‘identity’. It is possible that cells of the LRC also emit signals that diffuse to the right side of the blastula to suppress left identity in right-sided cells, the latter of which acquire right identity by default. Alternatively, a second signal, expressed by right cell lineages, might act locally to antagonize a response to Vg1 signaling emanating from the LRC. The results presented here support the latter model of an active signaling pathway on the right side that antagonizes left-sided signaling in establishment of the left-right axis in the Xenopus blastula (Fig. 4).
predominant right-sided nodal expression elicited by ectopic AVg alone, indicates that these two signaling pathways are antagonistic with respect to left-right development. Moreover, the Vg1-mediated attenuation of defects other than heart reversals that follow ectopic ALK2 signaling provides additional evidence of antagonism between these two TGFβ-related pathways. At present, the relationship of the BMP/ALK2 pathway, if any, to other previously identified right-sided signaling pathways that are involved in left-right patterning of the embryo is not known. In the chick, signaling molecules such as activin and FGF8 function as right-sided ‘determinants’ that are thought to repress right-sided nodal expression as well as activate right-sided cSnr expression (Boettger et al., 1999; Isaac et al., 1997; Levin et al., 1995, 1997; Levin and Mercola, 1998a). However, substantial evidence suggests that activin does not function as a right-sided determinant in Xenopus (reviewed by Ramsdell and Yost, 1998, 1999; see also below), and a recent study suggests that FGF8 functions as a left-sided determinant in mouse (Meyers and Martin, 1999). Thus, resolution of this issue will require an understanding of whether all aspects of the genetic left-right developmental pathway that operate upstream of nodal expression are functionally conserved among diverse vertebrate species (reviewed by Yost, 1999).

In evaluating a role for BMP ligands as part of the ALK2 pathway, it was found that when targeted to the same left cell lineages, BMP2 and BMP4 can mimic the effects of ALK2 signaling on heart looping. However, because of the reported trans-activity of TGFβ prodomains (Gentry and Nash, 1990; Gray and Mason, 1990) and the propensity of TGFβ family members to form heterodimers in vitro (Hazama et al., 1995; Suzuki et al., 1997a) and in vivo (Chang and Hemmati-Brivanlou, 1999; Israel et al., 1996; Nishimatsu and Thomsen, 1998; Sampath et al., 1990), it was important to test whether BMPs and Vg1 interact via related prodomains to inhibit Vg1 activity. Thus, a chimeric form of Vg1 containing the activin βB prodomain (AVg) was used in BMP-Vg1 coexpression experiments, and a chimeric form of activin-BMP4 was tested in single injection experiments. The ability of BMPs to inhibit AVg-mediated heart reversals is similar to that reported for coexpression of BMP4 with a BMP-Vg1 chimera (Hyatt and Yost, 1998), and the activin-BMP4 chimera elicits heart reversal rates similar to those reported for wild-type BMP2 and BMP4 (Hyatt and Yost, 1998). Thus, the results obtained with chimeric forms of BMP and Vg1 containing activin prodomains eliminate the possibility that ectopic BMP antagonizes endogenous or exogenous Vg1 via prodomain interactions, suggesting that BMPs do not interfere with Vg1 activity by a mechanism involving heterodimer formation or competition for components of a common precursor protein processing pathway.

A second mechanism by which BMP and Vg1 could serve as mutual antagonists is by competition for a common receptor, as is proposed to occur with two other TGFβ-related signaling molecules, activin and antivin (Thisse and Thisse, 1999). However, several lines of reasoning do not support this possibility. First, ectopic Vg1 and CA-ALK2 elicit heart reversals only when targeted to opposite sides of the blastula, raising the obvious point that Vg1 signaling does not occur via the type I ALK2 receptor. Second, activation of type I receptor kinase activity requires heterocomplex formation with type II receptors (reviewed by Yamashita et al., 1996), and it is possible that a common type II receptor could associate with both BMP and Vg1 type I receptors. However, this is contraindicated by previous findings that a truncated, dominant-negative type II activin receptor (IIb) capable of inhibiting Vg1 signaling (Kessler and Melton, 1995) elicits heart reversals only when targeted to the left (but not right) side of the blastula (Hyatt and Yost, 1998). Third, similar to ectopic BMPs and CA-ALK2, a downstream signal transducer of BMP/ALK2 signaling, Smad1, also elicits heart reversals when targeted to left cell lineages. Together, these results suggest that BMP-Vg1 antagonism occurs as a result of opposing, intracellular signal transduction pathways.

Whereas results obtained following ectopic activation of ALK2 signaling clearly support a role for this pathway in left-right patterning, the results obtained in experiments designed to inhibit ALK2 signaling in right cell lineages do not provide an unequivocal indication of its in vivo requirement. The low rates of heart reversals and the very modest alterations of nodal expression following tALK2 receptor expression are consistent with a role for endogenous ALK2 signaling in establishing early left-right molecular asymmetry, although by comparison, the effects of BMP ligand and CA-ALK2 expression on heart looping and nodal expression are much more pronounced. With respect to embryos exhibiting bilateral nodal expression, embryos injected with tALK2 RNA showed only a small patch of ectopic nodal expression in the right lateral plate mesoderm, compared to the robust right-sided nodal expression following injection with CA-ALK2 RNA. As discussed above, the relative quantitative differences in left- and right-sided nodal expression are strongly correlated with organ situs; that is, heart and gut orientation nearly always correspond to the side in which nodal expression is greater. In instances of equal bilateral expression, or of absent expression, organ situs is stochastic or ‘randomized’. The differing extent of
perturbations of nodal expression and heart orientation following CA-ALK2 expression compared to tALK2 expression might reflect the relative experimental ease of ectopic activation of a signaling pathway versus inhibition of the endogenous pathway in a whole animal-model system. Unfortunately, as with other genes that are involved in left-right development (including nodal; Conlon et al., 1994), mouse null mutations cannot substantiate a role for ALK2 signaling in left-right development, due to early embryonic lethality and/or failure to form the heart tube that occurs in the majority of ALK2−/− (Gu et al., 1999), BMP2−/− (Zhang and Bradley, 1996), BMP4−/− (Winnier et al., 1995), and BMP7−/− (Dudley et al., 1995) mice. However, given that ALK2 signaling was also found to attenuate Vg1 activity, and that Smad7 expression in right cell lineages results in heart reversals, these results together strongly implicate a role for the ALK2 pathway in modulating LRC function.

As with the role of BMPs in dorsoventral patterning, an endogenous role for the BMP/ALK2 pathway in left-right development may find further support as other components of the pathway become elucidated. In Xenopus, dorsoventral mesoderm patterning is mediated by the opposing activities of BMPs and activin, whereby BMP signaling induces ventral cell fates, and anti-BMP and activin signaling induces dorsal cell fates (reviewed by Lemaire and Kodjabachian, 1996). Complex extracellular regulatory mechanisms involving ligand-binding antagonists and proteases control the spatial extent of these signaling pathways (reviewed by Cho and Blitz, 1998; Lemaire and Kodjabachian, 1996). Moreover, limited expression of intracellular signal transduction proteins that are competitively utilized by both signaling pathways operates on a single-cell level to titrate a response to the simultaneous activation of both pathways (Candia et al., 1997). Thus, despite the lack of significant dorsoventral differences in BMP or activin mRNA and protein localization in the Xenopus egg or early embryo (Dohrmann et al., 1993; Hemmatti-Bri vanlou and Thomsen, 1995), other mechanisms facilitate restricted signaling activity of these ligands. By analogy to dorsoventral patterning processes, it is anticipated that BMP-Vg1 antagonism in left-right development that is proposed here involves similar levels of regulatory control and, moreover, that as these mechanisms become clarified, additional means of testing the role of the BMP/ALK2 pathway will be possible. Consistent with this idea, it also was found that expression of Xolloid, encoding a protease that liberates BMP from inactive BMP-chordin complexes (Piccolo et al., 1997), results in heart reversals when targeted to left cell lineages of the Xenopus blastula.

Lastly, it should be emphasized that very little is known about the Vg1 pathway or the mechanism by which Vg1 precursor is processed to a biologically active, mature form. Other than the type II activin receptor Iib (Kessler and Melton, 1995) and a recently reported potential role for Smad2 (Hoodless et al., 1999), components of the Vg1 signaling pathway, including its type I receptor(s), have yet to be identified. Whereas the results presented here do not identify components of the Vg1 pathway, they do eliminate the ALK2 receptor and its downstream signal transducers (Smad1) as mediators of Vg1 signaling. Moreover, coexpression studies with chimeric BMP and Vg1 ligands are inconsistent with the possibility that BMPs and Vg1 compete for utilization of a common precursor protein processing pathway, suggesting that the BMP proteolytic pathway is not involved in maturation of Vg1 ligand. Finally, the demonstrated antagonism between ALK2 signaling and Vg1 activity in the context of left-right patterning is the first indication of a molecular pathway that is capable of inhibiting Vg1-mediated responses in the early embryo, a finding which should serve as a useful entry point to define components of the intracellular pathway that are required for Vg1 signaling.

We thank Monique Judd, Cindy Rote and Jun Zhang for excellent technical assistance, Bill Branford and Jeff Essner for advice on whole-mount in situ hybridization, Dan Weeks for suggesting use of the CT3 antibody, and Alexander Tsudikov for expert assistance with statistical analyses. We are grateful to A. Blushan, K. W. Y. Cho, J. L. Christian, R. M. Harland, A. Hemmatti-Bri vanlou, D. A. Melton, G. H. Thomsen, W. Vale, and C. V. E. Wright for providing cDNA clones. The CT3 antibody was obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, and the Department of Biological Sciences, University of Iowa, under contract N01-HD-6-2915 from the NICHD. A. F. R. is an American Heart Association Western States Affiliate Postdoctoral Fellow and H. J. Y. is an Established Investigator of the American Heart Association. This work was supported by a grant from NIH/HLBI to H. J. Y.

Note added in proof

While the current manuscript was in press, it was reported that misexpression of a novel BMP ligand antagonist, encoded by caronte, antagonizes left-right development in the chick (Yokouchi et al., 1999; Esteban et al., 1999), concurring with our findings that a right-sided BMP/ALK2 signaling pathway is required to establish left-right asymmetry.


REFERENCES


Multiple TGF-beta signals: intracellular antagonism between activin/BVg1 and BMP-2/4 signaling mediated by Smads. Development 124, 4467-4480.


Graff, J. M., Thies, R. S., Song, J. J., Celeste, A. J. and Melton, D. A.


