INTRODUCTION

Conotruncal and aortic arch malformations are prevalent human birth defects. Septation of the outflow tract into aorta and pulmonary artery which, when defective, leads to persistent truncus arteriosus (PTA), involves a specific population of neural crest cells (NCCs), called ‘cardiac NCCs’. These cells migrate from the hindbrain rhombomeres (r) r6, r7 and r8 to populate the posterior (3rd, 4th and 6th) branchial arches, eventually contributing to the aortopulmonary septum and conotruncal ridges (Kirby, 1998; Jiang et al., 2000). Surgical ablation of premigratory cardiac NCCs in chick embryos produces a range of abnormal phenotypes including PTA, overriding aorta, and ventricular septal defects (Creazzo et al., 1998). Severe vitamin A deficiency in rodents can also lead to alterations of NCC derivatives, resulting in similar congenital malformations (Wilson and Warkany, 1949). The active derivatives of vitamin A, the retinoic acids (RAs), act as agonistic ligands for RA receptors (all-trans and 9-cis RAs for RARα, β and γ and retinoid X receptors (9-cis RAs for RXRα, β and γ), which bind as heterodimers to DNA motifs (RA response elements, RAREs) and thus regulate the transcriptional activity of target genes (reviewed by Mangelsdorf et al., 1994; Chambon, 1996). Compound inactivation of RARα and RARβ leads to various abnormalities, including abnormal patterns of branchial arch-derived great arteries and defective septation of the outflow tract (Mendelsohn et al., 1994; Ghyselinck et al., 1998). These

DEVELOPMENT AND DISEASE

The regional pattern of retinoic acid synthesis by RALDH2 is essential for the development of posterior pharyngeal arches and the enteric nervous system

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SUMMARY

Targeted inactivation of the mouse retinaldehyde dehydrogenase 2 (RALDH2/ALDH1a2), the enzyme responsible for early embryonic retinoic acid synthesis, is embryonic lethal because of defects in early heart morphogenesis. Transient maternal RA supplementation from E7.5 to (at least) E8.5 rescues most of these defects, but the supplemented Raldh2–/– mutants die prenatally, from a lack of septation of the heart outflow tract (Niederreither, K., Vermot, J., Messaddeq, N., Schuhbaur, B., Chambon, P. and Dollé, P. (2001). Development 128, 1019-1031). We have investigated the developmental basis for this defect, and found that the RA-supplemented Raldh2–/– embryos exhibit impaired development of their posterior (3rd-6th) branchial arch region. While the development of the first and second arches and their derivatives, as well as the formation of the first branchial pouch, appear to proceed normally, more posterior pharyngeal pouches fail to form and the pharyngeal endoderm develops a rudimentary, pouch-like structure. All derivatives of the posterior branchial arches are affected. These include the aortic arches, pouch-derived organs (thymus, parathyroid gland) and post-otic neural crest cells, which fail to establish segmental migratory pathways and are misrouted caudally. Patterning and axonal outgrowth of the posterior (9th-12th) cranial nerves is also altered. Vagal crest deficiency in Raldh2–/– mutants leads to agenesis of the enteric ganglia, a condition reminiscent of human Hirschprung’s disease. In addition, we provide evidence that: (i) wildtype Raldh2 expression is restricted to the posteriormost pharyngeal mesoderm; (ii) endogenous RA response occurs in both the pharyngeal endoderm and mesoderm, and extends more rostrally than Raldh2 expression up to the 2nd arch; (iii) RA target genes (Hoxa1, Hoxb1) are downregulated in both the pharyngeal endoderm and mesoderm of mutant embryos. Thus, RALDH2 plays a crucial role in producing RA required for pharyngeal development, and RA is one of the diffusible mesodermal signals that pattern the pharyngeal endoderm.

Key words: Retinoids, Branchial arches, Pharyngeal endoderm, DiGeorge syndrome, Hirschprung’s disease, Mouse mutant

INTRODUCTION

Conotruncal and aortic arch malformations are prevalent human birth defects. Septation of the outflow tract into aorta and pulmonary artery which, when defective, leads to persistent truncus arteriosus (PTA), involves a specific population of neural crest cells (NCCs), called ‘cardiac NCCs’. These cells migrate from the hindbrain rhombomeres (r) r6, r7 and r8 to populate the posterior (3rd, 4th and 6th) branchial arches, eventually contributing to the aortopulmonary septum and conotruncal ridges (Kirby, 1998; Jiang et al., 2000). Surgical ablation of premigratory cardiac NCCs in chick embryos produces a range of abnormal phenotypes including PTA, overriding aorta, and ventricular septal defects (Creazzo et al., 1998). Severe vitamin A deficiency in rodents can also lead to alterations of NCC derivatives, resulting in similar congenital malformations (Wilson and Warkany, 1949). The active derivatives of vitamin A, the retinoic acids (RAs), act as agonistic ligands for RA receptors (all-trans and 9-cis RAs for RARα, β and γ and retinoid X receptors (9-cis RAs for RXRα, β and γ), which bind as heterodimers to DNA motifs (RA response elements, RAREs) and thus regulate the transcriptional activity of target genes (reviewed by Mangelsdorf et al., 1994; Chambon, 1996). Compound inactivation of RARα and RARβ leads to various abnormalities, including abnormal patterns of branchial arch-derived great arteries and defective septation of the outflow tract (Mendelsohn et al., 1994; Ghyselinck et al., 1998). These
double mutant mice exhibit abnormal patterning of the post-
otic hindbrain and the caudal (3rd-6th) branchial arches (Dupé et al., 1999). In this respect, it is noteworthy that administration of a pan-RAR antagonist to cultured mouse embryos revealed a selective sensitivity of the caudal branchial region to inhibition of RA signaling during the period of NCC migration (Wendling et al., 2000).

Interestingly, Raldh2<sup>−/−</sup> null mutant embryos, which are severely impaired in their capacity to synthesize RA, die at midgestation (E9.5-10.5) because of severe defects of early heart morphogenesis (Niederreither et al., 1999; Niederreither et al., 2001). These mutant embryos also exhibit an altered development of trunk mesoderm derivatives, as well as growth and patterning defects of the posterior hindbrain region and an apparent lack of development of all branchial arches, with the exception of the first one (Niederreither et al., 1999; Niederreither et al., 2000). Several of these abnormalities have been shown to be rescued through maternal RA supplementation from embryonic day 7.5 (E7.5) to at least E8.5 (Niederreither et al., 1999; Niederreither et al., 2002). These rescued Raldh2<sup>−/−</sup> mutants consistently exhibit outflow tract septation defects (PTA), which most probably account for their death prior to (or at) birth, and extending the duration of RA supplementation (e.g. until E10.5 or 12.5) has no effect on the septation defect (Niederreither et al., 2001).

We have determined here the defective developmental events that lead to PTA in the RA-supplemented Raldh2<sup>−/−</sup> mutants, and have characterized a number of additional abnormalities exhibited by these rescued mutants. Most notably, we show for the first time that RA deficiency can lead to a Hirschsprung disease-like phenotype (agenesis of the enteric ganglia) due to vagal crest deficiency.

## MATERIALS AND METHODS

The generation of Raldh2-null mutant mice has been described previously (Niederreither et al., 1999). The defects investigated in this study were obtained after RA supplementation of the maternal food: all-trans-RA (Sigma) from a 5 mg/ml ethanol stock suspension was diluted in 50 ml water and mixed with 30 g powdered food (R03 breeding diet from UAR, Villemoisson, France) at a final concentration of 100 μg/g food (E7.5 to 8.5) or 250 μg/g (E8.5 onwards). The resulting food paste was provided daily to the pregnant mice in a Petri dish wrapped in aluminium foil. Most analyses (except histology, see below) were performed on embryos that were supplemented until the day of sacrifice. Extending the RA supplementation did not significantly improve the Raldh2<sup>−/−</sup> phenotype (see Results).

Whole-mount in situ hybridizations were performed as described previously (Décimo et al., 1995), using template plasmids cloned in our Institute or kindly provided by Drs G. Barsh (Stanford University, CA; kriessler/Mafβ), P. Gruss (MPI, Göttingen; Pax1, Pax9), R. Krumsäuf (Stowers Institute, Kansas City, MI; Hoxa2) G. Martin (UC San Francisco, CA; Fg/β). Whole-mount X-gal assays of embryos carrying a RARE-hsp65-lacZ reporter transgene, which harbors a tetramer repeat of the RARE<sub>β2</sub> RARE linked to the hsp65 minimal promoter, were performed as described previously (Rossant et al., 1991). Anti-neurofilament staining with the 2H3 monoclonal antibody (Developmental Studies Hybridoma Bank, Iowa City, IA) and in situ hybridization on cryosections were performed as described previously (Mark et al., 1993; Niederreither and Dollé, 1998).

## RESULTS

### RA-supplemented Raldh2<sup>−/−</sup> embryos exhibit defects of posterior branchial arch derivatives

Histological analysis was performed on E18.5 Raldh2<sup>−/−</sup> mutants after RA rescue from E7.5-E12.5, to see whether their heart septation defect (PTA; see Fig. 1B) correlated with defects of other branchial arch derivatives. The embryonic pharyngeal pouches give rise to several derivatives, including the thymus and parathyroid glands, which derive from the 3rd and 4th pouches, respectively (Larsen, 1997). Some of the RA-rescued Raldh2<sup>−/−</sup> mutants lacked the thymus gland (compare Fig. 1A and B). Others had ectopic thymic rudiments in their cervical region, adjacent to the thyroid tissue (Fig. 1C,F). Furthermore, no parathyroid glands were seen along the lateral wall of the thyroid lobes (compare Fig. 1D-F). Thus, defects of pharyngeal pouch-derived organs are associated with heart outflow tract septation defects in the RA-rescued Raldh2<sup>−/−</sup> mice. This condition is reminiscent of the defects seen in human DiGeorge syndrome (Scambler, 2000; Emanuel, 2001; Lindsay, 2001) and in recently generated mouse models for this syndrome (Lindsay et al., 2001; Merscher et al., 2001) (see Discussion).

When collected at E9.5, RA-rescued Raldh2<sup>−/−</sup> embryos exhibited a lack of discernible 3rd branchial arches, whereas their 1st and 2nd arches were of normal size (e.g. Fig. 3G-L). Embryonic intracardiac ink injections were performed at various developmental stages, to analyze aortic arch development in the rescued Raldh2<sup>−/−</sup> mutants. At E9.5, the mutant embryos appeared to lack the 3rd aortic arches, whereas the 2nd aortic arches were well formed (Fig. 2A,B). At E10.5 in wild-type embryos, ink injection mostly labels the 3rd and 4th aortic arches, as the 2nd arches are being remodelled (Fig. 2C). E10.5 Raldh2<sup>−/−</sup> embryos had no visible 3rd or 4th aortic arches, and the labeled arterial flow mostly passed through their 2nd arches (Fig. 2D). At E11.5, the 3rd, 4th and 6th aortic arches are labeled in wild-type embryos (Fig. 2E). In contrast, a single arterial vessel connected the aortic sac and the dorsal aortae in Raldh2<sup>−/−</sup> embryos (Fig. 2F). This abnormal arch was very poorly developed in some specimens (inset in Fig. 2F). The aortic arch defects were observed in mutants after short-term (E7.5-E9.5) or long-term (until day of analysis) RA supplementation.

### RA supplementation does not properly restore RA levels in the prospective branchial arch region of Raldh2<sup>−/−</sup> embryos

Raldh2 is known to be expressed in various mesodermal derivatives, from gastrulation onwards (Niederreither et al., 1997). We analyzed how its expression is related to branchial arch development in wild-type embryos. At E8.5 (10-12 somite pairs), when only the first branchial arch and first pharyngeal pouch are visible, Raldh2 was expressed in the splanchnic mesoderm up to a boundary located towards the posterior extremity of the foregut pocket (Fig. 2E, arrowhead). While Raldh2 expression levels decreased in the various trunk mesodermal derivatives (e.g. in somites) by ~E8.75, a specific expression domain persisted caudally to the foregut pocket (Fig. 2F). This domain remained distant from the developing 3rd pharyngeal pouch, i.e. was restricted to the posteriormost area of the prospective branchial arch region. Bisection of the
RALDH2 regulates branchial arch development

**Fig. 1.** Thymus and parathyroid defects in RA-rescued Raldh2–/– mutants. Coronal histological sections (stained with tetrachrome) of an E18.5 wild-type fetus (WT; A,D) and two Raldh2–/– fetuses (B,E and C,F) that were rescued by maternal RA supplementation from E7.5 to E12.5. (A) A representative section of the wild-type thymus gland. (D) Sections of the left and right thyroid lobes and the adjacent parathyroid glands. (B) No thymus was seen in the mediastinum of the first mutant (note the presence of a persistent truncus arteriosus). (C) An ectopic cervical thymus rudiment was found in the second animal. (E-F) This rudiment was connected to the thyroid tissue via a pouch-like structure (F, inset). No parathyroid glands were seen in the mutants. At, atrium; PTA, persistent truncus arteriosus; Pth, parathyroid; Th, thymus; Tr, trachea; Ty, thyroid.

**Fig. 2.** (A-F) Defects in aortic arch formation in RA-rescued Raldh2–/– embryos. Embryos collected at E9.5 (A,B), E10.5 (C,D) or E11.5 (E,F) were incubated in phosphate-buffered saline and injected intracardially with china ink. Compare the aortic arch patterns in wild-type (A,C,E) and Raldh2–/– (B,D,F) embryos (see text for details). The inset in F shows a detail of a different Raldh2–/– embryo. Arrowheads point to the single embryonic vessel connecting the aortic sac and the dorsal aorta. (G-I) Analysis of Raldh2 expression pattern in the wild-type embryonic pharyngeal region. Whole-mount in situ hybridization of an antisense Raldh2 riboprobe was performed on E8.5 (~10 somites, G) and E8.75 (~16 somites, H,I) wild-type embryos. The arrowhead in G points to Raldh2 expression boundary in the posterior foregut region. (J-L) Patterns of RA response in the branchial arch region of E8.5 wild-type (J) and Raldh2–/– (K,L) embryos carrying a RARE-hsp68-lacZ reporter transgene. Embryos are shown after whole-mount X-gal assay and medial bisection of their head and trunk region (inside views). a2-a6, aortic arches; as, aortic sac; b1-b3, branchial arches; da, dorsal aorta; ey, eye; fg, foregut; me, mesoderm; nt, neural tube; so, somites.
embryos revealed expression in the mesoderm underlying the ventral aspect of the foregut endoderm, rather than in the endoderm itself (Fig. 2G).

Interestingly, this expression pattern did not really match the spatial activity of a RA-inducible reporter transgene (Rossant et al., 1991) (see Materials and Methods). In wild-type E8.5 embryos, the reporter transgene was expressed at high levels along the foregut wall, up to the posterior edge of the developing 2nd arch (Fig. 2H). Furthermore, the RA-reporter transgene was activated in both the endodermal and mesodermal layers of the foregut pocket (Fig. 2H). RA-reporter activity also extended further rostrally in the dorsal (hindbrain-adjacent) mesenchyme up to the otocyst level (Fig. 2H), whereas Raldh2 transcripts did not reach the otocyst (Fig. 2E,F).

RA-reporter transgene activity was clearly weaker in the RA-rescued Raldh2–/– mutant embryos (Fig. 2I,J) although its spatial distribution was comparable to that seen in wild-type embryos (Fig. 2H). Downregulation was seen in both the endodermal and mesodermal layers of the foregut. In the most severe cases, expression was restricted to scattered cells in the branchial region and the hindbrain mesenchyme (Fig. 2J). However, these cells extended almost as rostrally as in wild-type embryos (Fig. 2H).

**Impairment of endodermal and mesodermal foregut gene expression in rescued Raldh2-null mutants**

The homeobox genes Hoxa1 and Hoxb1 are known to be RA-regulated, both in vitro and in vivo, through RAREs located in their regulatory regions (Gavalas et al., 1998). Expression of both genes was selectively downregulated in the foregut region of the RA-rescued Raldh2–/– embryos (Fig. 3A-F). Downregulation was seen in both the endoderm and mesoderm of the posterior foregut (compare Fig. 3C,E and D,F). Expression of both genes in the tail bud and posterior trunk tissues, as well as Hoxb1 expression in the 4th rhombomere (r4), was not detectably altered in the mutant embryos (Fig. 3A,B, and data not shown).

FGF8 is a signaling molecule expressed in specific regions of the wild-type branchial arch ectoderm and endoderm (Crossley and Martin, 1995; Wall and Hogan, 1995). Within the endoderm, Fgf8 expression is highest at the level of the developing pharyngeal pouches (Fig. 3I). Fgf8 was expressed at abnormally low levels along the posterior branchial arch region of E9.5 Raldh2–/– embryos, and its spatial distribution was altered. Scattered ectodermal Fgf8-expressing cells were observed in mutant embryos from the level of the 2nd-3rd branchial cleft down to ectopic posterior locations, almost reaching the level of the forelimb bud rudiment (compare Fig. 3G and H, bracket). Furthermore, there was almost no detectable Fgf8 expression in the mutant pharyngeal endoderm, except at the level of the developing 2nd pouch (compare Fig. 3I and J). The distribution and levels of expression of Fgf8 were unaltered in the first branchial arch, as well as in other craniofacial regions, of the mutant embryos (Fig. 3H,J, and data not shown).

![Fig. 3. Altered gene expression in the posterior pharyngeal region of RA-rescued Raldh2–/– embryos. (A-D) Whole-mount in situ analysis of Hoxb1 transcripts in E9 (~22 somites) wild-type (A,C) and Raldh2–/– (B,D) embryos. Profile views of the whole embryos (A,B) and enlargement of the posterior foregut region after medial bisection (C,D, inside views). (E,F) In situ hybridization analysis of Hoxa1 transcripts in the foregut area of E9 wild-type and Raldh2–/– embryos, respectively (viewed as in C,D). (G-J) In situ hybridization analysis of Fgf8 transcripts in E9.5 wild-type (G,I) and Raldh2–/– (H,J) embryos. External profile views (G,H) show Fgf8 ectodermal labeling, whereas internal views after bisection (I,J) reveal the endodermal labeling. Insets in (IJ) are coronal views which make it possible to distinguish the ectodermal and endodermal labeling. (K,L) In situ hybridization analysis of Pax9 expression in the foregut region of E9.5 (main panels) and E10.5 (insets; detail of the pharyngeal region caudally to the 2nd arch) wild-type and Raldh2–/– embryos, respectively. b1-b3, branchial arches; ec, ectoderm; en, endoderm; fg, foregut; me, mesoderm; p1-p3, pharyngeal pouches; r4, rhombomere 4; tb, tail bud.](image-url)
We also analyzed Pax1 and Pax9 transcript distributions in mutant embryos, as these genes are differentially expressed along the endoderm of the developing pharyngeal pouches (Müller et al., 1996). Both Pax1 (data not shown) and Pax9 (Fig. 3K,L) expression patterns confirmed the lack of a distinct 3rd pharyngeal pouch and the formation of a single, enlarged 2nd pouch in E9.5 mutant embryos (Fig. 3L, ‘p2’). Despite this abnormality, Pax9 was differentially expressed along the dorsoventral axis of the mutant 2nd pouch, in a pattern similar to that seen in the wild-type 2nd and 3rd pouches (compare Fig. 3K and L). Note that the overall size of the pharyngeal pouch region (from the anterior aspect of the 1st pouch to the posterior aspect of the abnormal 2nd pouch) was clearly reduced in E9.5 mutant embryos (Fig. 3K,L, brackets). Mutants collected at E10.5 after RA supplementation (from E7.5 to E9.5 or E10.5) similarly showed fused pouch-like rudiments caudally to their 2nd branchial arches (compare Fig. 3K and L). Note that the overall size of the pharyngeal pouch region (from the anterior aspect of the 1st pouch to the posterior aspect of the abnormal 2nd pouch) was clearly reduced in E9.5 mutant embryos (Fig. 3K,L, brackets). Mutants collected at E10.5 after RA supplementation (from E7.5 to E9.5 or E10.5) similarly showed fused pouch-like rudiments caudally to their 2nd branchial arches (compare Fig. 3K and L, insets).

Neural crest cell migration is abnormal in rescued Raldh2-null mutants

Several neural crest markers were used to investigate whether the abnormal development of posterior branchial arches in rescued Raldh2−/− embryos correlated with NCC defects. The EphA4 gene is normally expressed in r3 and r5, and in NCC emanating from the latter rhombomere (Nieto et al., 1992) (Fig. 4A). EphA4 was expressed at normal levels in r3 and r5 of Raldh2−/− embryos (compare Fig. 4A and B). However, the stream of NCC that colonized the 3rd arch in wild-type embryos had no counterpart in mutants (Fig. 4B). The EphA2 gene is expressed in wild-type embryos in both NCC and mesodermal cells of the post-otic region and 3rd-6th pharyngeal arches (Ruiz and Robertson, 1994) (Fig. 4C). EphA2-expressing cells were detected in Raldh2−/− embryos, but these cells remained confined dorsally to the foregut pocket (Fig. 4D, arrowhead).

Cellular RA binding protein 1 (Crabp1) gene transcripts mark migratory and early post-migratory NCC at all craniofacial levels (Maden et al., 1992). Crabp1-labeled cells colonized the 2nd arch, as well as more rostral facial regions of Raldh2−/− embryos (Fig. 2F,G), to the same extent as in wild-type embryos (Fig. 4E). Crabp1-positive cells were also found caudally to the 2nd arch in mutant embryos, but these cells failed to migrate along organized segmental pathways and extended more caudally than in wild-type embryos, eventually merging with the trunk and forelimb labeled cells (Fig. 4F,G, brackets). Crabp1 labeling also revealed abnormal patterns of connectivity between the post-otic hindbrain and the corresponding post-migratory NCC. In wild-type embryos, two main streams connect the hindbrain and the 3rd-4th arch NCC populations (Fig. 4H, arrowheads). Raldh2−/− embryos exhibited several ‘segmental-like’ streams extending from r6 down to the spinal cord levels (Fig. 4I).

Abnormal development of posterior cranial nerves in rescued Raldh2-null mutants

The patterning of cranial nerves, which develop within specific branchial arches, was analyzed at E11.5 in Raldh2−/− mutants that were RA-rescued from E7.5-E9.5, using anti-neurofilament immunostaining (Fig. 5). Patterning of the nerves that develop in the 1st and 2nd branchial arches (5th and 7th cranial nerves, respectively) was normal in most of the mutant embryos (Fig. 5B-D). All mutants, however, showed impaired development of the 9th (glossopharyngeal) and 10th (vagus) nerves, which normally develop within the 3rd and 4th branchial arches. Three examples of phenotypes of increasing severity are shown in Fig. 5B-D. In the less severe cases, separate 9th and 10th nerve tracts were formed, but their connections with the hindbrain were mingled (Fig. 5B). In addition, the distal sensory ganglia of these nerves, which normally derive from the 2nd and 3rd epibranchial placodes, were abnormally fused (Fig. 5B). In more severe cases, the axon bundles of presumptive 9th and 10th nerves merged in a single rudimentary trunk that followed an aberrant route (Fig. 5A).
or failed to extend towards the periphery (Fig. 5D, arrowhead). In such severe cases, a putative distal ganglion was formed (d9/10 in Fig. 5C,D), which had no connection with the proximal axonal trunk. This indicates that neuronal differentiation could proceed within the mutant epibranchial placodes, but that these neurons were unable to establish a connection with the hindbrain-derived nerve tracts. Altered outgrowth of the 11th (spinal accessory) and 12th (hypoglosseal) nerve tracts was also evident in mutants (Fig. 5B-D). The same range of abnormal phenotypes was observed in *Raldh2*–/– embryos when RA supplementation was extended up to E11.5 (data not shown).

**Hindbrain patterning is rescued in RA-supplemented *Raldh2*–/– embryos**

In the absence of RA supplementation, *Raldh2*–/– embryos exhibit severe growth and patterning defects of the posterior (prospective r3-r8) hindbrain region (Niederreither et al., 2000). These defects were partly reverted by maternal RA supplementation through oral gavage (Niederreither et al., 2000). Several rhombomeric markers were used to investigate hindbrain patterning in *Raldh2*–/– mutants after RA supplementation from E7.5 to E9.5. *Hoxb1* expression in r4 was comparable in both wild-type and *Raldh2*–/– embryos (Fig. 3A,B). As in wild-type embryos, *Hoxa2* was expressed at higher levels in r3 and (to a lesser extent) r5, and at low levels in r2 and r4 (Fig. 6A,B). Furthermore, *kreisler* expression in r5 and r6 was comparable in wild-type and *Raldh2*–/– embryos (Fig. 6C,D; note, however, that the *kreisler*-labeled NCCs, which normally migrate towards the 3rd branchial arch (Fig. 6C), are deficient in the mutant). We therefore conclude that hindbrain patterning is efficiently rescued in *Raldh2*–/– embryos through maternal dietary RA supplementation, and that the NCC and cranial nerve abnormalities described above are unlikely to result from defect(s) intrinsic to the hindbrain neuroepithelium.

**Rescued *Raldh2*–/– mutants lack enteric nervous system development**

The proto-oncogene *Ret* exhibits sequential expression within the NCC components of the developing cranial nerve ganglia. At E9.5, expression is detected in wild-type embryos within the facial-acoustic (7th-8th), glossopharyngeal (9th) and vagal (10th) nerve ganglia (Pachnis et al., 1993) (Fig. 6E). While *Ret* was normally expressed in the facial-acoustic ganglion complex of the RA-rescued *Raldh2*–/– mutants, very few positive cells were detected posteriorly to the otocyst, at the expected level of the glossopharyngeal and vagal ganglia (Fig. 6F, bracket).

At later stages, *Ret* is specifically expressed in various NCC populations of neural fate, including the vagal NCCs that will give rise to the ganglia of the enteric nervous system.
RALDH2 regulates branchial arch development (Pachnis et al., 1993). This cell population was analyzed in Raldh2−/− mutants by in situ hybridization of serial histological sections. At E10.5, Ret-positive prospective enteric ganglioblasts are migrating along the wall of the fore- and midgut of wild-type embryos (Fig. 7A). Only few Ret-positive presumptive ganglioblasts were seen in Raldh2−/− embryos after RA rescue from E7.5 to E9.5, and these cells were confined to the pharyngeal region and/or the nearby foregut wall (Fig. 7B). By E12.5, the vagal nerves are strongly labeled (Fig. 7C) and the enteric ganglioblasts are beginning to coalesce along the stomach and gut wall to form ganglia in wild-type embryos (Fig. 7E,G). In contrast, the stomach and gut wall of Raldh2−/− embryos was devoid of Ret-expressing cells (Fig. 7F,H). A few c-ret-expressing cells, arranged as rudimentary tracts, were detected at the expected location of the vagal nerves in mutants (compare Fig. 7C and D). Other domains of Ret expression were unaltered in Raldh2−/− embryos, both in the case of non-NCC (e.g. the ureteric bud or spinal cord motoneurons: Fig. 7A,B and C-F, respectively) and NCC-derived populations (e.g. the dorsal root or trigeminal ganglia: Fig. 7C-F and I,J, respectively).

This abnormal phenotype was confirmed by analyzing another marker of the developing enteric nervous system (Mash1) (Blaugrund et al., 1996) (data not shown). Extending the RA supplementation until E12.5 did not rescue the formation of enteric ganglia in Raldh2−/− mutants. In the best case, labeled cells were found along part of the foregut and stomach wall, but these cells did not colonize more posterior gut segments (data not shown).

**DISCUSSION**

RA-dependent development of posterior pharyngeal arches cannot be rescued in Raldh2-null mutants by maternal RA supplementation

Our analyses have revealed altered migration patterns of the post-otic hindbrain neural crest cells (NCC), which normally give rise to the cardiac NCC population, in the RA-rescued Raldh2−/− embryos. We show that these NCC defects are part of a complex range of alterations that encompass the whole posterior pharyngeal region. Whereas the first and second branchial arches form properly, development of the 3rd to 6th branchial arches and their derivatives (aortic arches, cranial nerves) is severely impaired in mutant embryos. Most notably, pharyngeal pouches fail to form within the posterior pharyngeal endoderm and development of the corresponding pouch-derived organs, such as the thymus and parathyroid glands, is compromised. Furthermore, a single (sometimes rudimentary) arterial vessel is formed instead of the 3rd to 6th aortic arches. This arterial defect likely accounts for the death of some of the rescued mutants prior to fetal stages. Indeed, PTA as an isolated malformation is not lethal until after birth (Larsen, 1993), whereas the observed aortic arch defect may compromise blood circulation in the mutant embryos as soon as their 1st and 2nd arches regress and are remodelled.

Using several hindbrain markers, we show that growth and patterning of the posterior rhombomeres, which is highly abnormal in unrescued Raldh2−/− embryos (Niederreither et al., 2000), is rescued by the present maternal RA supplementation.

**Fig. 7.** Lack of enteric nervous system development in RA-rescued Raldh2−/− mutants. In situ hybridization was performed with a 35S-labeled Ret probe on cryosections of wild-type (A,C,E,G,I) and Raldh2−/− (B,D,F,H,J) embryos. Each panel shows bright-field (left) and dark-field (right) views of the same section to show, respectively, the histology and the in situ hybridization signal (white dots). At E10.5, mutant embryos only show scattered Ret-labeled prospective ganglioblasts (arrows in B), which failed to colonize the midgut levels (compare A and B). E12.5 mutant embryos have rudimentary vagal nerve tracts (compare C and D, arrows) and their stomach and gut wall are devoid of Ret-positive ganglion cells (compare E,G and F,H). Other domains of Ret expression (e.g. in the trigeminal ganglia: compare I and J) are unaffected in mutants. fg, foregut; g5, trigeminal ganglion; mg, midgut; sc, spinal cord; st, stomach; ur, ureteric bud.
It is therefore likely that the abnormal NCC migration patterns observed in RA-rescued Raldh2-null mutants are secondary to the abnormal development of the pharyngeal region, rather than being the result of an intrinsic hindbrain or NCC defect.

Our data show that RALDH2 is responsible for producing RA which is required for proper development of the posterior branchial arch region. Previous studies have indicated that this embryonic region is particularly sensitive to RA deficiency. Incubation of early somite-stage (E8.0-8.5) cultured mouse embryos with a pan-RAR antagonist led to defects of the 3rd to 6th branchial arch region (Wendling et al., 2000), which are consistent with those seen in RA-rescued Raldh2-null mutants. Furthermore, vitamin A-deficient (VAD) quail embryos exhibit defects in the posterior pharyngeal region (Quinlan et al., 2002), which are reminiscent of the mouse phenotype in these experiments. For instance, both the VAD quail embryos and the rescued Raldh2 mouse mutants develop a rather normal first pharyngeal pouch, whereas only a single, abnormal second pouch is formed posteriorly. In agreement with these studies (Wendling et al., 2000; Quinlan et al., 2002), our data point to the pharyngeal endoderm as one of the main target tissues whose patterning is altered by conditions of impaired RA signaling.

We have shown that Raldh2 expression is mesoderm-specific, and is restricted to the posteriormost pharyngeal region during development of the posterior branchial arches. We propose that RALDH2 function is to produce locally high amounts of RA, which are indispensable to correctly pattern these branchial arches. The mesodermally produced RA would then diffuse and signal within the pharyngeal endoderm. This is illustrated by the fact that activity of a RARE-lacZ reporter transgene, as well as endogenous expression of RA target genes (such as Hoxal and Hoxb1), is induced in both the mesoderm and endoderm of the posterior pharyngeal region. In the rescued Raldh2-null mutants, RA brought transplacentally cannot mimic the levels and distribution of RA brought mesoderm and endoderm of the posterior pharyngeal region. In the rescued Raldh2-null mutants, RA brought transplacentally cannot mimic the levels and distribution of RA brought resulting from endogenous, RALDH2-mediated synthesis, transplacentally cannot mimic the levels and distribution of RA brought brings about a decrease of RA-reporter transgene activity in both the rescued Raldh2-null mutants. Furthermore, vitamin A-deficient (VAD) quail embryos exhibit defects in the posterior pharyngeal region (Quinlan et al., 2002), which are reminiscent of the mouse phenotype in these experiments. For instance, both the VAD quail embryos and the rescued Raldh2 mouse mutants develop a rather normal first pharyngeal pouch, whereas only a single, abnormal second pouch is formed posteriorly. In agreement with these studies (Wendling et al., 2000; Quinlan et al., 2002), our data point to the pharyngeal endoderm as one of the main target tissues whose patterning is altered by conditions of impaired RA signaling.

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We have also found that development of the 2nd branchial arches, which is deficient in the Raldh2-null mutants (Niederreither et al., 1999), is efficiently rescued by maternal RA supplementation. This indicates that 2nd arch development may require lower RA levels, which can be supplied maternally in the absence of RALDH2 function. Other lines of evidence, involving analysis of RAR compound mutant mice (Lohnes et al., 1999), VAD rat embryos (White et al., 2000) or pan-RAR antagonist-treated mouse embryos (Wendling et al., 2000), have indicated that development of the 2nd branchial arches is not as critically dependent on RA signaling as that of more posterior arches. Whether this differential requirement reflects binding to a different subset of receptors has been discussed elsewhere (Wendling et al., 2000). We postulate that RA produced by RALDH2 in the posterior pharyngeal mesoderm diffuses up to the level of the 2nd branchial arches, where its acts at relatively lower concentrations. This is supported by the pattern of activity of the RARE-lacZ reporter transgene which, in wild-type embryos, extends up to the developing 2nd arches, albeit at a much lower level than in the posterior pharyngeal region.

The rescued Raldh2-null phenotype resembles human DiGeorge syndrome and mouse Tbx1 knockout phenotypes

Conotruncal abnormalities are prevalent human birth defects occurring at a frequency as high as four per 10,000 births. As many congenital defects, the origins of PTA are often unknown. Poor nutrition during pregnancy – including vitamin A deficiency – may contribute to these malformations (Underwood and Arthur, 1996). The most frequent genetic cause of human conotruncal defects consists of heterozygous microdeletions of the chromosome 22q11 region, which lead to a spectrum of abnormalities of variable expressivity, known as DiGeorge/Velocardiofacial syndromes (DGS/VCFS) (reviewed by Scambler, 2000; Emanuel, 2001; Lindsay, 2001). Several engineered deletions of the corresponding mouse locus have implicated the T-box gene Tbx1 as a major determinant of the DGS/VCFS phenotypes (Jerome and Papaioannou, 2001; Lindsay et al., 2001; Merscher et al., 2001). Both haploinsufficient and null (Tbx1−/−) mutant mice fail to form the 3rd and 4th pharyngeal arches and have conotruncal defects (Lindsay et al., 2001; Jerome and Papaioannou, 2001; Merscher et al., 2001). Alterations in thymus and parathyroid morphology are also seen in these mutants (Jerome and Papaioannou, 2001; Merscher et al., 2001). According to its expression which is essentially restricted to the pharyngeal pouch endoderm, Tbx1 appears to function in a non-cell autonomous manner for branchial arch outgrowth, and NCC defects in these mutants have been attributed to a lack of guidance from the pouch endoderm (Vitielli et al., 2002).

The defects reported in the present study are clearly similar to those observed in posterior branchial arches (and their derivatives) in Tbx1 mutant mice, and therefore represent a new mouse model of the human DGS abnormalities. The pharyngeal region appears to be highly sensitive to alterations in RA levels, as a hypomorphic mouse Raldh2 mutation was found to selectively lead to DGS-like defects (Vermot et al., 2003), albeit less severe than in the present Raldh2-null mutants. As Tbx1 expression is not (or only mildly) affected in both the null (data not shown) and hypomorphic (Vermot et al., 2003) mutants, we do not consider it as a critical determinant of the Raldh2 phenotype. It rather seems that RA may act downstream (or in combination with) TBX1 to regulate expression of signaling molecules required for proper pharyngeal development. FGF8 could represent such a critical common downstream signal, as (i) its expression is altered in both Tbx1−/− and Raldh2−/− mutants (Vitelli et al., 2002) (this study), and (ii) its hypomorphic mutation also leads to a DGS-like phenotype (Abu-Issa et al., 2002; Frank et al., 2002).

Lack of vagal outgrowth leads to absence of the enteric nervous system

Retinoic acid has been implicated in the regulation of many aspects of neuronal development including specification of neuronal cell fate (Sockanathan and Jessell, 1998) and stimulation of neurite outgrowth (Hunter et al., 1991; Plum and Clagett-Dame, 1996; Corcoran and Maden, 1999). RA has also been found to promote survival and proliferation of neuronal
progenitors in NCC populations (Henion and Weston, 1994; Gale et al., 1996). We find that axonal growth is variably affected at the level of the posterior (9th-12th) cranial nerves in RA-rescued Raldh2−/− mutants (see Fig. 4), consistent with the idea that RA produced locally by RALDH2 in the pharyngeal mesoderm is required to stimulate their neurite outgrowth.

The enteric nervous system (ENS) is mainly derived from vagal NCCs, which migrate along the foregut mesenchyme to colonize the entire length of the gut and give rise to the majority of neurons and glia of the enteric ganglia (Le Douarin and Teillet, 1973; Young et al., 1998; Burns and Le Douarin, 1998). Among the signaling pathways involved in ENS development (for reviews, see Gershon, 1998; Taraviras and Pachnis, 1999), the RET receptor tyrosine kinase and its ligand GDNF (glial cell line-derived neurotrophic factor) are of critical importance. Both Ret and Gdnf knockout mice exhibit semi-dominant hypo- or aganglionosis of the gastrointestinal tract (Schuchardt et al., 1995; Pichel et al., 1996; Shen et al., 2002). RET mutations are also most frequently involved in cases of human Hirschprung’s disease (congenital megacolon or aganglionosis), the most frequent hereditary cause of intestinal obstruction (Gabriel et al., 2002). Here we show that the vagal defects in Raldh2−/− embryos lead to a similar gastrointestinal aganglionic phenotype. While few Ret-positive ENS progenitor cells are detected along the foregut wall at E10.5, they are apparently unable to colonize the gastrointestinal tract, leading to an absence of enteric ganglia. This is the first report of intestinal aganglionogenesis caused by an alteration in the retinoid signaling pathway. The possibility that such alterations could be involved in the pathogenesis of human Hirschprung’s disease, which is both variable in its expressivity and complex in its inheritance pattern, should therefore be considered.

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