

Embryonic retinoic acid synthesis is essential for heart morphogenesis in the mouse

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SUMMARY

Retinoic acid (RA), the active derivative of vitamin A, has been implicated in various steps of cardiovascular development, but its contribution to early heart morphogenesis has not been clearly established in a mammalian system. To block endogenous RA synthesis, we have disrupted the gene encoding RALDH2, the first retinaldehyde dehydrogenase whose expression has been detected during early mouse post-implantation development. We describe here the heart abnormalities of the RA-deficient *Raldh2* mutants that die in utero at gestational day 10.5. The embryonic heart tube forms properly, but fails to undergo rightward looping and, instead, forms a medial distended cavity. Expression of early heart determination factors is not altered in mutants, and the defect in heart looping does not appear to involve the *Nodal/Lefty/Pitx2* pathway. Histological and molecular analysis reveal distinct anteroposterior components in the mutant heart tube, although posterior chamber (atria and sinus venosus) development is severely impaired. Instead of

forming trabeculae, the developing ventricular myocardium consists of a thick layer of loosely attached cells. Ultrastructural analysis shows that most of the ventricular wall consists of prematurely differentiated cardiomyocytes, whereas undifferentiated cells remain clustered rostrally. We conclude that embryonic RA synthesis is required for realization of heart looping, development of posterior chambers and proper differentiation of ventricular cardiomyocytes. Nevertheless, the precise location of this synthesis may not be crucial, as these defects can mostly be rescued by systemic (maternal) RA administration. However, cardiac neural crest cells cannot be properly rescued in *Raldh2*^{-/-} embryos, leading to outflow tract septation defects.

Key words: Cardiomyocyte differentiation, Heart development, Left-right patterning, Mouse mutant, Myocardium, *Raldh2*, Retinaldehyde dehydrogenase, Retinoids, Tbx5

INTRODUCTION

Retinoic acid (RA), the active metabolite of vitamin A, is an important signaling molecule for the vertebrate embryo. Administration of excess RA has striking teratogenic effects on early heart development. According to the species, stage and mode of administration, excess RA can result in either lack of fusion of the paired cardiac primordia, impaired or reversed heart looping, and/or truncation of the posterior portion of the heart tube (prospective atrium) with abnormal expansion of anterior (prospective ventricle) structures (Osmond et al., 1991; Yutzey et al., 1994; Dickman and Smith, 1996; Drysdale et al., 1997). However, nutritional vitamin A deficiency (VAD) in rat (Wilson and Warkany, 1949), as well as RA receptor (RARs) and retinoid X receptor (RXRs) knockouts in mouse, lead to abnormal ventricular trabeculation, premature differentiation of the ventricular cardiomyocytes and defective outflow tract septation, while early stages of heart morphogenesis (i.e. looping of the heart tube and chamber determination) do not appear to be altered (Mendelsohn et al., 1994; Sucof et al., 1994; Kastner et al., 1994; Kastner et al.,

1997; Gruber et al., 1996; Ghyselinck et al., 1998; Mascrez et al., 1998). However, in quail embryos that develop under full VAD display early heart defects, including a highly distended heart cavity that loops imperfectly and lacks its caudalmost portion (inflow tract) and the associated omphalomesenteric vessels (Heine et al., 1985; Dersch and Zile, 1993; Zile et al., 2000). A similar complete VAD cannot be achieved in mammalian species, as it leads to maternal infertility. However, incubation of gastrulating mouse embryos in culture with synthetic RAR antagonists leads to altered heart looping and chamber formation (Chazaud et al., 1999; Tsukui et al., 1999).

Enzymatic RA synthesis is a two-step process in which retinol (vitamin A) is first oxidized into retinaldehyde by member(s) of the alcohol dehydrogenase (ADH) and/or short chain alcohol dehydrogenase/reductase (SDR) families, whereas conversion of retinaldehyde to RA is carried out by member(s) of the class I aldehyde dehydrogenase (ALDH) family (Duester, 2000; McCaffery and Dräger, 2000; Ulven et al., 2000). Among these, retinaldehyde dehydrogenase 2 (RALDH2) is a highly specific enzyme (Zhao et al., 1996) which is first expressed in the posterior mesoderm of the mouse

embryo during gastrulation (Niederreither et al., 1997), and then becomes restricted to posterior (inflow tract) structures of the developing heart (Moss et al., 1998). As (1) the expression pattern of this enzyme closely correlates with that of a highly sensitive RA-responsive reporter transgene (Rossant et al., 1991), and (2) other RALDH activities could not be detected before E8.5-9.5 (Duester, 2000; Ulven et al., 2000; Li et al., 2000), RALDH2 was proposed to be responsible for localized production of RA during early mouse embryogenesis (Niederreither et al., 1997). Accordingly, no expression of the above RA-responsive reporter transgene could be detected in *Raldh2* (*Aldh1a7* – Mouse Genome Informatics) gene knockout mutants, except in the developing eye (Niederreither et al., 1999).

We have previously reported that *Raldh2*^{-/-} embryos die at midgestation (E10.5) and exhibit major patterning defects along the anteroposterior (AP) body axis and developing hindbrain (Niederreither et al., 1999; Niederreither et al., 2000). Our initial analysis also revealed that E9.5 *Raldh2*^{-/-} embryos display an abnormal, dilated heart cavity, which apparently fails to undergo left-right (LR) looping morphogenesis (Niederreither et al., 1999). We have now further investigated the anatomical, cellular and molecular alterations presented by the *Raldh2*^{-/-} mutant heart. We find that AP and dorsoventral (DV) differentiation of the heart tube can proceed, at least to some extent, in the absence of LR looping, but atrial cavity outgrowth is severely impaired. Lack of RA synthesis further affects cardiomyocyte differentiation and trabeculation of the myocardial wall. We also show that early heart morphogenesis can be significantly rescued in *Raldh2*^{-/-} embryos by systemic (maternal) RA administration, which indicates that the existence of a precisely located source or sources of RA may not be mandatory for heart morphogenesis.

MATERIALS AND METHODS

The generation of *Raldh2* mutant mice has been described (Niederreither et al., 1999). For conventional histology, embryos were fixed in Bouin's fluid, paraffin-embedded, sectioned and stained with Hematoxylin/eosin (Mark et al., 1993). In situ hybridization with digoxigenin- or ³⁵S-labeled riboprobes were performed as described (Décimo et al., 1995; Niederreither and Dollé, 1998, respectively), using template plasmids cloned in our Institute or kindly provided by Drs M. Buckingham (Pasteur Institute, Paris; actin and myosin chains), V. Christoffels (University Amsterdam; *Irx*), C. Goridis (University Marseille-Luminy; *Pitx2*), E. Morrisey (University Pennsylvania, Philadelphia; *Gata5* and *Gata6*), E. Olson (University Texas, Dallas; *Gata4*, *Hand2*, *Mef2c*), V. Papaioannou (Columbia University, NY; *Tbx5*) and E. Robertson (Harvard University; *Nodal*). Scanning and transmission electron microscopy

Fig. 1. Morphology of the *Raldh2*^{-/-} mutant heart. Scanning electron micrographs of E9.5 wild-type (A) and *Raldh2*^{-/-} (B,C) embryos, after removal of the pericardial sac (ventral views; the posterior trunk of the wild-type embryo has been removed). lv, left ventricle; rv, right ventricle. Arrowhead in A indicates the demarcation between left and right ventricles; arrowhead in C indicates medial groove in the mutant heart (see Results).

was performed as described (Niederreither et al., 1999; Kastner et al., 1997, respectively). The *Wnt1-lacZ* (Echelard et al., 1994) and *Hoxa2-lacZ* (MNS4 construct; Maconochie et al., 1999) reporter lines were provided by the Jackson Laboratory (Bar Harbor, Maine) and Dr R. Krumlauf (Stowers Inst., Kansas City, MI), respectively. *lacZ* activity was revealed by conventional X-gal staining. Two procedures were used for RA rescue. All-*trans*-RA (Sigma) was either suspended in ethanol, diluted in sunflower oil (0.5 mg/ml) and administered orally (2.5 mg/kg body weight) to pregnant females twice a day from E6.5 to E10.5 dpc, or RA in suspension was directly mixed into powdered food (0.1 mg/g food at E7.5 and 0.2 mg/g food at later stages). The RA-containing food mixture (protected from light by aluminum foil) was left in the cage for ad libitum feeding and renewed each day until E10.5 or until the day of sacrifice.

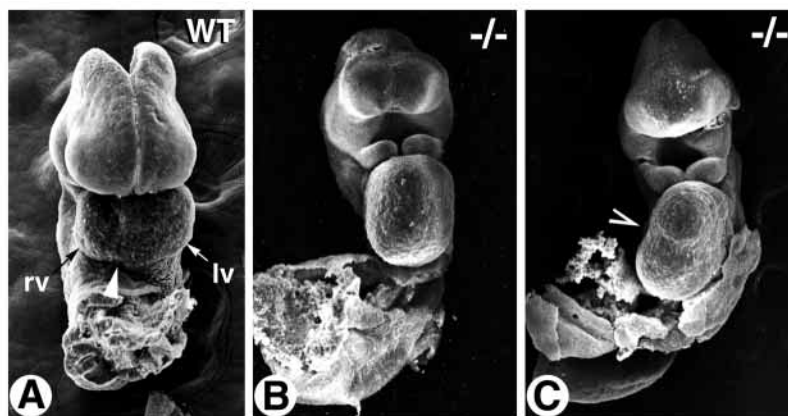
RESULTS

Heart morphology

We have previously reported that the heart of *Raldh2*^{-/-} embryos developed as a dilated cavity, with no obvious left-right (LR) asymmetry (Niederreither et al., 1999). Heart morphology was further analyzed on scanning electron micrographs of E9.0/E9.5 embryos after removal of the pericardial layer (Fig. 1). On ventral views, the *Raldh2*^{-/-} heart consisted of an ovoid cavity, whose main axis was aligned with the embryonic rostrocaudal axis, thereby contrasting with the LR orientation of the wild-type heart outer curvature (compare Fig. 1A-C). While some mutants showed no external signs of chamber formation (Fig. 1B), others displayed a medial groove that separated bulging rostral and caudal regions (Fig. 1C), whose identity was further investigated by serial histological analysis, and with the help of molecular markers.

Partial anteroposterior and dorsoventral morphogenesis in absence of left-right looping

Coronal sections of the E9.5 wild-type heart showed the distinct left and right ventricle cavities (Fig. 2B), followed rostrally by the non-trabeculated outflow tract (Fig. 2A,B) connecting with the aortic arches (Fig. 2D) via an aortic sac (Fig. 2C). A single, well-developed atrial cavity receiving blood inflow from the sinus venosus (Fig. 2C,D), is connected to the left ventricle by the atrioventricular canal (Fig. 2B,C,E). The atrial cavity, which developed from the caudal region of the linear heart tube, has been brought dorsally by the looping process (see Fig. 2E).



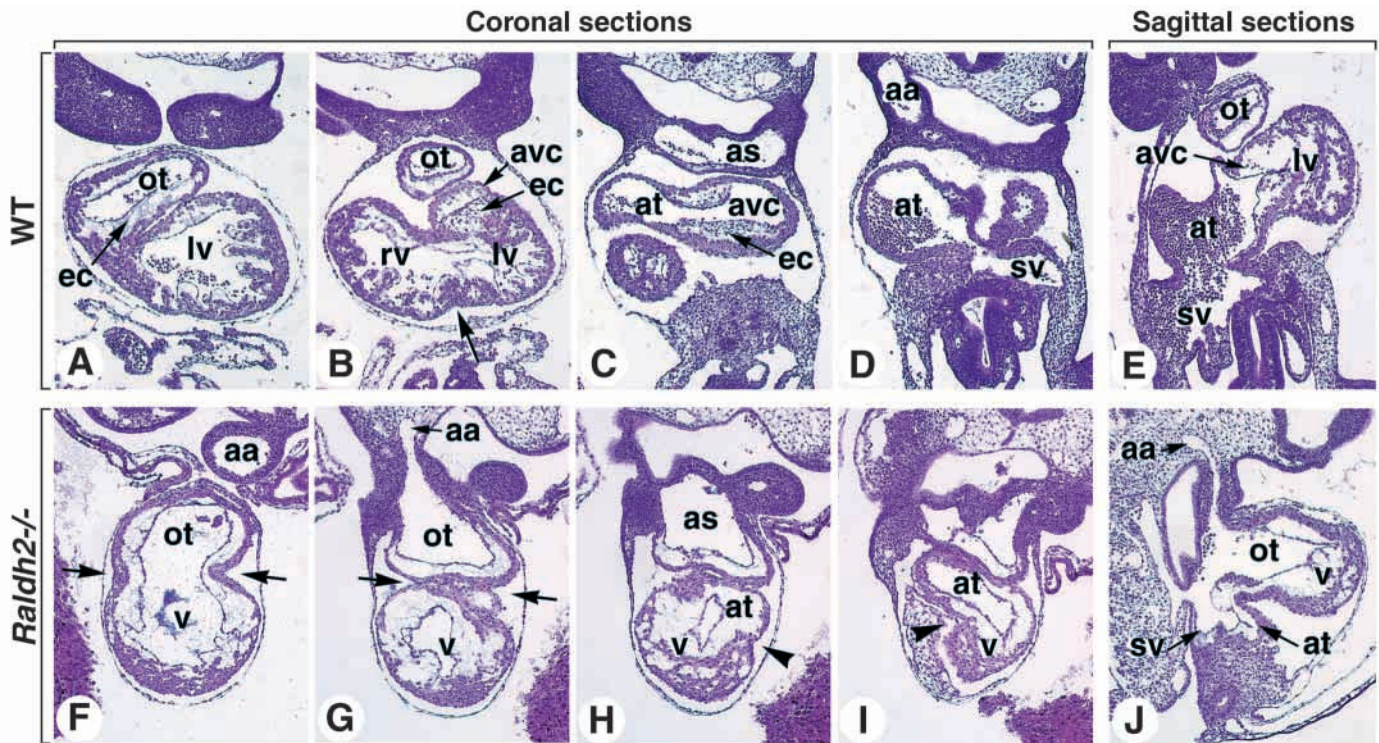


Fig. 2. Partial chamber development in the *Raldh2*^{-/-} mutant heart. (A-D) and (F-I) are serial coronal sections through the heart of E9.5 wild-type and *Raldh2*^{-/-} embryos, respectively. The sections progress from ventral to dorsal. (E) and (J) are representative sagittal sections of E9.5 wild-type and *Raldh2*^{-/-} hearts, respectively. Hematoxylin and Eosin staining. aa, aortic arch; as, aortic sac; at, atrial cavity; avc, atrioventricular canal; ec, endocardial cushion; lv, left ventricle; ot, outflow tract; sv, sinus venosus; rv, right ventricle; v, ventricle cavity. Arrow in B indicates left-right ventricle boundary; arrows in F,G indicate mutant heart medial groove; arrowheads in H,I indicate ventricle-atrial groove.

While some of the E9.5 *Raldh2* mutant hearts were too dilated to show clear histological landmarks (see Niederreither et al., 1999), others exhibited distinct features (Fig. 2F-J). Ventral sections showed a medial groove (Fig. 2F,G, arrows) separating an imperfectly trabeculated caudal region from a non-trabeculated rostral region. The rostral segment, which was connected with the aortic arches, may represent an indistinct outflow tract/aortic sac region (Fig. 2G,H). The 'trabecular' (see below) putative ventricle segment expanded caudally until another groove (Fig. 2H,I, arrowheads), separating it from a smaller posterior cavity, linked to the venous inflow tract (Fig. 2H-J). This abnormal posterior cavity probably represents a hypoplastic, indistinct atrial and sinus venosus portion (compare Fig. 2E,J; see below).

Thus, in the absence of RALDH2 function, the main heart chambers are arranged according to their normal AP sequence, although the poor development of the atria and sinus venosus indicates a specific function of this enzyme in the development of posterior heart structures (see below). Instead of achieving proper LR looping, the mutant heart tube forms a medial loop that bulges out of the trunk region (see Figs 1C, 2J). This abnormal anatomy can explain the lethality of the mutant embryos, as it results in an abnormal juxtaposition of the inflow and outflow circulations (see Fig. 2J), which is unlikely to allow efficient, unidirectional blood flow. In wild-type embryos, myocardial trabeculation occurs along the heart outer curvature, which derives from the ventral aspect of the linear heart tube, whereas the inner curvature remains smooth walled

and essentially gives rise to endocardial cushion tissue (Harvey, 1999; Christoffels et al., 2000a; and refs. therein). Endocardial cushions and ridges develop along the entire inner curvature, and morphogenetic movements will drive them to specific locations within the atrioventricular canal (Fig. 2B,C) and conotruncal segment (Fig. 2A; see Harvey, 1999; Mjaatvedt et al., 1999 for further description). *Raldh2*^{-/-} hearts show no distinct endocardial cushion tissue and do not form an atrioventricular canal (Fig. 2F-I, and data not shown). However, the presence of a ventrally restricted trabecular area (Fig. 2F-H,J) indicates that some dorsoventral specification has occurred. Further molecular evidence comes from the restricted expression of *Hand1* (Niederreither et al., 1999) and *Irx4* and *Irx5* (see below) in the ventral portion (outer curvature) of the *Raldh2*^{-/-} heart tube.

Molecular analysis

Expression of genes that are crucially involved in heart tube patterning and/or looping morphogenesis was analyzed by whole-mount in situ hybridization. Targeted disruption of the *Nkx2.5* homeobox gene, the earliest known marker of the heart field, severely impairs heart looping and AP chamber determination (Lyons et al., 1995; Biben and Harvey, 1997). *Raldh2*^{-/-} embryos, however, showed normal levels of *NKx2.5* expression throughout the heart myocardium, both at E8 (Fig. 3A,B) and E9 (data not shown). Expression of the *Mef2c* gene, the disruption of which prevents heart looping and right ventricle determination (Lin et al., 1997), was also unaffected

in *Raldh2*^{-/-} mutants (data not shown). The GATA4 transcription factor is required at earlier stages of heart development, for proper migration and fusion of the paired myocardial primordia (Kuo et al., 1997; Molkenin et al., 1997). Reduced levels of *Gata4* expression were observed in the developing heart of VAD quail embryos (Kostetskii et al., 1999). *Gata4* transcripts were expressed at normal levels in the E8 *Raldh2*^{-/-} heart tube (Fig. 3C,D), although the posterior areas of high *Gata4* expression were abnormally fused towards the midline in mutants (Fig. 3D, arrowhead). By E9, *Gata4* expression remained high in the posteriormost region of the mutant heart tube, but was abnormally weak in the remainder of the heart tube (Fig. 3E,F). Thus, RA synthesis appears to be required for the maintenance, rather than the proper initiation, of *Gata4* expression in the heart. No alteration in *Gata5* and *Gata6* expression levels was detected in the *Raldh2*^{-/-} heart (data not shown).

Two basic helix-loop-helix transcription factors, HAND1 and HAND2, have almost complementary expression domains in the caudal (prospective left ventricle and atrium) and cranial (prospective outflow tract, aortic sac and right ventricle) parts of the looping heart tube, respectively (Biben and Harvey, 1997; Fig. 3G). *Hand2* expression is gradually reduced in the right ventricle to persist, by E9, in the outflow tract and aortic sac only. We have previously shown that *Hand1* is expressed along the ventral/caudal portion of the *Raldh2*^{-/-} heart tube (Niederreither et al., 1999). In mutants, *Hand2* expression was appropriately restricted to the rostral part of the heart tube, gradually decreasing towards the medial groove (Fig. 3H, arrowhead; note that the additional arborescent labeling corresponds to normal endocardial *Hand2* expression; Biben and Harvey, 1997). *Hand2* expression was also normal in the dorsal mesocardium and lateral-ventral mesoderm, but was reduced in the first branchial arch of *Raldh2*^{-/-} embryos (Fig. 3H, and data not shown).

Of the five murine Iroquois-like (*Irx*) homeobox genes, three (*Irx3*, *Irx4* and *Irx5*) are expressed in the wild-type heart by E9.5 (Christoffels et al., 2000b; Fig. 3I,K). *Irx3* transcripts were not detected in the E9.5 *Raldh2*^{-/-} heart, although they were expressed in other regions of the mutant embryos, such as the neural tube (data not shown). *Irx4* is strongly expressed in wild type along the left and right ventricle myocardium and, to a lesser extent, in the atrioventricular canal and proximal outflow tract (Christoffels et al., 2000a; Christoffels et al., 2000b; Fig. 3I). In *Raldh2*^{-/-} embryos, *Irx4* was most strongly expressed in the postero-ventral portion of the bulging heart tube (Fig. 3J). *Irx5* expression is restricted in wild type to the ventral endocardium of the developing left and right ventricle and part of the atrial cavity (Christoffels et al., 2000b; Fig. 3K). In

mutants, its expression extended throughout the ventral side of the bulging heart tube (Fig. 3L). Both *Irx4* and *Irx5* expression patterns thus support the conclusion that the ventral (outer) curvature of the *Raldh2*^{-/-} heart tube is specified as ventricle. We found that another endocardial marker, the *Cyp26A1* RA-metabolizing enzyme, shows a normal pattern of expression in *Raldh2*^{-/-} mutants (data not shown).

To assess determination of posterior heart segments, we analyzed expression of the T-box gene *Tbx5*, which is expressed at high levels in wild type in the prospective atrial and sinus venosus regions, as soon as heart looping begins (Bruneau et al., 1999; Liberatore et al., 2000; Christoffels et al., 2000a; Fig. 4A,C). Both the expression levels and spatial distribution of *Tbx5* transcripts were abnormal in the posterior *Raldh2*^{-/-} heart tube (Fig. 4B,D). Only few cells exhibited high *Tbx5* expression (Fig. 4D, arrow). These had a patchy distribution, contrasting with the sharp caudal boundary seen in control embryos (compare Fig. 4A,C with 4B,D). Weaker *Tbx5* expression is seen in other wild-type heart segments, decreasing in a posterior-to-anterior direction (Christoffels et al., 2000a; Fig. 4A,E). Such a gradient of expression was also found in mutants (Fig. 4B,F). Thus, altered *Tbx5* expression is restricted to the posterior heart portion of *Raldh2*^{-/-} mutants.

Several transforming growth factor (TGF) β family members are involved in determination of LR handedness (*situs*) in the mouse (see Capdevila et al., 2000). The *Nodal* (Collignon et al., 1996), *Lefty1* (*Ebaf* – Mouse Genome Informatics) and *Lefty2* (*Leftib* – Mouse Genome Informatics; Meno et al., 1997) genes are transiently expressed in the left lateral plate mesoderm (LPM) at early somite stages (prior to heart looping). Both *Nodal* and the *Lefty* genes can be ectopically activated on the right side by exposure of

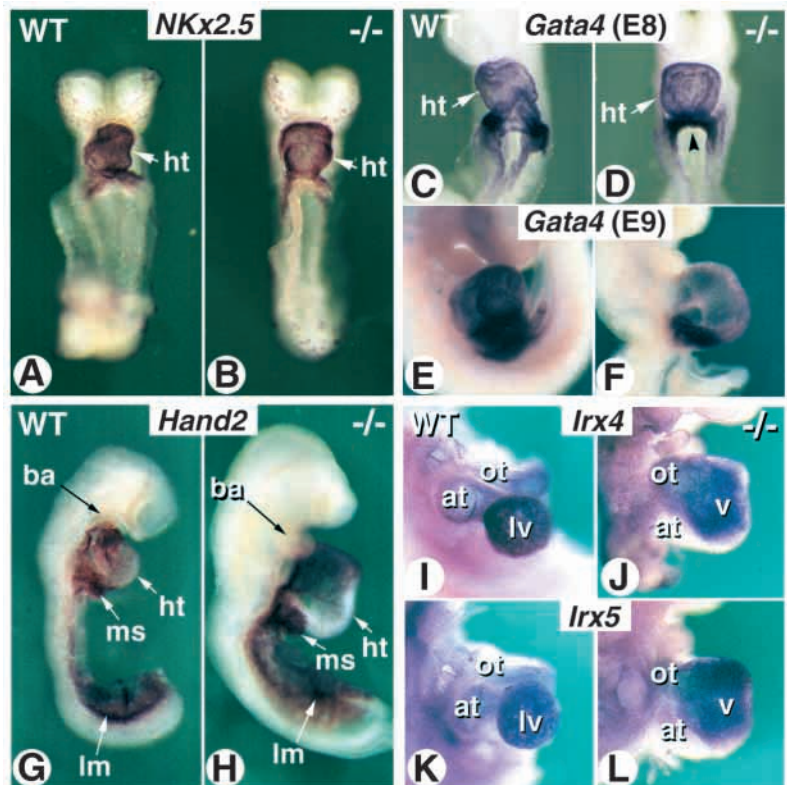


Fig. 3. Expression of heart-determining factors in *Raldh2*^{-/-} mutant embryos. Whole-mount in situ hybridization with *Nkx2.5* (A,B; E8, ventral views), *Gata4* (C,D, E8, ventral views; E,F, E9, profile views), *Hand2* (G,H; E9, profile views), *Irx4* (I,J) and *Irx5* (K,L, E9.5, profile views) riboprobes. at, atrial cavity; ba, branchial arch; ht, heart tube; lm, lateral mesoderm; lv, left ventricle; ms, mesocardium; ot, outflow tract; v, ventricle.

headfold-stage embryos to RA, whereas exposure to a pan-RAR inhibitor can prevent expression of these genes on the left side (Chazaud et al., 1999; Tsukui et al., 1999). We therefore anticipated that expression of *Nodal* and/or the Lefty genes would be downregulated in *Raldh2*^{-/-} embryos. However, *Nodal* transcripts were expressed at comparable levels throughout the left LPM of age-matched *Raldh2*^{-/-} and control embryos (Fig. 5A-D). *Nodal* was also expressed at normal levels in the node region of the *Raldh2*^{-/-} embryos (compare Fig. 5C with 5D). Similarly, no alteration of Lefty gene expression was seen in mutant embryos, using a full-length *Lefty1* probe that recognizes both *Lefty1* and *Lefty2* transcripts (data not shown). The *Pitx2* homeobox gene, a downstream effector of Nodal/Lefty signaling, shows persistent, left side-specific expression in the LPM and heart tube (Campioni et al., 1999; Fig. 5E), and its knockout generates laterality defects, including incomplete heart looping (Lin et al., 1999). However, *Pitx2* was expressed at normal levels in the left LPM and along the left curvature of the heart of *Raldh2*^{-/-} embryos, despite the lack of LR looping (compare Fig. 5E with 5F). Thus, the heart abnormalities of the *Raldh2*^{-/-} mutants do not seem to result from alteration of the *Nodal/Pitx2* signaling pathway.

Myofilament gene expression

To analyze whether the myocardium is able to express differentiation markers in the absence of RALDH2 function, in situ hybridization was performed on sections of E8.5 and E9.5 embryos with probes specific for cardiac actin, myosin heavy chain α (MHC α), and myosin alkali light chains MLC1a, MLC2a and MLC2v. Cardiac actin, MHC α and MLC1a were expressed throughout the *Raldh2*^{-/-} myocardium, at levels comparable with those found in control embryos (data not shown). The 'atrial' MLC2a chain is first expressed throughout the developing myocardium in wild type (Fig. 6A), downregulation outside of the atrial segment taking place progressively after E10 (Nguyễn-Trần et al., 1999). As in wild type, MLC2a was expressed throughout the heart segments of the *Raldh2*^{-/-} embryos (Fig. 6B, and data not shown). Expression levels were slightly lower than in control littermates at E8.5 (compare Fig. 6A with 6B), but were comparable at E9.5 (data not shown). MLC2v, however, is specifically activated in the ventricular chamber, thus representing the earliest known ventricular-specific marker (Nguyễn-Trần et al., 1999; Fig. 6C). Although reduced, MLC2v expression was restricted to the chamber that was histologically defined as ventricular in *Raldh2*^{-/-} mutants (Fig. 6D).

Fig. 5. Expression of *Nodal* and *Pitx2* in *Raldh2*^{-/-} mutant embryos. Ventral (A,B) and dorsal (C,D) views of E8.0 wild-type (A,C) and mutant (B,D) embryos hybridized with a *Nodal* riboprobe. (E,F) E8.5 wild-type and *Raldh2*^{-/-} embryos hybridized with a *Pitx2* probe. hf, headfold; ht, heart tube; lpm, lateral plate mesoderm; nr, node region.

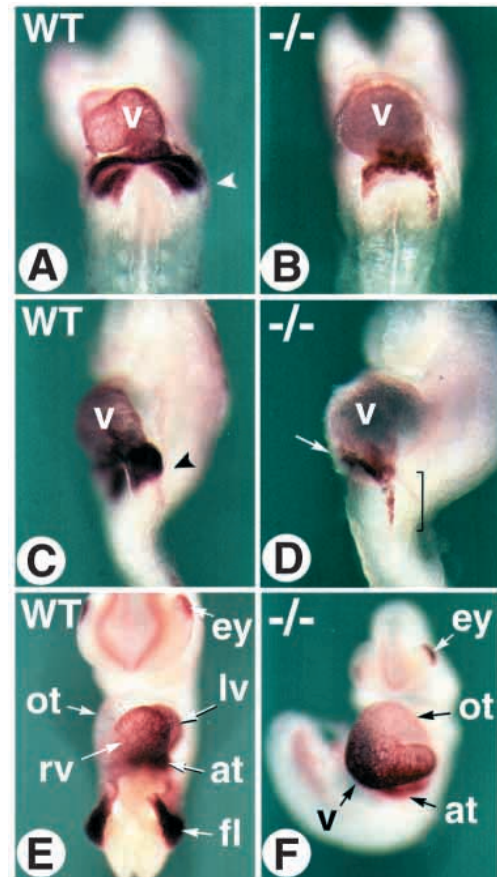
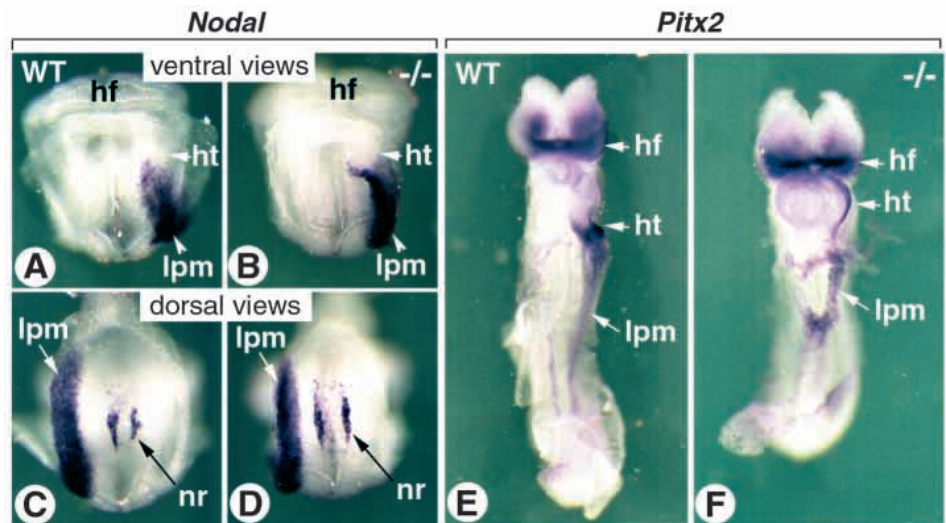


Fig. 4. Altered *Tbx5* expression in the posterior heart region of *Raldh2*^{-/-} embryos. Ventral (A,B) and profile (C,D) views of E8.5 embryos (early stage of heart looping in wild type). Arrowheads in A,C point to the sharp transcript boundary in the posterior heart region. The white arrow in D shows the few cells that exhibit high *Tbx5* expression, whereas the bracket indicates scattered *Tbx5*-expressing cells expanding caudally. (E,F) Ventral views of E9.0 embryos, showing *Tbx5* graded expression pattern in the ventricular region. (A,C,E) Wild-type embryos; (B,D,F) *Raldh2*^{-/-} embryos. at, atrium; ey, eye; fl, forelimb bud; lv, left ventricle; ot, outflow tract; rv, right ventricle; v, ventricle.



Cardiomyocyte differentiation

Myocardial architecture and cell morphology was analyzed on semi-thin (2 μm) sections prior to transmission electron microscope ultrastructural analysis. In E8.5 wild-type embryos, the developing ventricular myocardium is one to two cell layers thick, and some of the cardiomyocytes start to adopt a trabecular-like organization (Fig. 7A,C). The cardiomyocytes are characterized by their globular or cuboidal morphology, and their large, round nuclei (Figs 7C, 8A). The presumptive ventricular region of *Raldh2*^{-/-} littermates showed a characteristic alteration of myocardial cytoarchitecture. The myocardial wall was thicker than in wild type (compare Fig. 7C with 7D). Although most cells were densely packed, a number of large intercellular gaps were present (Figs 7D, 8B, asterisks). Cardiomyocytes from the outermost layer often had a flattened morphology (Figs 7D, 8B). However, a cluster of globular cardiomyocytes was found near the presumptive ventricle/outflow tract junction (Fig. 7B,E).

Upon ultrastructural analysis, the ventricular cardiomyocytes of E8.5 wild-type embryos appear mostly undifferentiated (Kastner et al., 1997; Barak et al., 1999; Fig. 8A,D). Some of these cells contain short and loose arrays of myofilaments, which have not acquired a sarcomeric organization (Fig. 8D, arrows). In contrast, many of the outermost cells of the *Raldh2*^{-/-} ventricular wall contained arrays of parallel striated myofilaments engaged in sarcomere assembly, as seen by the presence of dense Z-lines (the separations between individual sarcomeres; see Krstic, 1984) and intervening sarcoplasmic reticulum (Fig. 8E). Furthermore, a number of outermost cardiomyocytes had developed specialized step-like intercellular junctions called intercalated disks (IDs), bridging myofibrils from neighboring cells (Fig. 8E). These IDs do not appear before E9.5, and are never found in the subepicardial layer before E14.5, in wild-type embryos (data not shown; Kastner et al., 1997). As loss

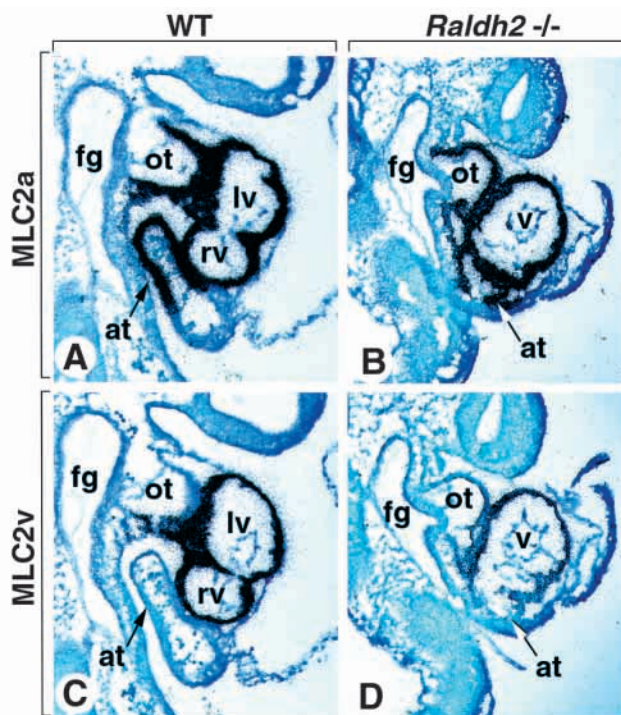


Fig. 6. Expression of the myosin light chain isoforms MLC2a and MLC2v in the *Raldh2*^{-/-} heart. Sagittal sections of E8.5 wild-type (A,C) and *Raldh2*^{-/-} (B,D) embryos hybridized with ³⁵S-labeled riboprobes for MLC2a (A,B) and MLC2v (C,D). Signal grains appear as black dots. Toluidine Blue counterstaining. at, atrial cavity; fg, foregut; lv, left ventricle; ot, outflow tract; rv, right ventricle; v, ventricle cavity.

of cell contact was otherwise seen along much of the cell surfaces (see Fig. 8B,E), IDs may be mainly responsible for

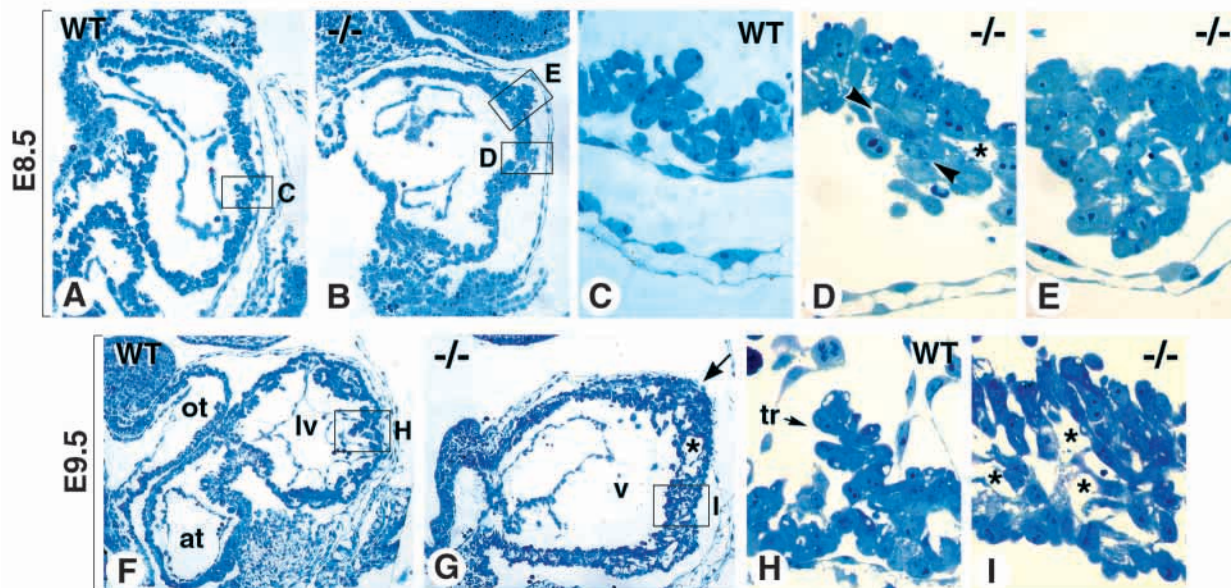


Fig. 7. Abnormal cytoarchitecture of the myocardial wall in *Raldh2*^{-/-} embryos. Semi-thin (2 μm) sections were performed according to a sagittal plane at E8.5 (A-E) and E9.5 (F-I). (A,C,F,H) Wild-type embryos; (B,D,E,G,I) *Raldh2*^{-/-} embryos. (C-E) and (H,I) are high-power magnifications of the boxed areas. Toluidine Blue staining. The arrowheads in D point to flattened myocardial cells, and the arrow in G to a cluster of undifferentiated cells. Asterisks indicate intercellular gaps. at, atria; lv, left ventricle; ot, outflow tract; tr, trabecula; v, ventricle.

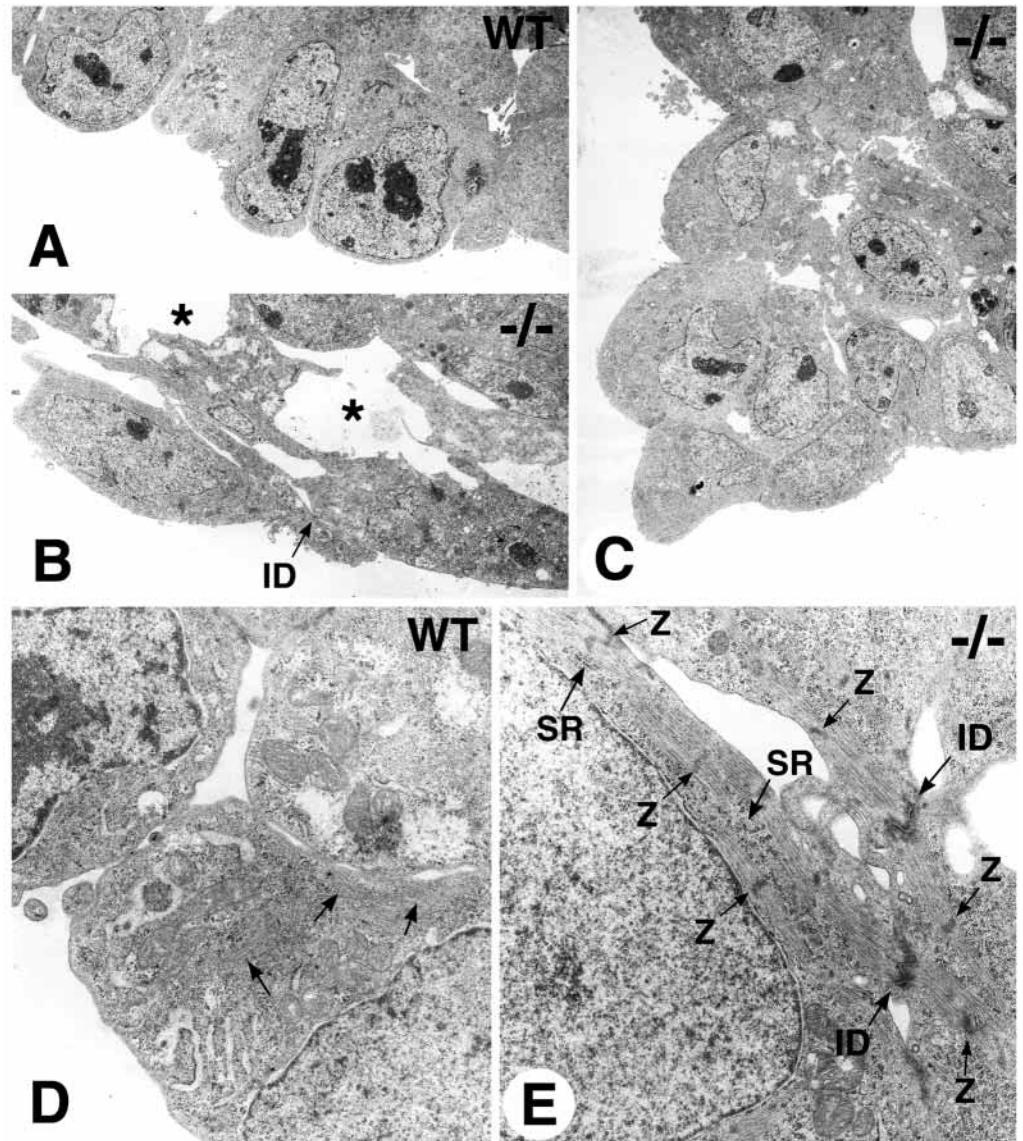


Fig. 8. Abnormal differentiation of the outer cardiomyocyte layer in the *Raldh2*^{-/-} myocardium. Transmission electron micrographs of the outermost layer of the ventricular myocardium in E8.5 wild-type (A,D) and *Raldh2*^{-/-} (B,C,E) embryos are shown at low (A-C) and high (D,E) magnifications. (C) The abnormal cell cluster shown in Fig. 6E. Asterisks in B indicate abnormal intercellular gaps. Thin arrows in D indicate to small, unorganized myofilament arrays. ID, intercalated disk; SR, sarcoplasmic reticulum; Z, Z-line.

maintaining integrity of the mutant myocardium. However, the abnormal rostral cell cluster (Fig. 7E) consisted of undifferentiated cardiomyocytes, devoid of myofilaments (Fig. 8C).

In E9.5 wild-type embryos, cardiomyocytes along the outer curvature of the ventricular chambers form thin trabeculae (Fig. 7F,H; see also Fig. 2A,B) that undergo sarcomeric differentiation, whereas most of the outermost (subepicardial) cardiomyocytes are either undifferentiated or poorly differentiated (data not shown; see Kastner et al., 1997). In *Raldh2*^{-/-} mutants, ventricular cardiomyocytes failed to establish an organized trabecular network (Fig. 7G,I; see also Fig. 2F-H). The thickened myocardial wall was characterized by large intercellular gaps (Figs 2H, 7G,I). Many cells were flattened and seemed to be maintained by their lateral edges, resulting in an aberrant 'horizontal' trabecular-like network (see Figs 2H, 7G,I). Ultrastructural analysis showed that most subepicardial cardiomyocytes exhibit sarcomeric differentiation, except for the presence of a rostral cluster of undifferentiated cardiomyocytes (Fig. 7G, arrow; and data not

shown). Mutant endocardial cells were ultrastructurally normal (data not shown).

Rescue of *Raldh2*^{-/-} embryos by maternal RA supplementation unveils cardiac neural crest defects

Maternal administration of subteratogenic doses of RA by oral gavage from E6.25 to E10.25 improves several aspects of the *Raldh2*^{-/-} phenotype, but does not allow survival after E12.5-E13.5 (Niederreither et al., 1999; Niederreither et al., 2000). Histological analysis at E12.5 revealed that the *Raldh2*^{-/-} heart phenotype was partially rescued under such conditions. Two well-formed left and right atrial cavities were present (compare Fig. 9A,B with 9E,F), contrasting with a single medial ventricle whose trabeculation was less extensive than in controls (compare Fig. 9A,B with 9E,F). Endocardial cushions had developed at the level of the atrio-ventricular canal (Fig. 9F) and outflow tract (Fig. 9E). Thus, these conditions may not fully rescue LR looping of the *Raldh2*^{-/-} heart tube.

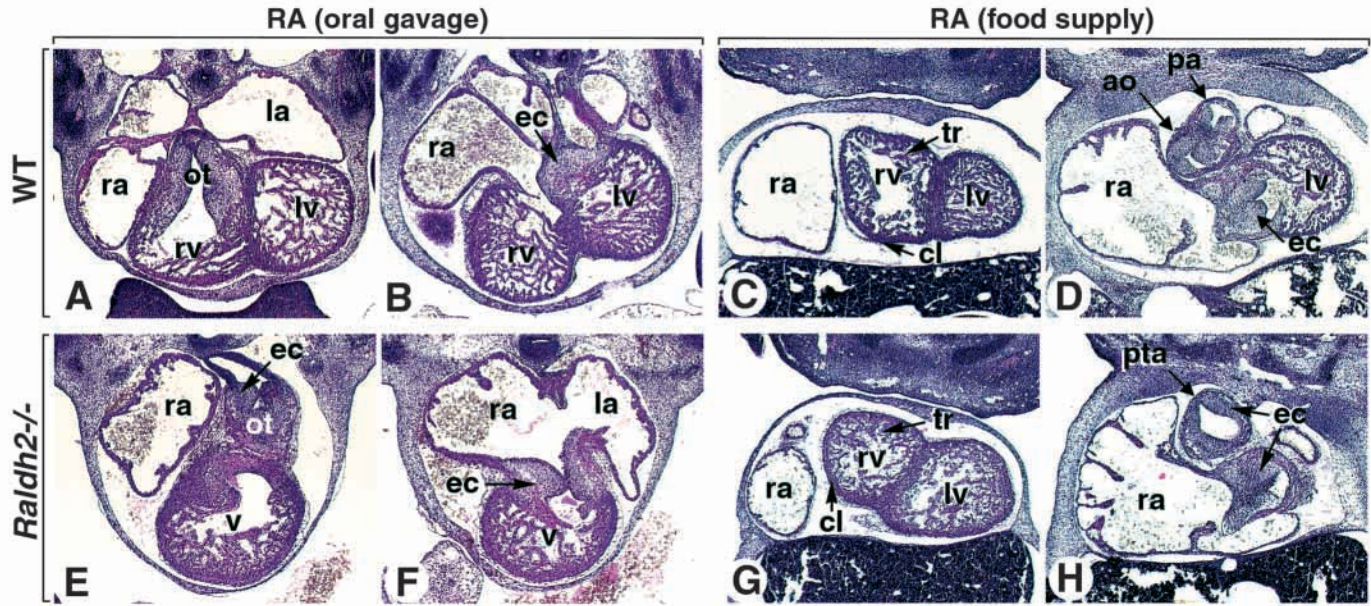


Fig. 9. Rescue of the *Raldh2*^{-/-} mutants by maternal RA supplementation leads to subsequent heart defects. (A,B,E,F) Comparative sections through the heart of E12.5 wild-type (A,B) and *Raldh2*^{-/-} (E,F) littermates whose mother was administered RA by oral gavage (E6.5 to E10.5; see Materials and Methods). This condition rescues atrial development, but leads to a single medial ventricle cavity in the mutant embryo. Note that the atrial communication seen in F is a normal feature at this stage, and is seen in other section planes in the control embryo. (C,D,G,H) Comparative sections through the heart of E12.5 wild-type (C,D) and *Raldh2*^{-/-} (G,H) littermates that received RA via maternal food supply (E7.5 to E10.5). This mode of administration rescues left-right ventricle development, but leads to persistent truncus arteriosus (unseptated outflow tract) in the *Raldh2*^{-/-} heart. The same phenotype was observed when RA was given until the day of sacrifice. ao, aorta; cl, compact myocardial layer; ec, endocardial cushion; la, left atrium; lv, left ventricle; ot, outflow tract; pa, pulmonary artery; pta, persistent truncus arteriosus; ra, right atrium; rv, right ventricle; tr, trabecular layer; v, ventricle cavity.

To see whether the incomplete phenotypic rescue may be due to the discontinuous mode of delivery, RA was administered in the food supply (see Materials and Methods). Viability of homozygous mutants could thus be rescued until E13.5-E14.5, and the resulting hearts had distinct left and right ventricles and interventricular septum (compare Fig. 9C with 9G). The mutant ventricular myocardium had well-developed compact and trabecular layers (compare Fig. 9C with 9G). However, these rescued hearts exhibited 'persistent truncus arteriosus' (PTA), i.e. lack of septation of the outflow tract into aorta and pulmonary trunk, and lack of development of the semilunar valves from the conotruncal endocardial cushion tissue (compare Fig. 9D with 9H).

Septation of the truncus arteriosus involves a specific population of hindbrain neural crest cells (NCCs), the 'cardiac NCCs', which migrate along the caudal aortic arches and contribute to the formation of the aorticopulmonary septum and conotruncal ridges (e.g. Kirby, 1999; Jiang et al., 2000). To analyze this population, RA-rescue experiments were performed on *Raldh2*^{-/-} mutants harboring *lacZ* transgenes as NCC markers. The *Wnt1-lacZ* transgene (Echelard et al., 1994) was used as a marker for migrating NCCs (Fig. 10A-F). RA-rescued *Raldh2*^{-/-} embryos analyzed at E10.5 showed a reduction in the stream of cells migrating from the caudal branchial arches (Fig. 10A,B, arrowheads), but no obvious decrease in transgene activity was seen otherwise. In slightly older mutants, the stream of cells migrating towards the outflow tract was reduced and poorly organized (compare Fig. 10C,D). Histological sections

confirmed a reduction of the cell stream colonizing the aortic wall (Fig. 10E,F). As the activity of the *Wnt1-lacZ* transgene fades after E10.5, we used a *Hoxa2-lacZ* transgene that contains regulatory elements driving expression in the NCCs (Maconochie et al., 1999) to confirm a deficiency in cells colonizing the heart outflow region in E11.5 mutants (Fig. 10G,H). Analysis of *Crabp1* gene transcripts as an endogenous marker for NCCs showed disorganized pathways of migration in the third to sixth branchial arch region, whereas more rostral NCC streams were well defined in E9.5 mutants (Fig. 10I,J). In contrast, expression of a myocardially expressed RA-responsive transgene (*RARE-hsp68-lacZ*; Rossant et al., 1991) was fully restored in the RA-supplemented *Raldh2*^{-/-} embryos (Fig. 10K,L), indicating that its restricted expression does not solely depend on the distribution of locally synthesized RA.

DISCUSSION

RA is essential for heart posterior chamber development

The embryonic heart tube is molecularly patterned prior to looping, but the nature of DV and AP patterning signals that trigger chamber specification is still unclear (for reviews, see Olson and Srivastava, 1996; Harvey, 1999). Administration of excess RA to chick embryos truncates the anterior portion of the heart tube and enlarges its caudal portion (Osmond et al., 1991; Yutzey et al., 1994; Dickman and Smith, 1996; Xavier-

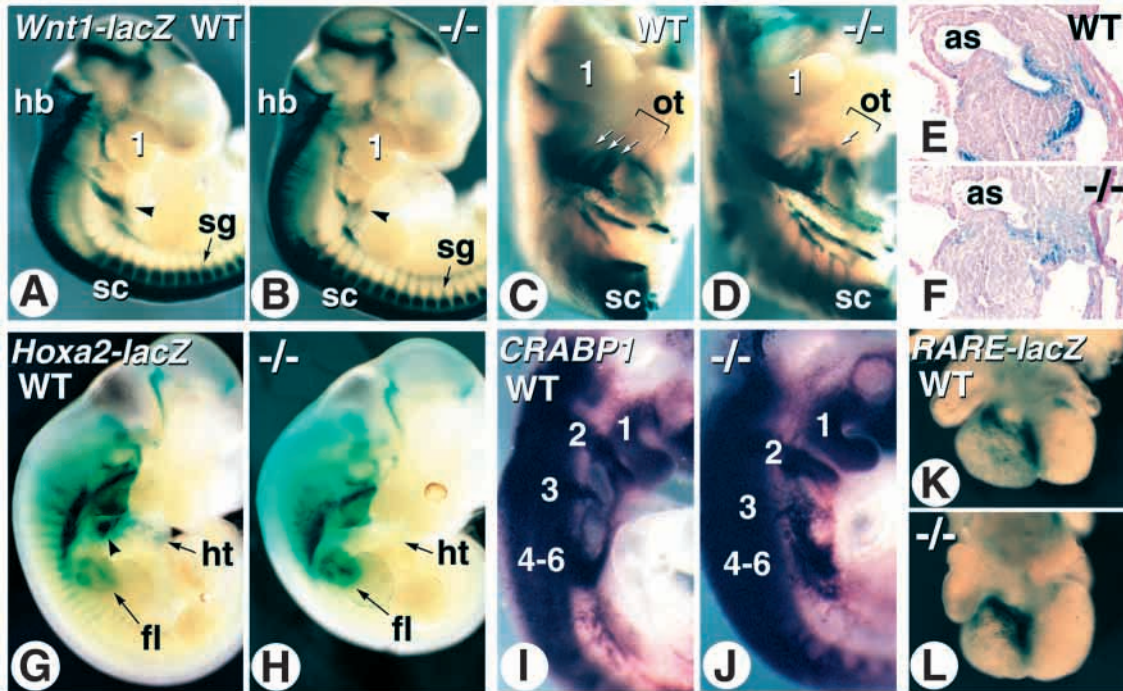


Fig. 10. Altered patterns of cardiac neural crest migration in the RA-rescued *Raldh2*^{-/-} mutants. (A-F) *Wnt1-lacZ* transgene activity in wild-type (A,C,E) and *Raldh2*^{-/-} (B,D,F) E10.5 embryos supplemented with RA via maternal food supply from E7.5 until day of sacrifice. Two littermate pairs are shown in toto (A,B) or after partial dissection to expose the outflow tract area (C,D). (E,F) Comparative histological sections (safranin counterstaining). Note the selective depletion (A,B, arrowheads) and/or disorganization (C,D, arrows) of the stream of cells colonizing the outflow tract. (G,H) *Hoxa2-lacZ* transgene activity in littermate E11.5 wild-type (G) and *Raldh2*^{-/-} (H) embryos similarly supplemented with RA. Arrowhead in G points to the strong labeling of the wild-type heart outflow wall. (I,J) *Crabp1* transcript distribution in E9.5 wild-type (I) and *Raldh2*^{-/-} (J) RA-supplemented embryos, showing disorganized neural crest streams in the mutant third to sixth branchial arch region. (K,L) Comparable *RARE-hsp68-lacZ* transgene activity in dissected hearts from E12.5 wild-type (K) and *Raldh2*^{-/-} (L) RA-supplemented embryos. as, aortic sac; hb, hindbrain; ot, outflow tract; sc, spinal cord; sg, spinal ganglia; 1-6, branchial arches 1-6.

Neto et al., 1999). As these RA treatments induce a rostral expansion of expression of posterior chamber markers (Yutzey et al., 1994; Liberatore et al., 2000) and, in addition, administration of an aldehyde dehydrogenase inhibitor diminishes expression of an atrial-specific transgene (Xavier-Neto et al., 1999), it has been proposed that RA signaling may be involved in the determination of posterior heart chamber fate. Supporting this hypothesis, both RALDH2 protein and RA-responsive transgene activity are specifically detected in the posterior region of the mouse embryonic heart tube (Moss et al., 1998). Our present data show that the posteriormost segments of the heart tube (putative atria and sinus venosus) are markedly reduced in size in *Raldh2*^{-/-} embryos. Furthermore, expression of *Tbx5*, which marks the prospective atria and sinus venosus, is reduced and shows an ill-defined posterior boundary in the mutants.

We conclude that endogenous RA is required in the mouse for proper development of posterior chambers. During heart tube formation, cardiogenic cells migrate caudally along the extracellular matrix (ECM) (Linask and Lask, 1988); RA may thus regulate the cell-ECM interactions (Osmond et al., 1991; Dickman and Smith, 1996) required for the correct allocation of migrating cells towards the prospective atrial region. Alternatively, retinoids may control the expression of atrial-specific growth factors (as suggested by MySM3 transgenic reporter gene expression; Xavier-Neto et al., 1999).

RA is dispensable for left side-specific expression of situs-determining genes, but is required to generate a loopable heart

Morphogenetic looping of the linear heart tube in a rightward direction is required to establish the definitive spatial arrangement of the developing chambers (reviewed by Olson and Srivastava, 1996; Harvey, 1999). The direction of heart looping (i.e. the heart situs) depends on determinants of the embryonic LR axis (reviewed by Capdevila et al., 2000), which induce asymmetrical expression of the TGF β -related genes *Lefty* and *Nodal* in the left lateral plate mesoderm (LPM) at early somite stages (Collignon et al., 1996; Meno et al., 1997). A role for retinoids in LR situs determination has been inferred from the observation that short-term administration of RA to gastrulating/early headfold stage embryos can randomize heart situs and induce ectopic, right-sided expression of *Lefty* and *Nodal* genes (Chazaud et al., 1999; Tsukui et al., 1999; Wasiak and Lohnes, 1999). Moreover, and conversely, treatments at the same stage with synthetic pan-RAR antagonists inhibit the normal expression of *Lefty*, *Nodal* and *Pitx2* in the left LPM, and also lead to situs randomization (Chazaud et al., 1999; Tsukui et al., 1999). Long-term treatments (until early somite stages) generated additional abnormalities of heart chamber formation that are associated with impaired looping (Chazaud et al., 1999), which externally resembled those exhibited by *Raldh2*^{-/-} mutants. Thus, it was concluded that in the mouse,

embryonic RA may act at distinct developmental stages to control situs determination and heart looping and/or chamber determination.

In VAD quail embryos, the heart develops asymmetrically and often bends towards the wrong side of the body. However, these reversed quail hearts never develop as perfect mirror-images of the normal heart and, thus, cannot be considered as true situs inversus (Zile et al., 2000). In these embryos, *Lefty*, *Nodal* and *Pitx2* are always expressed on the left side, but the two latter genes show transient alterations of their expression levels, especially towards the heart region (Zile et al., 2000). Although the lack of LR asymmetry of heart looping seen in *Raldh2*^{-/-} embryos may appear as more severe than in the VAD quail embryos, we found that *Lefty*, *Nodal* and *Pitx2* were expressed at normal levels on the left side of mouse mutant embryos. Thus, both animal models lead us to the conclusion that endogenous RA is not mandatory for setting up left side-specific expression of genes involved in situs determination, but in some way is instrumental for proper development of a 'loopable' heart. The different outcomes on the levels of expression of *Nodal* and *Pitx2* could reflect an evolutionary difference in the regulation of these genes, which would be less susceptible to RA deficiency in mammals. Alternatively, the mouse *Raldh2*^{-/-} embryos may contain low levels of RA, which could not be detected using a sensitive RA-responsive transgene (Niederreither et al., 1999), but may be sufficient to maintain normal expression levels of *Nodal* and *Pitx2*. Although the other known RALDH activities are not detected prior to somitogenesis (Ulven et al., 2000; Li et al., 2000; Duester, 2000), one cannot exclude that another enzyme, perhaps a nonspecific ALDH, may oxidize retinaldehyde at low efficiency. The mammalian embryo may also receive some RA of maternal origin, either by diffusion from maternal blood, which contains circulating RA, or from uterine tissues which express RA-synthesizing enzymes (Zheng et al., 1999; Vermot et al., 2000). Such low levels of RA could also explain other phenotypic differences; for instance, *Raldh2*^{-/-} embryos exhibit less severe downregulation of *Gata4* expression and less severe posterior heart phenotype than VAD quail embryos, which usually lack the whole inflow tract and associated omphalomesenteric vessels.

If endogenous RA is not mandatory for setting up left side-specific expression of genes (*Lefty*, *Nodal*, *Pitx2*) involved in situs determination, why then is the expression of these genes repressed upon short-term treatment with the specific pan-RAR antagonist BMS493 at the early headfold stage, which subsequently leads to heart situs randomization (Chazaud et al., 1999)? The most likely and simple explanation would be that, at this stage, RA is not crucially implicated in the physiological control of expression of these genes, even though they can be activated by nonphysiological levels of RA, as demonstrated by their ectopic right-sided expression upon RA administration, which also results in situs randomization (see above). Note in this respect that *Lefty1* was isolated as a RA-inducible gene in cultured P19 embryonal carcinoma cells (Oulad-Abdelghani et al., 1998). We therefore propose that the results of pan-RAR antagonist treatments (Chazaud et al., 1999; Tsukui et al., 1999) do not provide a demonstration that RA is required for *Lefty/Nodal* left-sided expression at this stage, but rather reflect the potential of co-repressor(s) that are tightly associated with RARs in the presence of antagonists

(Klein et al., 2000; H. Gronemyer and P. C., unpublished results), to repress transcription of these genes. Under physiological conditions, RA would not control the left-sided expression of these genes, although the accessibility of their promoter regulatory elements to retinoid receptors can result either in their nonphysiological symmetrical expression, in the case of RA treatment, or in repression, in the case of pan-RAR antagonist treatment. We therefore conclude that RA, most probably, plays similar roles in quail and mouse heart morphogenesis and looping, not being crucially involved in LR situs determination, but being required for generating a 'loopable' heart.

RA is important for differentiation of ventricular cardiomyocytes

VAD in rat (Wilson and Warkany, 1949), as well as RAR α , RAR α /RAR γ , RXR α and RXR α /RXR β gene knockouts in mouse (Mendelsohn et al., 1994; Sucov et al., 1994; Kastner et al., 1994; Kastner et al., 1997; Mascrez et al., 1998) all result in defective ventricular wall outgrowth. At the ultrastructural level, these knockout mice exhibit precocious signs of differentiation, as well as reduced mitotic indexes, in the external (subepicardial) cardiomyocyte layer (Kastner et al., 1997). In the case of RXR α , this abnormality has been found to be non cell-autonomous, as it does not occur if this receptor is specifically inactivated in the ventricular cardiomyocyte lineage (Chen et al., 1998). Myocardial defects may be, to some extent, secondary to placental failure, as both RXR α (Sapin et al., 1997a) and RXR α /RXR β (Wendling et al., 1999) mutants exhibit impaired development of the placental labyrinthine exchange zone. Placental RXR function may in fact involve the action of peroxisome proliferator-activated receptor PPAR γ /RXR heterodimers, rather than RAR/RXR heterodimers, as PPAR γ mutants (Barak et al., 1999) display placental and myocardial defects similar to those of RXR α /RXR β mutants (Wendling et al., 1999). Interestingly, the myocardial defects are rescued in PPAR γ aggregation chimeras by the presence of wild-type placental cells (Barak et al., 1999).

Raldh2^{-/-} embryos exhibit, as early as E8.5, ultrastructural signs of differentiation of subepicardial ventricular cardiomyocytes, which are similar to those described in VAD and RAR/RXR mutants. In addition, the formation of ventricular trabeculae is severely impaired in the *Raldh2*^{-/-} embryos. Because of their early death, it is difficult to assess whether placental defects may contribute to the *Raldh2*^{-/-} myocardial phenotype. We note, however, that *Raldh2* does not appear to be expressed in the developing placenta (Sapin et al., 1997b). Another characteristic of the *Raldh2*^{-/-} heart is the presence of an abnormal cluster of undifferentiated cardiomyocytes at the rostral edge of the developing ventricle, suggesting that RA may act within the heart to regulate cell-to-cell interactions allowing cardiomyocyte precursors to colonize the entire ventricular wall and/or initiate trabeculae formation. Lack of trabeculation could be linked to a defect in cell adhesion, as the mutant cardiomyocytes, although connected laterally by specialized structures (intercalated disks), are otherwise separated by large gaps. Retinoids regulate the expression of several components of the mammalian extracellular matrix including lamininB1, fibronectin and type IV collagen (reviewed by Means and

Gudas, 1995), whose expression levels will have to be analyzed in the mutant embryos.

RA rescue of early heart morphogenesis reveals cardiac neural crest defects

We found that maternally supplied RA from E7.5 to E10.5 could rescue several aspects of heart development in *Raldh2*^{-/-} mutants, including atrial outgrowth, looping morphogenesis and ventricular trabeculation. Thus, precisely located source(s) of RA synthesis do not appear to be crucial for these events to occur. Rescue of heart looping was more efficient when RA was provided in the food supply, rather than by oral gavage, indicating a need for steady RA levels for looping to proceed correctly. Both modes of RA administration, however, consistently led to a defect of septation of the mutant outflow tract, leading to persistent truncus arteriosus (PTA). High incidence of PTA is also seen in VAD rat fetuses (Wilson and Warkany, 1949) and *RARα/RARβ*, *RARα/RARδ* or *RXRα/RARβ* compound mutants (Mendelsohn et al., 1994; Ghyselinck et al., 1998). As these septation defects were observed in *Raldh2*^{-/-} mutants, even when RA was administered continuously in the food supply until the day of sacrifice (E12.5-E13.5), they probably result from lack of an appropriately located source of RA synthesis.

Two cell types populate the differentiating outflow tract: (1) endocardium-derived cells that undergo epithelial-to-mesenchymal transformation, especially at the level of the conotruncal cushions that will form the semilunar valves; and (2) hindbrain-derived ('cardiac') NCCs that migrated along the caudal pharyngeal arch arteries to reach the outflow tract (Kirby, 1999; Jiang et al., 2000). Surgical ablation of cardiac neural crest in chick results in outflow tract defects, including PTA (Kirby, 1999). Epstein et al. showed impaired migration and/or reduced numbers of cardiac NCCs in *Pax3/Splotch* mutant embryos (Epstein et al., 2000), which exhibit PTA and die at the same gestational stage as the RA-rescued *Raldh2*^{-/-} mutants. Using *lacZ* reporter transgenes, we provide evidence that this cell population is reduced and/or hindered in its migratory properties in RA-rescued *Raldh2* embryos. The source of the RA signal(s) that influence cardiac NCC development remain to be determined. We favor the idea that cardiac NCCs are abnormally patterned before their migration, as they derive from a hindbrain region that is specifically altered in *Raldh2*^{-/-} mutants (Niederreither et al., 2000). However, RALDH2 is also expressed in lateral plate mesoderm and somites during cardiac NCC migration (Niederreither et al., 1997; Berggren et al., 1999), and in part of the conotruncus (Moss et al., 1998). Hindbrain cell transplantation techniques, which have recently been developed for mouse embryos (Trainor and Krumlauf, 2000), may help define whether the *Raldh2*^{-/-} mutation affects premigratory hindbrain NCC and/or the cellular environment encountered during their migration.

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