Development 127, 1553-1562 (2000)
Printed in Great Britain © The Company of Biologists Limited 2000
DEV2533

Retinoid signaling is essential for patterning the endoderm of the third and fourth pharyngeal arches

Olivia Wendling, Christine Dennefeld, Pierre Chambon and Manuel Mark*

Institut de Génétique et de Biologie Moléculaire et Cellulaire (IGBMC), CNRS/INSERM/ULP/Collège de France, BP 163, 67404 Illkirch Cedex, CU de Strasbourg, France
*Author for correspondence (e-mail: marek@igbmc.u-strasbg.fr)

Accepted 3 February; published on WWW 21 March 2000

SUMMARY

The requirement of retinoic acid (RA) in the initial formation of the pharyngeal arches was investigated by treating headfold-stage mouse embryos with a pan-RAR antagonist in vitro and in vivo. This results in a complete absence of mesenchyme, arteries, nerves and epibranchial placodes of the 3rd and 4th pharyngeal arches, complete agenesis of the 3rd and 4th pouches and consistent lack of the 6th arch artery. Mesodermally derived endothelial cells are absent from the 3rd and 4th pharyngeal arch region and the distribution domain of *EphA2* transcripts in mesodermal cells is shifted caudally. In situ hybridization with *CRABPI*, *kreisler* and *EphA4* probes and the pattern of expression of a *Wnt1-lacZ* transgene show that neural crest cells (NCC) normally destined to the 3rd and 4th arches migrate ectopically. Most interestingly, the appearance of the 3rd and 4th arches is prevented by the antagonist only during a very narrow window of time, which does not correspond to the period of post-otic NCC migration. Both the timing of appearance and the nature of the defects in RAR antagonist-treated embryos indicate that migrating NCC and mesodermal cells destined to the caudal pharyngeal arches do not represent primary targets of RA action. Alterations in the endodermal expression pattern of *Hoxa1*, *Hoxb1*, *Pax1*, *Pax9*, *Fgf3* and *Fgf8* in response to the antagonist-induced block in RA signal transduction demonstrate for the first time that RA signaling is indispensable for the specification of the pharyngeal endoderm and suggest that this signaling is necessary to provide a permissive environment locally for the migration of NCC and mesodermal cells. Our study also indicates that the formation of the 2nd pharyngeal arch and that of the 3rd and 4th pharyngeal arches probably involve distinct RA-dependent developmental processes.

Key words: Retinoic acid receptor, Endoderm, Pharynx, Neural crest, *Hox* gene, *Pax* gene, Fgf, Embryo culture, Mouse

INTRODUCTION

Pharyngeal arches (PA) are transient bulges of the embryonic surface that develop in a cranial to caudal sequence between the head and the heart and are separated by evaginations of the pharyngeal endoderm, the pharyngeal pouches. Adult organs originating from caudal (i.e. post-otic) PA and 3rd pouch, which include the thymus, parathyroid glands and arteries destined to the head and lungs, are all partially derived from neural crest cells (NCC) (Larsen, 1993; Le Douarin, 1982).

Transduction of retinoic acid (RA) signals by nuclear receptors, the RARs and RXRs, plays key roles in development (Kastner et al., 1995, 1997; Chambon, 1996; Mascrez et al., 1998). Mouse fetuses carrying targeted inactivations of both the RARα and RARβ genes recapitulate the defects generated in the chick by ablation of large portions of the neural crest destined to the caudal pharynx, namely thymus and parathyroid gland ageneses or ectopias, aberrant pattern of the great cephalic arteries, and absence of the pulmonary arteries and aortico-pulmonary septum (Bockman et al., 1989; Kirby and Waldo, 1990; Mendelsohn et al., 1994; Ghyselinck et al., 1997). RARα$^{-/-}$ / RARβ$^{-/-}$ embryos have fused 3rd and 4th PA but do not display obvious defects in the number and migration paths of NCC (Dupé et al., 1999). The defects of RARα$^{-/-}$ / RARβ$^{-/-}$ mutants do not reflect a state of complete retinoic acid (RA) deficiency, possibly because the loss of these two receptors is partially compensated by RARγ (Dupé et al., 1999). The generation of RARα$^{-/-}$ / RARβ$^{-/-}$ / RARγ$^{-/-}$ mutants is technically difficult and, additionally, such triple null mutants would probably die prior to the onset of organogenesis, as RA and RARs are present in embryos as early as E6.5 (Ang et al., 1996; Ang and Duester, 1997). Thus, in order to study the formation of the embryonic pharynx in a situation where retinoid signaling is severely impaired, we designed a culture system in which wild-type mouse embryos, collected at the headfold stage, were exposed to a pan-RAR antagonist BMS493, which competitively blocks the activation of all three RAR isotypes (α, β and γ) by agonistic retinoids.

MATERIALS AND METHODS

Embryo culture, retinoid treatments and mouse lines

Embryos were collected at various time points between the early

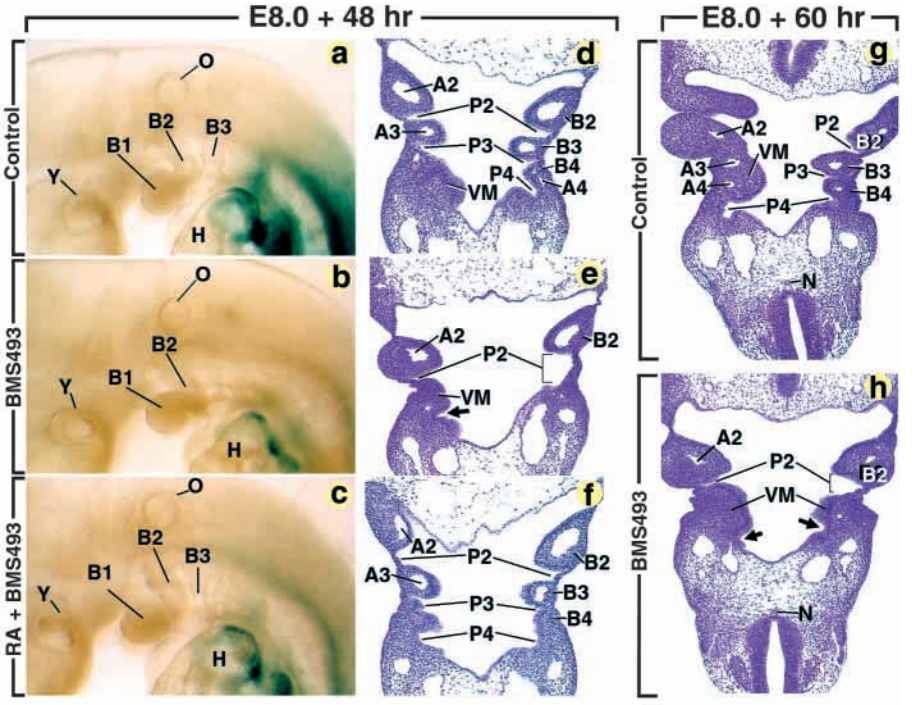

Fig. 1. External aspect (a-c) and histological analysis (d-h) of the pharyngeal region of cultured embryos. With the exception of (f), the plane of the histological sections is such that the left side of the embryo is always slightly more ventral than its right counterpart. A2-A4, pharyngeal arch arteries 2-4; B1-B4, pharyngeal (branchial) arches 1-4; H, heart; N, notochord; O, otocyst; P2-P4, pharyngeal pouches 2-4; VM, ventral mesenchyme of the pharynx; Y, eye. The large arrows point to an abnormal evagination of the pharyngeal endoderm marking the end of the 2nd pouch in BMS493-treated embryos. Magnification: ×30 (d-h).

headfold, presomite stage and the 14-somite stage and cultured as described by Copp and Cockroft (1990). All-*trans*-RA (Sigma) or the pan-RAR synthetic retinoid antagonist BMS493 (Bristol-Myers-Squibb, Princeton, NJ, USA), diluted in ethanol, was added to the culture medium to 0.1%. In control embryo cultures, the ethanol vehicle was added at the same final dilution. For in vivo treatments, a solution of BMS493 at 10^{-2} M in ethanol was diluted in sunflower oil (1:2.5 v/v) and administered by oral gavage twice at 10-hour intervals to pregnant females at a concentration of 10 mg/kg body mass. Embryos heterozygous for the *mRARβ2-lacZ* (Mendelsohn et al., 1991), the *RAREhsplacZ* (Rossant et al., 1991) and the *Wnt1-lacZ* (Echelard et al., 1994) transgenes were processed for X-gal staining as described by Mendelsohn et al. (1991).

Histology, histoenzymology, whole-mount immunohistochemistry and in situ hybridisation

Histology was performed according to standard procedures. Whole-mount in situ hybridisation (ISH) was performed according to Décimo et al. (1995) using digoxygenin-labeled riboprobes for *Hoxa1*, *Hoxb1*, *Pax1*, *Pax9*, *Fgf8*, *Fgf3*, *EphA4*, *EphA2*, *kreisler*, *CRABPI* and *Twist*. Whole-mount immunohistochemistry using the 2H3 neurofilament-specific antibody (Developmental Studies Hybridoma Bank), the anti-Phox2a epibranchial placode-specific antibody (Tiveron et al., 1996), and the anti-PECAM-1 (Pharmingen, San Diego, CA, USA), as well as whole-mount TUNEL labeling, were used as described (Mark et al., 1993; Davis et al., 1991; Schlaeger et al., 1995; Conlon et al., 1995). TUNEL staining on histological sections was performed according to Ghyselinck et al. (1998) with a 3-minute proteinase K treatment.

Ink injections

Embryos cultured for 48 and 60 hours were injected with Pelikan Ink n°17 via the yolk-sac vasculature, using a 1 mm capillary tube, then fixed and cleared according to Waldo et al. (1990).

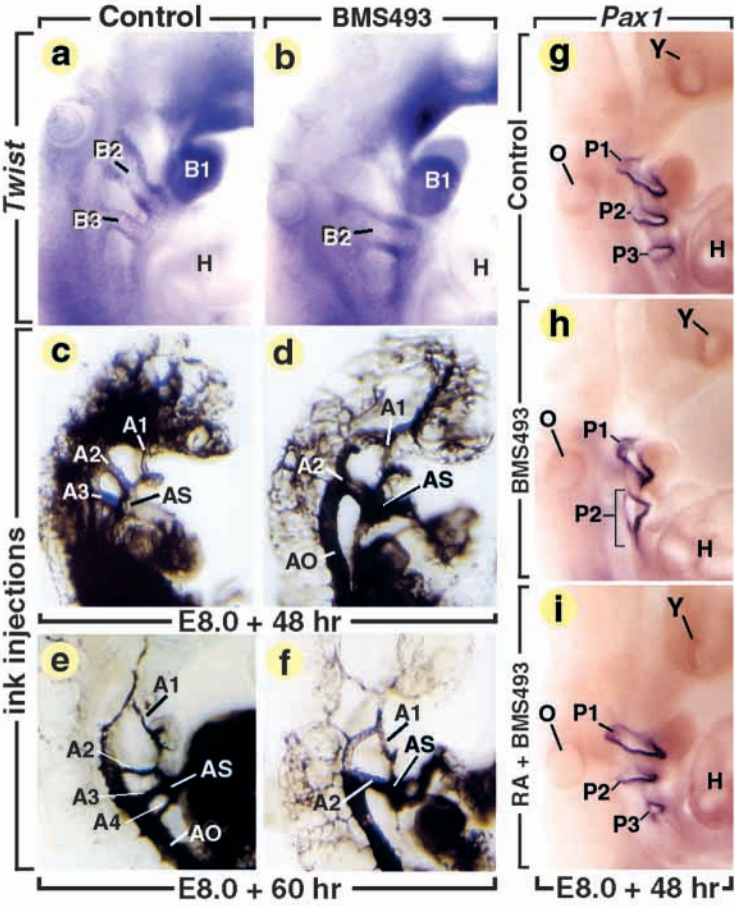

Fig. 2. Absence of arch mesenchyme (a,b), arch arteries (c-f) and branchial pouches (g-i) in BMS493-treated embryos. A1-A4, pharyngeal arch arteries 1-4; AO, aorta; AS, aortic sac; B1-B3, pharyngeal arches 1-3; H, heart; O, otocyst; P1-P3, pharyngeal pouches 1-3; Y, eye.

RESULTS

Unless otherwise mentioned, embryos collected at E7.5 (i.e. early headfold, presomite stage), E8.0 i.e. 2-4 somite stages (ss), E8.5 (10-14 ss) were cultured in a medium supplemented with either BMS493 at a concentration of 10^{-6} M or vehicle alone for 6-60 hours. For the sake of simplicity, these cultured embryos are referred to as Ex+y hours BMS493-treated embryos or controls respectively, x corresponding to the age of the embryos in days at the time of explantation and y to the hours spent in culture.

The competition between retinoic acid and its antagonist can be monitored in *mRARβ2-lacZ* transgenic embryos

The effects of the RA antagonist were tested on cultured embryos harboring an RA-inducible *mRARβ2-lacZ* transgene (Mendelsohn et al., 1991). A dose-response curve was established on E8.5+36 hours embryos treated with BMS493 at 10^{-5} M, 4×10^{-6} M, 10^{-6} M, 10^{-7} M, 10^{-8} M and 10^{-9} M. The last three concentrations did not affect the extent or the intensity of β-galactosidase staining. However, expression of the transgene was strongly decreased at 10^{-6} M and abolished at 4×10^{-6} M (data not shown). Even at this latter concentration, the embryos did not show external malformations. However, exposure to BMS493 at 10^{-5} M resulted in severe morphological alterations, which varied greatly between embryos and could not be prevented by simultaneous administration of RA, thus raising the possibility that they were caused by toxic (e.g. detergent-like) effects of the retinoid (data not shown). Therefore, all subsequent cultures were carried out in the presence of BMS493 at 10^{-6} M: at this antagonist concentration, the *RARβ2* promoter activity was already decreased after 6 hours and this decrease was reverted by the simultaneous addition to the culture medium of RA at 10^{-7}M (Fig. 1a-c and data not shown).

Long-term treatments with BMS493 of early somite stage embryos result in defects restricted to the caudal portion of the pharynx, the fifth rhombomere and the lungs

The external morphology of the vast majority (80%) of E8.0+24 hours (n=70) and E8.0+48 hours (n=110) controls was identical to that of E8.75 and E9.5 embryos in vivo, respectively (Kaufman, 1992; Van Maele-Fabry et al., 1997 and references therein). Dysmorphogenetic embryos exhibiting abnormal body turning, forebrain hypoplasia or caudal truncations, which occurred at the same frequency in the presence and absence of BMS493 (about 20%), were discarded. The majority (70%) of E8.0 embryos cultured for 60 hours (n=20) were dead. The surviving E8.0+60 hours embryos were comparable to E9.75 embryos in vivo. Embryos treated with BMS493 at E8.0 differed from controls only by the constant agenesis of the 3rd and 4th PA, enlargement of the second branchial pouch and near-absence of primary lung buds, as well as frequent increase of the size of rhombomere 5. The lungs and hindbrain defects will be described elsewhere.

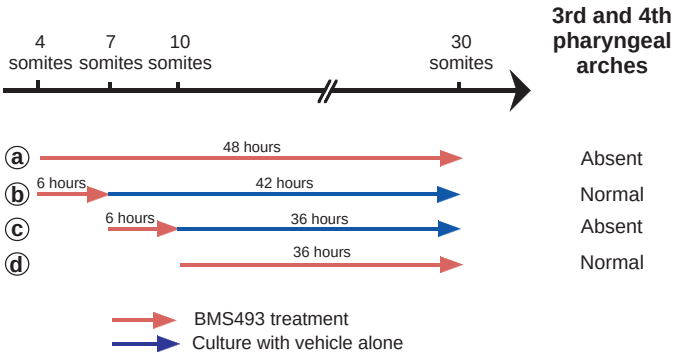

Fig. 3. Effect of embryo culture conditions on the formation of the 3rd and 4th arches (see the text for further details).

Treatments at E8.0 selectively impair the formation of the 3rd and the 4th pharyngeal arches, arch arteries and branchial pouches

Externally, all E8.0+48 hours controls displayed bulges corresponding to the first three PA (B1-B3, Figs 1a, 2a). On histological sections, the 3rd PA (B3, Fig. 1d) was delimited by the 2nd and the 3rd branchial pouches (P2 and P3), and contained an arch artery (A3). This histological analysis also revealed the presence of a 4th PA (B4, Fig. 1d), containing an artery in the process of canalisation (A4) and delimited caudally by a 4th pouch (P4). In E8.0+48 hours BMS493-treated embryos (n=110),

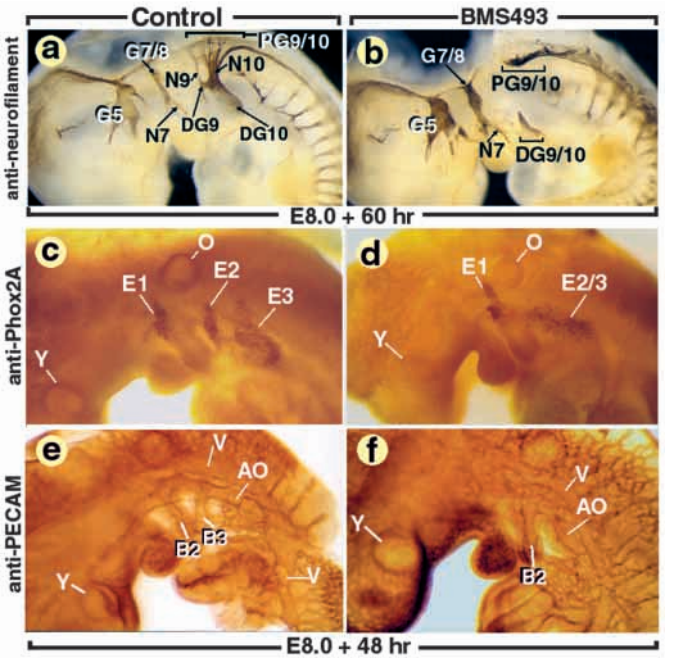

Fig. 4. Comparison of immunostained cranial nerves, epibranchial placodes and cephalic vessels in control and BMS493-treated embryos. AO, aorta; B2 and B3, branchial arches 1 and 3; DG9 and DG10, distal ganglia of the glossopharyngeal (IX) and vagus (X) nerves, respectively; E1-E3, epibranchial placodes 1-3; G5 and G7/8, trigeminal and facial-acoustic ganglia, respectively; DG9/10, abnormal fusion of the distal ganglia of cranial nerves IX and X; N7, N9, N10, facial, glossopharyngeal and vagus nerves; O, otocyst; PG9/10, common superior ganglion of the IXth and Xth nerves; V, cardinal vein; Y, eye.

the bulge of the 3rd PA was never seen (Figs 1b, 2b). Histological analysis of seven embryos revealed a complete, bilateral, absence of 3rd and 4th PA, corresponding arch arteries and branchial pouches (compare Fig. 1d and e). Moreover, the treated embryos consistently showed a markedly enlarged 2nd pouch (P2, Fig. 1e). The absence of mesenchyme corresponding to the 3rd PA and the enlargement of the 2nd pouch in BMS493-treated embryos was confirmed by whole-mount ISH using *twist* and *Pax1* probes, respectively (compare Fig. 2a,b,g,h). The first two PA (B1 and B2) of the BMS493-treated embryos were indistinguishable from their counterparts in controls (Fig. 1a,b,d,e).

In order to rule out the possibility that the alterations observed in E8.0+48 hours BMS493-treated embryos reflected regionally restricted developmental retardations, embryos were analyzed after 60 hours in culture. In E8.0+60 hours controls, the 4th PA was externally visible (data not shown), the 4th arch artery (A4, Fig. 1g) was now connected to the dorsal aorta and aortic sac, and the 4th pouch (P4) had enlarged. In contrast, the pharyngeal region of E8.0+60 hours BMS493-treated embryos did not show any morphological changes (compare Fig. 1e and h). Ink injections allowed further investigation of the pattern of arch arteries (Fig. 2c-f). The first three (A1-A3) and the first four (A1-A4) arch arteries were readily visualized in controls cultured for 48 and 60 hours, respectively. Injections of BMS493-treated embryos confirmed the absence of the 3rd and 4th arch arteries and also showed an enlargement of the 2nd arch artery (A2, Fig. 2d,f), probably reflecting an attempt to functionally compensate the absence of the 3rd and 4th arch arteries.

BMS493-induced defects of the caudal pharynx can be prevented by all-trans retinoic acid and are determined during a narrow period of time

70% of the E8.0 embryos simultaneously exposed to 10^{-6} M of BMS493 and 10^{-7} M RA for 48 hours (n=21) developed 3rd and 4th PA and pouches (Figs 1c,f, 2i). That an RAR physiological ligand could prevent defects caused by the synthetic antagonist demonstrates that they arose as a consequence of impaired retinoid signaling. Moreover, the intensity of β-galactosidase staining was increased in the 'rescued embryos' (Fig. 1b,c), indicating that the competition between RA and its antagonist most probably took place at the transcriptional level, as expected.

Embryos were treated with BMS493 for 6 hour periods (during which 2-3 additional pairs of somites were formed), then washed and cultured for additional 36 or 42 hour periods in medium without added retinoids in order to reach approximately 30 ss. At least three groups of 4-5 embryos were analyzed in each culture experiment. Embryos treated for 6 hours always developed normal 3rd and 4th PA and pouches when collected at 3-4 ss (Fig. 3b), in contrast to embryos collected at 7-8 ss, in which these PA always failed to form (Fig. 3c). In embryos explanted at 10-11 ss and exposed to BMS493 throughout the 36 hour culture period, normal 3rd and 4th PA structures were formed (Fig. 3d and data not shown). Thus the RA antagonist can disturb the ontogeny of the post-otic pharynx only during a narrow window of time, between 7-10 ss.

Pan-RAR antagonist treatments at E8.0 selectively impair the formation of the post-otic branchial nerves and of their distal ganglia

The patterning of the nerves and ganglia related to the 1st and 2nd PA (e.g. G5, G7/8 and N7; Fig. 4a,b), were indistinguishable in control (n=3) and BMS493-treated (n=3) embryos. The IXth and the Xth nerves, which develop within the 3rd and 4th PA, respectively, were fused in controls (N9 and N10; Fig. 4a), mimicking a condition rarely observed in normal embryos in vivo (Ghyselinck et al., 1997). In BMS493-treated embryos, the bundle of axons corresponding to IXth and Xth nerves, was absent or appeared markedly thinner than in controls (Fig. 4b, and data not shown); the common proximal ganglion of the IXth and Xth nerves, which is exclusively derived from NCC, was normally developed (PG9/10, Fig. 4a,b), in contrast to the distal ganglia of these nerves, which were small, fused and never connected to the rhombencephalon (DG9/10, compare Fig. 4a and b). Neurons of the distal ganglia of the IXth and Xth nerves arise from ectodermal thickenings, the 2nd and 3rd epibranchial placodes (E2 and E3; Fig. 4c) located at the anterior margin of the 3rd and 4th PA, respectively (Begbie et al., 1999 and references therein). Immunostaining of E8.0+48 hours BMS493-treated embryos with antibodies against Phox2a showed a fusion of the 2nd and 3rd placodes and a decrease in the number of their neuronal cells (E2/3; Fig. 4d). The 1st epibranchial placode was unaffected by the treatment (E1; Fig. 4c,d).

Neural crest cells and angioblasts are not quantitatively deficient, but fail to colonize the lateral portion of the caudal pharynx

The absence of caudal PA in BMS493-treated embryos might reflect a deficiency in post-otic NCC since these cells normally provide mesenchyme to the 3rd and 4th PA (Lumsden et al., 1991). Therefore, the status of the rhombencephalic NCC was analysed by ISH with probes to cellular retinoic acid binding protein one (*CRABPI*), *kreisler* and *EphA4* and by studying apoptosis in the PA using the TUNEL technique. These experiments were carried out on embryos cultured for 24 or 30 hours, i.e., prior to and at the onset of formation of the 3rd PA, respectively and, in any event, before the earliest appearance of the 4th PA.

CRABPI is a general marker of migrating and early post-migratory NCC (Maden et al., 1992) which, in E8.0+24 hours controls, is detected in three streams populating the 1st PA (B1, Fig. 5a), the 2nd PA (B2) and the prospective 3rd and 4th PA (B3/4). In E8.0+24 hours BMS493-treated embryos, NCC were as numerous as in controls (Fig. 5a,b), but the posterior stream migrated more caudally (Fig. 5b). *EphA4* and *kreisler* are expressed in NCC destined for the 3rd PA and originating from rhombomeres 5 and 6 (R5 and R6), respectively (Nieto et al., 1992; Cordes and Barsh, 1994). ISH for these two markers on E8.0+30 hours BMS493-treated embryos showed similar caudal deviations in the migration paths of NCC (Fig. 5c,d,g,h).

In situ detection of DNA nicks was performed at E8.0+24 hours and E8.0+48 hours, i.e. at developmental stages when NCC have already left the hindbrain neurectoderm, prior and after the appearance of the 3rd PA, respectively. The number of apoptotic cells was increased in the ectoderm overlying the dorsal portion of the 2nd branchial pouch of E8.0+24 hours BMS493-treated embryos (arrowheads in Fig. 5i,j). In contrast, BMS493-induced changes in the amount of apoptotic cells were not observed in the mesenchyme after

24 hours and 48 hours in culture (Fig. 5k,l and data not shown).

The mesodermally derived angioblasts which, like NCC, are endowed with the capacity to extensively invade embryonic tissues, provide the primitive scaffolding of the arch arteries (Noden, 1991). Immunostaining for detection of PECAM-1 demonstrated a normal arrangement of endothelial cells in the portions of the aorta and anterior cardinal vein adjacent to the caudal pharynx in both E8.0 controls and BMS493-treated embryos (AO and V; Fig. 4e,f), thereby ruling out the possibility that the lack of the 3rd and 4th arch arteries could reflect a generalized failure of angiogenesis in the head region. The expression domain of *EphA2* (Ruiz and Robertson, 1994) at E8.0+30 hours includes migrating post-otic NCC and mesodermal cells. Interestingly, this domain was shifted caudally in BMS493-treated embryos, supporting the view that both NCC and mesodermal cells are unable to colonize the caudal-lateral portion of the pharynx (Fig. 5e,f). Interactions between the *Eph* receptor tyrosine kinases and their membrane-bound ligands, ephrins, have been proposed to regulate the targeted migration of branchial NCC (Smith et al., 1997; Helbling et al., 1998). In this context it is noteworthy that in BMS493-treated embryos, which show aberrant migration of *EphA4*- and *EphA2*-expressing NCC and/or paraxial mesodermal cells, the expression domain of ephrin-A5, an EphA ligand (reviewed in O'Leary and Wilkinson, 1999), was not altered (data not shown).

The endoderm of the caudal pharynx responds to physiological concentrations of RA

The *mRARβ2-lacZ* transgene used to monitor the general level of retinoid responsiveness in cultured embryos is not expressed in the pharynx. In order to provide direct evidence that pharyngeal tissues respond to RA, we used transgenic embryos harbouring three copies of a retinoic acid response element (RARE) inserted upstream of a *hsplacZ* construct (Rossant et al., 1991). In these embryos, the pattern of β-galactosidase activity matches closely the distribution of endogenous RA (Ang et al., 1996; Wagner et al., 1992; Maden et al., 1998). *RAREhsplacZ* embryos collected at E7.5 were cultured for 24 hours to reach the 8-10 ss. In control embryos, strong β-galactosidase activity was detected in the endoderm (E; Fig. 6a) of the caudal pharynx, but not in its anterior counterpart. The staining of the endoderm was abolished in BMS493-treated embryos, demonstrating that the hsp promoter was not constitutively active in this tissue (Fig. 6b). Conversely, simultaneous addition of 10^{-7}M RA and 10^{-6}M BMS493 to the culture medium prevented the inhibition of β-galactosidase activity in the pharyngeal endoderm (Fig. 6c). These results indicate that the cells in the prospective 3rd and 4th PA region respond to physiological concentrations of RA, and that the absence of these PA in BMS493-treated embryos results from a local inhibition of RA signaling.

The endoderm of the caudal pharyngeal arches is not correctly specified in BMS493-treated embryos

The products of the 3′ *Hox* genes are essential for the morphogenesis of the pharynx (reviewed in Rijli et al., 1998). Expression of *Hoxa1* and *Hoxb1* in the foregut endoderm and its associated mesoderm and ectoderm is regulated through RAREs (Frasch et al., 1995; Huang et al., 1998). In E8.0+24 hours and E8.0+30 hours controls, *Hoxa1* and *Hoxb1* transcripts were detected only in the caudal pharynx (Fig. 6d,f), in accordance with in vivo data (Murphy and Hill, 1991). In BMS493-treated embryos, the pharyngeal expression of *Hoxb1* was undetectable and that of *Hoxa1* was markedly decreased (Fig. 6e,g).

Pax1 and *Pax9* are paired-box-containing genes whose expressions in the endoderm correlate with focal increases in cell proliferation that are responsible for the growth of the branchial pouches (Müller et al., 1996). The expression patterns of *Pax1* and *Pax9* in E8.0+30 hours and E8.0+48 hours controls were comparable to those reported in vivo (Figs 2g, 7c and data not shown) (Wallin et al., 1996; Peters et al., 1998). In E8.0+30 hours BMS493-treated embryos, the domain of strong *Pax9* expression in the 2nd pouch was broader than in controls (data not shown). These data and the patterns of *Fgf8* expression at E8.0+24 hours (see the text below and Fig. 7a) indicate that the enlargement of the 2nd pouch represents the first detectable morphological defect caused by inhibition of RA signaling, the absence of the 3rd PA being consistently observed only after 48 hours in culture. In E8.0+48 hours BMS493-treated embryos, *Pax9* was only weakly expressed at the axial level corresponding to the 3rd pouch (large arrows in Fig. 7c). Additionally, the caudal extremity of the lateral pharyngeal endoderm displayed a persistant expression of *Pax1* whereas the 4th pouch, its counterpart in the control embryos, does not express *Pax1* (Fig. 2h and data not shown).

Thus, BMS493-induced alterations in expression patterns of *Hoxa1*, *Hoxb1*, *Pax1* and *Pax9* are consistent with the histological observation that the pharyngeal endoderm caudal to the 2nd PA has adopted a fate resembling that of a normal 2nd pouch.

Fgf8 and *Fgf3* signalings are locally altered in the pharyngeal endoderm following treatment with BMS493

The expression pattern of *Fgf8* in the developing PA has suggested that this signaling molecule could trigger the formation of the pharyngeal pouches, as well as interactions between endoderm or ectoderm and migrating NCC (Crossley and Martin, 1995; Wall and Hogan, 1995). When compared to controls, E8.0+24 hours BMS493-treated embryos showed an enlarged domain of *Fgf8* expression in the endoderm caudal to the 2nd PA (P2; Fig. 7a). In contrast, the strong *Fgf8* expression in the prospective 3rd pouch of E8.0+30 hours controls was undetectable following treatment with BMS493 (brackets in Fig. 7b). Interestingly, this treatment did not affect the intensity nor the pattern of *Fgf8* expression in the PA ectoderm. The transient *Fgf3* expression in the endoderm of the caudal-lateral pharynx (Wilkinson et al., 1989) was also strongly decreased in E8.0+30 hours BMS493-treated embryos (data not shown). These data indicate that treatment with BMS493 inhibits the synthesis by endodermal cells of secreted factors thought to play important roles in their specification and/or in signaling pathways controlling cell migration within the prospective PA.

BMS493 treatment at the presomitic stage in utero does not affect the formation of the first two pharyngeal arches, but the 3rd and 4th arches are lacking

When compared to E9.0 embryos in vivo, E7.5+48 hours BMS493-treated embryos (n=70) displayed multiple external

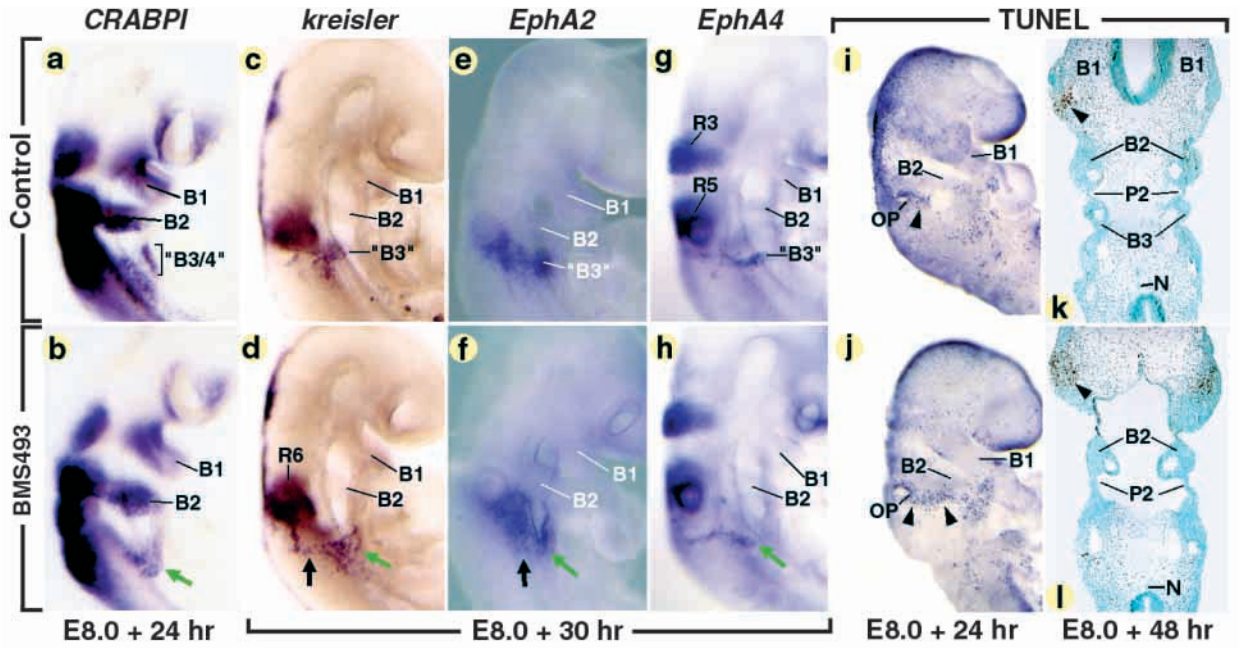

Fig. 5. Abnormal cell migrations and apoptosis in BMS493-treated embryos. Whole-mount ISH (a-h) for detection of migrating neural crest cells, (a-d, g, h) and/or mesodermal cells (e,f), and Tunel staining of whole embryos (i,j) and of histological sections (k,l). B1-B3 branchial arches 1-3; 'B3', prospective branchial arch 3; 'B3/4', prospective branchial arches 3 and 4; N, notochord; OP, otic placode; P2, 2nd pharyngeal pouch; R3, R5 and R6, rhombomeres 3, 5 and 6. The large black and green arrows point to NCC (or mesodermal cells) migrating caudally or accumulating in the ventral region of the pharynx, respectively. The arrowheads indicate apoptotic cells in the ectoderm (i, j) or in the mesenchyme (k,l).

malformations (data not shown). The majority of these abnormalities were also observed in E7.5+48 hours controls (n=70), albeit at lower frequencies. The 3rd PA was consistently absent in all treated embryos, but only in two control embryos. In contrast, the first two PA were not conspicuously altered following BMS493 treatment.

As cultures of presomitic stage embryos resulted in relatively high rates of spontaneous malformations, we complemented our observations by in vivo experiments performed with mice harbouring a *Wnt1-lacZ* transgene that is expressed in migrating and post-migratory NCC (Echelard et al., 1994). BMS493 was administrated twice to two pregnant mice at both E7.0 and E7.5, i.e. prior to the appearence of the 1st PA and to the onset of NCC migration (Serbedzija et al., 1992 and references therein), whereas two control mice were given the vehicle (i.e. ethanol and oil) only. At E10.5, ten living embryos, and six living embryos and four resorptions, were recovered from the two BMS493-treated dams. Control embryos were morphologically normal, whereas all embryos exposed to BMS493 in utero showed identical phenotypes: the first two PA were normal (B1 and B2; Fig. 8a,b), the 2nd branchial pouch (P2) extended caudally and NCC accumulated at its ventral side (large arrow in Fig. 8b); the 3rd and 4th PA were absent (compare Fig. 8a and b, and data not shown). Quite strikingly, the 3rd, 4th and 6th arch arteries (A3, A4 and A6; Fig. 8c) were bilaterally absent, so that the only connection between the aortic sac (AS; Fig. 8c,d) and aortas was represented by an enlarged 2nd arch artery (A2; Fig. 8d). These findings strongly suggest that the in vivo formation of the 1st and 2nd PA and

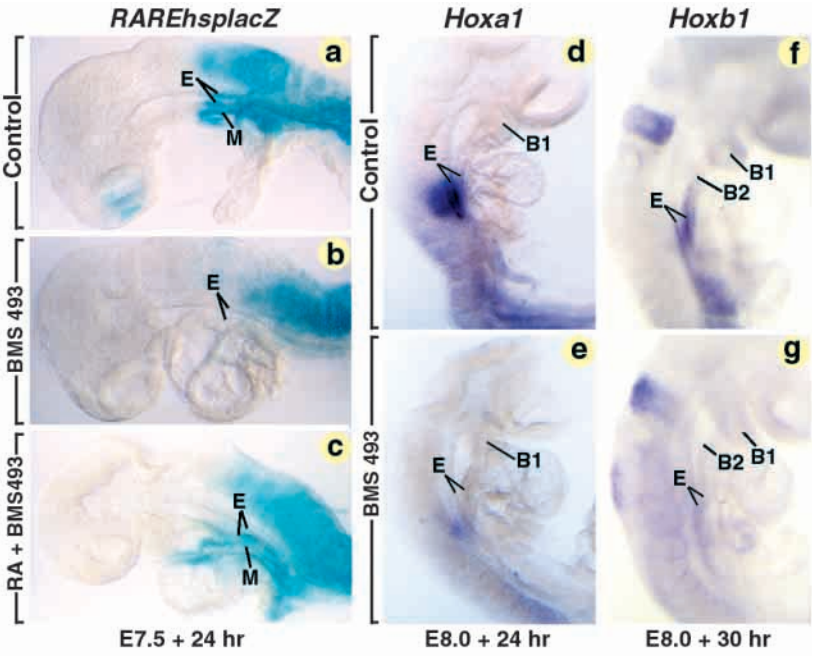

Fig. 6. Distribution of the *RAREhsplacZ* transgene and of *Hoxa1* and *Hoxb1* transcripts in control and retinoid-treated embryos. The embryos were cut into halves and the medial side is displayed. B1 and B2, branchial arches 1 and 2; E, endoderm; M, dorsal mesocardium.

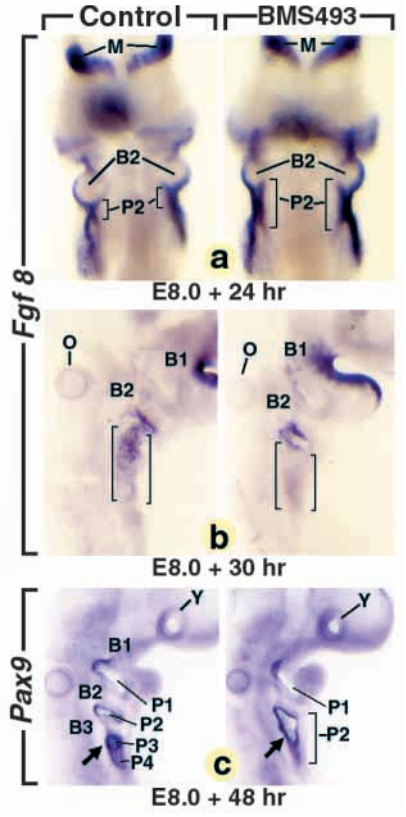

Fig. 7. Distribution of *Fgf8* and *Pax9* transcripts in the endoderm of the pharyngeal arches. In (b, c) the embryos were cut into halves and the medial side is displayed; note that the ectoderm was completely removed in the region of the 2nd and 3rd pharyngeal arches after staining in b. (a) Dorsal view, the others are lateral. B1-B3, branchial arches 1-3; M, midbrain; O, otocyst; P1-P4, pharyngeal pouches 1-4; Y, eye. The brackets in b encompass the lateral pharyngeal endoderm. The large arrow in c points to the level of the 3rd pharyngeal pouch.

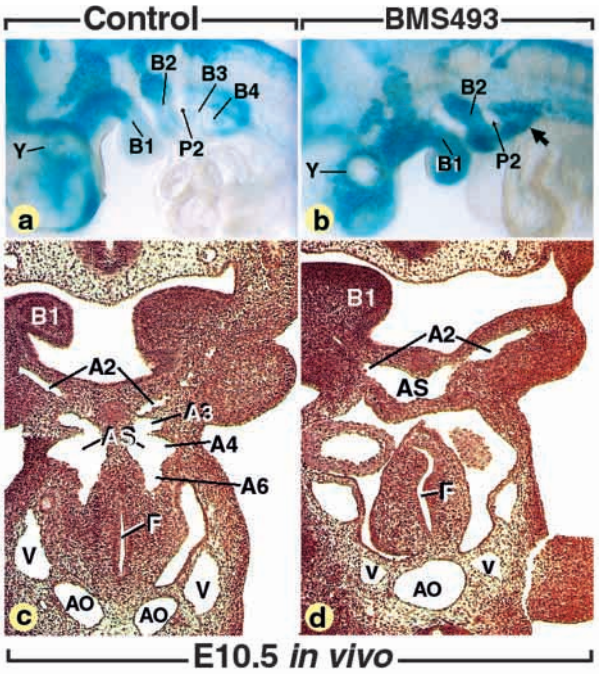

Fig. 8. Malformations induced by in utero treatments with BMS493. (a,b) External views of *Wnt1-lacZ* transgenic embryos stained for detection of β-galactosidase activity. (c,d) Frontal histological sections at comparable levels of the pharynx of control and BMS493-treated embryos. A2-A4, A6, pharyngeal arch arteries 2-4, 6; AO, aorta; AS, aortic sac; B1-B3, branchial arches 1-3; F, foregut; P2, pharyngeal pouch 2; V, cardinal vein. The large arrow in b indicates an accumulation of NCC in the ventral pharynx. Magnification: ×60 (c,d).

of the 1st branchial pouch require less RA than that of the 3rd and 4th PA and branchial pouches.

DISCUSSION

Unliganded RARs can transrepress the basal promoter activity of target genes in transfected cells through binding to corepressors (reviewed in Chambon, 1996). The fact that the pan-RAR antagonist BMS493 stabilizes the association of RAR/RXR heterodimers with such corepressors in vitro (our unpublished results) raises the possibility that it could not only block the effect of agonistic retinoid signals, but also act as an artificial silencing signal in our cultured embryos. However, this is most probably not the case, as defects in the pharyngeal arches (PA) and pouches similar to those observed here upon BMS493-treatment of wild-type embryos are exhibited by embryos lacking RARs (Dupé et al., 1999), RXRs (Wendling et al., 1999) or their physiological agonistic ligands (White et al., 1998; Niederreither et al., 1999). Therefore, our present study of embryos with a block in retinoid signal transduction (BRST) definitively demonstrates that retinoid signaling is essential for the formation of 3rd and 4th PA structures during a narrow developmental window corresponding to the 7-10 somite stages.

Migrating post-otic neural crest cells and angioblasts are not primary targets of the RA signal

In normal mouse embryos, neural crest cells (NCC) arising from the post-otic hindbrain yield part of the mesenchyme of the 3rd and 4th PA and all the cells of the common proximal ganglion of the IXth and Xth nerves. Later in ontogeny, NCC of these two PA differentiate into connective tissues of the neck glands (e.g. parathyroid and thymus) and smooth muscle cells of the great cephalic arteries and aortico-pulmonary septum (Le Douarin, 1982). The present data indicate that a block in retinoid signaling (BRST) specifically alters the migratory behaviour of post-otic mesenchymal NCC, which are derouted towards the most caudal portion of the pharynx, instead of invading the 3rd and 4th PA. In the post-otic region of the embryonic head, the choice of the migration path is not an NCC-autonomous property (Couly et al., 1998). Therefore, the ectopic, caudal, migration of mesenchymal NCC observed in embryos with a BRST must reflect the existence of RA-dependent guidance cues emanating from the other tissues of the PA. Along the same lines, we note that neurogenic post-otic NCC, which do not interact with PA, migrate normally in embryos with BRST, as assessed from the normal position and size of the common proximal ganglion of the IXth and Xth nerves.

We have previously concluded that the defects in the neck

glands and cephalic arteries observed in all RARα$^{-/-}$/RARβ$^{-/-}$ mutant fetuses cannot be accounted for by a decrease in the number of NCC destined to invade the 3rd and 4th PA (Ghyselinck et al., 1997; Dupé et al., 1999). The present analysis of the *CRABPI*, *kreisler* and *EphA4* transcript distribution, of the expression of the *Wnt1-lacZ* NCC-specific transgene and of apoptosis in NCC, reveals that a severe BRST that leads to a complete agenesis of these two PA (1) has no obvious effect on the production of NCC by the post-otic hindbrain neurectoderm, (2) does not affect the survival of migratory and early post-migratory NCC and, most importantly, (3) acts during a very narrow critical developmental period (7-10 ss), which does not coincide with that of post-otic NCC migration as the last NCC are leaving this region of the neural tube only at the 14-somite stage (Serbedzija et al., 1992). Moreover, a severe BRST completely inhibits the formation of all 3rd, 4th and 6th arch arteries. This inhibition cannot be accounted by a generalized failure of angiogenesis, but most probably reflects the inability of the mesodermally derived angioblasts to populate the region of the pharynx caudal to the 2nd PA. Altogether these data indicate that the migrating post-otic NCC and angioblasts do not represent primary targets of RA action.

RA signaling plays an essential role in the specification of the pharyngeal endoderm by regulating the expression of *Hoxa1* and *Hoxb1*

The 3rd and 4th pharyngeal pouches are hypoplastic in RARα$^{-/-}$/RARβ$^{-/-}$ mutant embryos (Dupé et al., 1999), but absent here in embryos treated with the pan-RAR antagonist, demonstrating that RA signaling is more severely impaired in this latter condition. The molecular marker analyses of these treated embryos with *Hoxa1*, *Hoxb1*, *Pax1*, *Pax9*, *Fgf3* and *Fgf8* probes support the conclusion that the single lateral evagination of the endoderm caudal to the 2nd PA is analogous to an enlarged 2nd pouch.

It is well established that *Hox* gene products impart distinct morphological identities to neurectodermal and mesectodermal derivatives (for reviews, see Krumlauf, 1993; Mark et al., 1997; Favier and Dollé, 1997; Rijli et al., 1998), but their role in patterning endodermal structures has received little attention. In embryos with BRST, *Hoxa1* expression is downregulated and *Hoxb1* expression is abolished in the endoderm of the caudal pharynx at developmental stages preceding the appearance of the 3rd and 4th pharyngeal pouches. Both *Hoxa1* and *Hoxb1* contain retinoic acid response elements (RAREs) that are functional in vivo (Marshall et al., 1994; Studer et al., 1994; Dupé et al., 1997; Huang et al., 1998). In addition one of the *Hoxb1* RARE is indispensable for initiating expression of this gene in the pharyngeal endoderm at E8.0 (Huang et al., 1998). Altogether these data strongly suggest that the RA-dependent expression of *Hoxa1* and *Hoxb1* in the endoderm is crucial for the specification of the 3rd and 4th pharyngeal pouches and that the endoderm is a primary target for endogenous RA. Along these lines it is noteworthy that *Hoxa1*$^{-/-}$/*Hoxb1*$^{-/-}$ fetuses consistently exhibit an agenesis of the thymus and parathyroid glands as a result of abnormal patterning of their pharyngeal pouches (Rossel and Capecchi, 1999). The decrease in *Pax1* and *Pax9* expression in the caudal portion of the pharyngeal endoderm of embryos treated with the pan-RAR antagonist is in keeping with our proposal that this tissue is a primary target of RA action, as the expressions of both of these genes in the endoderm are regulated cell-autonomously, as demonstrated by heterotopic grafting of prospective foregut endoderm (Müller et al., 1996). Interestingly, *Pax9* is indispensable for the formation of the thymus and parathyroid glands (Peters et al., 1998) and agenesis of these glands is observed in RARα$^{-/-}$ / RARβ$^{-/-}$ fetuses (Ghyselinck et al., 1997). Thus, it is most likely that the lack of the *Pax9* expression at the level of the presumptive 3rd pouch under conditions of impaired RA signaling accounts for the cases of thymus and parathyroid gland agenesis observed in RARα$^{-/-}$ / RARβ$^{-/-}$ fetuses.

The ectopic migration of post-otic NCC in embryos with BRST could be caused by a lack of RA-dependent guidance cues originating from the endoderm, e.g. *Fgf3* and *Fgf8* are specifically and strongly downregulated in the endoderm of the caudal pharynx prior to the formation of the 3rd and 4th PA. Alternatively, the misshapen pharyngeal endoderm might prevent the normal ventral movement of the neural crest.

Epibranchial placodes are induced by the endoderm (Begbie et al., 1999). Therefore, the extensive apoptosis in the ectoderm corresponding to the prospective 2nd epibranchial placode, the subsequent fusion of the 2nd and 3rd placodes, the decrease in the number of their neurons, and the hypoplasia of the distal ganglia of the IXth and Xth nerves, are probably all related to the improper specification of the pharyngeal endoderm in embryos with BRST.

The formation of the 2nd pharyngeal arch and that of the 3rd and 4th pharyngeal arches are likely to involve distinct RA-dependent developmental processes

Previous studies have shown that the formation of the first PA and of its derivatives appear essentially independent of RA. In contrast, RA is clearly required for the formation of the 2nd and more caudal PA (Lohnes et al., 1994; Mark et al., 1995; Maden et al., 1996; Niederreither et al., 1999).

Under our culture conditions, BRST induces rapid changes in the expression patterns of the *mRARβ2-lacZ* transgene and of several endogenous genes (Chazaud et al., 1999; O. W., P. C. and M. M., unpublished data). Thus, the finding that blocking the RA signal transduction prior to the earliest appearance of the foregut (i.e. at E7.5) has no effect on the subsequent formation of the 2nd PA suggests that this formation is less dependent on RA than that of more caudal PA. This view is supported by several other lines of evidence: (1) RALDH2, a major RA-synthesizing enzyme, has an anterior expression boundary that is immediately adjacent to the prospective 4th PA, but distant from the prospective 2nd PA (Niederreither et al., 1997); (2) endogenous RA induces the expression of two distinct RA-responsive transgenes in the caudal, but not in the rostral pharyngeal region (Rossant et al., 1991; Balkan et al., 1992; the present report); in both of these transgenic mouse lines, the availability of RA is a limiting factor, as cells in the 2nd PA express both *lacZ* reporters in response to teratogenic concentrations of RA; (3) compound knockouts of either RARα and RARβ or RARα and RARγ cause drastic alterations of the 3rd and 4th PA, but have no apparent effect on the 2nd PA; and (4) RA deficiency begun at the time of conception in rat embryos leads to agenesis of the 3rd and 4th PA, without altering the formation of the 2nd PA (White et al., 1998). It is clear, however, that the formation of the 2nd PA

requires RA, as it is totally inhibited in RALDH2-null mutant mice (Niederreither et al., 1999) as well as in vitamin A-deficient quails (Maden et al., 1996). In our culture system, the agenesis of the 2nd PA induced by high BMS493 concentrations (i.e. 10^{-5}M; data not shown) is difficult to interpret because of possible non-specific embryotoxic effects.

In any event, the above observations strongly suggest that the formation of the 2nd PA and that of the 3rd and 4th PA rely on distinct subsets of RA receptors, RA-responsive elements and developmental mechanisms. Along these lines, it is noteworthy that: (1) RARβ is apparently expressed at higher levels in the endoderm of the caudal pharynx than in its rostral counterpart (Ruberte et al., 1991; Smith, 1994); (2) distinct RA-responsive elements control the expression of *Hoxb1* in the endoderm and in NCC migrating from rhombomere 4 (Huang et al., 1998 and references therein) and (3) the extensive apoptosis reported in the NCC destined to the 2nd and more posterior PA in RALDH2-null mutant mice and vitamin A-deficient quail embryos (Niederreither et al., 2000; Maden et al., 1996) is not observed in our embryos with BRST.

Implication for CATCH22 syndromes

Absent or hypoplastic thymus, absent or hypoplastic parathyroid glands, atresia of the aortic arch and absent or abnormal aorticopulmonary septum are observed in human CATCH22 syndromes characterized by monoallelic microdeletion of chromosome 22q11.2. That this spectrum of defects can be recapitulated in the chick by NCC ablations has popularized the belief that genes regulating cell-autonomous functions in NCC are deleted in the CATCH22 syndromes (Kirby and Waldo, 1990; Yamagishi et al., 1999 and references therein). We have shown here that, even though RARα$^{-/-}$ / RARβ$^{-/-}$ mouse fetuses also display a typical 'NCC ablation phenotype' (Mendelsohn et al., 1994; Ghyselinck et al., 1997; Mark et al., 1998) closely matching that of the CATCH22 syndrome, severe impairement of RA-signaling affects NCC only secondarily, whereas it has direct effects on the endoderm contributing to the thymus and parathyroid gland. Thus our present study raises the possibilities that (1) in CATCH22 syndromes, the causal lesion might affect the endoderm and (2) RA target genes expressed in the endoderm may be deleted in these syndromes.

We thank Drs P. Reczek and C. Zusi for the gift of BMS493; Drs D. Wilkinson, G. Martin, A. P. McMahon, R. Balling, G. Barsh, P. Gruss and J.-F. Brunet for providing us with the cDNAs probes or antibodies; J. Rossant for the *RAREhsplacZ* mice; Drs S. Ward, S. Viville, M. Kirby, N. B. Ghyselinck and F. Rijli for technical advice and helpful discussions; B. Weber, C. Hummel, I. Tilly and G. Duval for technical assistance. This work was supported by funds from the Centre National de la Recherche Scientifique (CNRS), the Institut National de la Santé et de la Recherche Médicale (INSERM), the Hôpital Universitaire de Strasbourg, the Collège de France, the Institut Universitaire de France, the Association pour la Recherche sur le Cancer (ARC), the Fondation pour la Recherche Médicale (FRM), the Ligue Nationale contre le Cancer, the Human Frontier Science Program, Bristol-Myers Squibb and EU Contract FAIR-CT97-3320. O.W. was supported by a Ligue contre le cancer fellowship.

REFERENCES

Ang, H. L., Deltour, L., Hayamizu, T. F., Zgombic-Knight, M. and Duester, G. (1996). Retinoic acid synthesis in mouse embryos during gastrulation and craniofacial development linked to class IV alcohol dehydrogenase gene expression. *J. Biol. Chem.* **271**, 9526-9534.

Ang, H. L. and Duester, G. (1997). Initiation of retinoid signaling in primitive streak mouse embryos: Spatiotemporal expression patterns of receptors and metabolic enzymes for ligand synthesis. *Dev. Dyn.* **208**, 536-543.

Balkan, W., Colbert, M., Bock, C. and Linney, E. (1992). Transgenic indicator mice for studying activated retinoic acid receptors during development. *Proc. Natl. Acad. Sci. USA* **89**, 3347-3351.

Begbie, J., Brunet, J.-F., Rubenstein, J. L. R. and Graham, A. (1999). Induction of the epibranchial placodes. *Development* **126**, 895-902.

Bockman, D. E., Redmond, M. E. and Kirby, M. L. (1989). Alteration of early vascular development after ablation of cranial neural crest. *Anat. Rec.* **225**, 209-217.

Chambon, P. (1996). A decade of molecular biology of retinoic acid receptors. *FASEB J.* **10**, 940-954.

Chazaud, C., Chambon, P. and Dollé, P. (1999). Retinoic acid is required in the mouse embryo for left-right asymmetry determination and heart morphogenesis. *Development* **126**, 2589-2596.

Conlon, R. A., Reaume, A. G. and Rossant, J. (1995). *Notch1* is required for the coordinate segmentation of somites. *Development* **121**, 1533-1545.

Copp, A. J. and Cockroft, D. L. (1990). *Postimplantation Mammalian Embryos. A Practical Approach.* Oxford University Press.

Cordes, S. P. and Barsh, G. S. (1994). The mouse segmentation gene *kr* encodes a novel basic domain-leucine zipper transcription factor. *Cell* **79**, 1025-1034.

Couly, G., Grapin-Botton, A., Coltey, P., Ruhin, B. and Le Douarin, N. M. (1998). Determination of the identity of the derivatives of the cephalic neural crest: incompatibility between *Hox* gene expression and lower jaw development. *Development* **125**, 3445-3459.

Crossley, P. H. and Martin, G. R. (1995). The mouse *Fgf8* gene encodes a family of polypeptides and is expressed in regions that direct outgrowth and patterning in the developing embryo. *Development* **121**, 439-451.

Davis, C. A., Holmyard, D. P., Millen, K. J. and Joyner, A. L. (1991). Examining pattern formation in mouse, chicken and frog embryos with an *En*-specific antiserum. *Development* **111**, 287-298.

Décimo, D., Georges-Labouesse, E. and Dollé, P. (1995). In situ hybridization to cellular RNA. In *Gene Probes, A Practical Approach*, vol. II (ed. B. D. Hames and S. J. Higgins), pp. 183-210. New York: Oxford University Press.

Dupé, V., Davenne, M., Brocard, J., Dollé, P., Mark, M., Dierich, A., Chambon, P. and Rijli, F. M. (1997). In vivo functional analysis of the *Hoxa1* 3′ retinoic acid response element (3′RARE). *Development* **124**, 399-410.

Dupé, V., Ghyselinck, N. B., Wendling, O., Chambon, P. and Mark M. (1999). Key roles of retinoic acid receptors alpha and beta in the patterning of the caudal hindbrain, pharyngeal arches and otocyst in the mouse. *Development* **126**, 5051-5059.

Echelard, Y., Vassileva, G. and McMahon, A. P. (1994). Cis-acting regulatory sequences governing Wnt-1 expression in the developing mouse CNS. *Development* **120**, 2213-2224.

Favier, B. and Dollé, P. (1997). Developmental functions of mammalian Hox genes. *Mol. Hum. Reprod.* **3**, 115-131.

Frasch, M., Chen, X. and Lufkin, T. (1995). Evolutionary-conserved enhancers direct region-specific expression of the murine *Hoxa1* and *Hoxa-2* loci in both mice and *Drosophila. Development* **121**, 957-974.

Ghyselinck, N. B., Dupé, V., Dierich, A., Messaddeq, N., Garnier, J. M., Rochette-Egly, C., Chambon, P. and Mark M. (1997). Role of the retinoic acid receptor beta (RARβ) during mouse development. *Int. J. Dev. Biol.* **41**, 425-447.

Ghyselinck, N. B., Wendling, O., Messaddeq, N., Dierich, A., Lampron, C., Décimo, D., Viville, S., Chambon, P. and Mark, M. (1998). Contribution of retinoic acid receptor β isoforms to the formation of the conotruncal septum of the embryonic heart. *Dev. Biol.* **198**, 303-318.

Helbling, P. M., Tran, C. T. and Brändli, A. W. (1998). Requirement for EphA receptor signaling in the segregation of *Xenopus* third and fourth arch neural crest cells. *Mech. Dev.* **78**, 63-79.

Huang, D., Chen, S. W., Langston, A. W. and Gudas, L. J. (1998). A conserved retinoic acid responsive element in the murine *Hoxb1* gene is required for expression in the developing gut. *Development* **125**, 3235-3246.

Kastner, P., Mark, M. and Chambon, P. (1995). Nonsteroid nuclear receptors: what are genetic studies telling us about their role in real life? *Cell* **83**, 859-869.

Kastner, P., Mark, M., Ghyselinck, N. B., Krezel, W., Dupé, V., Grondona, J. M. and Chambon, P. (1997). Genetic evidence that the retinoid signal is

transduced by heterodimeric RXR/RAR functional units during mouse development. *Development* **124**, 313-326.
Kaufman, M. H. (1992). *The Atlas of Mouse Development*. Academic Press.
Kirby, M. L. and Waldo, K. L. (1990). Role of neural crest in congenital heart disease. *Circulation* **82**, 332-340.
Krumlauf, R. (1993). Hox genes and pattern formation in the branchial region of the vertebrate head. *Trends Genet.* **9**, 106-112.
Larsen, W. (1993). *Human Embryology*. New York: Churchill Livingstone Inc.
Le Douarin, N. (1982). *The Neural Crest*. Cambridge: Cambridge University Press.
Lohnes, D., Mark, M., Mendelsohn, C., Dollé, P., Dierich, A., Gorry, P., Gansmuller, A. and Chambon, P. (1994). Function of the retinoic acid receptors (RARs) during development. (I) Craniofacial and skeletal abnormalities in RAR double mutants. *Development* **120**, 2723-2748.
Lumsden, A., Sprawson, N. and Graham, A. (1991). Segmental origin and migration of neural crest cells in the hindbrain region of the chick embryo. *Development* **113**, 1281-1291.
Maden, M., Horton, C., Graham, A., Leonard, L., Pizzey, J., Siegenthaler, G., Lumsden, A. and Eriksson, U. (1992). Domains of cellular retinoic acid-binding protein I (CRABP I) expression in the hindbrain and neural crest of the mouse embryo. *Mech. Dev.* **37**, 13-23.
Maden, M., Gale, E., Kostetskii, I. and Zile, M. (1996). Vitamin A-deficient quail embryos have half a hindbrain and other neural defects. *Curr. Biol.* **6**, 417-426.
Maden, M., Sonneveld, E., van der Saag, P. T. and Gale, E. (1998). The distribution of endogenous retinoic acid in the chick embryo: implications for developmental mechanisms. *Development* **125**, 4133-4144.
Mark, M., Lufkin, T., Vonesch, J-L., Ruberte, E., Olivo, J-C., Dollé, P., Gorry, P., Lumsden, A. and Chambon, P. (1993). Two rhombomeres are altered in *Hoxa1* mutant mice. *Development* **119**, 319-338.
Mark, M., Lohnes, D., Mendelsohn, C., Dupé, V., Vonesch, J-L., Kastner, P., Rijli, F. M., Bloch-Zupan, A. and Chambon, P. (1995). Roles of retinoic acid receptors and of *Hox* genes in the patterning of the teeth and of the jaw skeleton. *Int. J. Dev. Biol.* **39**, 111-121.
Mark, M., Rijli, F. M. and Chambon, P. (1997). Homeobox genes in embryogenesis and pathogenesis. *Ped. Res.* **42**, 421-429.
Mark, M., Ghyselinck, N. B., Kastner, P., Dupé, V., Wendling, O., Krezel, W., Mascrez, B. and Chambon, P. (1998). Mesectoderm is a major target of retinoic acid action. *Eur. J. Oral Sci.* **106**, 24-31.
Marshall, H., Studer, M., Popperl, H., Aparicio, S., Kuroiwa, A., Brenner, S. and Krumlauf, R. (1994). A conserved retinoic acid response element required for early expression of the homeobox gene *Hoxb1*. *Nature* **370**, 567-571.
Mascrez, B., Mark, M., Dierich, A., Ghyselinck, N. B., Kastner, P. and Chambon, P. (1998). The RXRα ligand-dependent activation function 2 (AF-2) is important for mouse development. *Development* **125**, 4691-4707.
Mendelsohn, C., Ruberte, E., LeMeur, M., Morriss-Kay, G. and Chambon, P. (1991). Developmental analysis of the retinoic acid-inducible RARβ2 promoter in transgenic animals. *Development* **113**, 723-734.
Mendelsohn, C., Lohnes, D., Décimo, D., Lufkin, T., Lemeur, M., Chambon, P. and Mark, M. (1994). Function of the retinoic acid receptors (RARs) during development. (II). Multiple abnormalities at various stages of organogenesis in RAR double mutants. *Development* **120**, 2749-2771.
Müller, T. S., Ebensperger, C., Neubüser, A., Koseki, H., Balling, R., Christ, B. and Wilting, J. (1996). Expression of avian Pax1 and Pax9 is intrinsically regulated in the pharyngeal endoderm, but depends on environmental influences in the paraxial mesoderm. *Dev. Biol.* **178**, 403-417.
Murphy, P. and Hill, R. E. (1991). Expression of the mouse labial-like homeobox-containing genes, *Hox 2.9* and *Hox 1.6*, during segmentation of the hindbrain. *Development* **111**, 61-74.
Niederreither, K., McCaffery, P., Dräger, U. C., Chambon, P. and Dollé, P. (1997). Restricted expression and retinoic acid-induced downregulation of the retinaldehyde dehydrogenase type 2 (RALDH-2) gene during mouse development. *Mech. Dev.* **62**, 67-78.
Niederreither, K., Subbarayan, V., Dollé, P. and Chambon, P. (1999). Embryonic retinoic acid synthesis is essential for early mouse post-implantation development. *Nature Genet.* **21**, 444-448.
Niederreither, K., Vermot, J., Schuhbaur, B., Chambon, P. and Dollé, P. (2000). Retinoic acid synthesis and hindbrain patterning in the mouse embryo. *Development* **127**, 75-85.
Nieto, M. A., Gilardi-Hebenstreit, P., Charnay, P. and Wilkinson, D. G. (1992). A receptor protein tyrosine kinase implicated in the segmental patterning of the hindbrain and mesoderm. *Development* **116**, 1137-1150.
Noden, D. M. (1991). Cell movements and control of patterned tissue assembly during craniofacial development. *J. Craniofac. Genet. Dev.* Biol. **11**, 192-213.
O'Leary, D. D. and Wilkinson D. G. (1999). Eph receptors and ephrins in neural development. *Curr. Opin. Neurobiol.* **9**, 65-73.
Peters, H., Neubüser, A., Kratochwil, K. and Balling, R. (1998). *Pax9*-deficient mice lack pharyngeal pouch derivatives and teeth and exhibit craniofacial and limb abnormalities. *Genes Dev.* **12**, 2735-2747.
Rijli, F. M., Gavalas, A. and Chambon, P. (1998). Segmentation and specification in the branchial region of the head: the role of the *Hox* selector genes. *Int. J. Dev. Biol.* **42**, 393-401.
Rossant, J., Zirngibl, R., Cado, D., Shago, M. and Giguère, V. (1991). Expression of a retinoic acid response element-*hsplacZ* transgene defines specific domains of transcriptional activity during mouse embryogenesis. *Genes Dev.* **5**, 1333-1344.
Rossel, M. and Capecchi, M. R. (1999). Mice mutant for both *Hoxa1* and *Hoxb1* show extensive remodeling of the hindbrain and defects in craniofacial development. *Development* **126**, 5027-5040.
Ruberte, E., Dolle, P., Chambon, P. and Morriss-Kay, G. (1991). Retinoic acid receptors and cellular retinoid binding proteins. II. Their differential pattern of transcription during early morphogenesis in mouse embryos. *Development* **111**, 45-60.
Ruiz, J. C. and Robertson, E. J. (1994). The expression of the receptor-protein tyrosine kinase gene, *eck*, is highly restricted during early mouse development. *Mech. Dev.* **46**, 87-100.
Schlaeger, T. M., Qin, Y., Fujiwara, Y., Magram, J. and Sato, T. N. (1995). Vascular endothelial cell lineage-specific promoter in transgenic mice. *Development* **121**, 1089-1098.
Serbedzija, G. N., Bronner-Fraser, M. and Fraser, S. E. (1992). Vital dye analysis of cranial neural crest cell migration in the mouse embryo. *Development* **116**, 297-307.
Smith, A., Robinson, V., Patel, K. and Wilkinson, D. G. (1997). The EphA4 and EphB1 receptor tyrosine kinases and ephrin-B2 ligand regulate targeted migration of branchial neural crest cells. *Curr. Biol.* **7**, 561-570.
Smith, S. M. (1994). Retinoic acid receptor isoform β2 is an early marker for alimentary tract and central nervous system positional specification in the chicken. *Dev. Dyn.* **200**, 14-25.
Studer, M., Popperl, H., Marshall. H., Kuroiwa, A. and Krumlauf, R. (1994). Role of a conserved retinoic acid response element in rhombomere restriction of *Hoxb1*. *Science* **265**, 1728-1732.
Tiveron, M.-C., Hirsch, M.-R. and Brunet, J.-F. (1996). The expression pattern of the transcription factor Phox2 delineates synaptic pathways of the autonomic nervous system. *J. Neurosci.* **16**, 7649-7660.
Van Maele-Fabry, G., Clotman, F., Gofflot, F., Bosschaert, J. and Picard, J. J. (1997). Postimplantation mouse embryos cultured in vitro. Assessment with whole-mount immunostaining and *in situ* hybridisation. *Int. J. Dev. Biol.* **41**, 365-374.
Wagner, M., Han, B. and Jessel, T. M. (1992). Regional differences in retinoid release from embryonic neural tissue detected by an in vitro reporter assay. *Development* **116**, 55-66.
Waldo, K. L., Willner, W. and Kirby, M. L. (1990). Origin of the proximal coronary artery stems and a review of ventricular vascularization in the chick embryo. *Am. J. Anat.* **188**, 109-120.
Wall, N. A. and Hogan, B. L. M. (1995). Expression of bone morphogenetic protein-4 (BMP-4), bone morphogenetic protein-7 (BMP-7), fibroblast growth factor-8 (FGF-8) and sonic hedgehog (SHH) during branchial arch development in the chick. *Mech. Dev.* **53**, 383-392.
Wallin, J., Eibel, H., Neübuser, A., Wilting, J., Koseki, H. and Balling, R. (1996). *Pax1* is expressed during development of the thymus epithelium and is required for normal T-cell maturation. *Development* **122**, 23-30.
Wendling, O., Chambon, P. and Mark, M. (1999). Retinoid X receptors are essential for early mouse development and placentogenesis. *Proc. Natl. Acad. Sci. USA* **96**, 547-551.
White, J. C., Shankar, V. N., Highland, M., Epstein, M. L., DeLuca, H. F. and Clagett-Dame, M. (1998). Defects in embryonic hindbrain development and fetal resorption resulting from vitamin A deficiency in the rat are prevented by feeding pharmacological levels of all-trans-retinoic acid. *Proc. Natl. Acad. Sci. USA* **95**, 13459-13464.
Wilkinson, D. G., Bhatt, S. and McMahon, A. P. (1989). Expression pattern of the FGF-related proto-oncogene *int-2* suggests multiple roles in fetal development. *Development* **105**, 131-136.
Yamagishi, H., Garg, V., Matsuoka, R., Thomas, T. and Srivastava, D. (1999). A molecular pathway revealing a genetic basis for human cardiac and craniofacial defects. *Science* **283**, 1158-1160.