Retinoic acid is required in the mouse embryo for left-right asymmetry determination and heart morphogenesis

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SUMMARY

Determination of the left-right position (situs) of visceral organs involves *lefty*, *nodal* and *Pitx2* genes that are specifically expressed on the left side of the embryo. We demonstrate that the expression of these genes is prevented by the addition of a retinoic acid receptor pan-antagonist to cultured headfold stage mouse embryos, whereas addition of excess retinoic acid leads to their symmetrical expression. Interestingly, both treatments lead to randomization of heart looping and to defects in heart anteroposterior patterning. A time course analysis indicates that only the newly formed mesoderm at the headfold-presomite stage is competent for these retinoid effects. We conclude that retinoic acid, the active derivative of vitamin A, is essential for heart situs determination and morphogenesis.

Key words: Retinoids, Mouse development, Heart looping, *lefty*, *nodal, Pitx2*

INTRODUCTION

All vertebrates develop with left-right (L-R) asymmetry. In the last few years, a number of molecules have been shown to be involved in L-R patterning (reviewed by Harvey, 1998; Ramsdell and Yost, 1998). In chick embryos, the activin pathway, as well as *sonic hedgehog (shh)*, appear to be the earliest L-R asymmetric signals operating in Hensen's node region during gastrulation (Levin et al., 1995; 1997; Pagan-Westphal and Tabin, 1998). In the mouse, activin receptor II B inactivation leads to lateral asymmetry defects (Oh and Li, 1997), whereas *shh* knockout does not result in laterality defects (Chiang et al. 1996). Two other mouse mutations, *inversion of embryonic turning (inv)* and *inverted viscerum (iv)*, lead to situs inversus and randomized situs, respectively, and appear to act upstream of all other known asymmetrical markers (reviewed by Supp et al., 1998). However, the *inv* and *iv* genes, which have recently been cloned, are expressed ubiquitously or symmetrically within the node (Supp et al., 1997; Morgan et al., 1998; Mochizuki et al., 1998). Thus, it remains unclear how these genes control L-R axis determination. At the beginning of somitogenesis, the TGFβ-related secreted molecules Lefty1, Lefty2 and Nodal, which are specifically expressed on the left side of the embryo, have been shown to play a role in vertebrate L-R determination (Collignon et al., 1996; Lowe et al., 1996; Meno et al., 1996; 1997; 1998; Oulad-Abdelghani et al., 1998). The Pitx2 homeodomain transcription factor that acts downstream of these genes, is coexpressed with them in the left lateral plate mesoderm (LPM). However, at later stages, only *Pitx2* expression persists in the left side of the heart and of some visceral organs (Ryan et al., 1998; Yoshioka et al., 1998; Logan et al., 1998; Piedra et al., 1998; St Amand et al., 1998; reviewed by Harvey, 1998).

Retinoic acid (RA), the active derivative of vitamin A, plays numerous roles during embryogenesis (reviewed by Sporn et al., 1994; Kastner et al. 1995). RA-induced asymmetry defects in mammalian (hamster, rat and mouse) embryos have been reported (reviewed by Fujinaga, 1997). Excess RA administration (Smith et al., 1997), as well as vitamin A deficiency (Dersch and Zile, 1993; Twal et al., 1995), can cause situs inversus in avian embryos, but it is not yet known when and how retinoids act on L-R patterning. We have previously cloned *lefty1* as an RA-inducible gene in embryonal carcinoma cells and showed that RA induces its overexpression in the visceral endoderm of 6.5 days post-coitum (dpc) mouse embryos (Oulad-Abdelghani et al., 1998). During early somitogenesis (4- to 8-somite stage), *lefty* genes are transiently expressed on the left side of lateral plate mesoderm (LPM) and left half of the neural tube presumptive floor plate (FP), with a predominant expression of *lefty1* in the FP and *lefty2* in the LPM (Meno et al., 1996; 1997; Oulad-Abdelghani et al., 1998). We show here that RA controls the expression of these genes during L-R patterning. Furthermore, we demonstrate that RA is necessary for heart situs determination at the time of node formation, and acts on cells that have just undergone gastrulation. We also show that retinoids are necessary for heart morphogenesis along the anteroposterior axis.

MATERIALS AND METHODS

Embryo culture and retinoid treatments

Mouse embryos were collected at various time points during the eighth day of gestation, staged according to Downs and Davies (1993) and kept in culture as described by New (1990) for 6-30 hours. All-*trans* RA (10^{-6} or 10^{-7} M; Sigma, St-Louis, MO) or the BMS493 (10^{-6} M) pan-RAR synthetic retinoid antagonist (Bristol-Myers-Squibb, Princeton, NJ), diluted in ethanol, were added to the culture medium. In control embryo cultures, the ethanol vehicle was added at the same dilution (0.1%). In the case of short-term (3-4 hours) retinoid treatments, the embryos were washed in Tyrode's buffer at the end of the treatment and reincubated with fresh culture medium until they reached the adequate stage for analysis. Embryos heterozygous for the mRARβ2-*lacZ* transgene were processed for X-gal staining as described by Mendelsohn et al. (1991).

Whole-mount in situ hybridization

The 1.6 kilobase (kb) *lefty1* cDNA (Oulad-Abdelghani et al., 1998) cloned in pBluescriptSK (Stratagene, Palo Alto, CA) was used as template for T7 polymerase in vitro transcription reactions with digoxigenin-11-UTP or fluorescein-12-UTP (Boehringer, Indianapolis, IN) to generate antisense riboprobes. As this probe cross-hybridizes with *lefty2* gene transcripts (Meno et al., 1997), in situ hybridizations were also performed using probes (kindly provided by Dr H. Hamada, Osaka, Japan) that are specific for the *lefty1* and *lefty2* gene transcripts. The *nodal*, *Hand1*, *NKx2.5* and *Pitx2* cDNA clones were kindly provided by M. Kuehn (NIH, Bethesda), G. Gradwohl and P. Kastner (IGBMC, Strasbourg) and C. Goridis (IBDM, Marseille). Whole-mount ISH was carried out as described by Décimo et al. (1995). For double labellings, digoxigenin- and fluorescein-labelled probes were hybridized simultaneously. The fluorescein probe was revealed with an anti-fluorescein antibody coupled with alkaline phosphatase, and a substrate giving a blue color (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 50 mM MgCl$_2$, 0.1% Tween 20, 225 μM potassium ferricyanide, 450 μM potassium ferrocyanide, 17.5 μg/ml BCIP (Boehringer). The first antibody was inactivated by incubation for 10 minutes in 0.1 M glycine-HCl (pH 2.2). The second probe was revealed with an anti-digoxigenin antibody coupled with alkaline phosphatase, with NBT/BCIP substrates giving a purple color. For histological analysis, the hybridized embryos were postfixed for 10 minutes in 4% paraformaldehyde, embedded in paraffin wax and cut at 7-10 μm.

RESULTS

lefty and *nodal* expression is controlled by RA

Before investigating whether RA excess or deficiency could affect the asymmetric expression of *lefty* genes, we tested the response of in vitro cultured embryos to retinoids using a mRARβ2-*lacZ* reporter transgene that contains an RA response element (Mendelsohn et al., 1991). Transgenic embryos were collected at the headfold (HF) stage (7.5 dpc) and cultured for 8 to 10 hours, i.e. until the 4- to 8-somite stage, in the presence of 10^{-6} or 10^{-7} M RA (Fig. 2A, a) or vehicle (ethanol, control embryos). RA treatment strongly enhanced the level of *lacZ* expression both quantitatively and qualitatively (compare Fig. 1A and B; note the rostral shift of
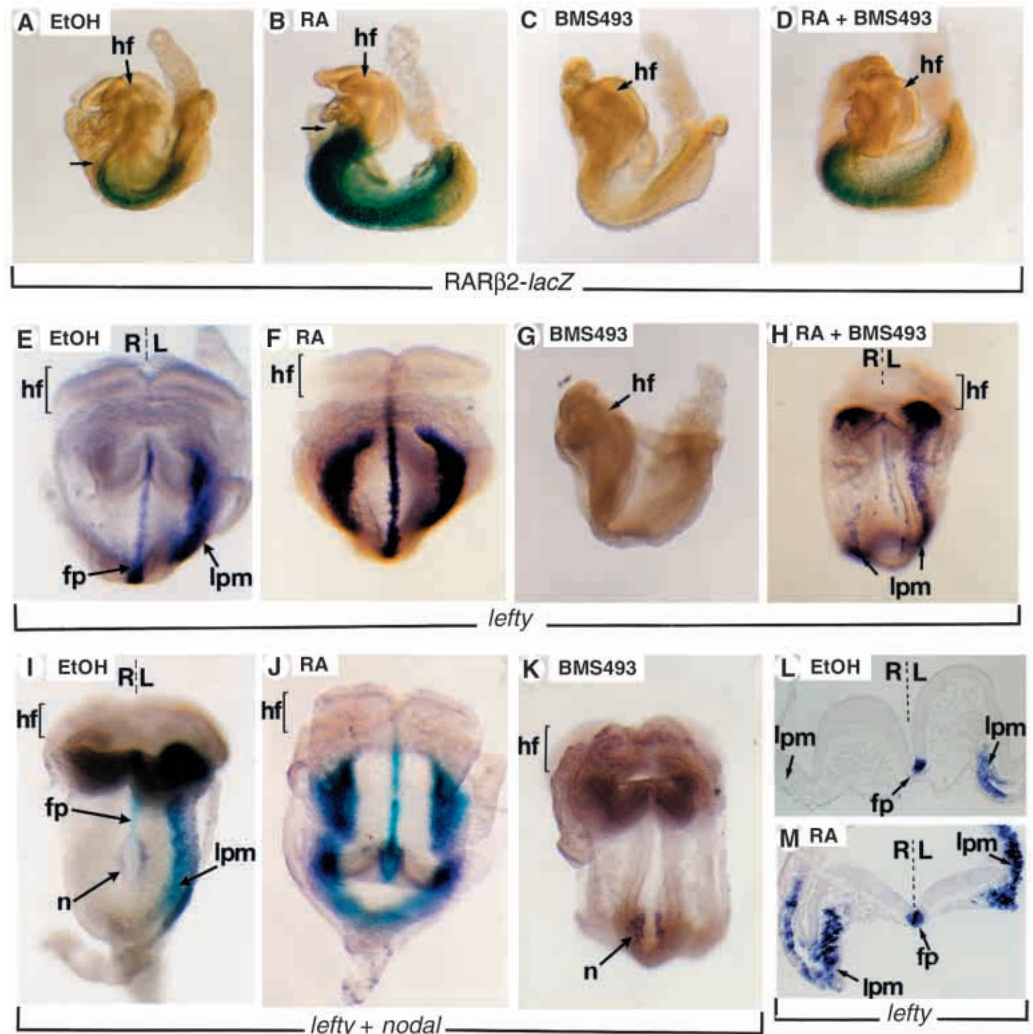

Fig. 1. Effects of retinoids on RARβ2-*lacZ* transgene expression and on endogenous *lefty* and *nodal* expression. Embryos were collected at the headfold stage and cultured for 8-10 hours in the presence of all-*trans* retinoic acid (RA), the pan-RAR synthetic retinoid antagonist BMS493, or ethanol (EtOH, vehicle) alone. (A-D) X-gal staining of mRARβ2-*lacZ* transgenic embryos. The thin arrows in A and B point to the anterior boundary of transgene expression. (E-H) Detection of *lefty* transcripts by whole-mount ISH. (I-K) Double ISH with *lefty* (blue) and *nodal* (purple) probes. (A,E,I) Control embryos cultured in 0.1% ethanol. (B,F,J) Embryos treated with 10^{-6} M RA. (C,G,K) Embryos treated with 10^{-6} M BMS493. (D,H) Embryos treated with 10^{-6} M BMS493 and 10^{-6} M RA. (L,M) Sections through the floor plate of control and RA-treated embryos, respectively. fp, floor plate; hf, headfold; L, left side; lpm, lateral plate mesoderm; n, node; R, right side.

Table 1. Effects of retinoids on *lefty*, *nodal* and *Pitx2* expression in lateral plate mesoderm

Treatment	*lefty* and *nodal*					*Pitx2*			
	L	L=R	L>R	R>L	Abs.	L	L=R	L>R	Abs.
Ethanol	32 100%	0	0	0	0	11 100%	0	0	0
RA (10^{-6} or 10^{-7} M)	0	10 25%	29 71%	2 4%	0	15 65%	9 35%	0	0
BMS493 (10^{-6} M)	0	0	0	0	24 100%	0	0	0	12 100%
BMS493 + RA (10^{-6} M)	0	3 25%	9 75%	0	0	n.d.	n.d.	n.d.	n.d.

Treatments were performed from HF stage until 4 to 10 somite-stages.
L: expression on the left side only; L=R: perfect mirror image expression; L>R: expression on both sides, but stronger on the left; Abs.: absence of expression; n.d.: not determined.

the *lacZ* expression boundary, thin arrows). On the other hand, *lacZ* expression was abrogated by a synthetic antagonist (BMS493, 10^{-6} M) (G. Einsenmann, H. Gronemeyer and P. C., unpublished data) of all three retinoic acid receptors (RARα, β and γ) (Figs 1C; 2A, b; O. Wendling, P. C. and M. Mark, unpublished data). This inhibition was specific, as it was relieved by the simultaneous administration of 10^{-6} M RA (Fig. 1D).

Under the same conditions (Fig. 2A, a), the addition of RA induced an ectopic expression of *lefty* on the right side of the embryo, as a mirror-image of the normal domains, in both LPM and FP (compare Fig. 1E with F and L with M; and data not shown). All RA-treated embryos (*n*=29) showed ectopic expression on the right side, but only 25% as a perfect mirror-image (Fig. 1F), the remaining 75% having a weaker expression on the right side (Table 1; data not shown). This right-sided expression was observed both with a *lefty1* full-length cDNA probe that cross-hybridizes with *lefty2* (Meno et al., 1997) and with *lefty1*- and *lefty2*-specific probes (see Materials and Methods). In contrast, a full inhibition of *lefty* gene expression was observed in all embryos treated with the pan-RAR antagonist BMS493 (*n*=14; Figs 1G,K, 2A, b). This inhibition was relieved in the presence of 10^{-6} M RA (Fig. 1H), thus demonstrating that expression of *lefty* genes in control embryos (Fig. 1E) is dependent on RAR activation by endogenous retinoids. In this latter experiment, only about 25% of the embryos expressed *lefty* in a perfect mirror-image, the others exhibiting weaker expression on the right side (Fig. 1H and data not shown; Table 1). At the 4- to 8-somite stage, *nodal* is coexpressed with *lefty* genes in the left LPM and is also expressed in the node (Fig. 1I; Collignon et al., 1996; Lowe et al., 1996). *nodal* was ectopically activated in the right LPM of all RA-treated embryos (*n*=11; Fig. 1J). The BMS493 antagonist inhibited *nodal* expression in the LPM, but, interestingly, not in the node (*n*=10; Fig. 1K). As observed for *lefty*, this inhibition of *nodal* expression in the LPM was relieved by RA addition (data not shown).

RA is required for heart patterning and situs determination

We then analyzed whether alterations in *lefty* and *nodal* asymmetric expression, following RA or pan-RAR antagonist addition, might correlate with abnormalities in heart looping.

Mouse embryos were collected at the HF stage and cultured for 20-30 hours in order to allow the heart to develop. RA (10^{-6} or 10^{-7} M) or BMS493 (10^{-6} M) were present throughout the culture period (long-term treatments, Fig. 2A, c) or only during the first 3-4 hours (short-term treatments; Fig. 2A, d). About half of the embryos showed reversed (leftward) heart looping after short-term treatments (Table 2; Fig. 2E), whereas only ~15% of the control embryos cultured in the presence of ethanol (vehicle) showed this reversion (Table 2). Thus, both agonist and antagonist retinoid treatments led to randomization of the heart situs. Long-term RA treatments generated additional abnormalities of the heart tube, i.e. an incomplete fusion of the cardiac tubes and a posteriorization characterized by an enlarged atrium and reduced or missing pulmonary (right) ventricle and outflow tract (Table 2; Fig. 2C; and data not shown), as previously shown in zebrafish and chicken embryos (Osmond et al., 1991; Stainier and Fishman, 1992; Yutzey et al., 1994). In contrast, long-term BMS493 treatments led to an anteriorisation characterized by smaller systemic (left) ventricle and atrium, and a large pulmonary ventricle with, in the most severe cases, a single dilated ventricle cavity (Table 2; Fig. 2D). However, short-term treatments with either RA or BMS493 (Fig. 2A, d) produced little, if any, of these additional heart abnormalities (Table 2; Fig. 2E; data not shown).

The heart defects induced by long-term retinoid treatments were further characterized by the analysis of *Hand1* expression, a marker of the systemic ventricle in the posterior part of the heart tube (Biben and Harvey, 1997; Thomas et al., 1998; Fig. 2B) and implicated in heart morphogenesis (Srivastava et al., 1995; Firulli et al., 1998; Riley et al., 1998). In RA-treated embryos, *Hand1* was expressed in the most rostral part of the heart tube (Fig. 2C, and data not shown), indicating that the anteriormost region of the tube was missing. In BMS493-treated embryos, *Hand1* was not expressed in the dilated ventricle, suggesting that this abnormal cavity has a rostral – probably pulmonary ventricle – identity (Fig. 2D). We conclude that an agonist retinoid signal is independently required for heart situs determination and the patterning of heart chambers.

RA is transiently required at the headfold stage for normal heart looping

Embryos exposed to retinoids for short-term treatments (3-4

hours) and further cultured until the 4- to 8-somite stage were used to determine the embryonic stage at which *lefty* expression becomes sensitive to retinoids. No ectopic *lefty* expression was observed following an early RA exposure at the early allantoic bud (EAB) stage (*n*=5; Fig. 2A, e), whereas *lefty* expression was ectopically induced upon RA treatment at the HF (Fig. 2A, f; data not shown). RA treatments of older embryos (just before somitogenesis or at the 1- to 3-somite stages) led to an ectopic activation of *lefty* on the right side, but only in the posterior region of the embryo (*n*=7; Fig. 2A, h and 2F), revealing that only newly formed mesoderm was competent to respond to RA at this stage. Short-term treatments with the pan-RAR antagonist at the HF stage consistently inhibited *lefty* expression (*n*=5; Fig. 2A, g), whereas treatments at the presomite/early somite stages did not lead to a significant inhibition (Fig. 2A, i; data not shown). Accordingly, heart looping, which was randomized after short-term treatments of HF stage embryos by either RA or BMS493 addition (Table 2; Fig. 2A, d and 2E), remained normal when the embryos were treated at the presomite/early somite stage (Fig. 2A, j; data not shown). In another experiment (Fig. 2A, k), HF stage embryos were exposed to RA for 3-4 hours, washed and incubated in the presence of BMS493 until the onset of *lefty* expression (5-6 hours later). Under these conditions, an ectopic expression of *lefty* could still be observed on the right side of the LPM (*n*=7; data not shown), thus supporting the idea that *lefty* expression might not be directly induced by RA.

Differential effect of RA on the expression of genes involved in heart morphogenesis

We analysed the effects of retinoids on the expression of possible upstream and downstream effectors of *lefty* and *nodal*. As *HNF3-β* and *shh* have been implicated in left-right determination (Collignon et al., 1996; Levin et al., 1995; Pagan-Westphal et al., 1998; Dufort et al., 1998), we analyzed their expression in embryos treated for 4 hours with retinoids at the HF stage. The expression of both genes was not modified upon RA or BMS493 treatment (data not shown) although the expression is already symmetrical in the normal embryos. *shh* is expressed asymmetrically on the left side of the node in avian embryos (Levin et al., 1995; Pagan-Westphal et al., 1998), but such an asymmetric expression could not be detected in the mouse embryo. Interestingly, vitamin A-deficient quail embryos exhibit normal asymmetric expression of *shh* (Chen et al., 1996), suggesting that endogenous RA acts in parallel (or downstream) of *shh* to regulate *nodal* and *lefty* expression.

The transcription factor *Pitx2* is expressed in the head mesenchyme, as well as in the left LPM (see Fig. 3A), together with *lefty-2* and *nodal* but, unlike these genes, its expression
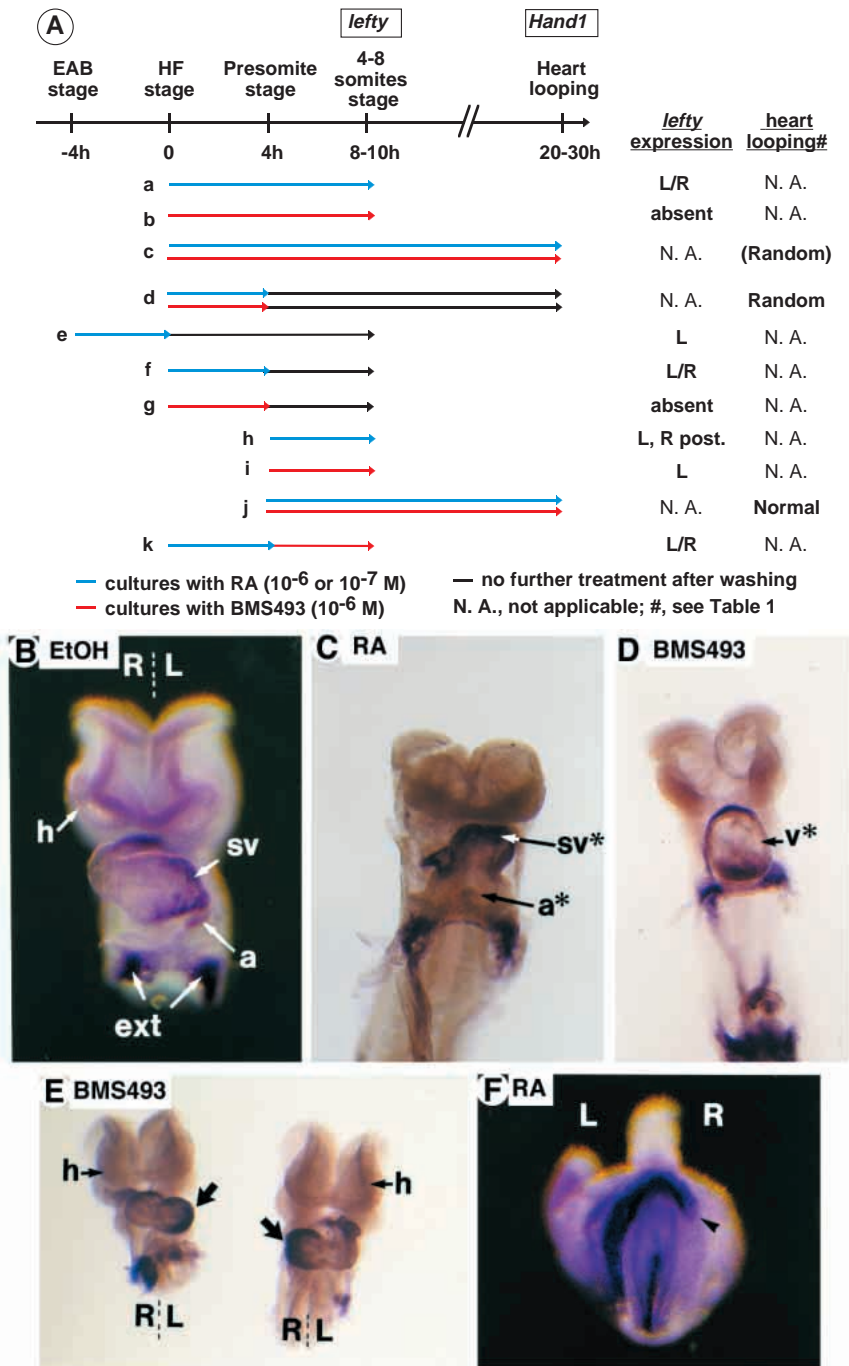

Fig. 2. (A) Embryo culture conditions. Embryos were collected at the 4- to 8-somite stage to analyze *lefty* expression, or 1 day after the beginning of the culture to analyze heart looping. (B-D) Heart morphology and *Hand1* expression pattern after long-term treatments (20-30 hours) with 0.1% ethanol (B), 10^{-6} M RA (C) or 10^{-6} M BMS493 (D). (E) Opposite orientations of heart looping, visualized by the asymmetric expression of *Hand1* in the systemic (left) ventricle (arrows) in two embryos analyzed after short-term administration of BMS493 (A, d). (F) Restricted ectopic induction of *lefty* in the posterior right-side mesoderm (arrowhead) of an embryo after exposure to RA at the presomite-early somite stage (A, h). a, atrium; a*, enlarged atrium; EAB, early allantoic bud; ext, extraembryonic tissues; h, head; HF, headfold; L, left; R, right; sv, systemic ventricle; sv*, abnormal systemic ventricle; v*, abnormal dilated ventricle cavity.

Table 2. Summary of retinoid-induced defects on heart situs and morphogenesis

| Ligand addition | Duration of treatment‡ (hours) | Heart looping | | Additional heart abnormalities |
		Normal situs	Reversed situs	
EtOH	20-30	16	2 (11%)	none
0.1%	3-4	25	5 (16%)	none
RA	20-30	4	4 (50%)	++++*
10^{-6} M	3-4	15	10 (40%)	++¶
RA	20-30	9	7 (43%)	+++*
10^{-7} M	3-4	12	8 (40%)	+¶
BMS493	20-30	12	10 (45%)	+++§
10^{-6} M	3-4	13	10 (43%)	+§

‡See Fig. 2A.

*These RA treatments induced posteriorization of the heart tube with enlarged atrial cavity and truncation of anterior structures (outflow tract, pulmonary ventricle), incomplete fusion of the heart tubes in some of the most severe cases (++++). In these cases, the sideness of heart looping could not be determined.

¶The posteriorisation defects were milder than in *.

§BMS493 treatment induced anteriorization of the heart tube (enlarged pulmonary ventricle, smaller systemic ventricle and atrium), reduced or absent septation between heart cavities and absence of looping in the most severe cases. These defects were most apparent after long-term BMS493 treatment.

lasts until heart looping (Ryan et al., 1998; Yoshioka et al., 1998; Logan et al., 1998; Piedra et al., 1998; St Amand et al., 1998). At later stages, *Pitx2* is expressed on the left side of most asymmetrical organs including the heart (see Fig. 3D) and gut (not shown). Expression and functional studies involving the ectopic expression of *nodal* or *lefty* or the knockout of *lefty1*, have shown that *Pitx2* acts downstream of these genes (Ryan et al., 1998; Yoshioka et al., 1998; Logan et al., 1998; Piedra et al., 1998; Meno et al., 1998). Two different effects on *Pitx2* expression were observed in retinoid-treated embryos. Prior to heart looping, *Pitx2* expression was inhibited by the antagonist BMS493 in the LPM, but not in the head mesenchyme (Fig. 3C). Embryos (*n*=12) treated at the HF stage with 10^{-6} M BMS493 and analyzed after 8-14 hours exhibited a lack of *Pitx2* signal in the LPM (Fig. 3C; Table 1). Upon RA treatment (10^{-7} M for 8-14 hours), *Pitx2* was ectopically induced in the right LPM in a fraction (9/26) of the embryos (Fig. 3B; Table 1). These embryos may correspond to the RA-treated embryos that exhibit a perfectly symmetrical expression of *lefty* and *nodal* (see above). We also noticed that the symmetrical *Pitx2* expression in RA-treated embryos was weaker than the asymmetrical expression in control embryos (Fig. 3A,B), suggesting that a mechanism of amplification of L-R asymmetrical expression operates in the embryo.

When analyzed at later stages during heart looping (20-30 hours in culture; Fig. 2A, d), *Pitx2* expression was no longer inhibited by the BMS493 treatment, nor symmetrically expressed upon RA treatment. In both cases, it was expressed on either the left or the right side of the heart and vitelline vein, according to the sideness of heart looping (Fig. 3E,F and data not shown, *n*=9). Note that bilateral expression of *lefty*, *nodal* and *Pitx2* never prevented heart looping in our embryos, whereas bilaterally symmetric, unlooped hearts were observed in chick embryos expressing these genes symmetrically (Levin et al., 1997; Logan et al., 1998).

The *Hand1* gene is involved in heart looping (Srivastava et al., 1995; Firulli et al., 1998; Riley et al., 1998) and is expressed, shortly prior to and during looping, in the rostralmost part of the heart tube (aortic sac), as well as in the prospective systemic ventricle, with a higher intensity along its left side (Fig. 3G; Biben and Harvey, 1997; Thomas et al., 1998). The side of *Hand1* predominant expression was randomized by both RA and BMS493 treatments (Fig. 2A, d), thus correlating with randomization of heart looping (Fig. 3H,I and data not shown), as it is the case for *Pitx2* late expression. The observation that *Hand1* and *Pitx2* late expression was not repressed in the presence of BMS493, indicates that retinoids, as well as *nodal* and *lefty* expression (see above), are not required for *Hand1* and *Pitx2* expression at this stage of development. Thus, another signal may induce heart-specific expression of these genes. However, a retinoid signal (as well as *lefty* and *nodal* expression) is required for correct situs determination and, therefore, for left-sided expression of these genes.

The *Nkx2.5* gene, which is required to generate that predominant expression of *Hand1* during heart looping (Biben and Harvey, 1997), is symmetrically expressed in both right and left precardiac tubes (Fig. 3J). *Nkx2.5* expression was not altered by RA or BMS493 treatment (Fig. 2A, a and b; Fig. 3J,K and data not shown). Note that *Nkx2.5* and *lefty* (as well as *nodal)* are coexpressed in the region corresponding to the prospective systemic ventricle (left in normal embryos, left or right in RA-treated embryos) where *Hand1* will be asymmetrically expressed (Fig. 3J,K).

DISCUSSION

Our study reveals that retinoid signaling is essential for two aspects of mouse heart development. First, it is essential for determination of its situs. Situs inversus has been reported in RA-treated chick and vitamin A-deficient quail embryos (Dersch and Zile, 1993; Twal et al., 1995; Smith et al., 1997). Thus, the retinoid signal acts upstream of *lefty*, *nodal* and *Pitx2* expression, similarly to the *iv* and *inv* genes whose mutations result in situs inversus and/or situs randomization (reviewed by Supp et al., 1998). This raises the question as to whether the expression of these latter genes could be controlled by retinoids. In any event, the effect of the pan-RAR antagonist shows that a retinoid agonistic signal is required for proper heart situs determination, while the randomization of heart looping as well as the symmetrical expression of *lefty*, *nodal* and *Pitx2* subsequent to RA treatment, indicates that both sides can respond to this signal. It appears therefore that the retinoid signal has to be asymmetrically present or mediated on the left side of the embryo. In the majority of RA-treated embryos, *lefty* expression was stronger on the left side than on the right (ectopic) side. This observation suggests that RA may not be a determining, but a permissive factor, probably synergizing with the action of left-restricted factors.

RA has been detected in the node region (Hogan et al., 1992), but there is no evidence at the present time that retinol or RA could be preferentially localized on the left side of the embryo. Enzymes which might be involved in RA synthesis (ADH-4, ALDH-1 and RALDH-2; Ang and Duester, 1997; Zhao et al., 1996; Niederreither et al., 1997) and degradation (P450 RAI, Fujii et al., 1997), as well as RA binding proteins (CRABPs, Ruberte et al., 1991), do not appear to be

asymmetrically distributed at the HF stage. At this stage, RARα is preferentially expressed in the node, and RARβ and RARγ in newly formed mesoderm, but the distribution of their transcripts appears to be symmetrical (unpublished observations). However, a translational control of their synthesis (Reynolds et al., 1996), or a post-transcriptional control of their activity by phosphorylation (Taneja et al., 1997) cannot be excluded.

The present effect of retinoids on heart looping is restricted to a critical period. At the headfold stage, but not earlier, there is a critical retinoid signaling window that controls the future expression of *lefty*, as well as the sidedness of heart looping. The headfold stage corresponds to the appearance of the node. Several lines of evidence have indicated that this transient structure is involved in L-R asymmetry (reviewed by Harvey et al., 1998), most likely through the action of motile cilia that generate a leftward flow of extraembryonic fluid (Nonaka et al., 1998). Retinoid agonist or antagonist treatments performed at later stages, just before somitogenesis, did not lead to randomized heart

looping, showing that, at that stage, cardiac mesoderm is already determined for the situs. However, an RA treatment at the presomite stage led to symmetrical expression of *lefty* in
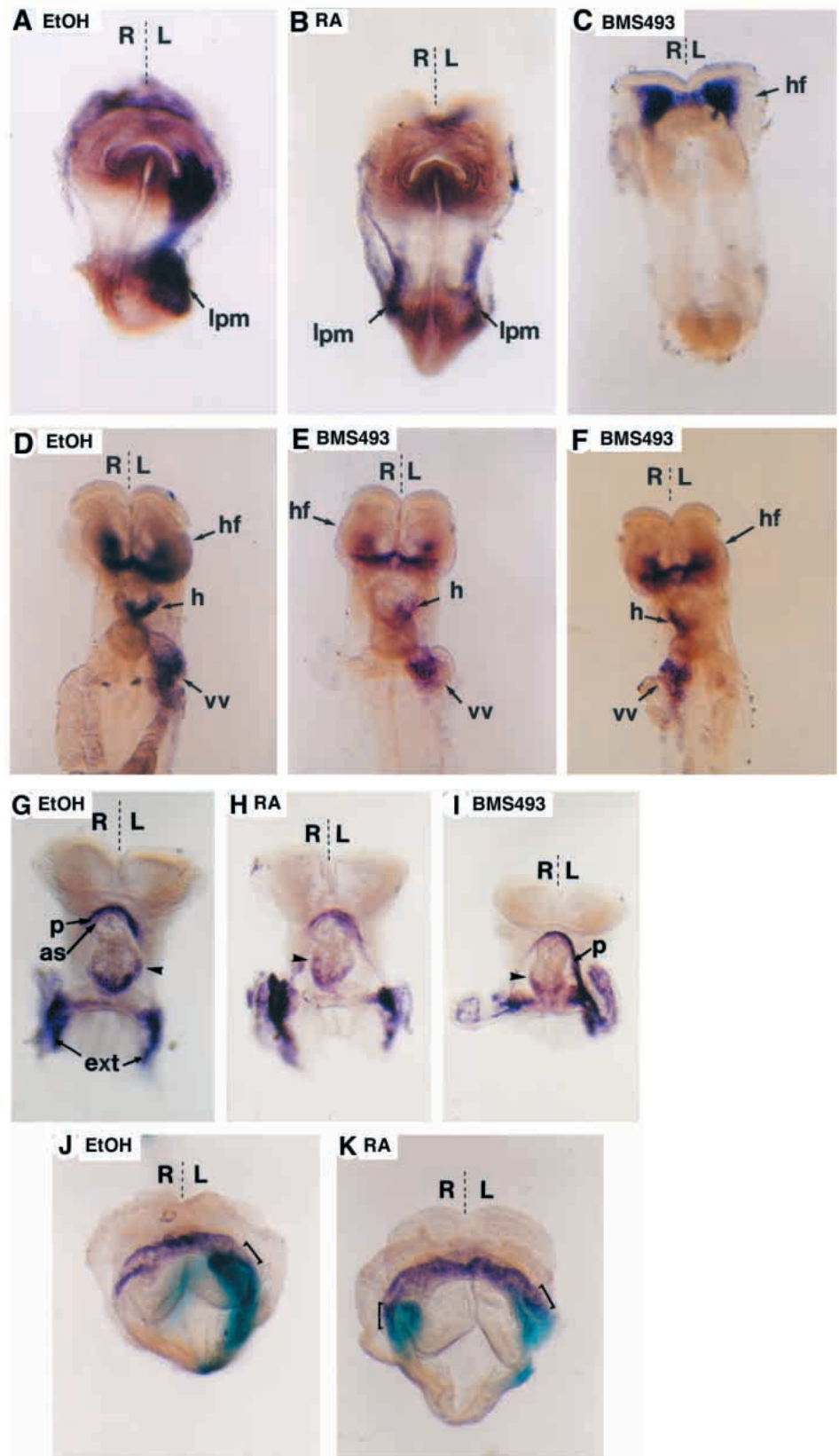

Fig. 3. Effects of retinoids on *Pitx2*, *Hand1* and *Nkx-2.5* expression. (A-C) *Pitx2* expression (ventral views) in embryos cultured from the HF stage during 14 hours with 0.1% ethanol (A), 10^{-7} M RA (B) or 10^{-6} M BMS493 (C). Note that the bilateral expression in the RA-treated embryo (B) is not as strong as the left-sided expression in the control (A). (D-F) Randomized expression of *Pitx2* in 12- to 15-somite stage embryos (ventral views) after short term administration (see Fig. 2A, d) of 10^{-6} M BMS493 (E,F). The control embryo (D) was administered ethanol only. G-I: *Hand1* expression in 12- to 15-somite stage embryos after short-term administration of 0.1% ethanol (G), 10^{-7} M RA (H) or 10^{-6} M BMS493 (I). The arrowheads point to the side of *Hand1* predominant expression within the prospective systemic ventricle. (J,K) Double ISH with *lefty* (blue) and *Nkx2-5* (purple) on embryos treated with 0.1% ethanol (J) and 10^{-6} M RA (K). *Nkx2-5* is expressed in heart progenitor cells and *lefty* in lateral plate mesoderm. Note the cells coexpressing both genes at the basis of the heart tubes (brackets). as, aortic sac; ext, extraembryonic tissues; h, heart; hf, headfold; L, left; lpm, lateral plate mesoderm; p, pericardium; R, right; vv, vitelline vein.

more posterior regions of the mesoderm, suggesting that retinoids act during, or shortly after, migrating mesodermal cells have left the primitive streak. Whether this symmetrical expression of *lefty* in posterior mesoderm could affect the situs of posterior organs, such as lungs, cannot be tested with the whole embryo culture system.

Retinoid signaling is also essential for anteroposterior patterning of the heart chambers, as its blockage by a pan-RAR antagonist leads to an anteriorization of these structures, whereas RA excess leads to their posteriorization. These data are in agreement with previous observations made in non-mammalian species (Heine et al., 1985; Osmond et al., 1991 Stainier and Fishman, 1992; Yutzey et al., 1994). These anteriorizations and posteriorizations were generated upon long-term (20-30 hours) treatments only, indicating that a spatially restricted RA signal is required for heart tube patterning. Interestingly, the RA-synthesizing enzyme Raldh2 is specifically expressed in the caudal portion of the differentiating heart tube (prospective atria and sinus venosa) where RA is apparently present (Moss et al., 1998). Thus, treatment with the BMS493 antagonist may block the RA signal required for the development of heart posterior structures (leading to anteriorization), whereas, on the contrary, RA excess would favor the development of posterior structures at the expense of anterior structures (leading to posteriorization). It appears therefore that a localized RA production, possibly through a posterior to anterior gradient, is important for patterning along the linear heart tube. Interestingly, the heart phenotype of *Raldh2* knockout embryos that includes the absence of looping and chamber morphogenesis (Niederreither et al., 1999), resembles that of embryos treated with the retinoid antagonist.

In conclusion, our study reveals that alterations of retinoid signaling affect the L-R situs as well as heart morphogenesis in mouse embryos. Congenital cardiac diseases are frequent in humans (about 1 and 10% of live and still births, respectively), and a number of syndromes include situs and/or chamber defects (reviewed by Payne et al., 1995; Goldstein et al., 1998). The genetic basis of most of these syndromes is unknown. Our data suggest that mutations in genes encoding components of the retinoid signaling pathway could be involved in their etiology, as well as in some cases of early embryonic lethality. In this respect, dietary vitamin A deficiency could represent a major epigenetic (environmental) factor increasing the penetrance and/or expressivity of such mutations.

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