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FUNCTION OF RETINOID NUCLEAR RECEPTORS: Lessons from Genetic and Pharmacological Dissections of the Retinoic Acid Signaling Pathway During Mouse Embryogenesis

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■ **Abstract** Retinoic acid (RA) is involved in vertebrate morphogenesis, growth, cellular differentiation, and tissue homeostasis. The use of in vitro systems initially led to the identification of nuclear receptor RXR/RAR heterodimers as possible transducers of the RA signal. To unveil the physiological functions of RARs and RXRs, genetic and pharmacological studies have been performed in the mouse. Together, their results demonstrate that (a) RXR/RAR heterodimers in which RXR is either transcriptionally active or silent are involved in the transduction of the RA signal during prenatal development, (b) specific RXRα/RAR heterodimers are required at many distinct stages during early embryogenesis and organogenesis, (c) the physiological role of RA and its receptors cannot be extrapolated from teratogenesis studies using retinoids in excess. Additional cell type–restricted and temporally controlled somatic mutagenesis is required to determine the functions of RARs and RXRs during postnatal life.

INTRODUCTION

Clinical findings and experimental approaches have revealed that vitamin A (retinol) and its active derivatives (collectively referred to as retinoids) exert a wide variety of effects on vertebrate embryonic body shaping and organogenesis, tissue homeostasis, cell proliferation, differentiation, and apoptosis (reviewed in 1–4). Following Hale's initial demonstration that vitamin A deficiency (VAD) induces congenital ocular malformations (5), Warkany and his collaborators showed that a large array of congenital malformations affecting the ocular, cardiac, respiratory, and urogenital systems (collectively referred to as the fetal VAD syndrome) occurred in fetuses from VAD rats (reviewed in 6). Vitamin A is also indispensable

throughout postnatal development and adult life for growth, survival, reproduction, vision, as well as for the homeostasis of numerous tissues: Growth retardation, widespread squamous metaplasia of glandular and transitional epithelia, as well as degeneration of the testis, of the retina, and of motoneurons, are hallmarks of the postnatal VAD syndrome (7, 8). All-trans retinoic acid (RA) and its 9-cis isomer (9-cis RA), the most active biological retinoids, can prevent and rescue the defects caused by VAD in adults, with, however, the exceptions of night-blindness and degeneration of the retina (3, 9). RA can also replace vitamin A during embryogenesis, at least at certain stages and in certain organs (10, 11).

How this simple molecule can exert such pleiotropic effects was a long-standing question that found its solution with the discovery of two classes of RA-binding transcriptional regulators belonging to the nuclear receptors (NRs) superfamily, the retinoic acid receptors (the multiple isoforms of the RARα, β, and γ isotypes), which bind all-trans and 9-cis RA, and the rexinoid receptors (the multiple isoforms of the RXRα, β, and γ isotypes), which bind 9-cis RA only (reviewed in 12, 13). RARs and RXRs exhibit the conserved structure of NRs, organized into A to F regions. For each RAR isotype, several isoforms harboring distinct N-terminal A regions arise from the differential usage of two promoters (of which the downstream one, P2, initiating transcription of isoform 2, is RA inducible) and alternative splicing. There are two major isoforms for RARα (α1 and α2) and for RARγ (γ1 and γ2), and four major isoforms for RARβ (β1 to β4). Similarly, at least two isoforms have been identified for RXRα (α1 and α2), RXRβ (β1 and β2) and RXRγ (γ1 and γ2) (12, 13, and references therein).

WHAT HAVE WE LEARNED ABOUT RETINOID RECEPTOR FUNCTIONS FROM IN VITRO STUDIES?

In vitro studies, performed either in cell-free systems or transfected cells in culture, revealed that RAR/RXR heterodimers could control the transcription of RA-target genes through binding to DNA response elements (RARE) and unveiled the functions of the A to F regions (12, 14). The amino-terminal A/B region contains the ligand-independent transcriptional activation function (AF-1) and several phosphorylation sites for proline-dependent kinases, including cyclin-dependent kinases, MAP kinases, and stress kinases (reviewed in 15, 16). The centrally located C region, which belongs to the DNA-binding domain (DBD), also contains a surface for RAR/RXR interaction. The E region includes the ligand-binding domain (LBD) and surfaces for RXR/RAR heterodimerization and binding of transcriptional silencing corepressors. The E region also contains the ligand-dependent transcriptional activation function (AF-2) that requires the so-called AF-2AD core, which corresponds to the α-helix 12 located at the C-terminal end of the LBD (12). Binding of an agonistic ligand induces a transconformation of the LBD, which involves α-helix 12 and results in the generation of a surface that allows the binding of coactivators while corepressors are concomitantly released (12, 17–20, and

references therein). In vitro studies have shown that the AF-1 and AF-2 of a given receptor isoform can cooperate and exhibit some transcriptional specificity that depends on the cell-type and the promoter context of RA-target genes (21, 22). They also showed that RXRs can function as promiscuous heterodimerization partners for numerous nonsteroidal NRs other than RARs (reviewed in 12, 14, 23).

The synergism between liganded RAR and RXR on the transcriptional activation of target genes in vitro indicated that RXRs are not a priori transcriptionally silent partners (12, and references therein). However, in RXR/RAR heterodimers, the ligand-dependent transcriptional activity of RXR appeared in general to be subordinated to the binding of an agonistic ligand to its RAR partner (in 12, 14, 24, and references therein). This subordination may exist in the case of some other RXR/NR heterodimers, as binding of the cognate ligand to the thyroid hormone (TRs) and vitamin D3 (VDR) receptors could also be a prerequisite for the RXR partner to respond to its agonistic ligand. It was assumed that such subordinated heterodimers may prevent the promiscuous activation of these signaling pathways by the RXR ligand (9-cis RA) on its own. On the other hand, in the case of several other RXR/NRs heterodimers [e.g., farnesoids (FXR), oxysterols (LXRs) and peroxisome proliferator–activated receptors], RXR agonists can activate transcription on their own. Such permissive heterodimers may integrate retinoid signaling into other signaling pathways. A molecular mechanism accounting for RXR subordination and permissivity in heterodimers has been recently proposed (20). However, the finding of permissive heterodimers raises the question as to whether 9-cis RA could actually be a physiological ligand for RXRs. Indeed, assuming that 9-cis RA might be the ligand activating RXRs in these permissive heterodimers would create a problem of promiscuity, as it would result in concomitant activation of the RXR/RAR-mediated retinoid signaling pathway (25). For instance, in the same cell this would not permit the simultaneous occurrence of 9-cis RA-induced transcriptional activation events mediated by permissive RXR/NR heterodimers and transcriptional repression events mediated by RXR/RAR heterodimers. Interestingly, long-chain polyunsaturated fatty acids can bind RXR with a low efficiency and activate RXR in cell-based assays (26–28). These observations raise the possibility that the RXR subunit of heterodimers could be bound to low-affinity ligands to maximally sensitize (through synergistic effects) the transcriptional activity of the heterodimer to discrete variations in the concentration of the cognate ligand of its heterodimeric partner (25).

The initial suggestion that each RAR and RXR isoform may perform unique functions (13) came from their high degree of conservation among vertebrate species (14, 29, and references therein) and from their selective expression patterns in embryonic and adult tissues (12, 13, and references therein). Moreover, results from in vitro studies led to the proposal that a combinatorial mechanism in which multiple actors (i.e., at least 48 distinct RAR/RXR heterodimers) differentially transduce the retinoid signal to selectively control expression of distinct RA-target genes accounts for the pleiotropic effects of RA (12, 13). However, a genetic dissection of the retinoid signaling pathway was obviously required to

determine the physiological functions of each retinoid receptor and to investigate the relevance in vivo of the signal transduction mechanisms characterized in vitro. The present review focuses on three main questions: (*a*) Are RARs and RXRs involved in the transduction of RA signals? (*b*) To what extent do the observations made in vivo support the molecular mechanisms deduced from in vitro studies? (*c*) What are the developmental events controlled by RARs and RXRs? We summarize here and discuss the developmental phenotypes induced by mutations that have been introduced at loci encoding RARs and RXRs, and compare them to the phenotypes resulting either from VAD or from administration of pharmacological doses of selective agonistic or antagonistic ligands for RARs and RXRs.

RARs AND RXRs ARE ESSENTIAL FOR RETINOIC ACID SIGNALING DURING DEVELOPMENT

Signaling Through RARs Is Indispensable for Embryonic Patterning and Shaping and for Organogenesis

Rara-, Rarb-, and *Rarg*-null mutant mice are viable. They display some aspects of the fetal and postnatal VAD syndromes, as well as few additional congenital malformations (Table 1). However, their abnormalities are restricted to a subset of tissues normally expressing these receptors, probably reflecting the existence of functional redundancies between RARs (extensively discussed in 3, 30–32). To test this hypothesis, mutants lacking two RAR isotypes (*Rara/b-*, *Rara/g-*, and *Rarb/g*-null mutants) or several isoforms belonging to distinct isotypes were generated. For the sake of clarity, Tables 2 and 3 list only abnormalities displayed by compound null mutants lacking a couple of RAR isotypes (all isoforms deleted). Similar, albeit often less penetrant, abnormalities are displayed by compound null mutants lacking a couple of isoforms (3, 33–36).

Rara/b-, *Rara/g-*, and *Rarb/g*-null mutants die in utero or at birth from severe developmental defects that include the complete spectrum of malformations belonging to the fetal VAD-induced syndrome (6) (Table 2). *Rara-*, *Rarb-*, or *Rarg*-null (Table 1) and compound *Rara/b-*, *Rara/g-*, and *Rarb/g*-null mutants (Table 3) exhibit congenital abnormalities that were not described in Hale's and Warkany's pioneering studies (5, 6), ranging from agenesis of the Harderian gland to skeletal defects of the skull, face, vertebrae, and limbs (31, 33, 35, 37–39). The occurrence of these nonVAD defects in the null mutant mice is most probably accounted for by the difficulty to achieve, by dietary deprivation, a state of severe VAD compatible with pregnancy. In fact, almost all these nonVAD defects have been subsequently duplicated in rodent embryos (*a*) deficient in vitamin A, but supplemented with RA (10, 11, 40); (*b*) lacking the RA-synthesizing retinaldehyde dehydrogenases RALDH2 (41–46) or RALDH3 (47); and (*c*) treated with synthetic retinoids having RAR antagonistic activities (48–50).

Altogether these studies demonstrate that RA-liganded RARs play crucial roles at many distinct stages of the embryonic patterning and organogenesis. The severe malformations found in *Rara/g*-null embryos (49) are similar to those of

TABLE 1 Postnatal manifestations of germ line ablation of *Rar* and *Rxr* genes. CD: congenital defects; PnVAD: abnormalities present in postnatal vitamin A–deficiency (7); fetal VAD: abnormalities present in vitamin A–deficiency during pregnancy (6). *Rara1*, *Rara2*, *Rarb1/3*, *Rarb2/4*, *Rarg1*, and *Rarg2* refer to isoform-specific ablations

Null mutation	Abnormalities	References
Rara1	None	(83, 158)
Rara2	None	(M. Mark, N.B. Ghyselinck & P. Chambon, unpublished results)
Rara	Growth retardation (PnVAD); male sterility (testis degeneration #; PnVAD); impaired alveolar formation. CD: webbed digits; homeotic transformations and malformations of cervical vertebrae; pterygoquadrate cartilage; malformation of the squamosal bone; malformed laryngeal (i.e., cricoid) cartilage	(31, 35, 69, 89)
Rarb1/3	None	(36)
Rarb2/4	CD: persistence and hyperplasia of the primary vitreous body (fetal VAD)	(34)
Rarb	Growth retardation (PnVAD); behavioral defects; altered alveolar formation. CD: homeotic transformations and malformations of cervical vertebrae; persistence and hyperplasia of the primary vitreous body (fetal VAD)	(31, 67, 70, 159, 160)
Rarg1	Growth deficiency (PnVAD). CD: malformations of cervical vertebrae #; malformed laryngeal (i.e., cricoid) cartilage; abnormal differentiation of granular keratinocytes #	(35; M. Mark, N.B. Ghyselinck & P. Chambon, unpublished results)
Rarg2	None	(38)
Rarg	Growth deficiency (PnVAD); male sterility [squamous metaplasia of the seminal vesicle and prostate gland epithelia # (PnVAD)]; squamous metaplasia of other epithelia (PnVAD); impaired alveolar formation. CD: webbed digits; homeotic transformations and malformations of cervical vertebrae #; malformed laryngeal cartilages and tracheal rings #; agenesis of the Harderian glands; agenesis of the metoptic pillar of the skull; abnormal differentiation of granular keratinocytes #	(31, 38, 68, 154; M. Mark, N.B. Ghyselinck & P. Chambon, unpublished results)
Rarb/Rarg1	Hydronephrosis	(35)

(*Continued*)

TABLE 1 (*Continued*)

Null mutation	Abnormalities	References
Rarb2/4/Rarg2	Dysplasia and degeneration # of the retina. CD: persistence and hyperplasia of the primary vitreous body (fetal VAD); blepharophimosis #; partial agenesis of the sclera and choroid #	(34)
Rarb1/3/Rarg	Degeneration of the retina. CD: persistence and hyperplasia of the primary vitreous body (fetal VAD); partial agenesis of the sclera and choroid #	(36)
$Rxra^{+/-}$	Growth retardation (PnVAD). CD: webbed digits	(30, 76)
Rxrb	Male sterility # (defective spermatogenesis); abnormal lipid metabolism in Sertoli cells; behavioral defects	(85, 159)
Rxrg	Metabolic and behavioral defects	(82, 159–161)

#: These abnormalities are completely penetrant.

embryos lacking RALDH2 (41), thereby reflecting early roles of RA-liganded RAR signaling in axial rotation, segmentation and closure of the hindbrain, formation of otocysts, development of pharyngeal arches and forelimb buds, as well as in closure of the primitive gut. RARs are also indispensable for the ontogenesis of almost all the structures derived from mesectodermal cells, i.e., the cranial neural crest cells (NCC) that give rise to mesenchymal derivatives (reviewed in 3, 51, 52). RARs are involved in antero-posterior patterning of the somitic mesoderm and hindbrain neuroectoderm (31, 37, 38, 49, 53), notably through controlling expression of homeobox genes (53–58). RARs are also involved in the establishment of the antero-posterior axis of the limbs (32, 37, 59). RARs are required for the development of a large number of ocular structures and for histogenesis of the retina (31, 34, 38), for cardiomyocyte differentiation (60), as well as for the control of apoptosis in the frontonasal and interdigital mesenchymes (31, 37, 59, 61) and in the conotruncal segment of the embryonic heart (36) (Tables 1 to 3). RARs control the formation of the genital ducts and ureters (31, 39, 62), as well as epithelial-mesenchymal interactions in the kidney, through expression of the receptor tyrosine kinase *Ret* (63, 64). In the developing respiratory tract, RA-liganded RARs are necessary for the morphogenesis of the nasal cavities and for their communication with the more caudal airways (31, 47) (Tables 2 and 3). RARs regulate lung branching morphogenesis (65, 66) and lung alveoli septation (67–70), and they are also required for the partitioning of the primitive foregut into esophagus and trachea (31, 39, 65) (Tables 1–3).

RARs Have Been Instrumental to the Phylogenesis of Mesectodermal Derivatives

In addition to the dramatic craniofacial skeletal deficiencies affecting *Rara/g*-null mutants (37), subtle defects that often alter the shape of a single skeletal piece are

TABLE 2 Abnormalities of the fetal vitamin A deficiency (VAD) syndrome (6) present in *Rarb*-null mutants (Aβ), *Rxra*-null mutants, and in compound *Rara/b*-, *Rara/g*-, and *Rarb/g*-null mutants (AαAβ, AαAγ, and AβAγ, respectively). From References 31, 37, 76. Note that most of the abnormalities seen in *Rara/b*-null mutants occur at similar frequencies in *Rara/b2* mutants (39)

Abnormalities of the fetal VAD syndrome	Genotypes of *Rar*-null mutants showing similar abnormalities	Abnormalities observed in *Rxra*-null mutants
Respiratory system defects		
• Agenesis or hypoplasia of the left lung	Aα/Aβ#	No
• Hypoplasia of the right lung	Aα/Aβ#	No
• Agenesis of the esophago-tracheal septum	Aα/Aβ#	
• Diaphragmatic hernia	Aα/Aβ	No
Hypoplasia of the ventricular myocardium	Aα/Aγ	Yes #
Heart outflow tract defects		
• Persistent truncus arteriosus	Aα/Aβ#, Aα/Aγ#	No
• High interventricular septal defect or double outlet right ventricle (E18.5)/conotruncal septum defect (E14.5)	Aα/Aβ#, Aα/Aγ#, Aβ/Aγ	Yes
• Abnormal great arteries derived from aortic arches	Aα/Aβ#, Aα/Aγ#, Aβ/Aγ	No
Kidney hypoplasia	Aα/Aβ#, Aα/Aγ#	No
Ureteral defects		
• Agenesis	Aα/Aβ, Aα/Aγ#	No
• Ectopia	Aα/Aβ, Aβ/Aγ	No
Genital tract defects		
-Female		
• Agenesis of the oviduct and uterus (E18.5)/ agenesis of the Müllerian duct (E14.5)		
-Complete	Aα/Aβ#	No
-Partial	Aα/Aγ#	No
• Agenesis of the cranial vagina	Aα/Aβ#, Aα/Aγ#	NA
-Male		
• Agenesis or dysplasia of the vas deferens	Aα/Aγ#	NA
• Agenesis of the seminal vesicles	Aα/Aγ#	NA
Ocular defects		
• Coloboma of the retina	Aα/Aγ#	No
• Coloboma of the optic disc	Aβ/Aγ, Aα/Aγ#	Yes
• Persistence et hyperplasia of the primary vitreous body (PHPV)	Aβ, Aα/Aβ#, Aβ/Aγ# Aα/Aγ#	Yes #
• Hypoplasia of the conjunctival sac	Aβ/Aγ#, Aα/Aγ#	Yes #
• Thickening of the corneal stroma	Aβ/Aγ#, A α/Aγ	Yes #
• Ventral rotation of the lens	AβAγ#	Yes #
• Shortening of the ventral retina	AβAγ#	Yes #

#: This abnormality is completely penetrant.

NA: Not applicable as the corresponding structure is normally not found at E14.5, the time around which *Rxra*-null mutants die.

TABLE 3 Abnormalities absent from the fetal vitamin A deficiency (VAD) syndrome (6) are found in *Rara*-, *Rarb*-, and *Rarg*-null mutants (Aα, Aβ, Aγ), and in compound *Rara/b*-, *Rara/g*-, and *Rarb/g*-null mutants (Aα/Aβ, Aα/Aγ, and Aβ/Aγ). From References 31, 37, 76. Note that most of the abnormalities seen in *Rara/b*-null mutants occur at similar frequencies in *Rara/b2*-null mutants (39)

Congenital abnormalities not associated with the fetal VAD syndrome	**Genotypes of *Rar*-null fetuses showing these defects**
Nervous system defects	
• Exencephaly	Aα/Aγ
• Agenesis of the corpus callosum	Aα/Aγ#
Skeletal defects	
• Agenesis or multiple malformations of cranial skeletal elements	Aα/Aγ#
• Homeotic transformation and malformations of cervical vertebrae	Aα, Aβ, Aγ Aα/Aγ#, Aα/Aβ #, Aβ/Aγ #
• Agenesis and malformations of limb bones	Aα/Aγ#
• Reappearence of atavistic skeletal elements	
-Pila antotica	Aα/Aγ#
-Ptrerygoquadrate cartilage	AαAα/Aγ#, Aα/Aβ
Eye defects	
• Corneal-lenticular stalk	Aα/Aγ
• Agenesis of the lens	Aα/Aγ
Glandular defects	
• Agenesis or dysplasia, of the sub-maxillary and sub-lingual glands and/or of their excretory ducts	Aα/Aγ#, Aβ/Aγ#
• Agenesis of the Harderian gland	Aγ, Aα/Aγ#, Aβ/Aγ#
• Agenesis or ectopia of the thyroid, thymus and parathyroid glands	Aα/Aβ, Aα/Aγ
Other defects	
• Webbed digits	Aα, AγA$\beta^{+/-}$/Aγ#, Aβ/Aγ#
• Abnormal laryngeal cartilages and tracheal rings	Aγ#, Aα/Aγ#, Aα/Aβ#, Aβ/Aγ#
• Kidney agenesis	Aα/Aγ
• Agenesis of the anal canal	Aα/Aβ#

#: This abnormality is completely penetrant.

observed in several *Rar*-null mice, including a cartilaginous or osseous connection between the incus middle ear bone and the alisphenoid bone (the pterygoquadrate element), a cartilaginous wall separating the trigeminal ganglion from the brain (the pila antotica), agenesis of the rostral ethmoturbinate and maxillary sinus, and absence of the pila metoptica (Table 3) (31, 37, 52). The pterygoquadrate element and the pila antotica, which were lost during evolution from reptiles to mammals,

represent atavistic features (Figure 1) (discussed in 51, 52). Along the same lines, ethmoturbinate bones and paranasal sinuses (such as the maxillary sinus) are typical mammalian features not present in reptiles (71). It is thus conceivable that agenesis of these nasal structures in *Rar*-null mutants also mimics an atavistic condition. The pila metoptica is lacking in monotremes and marsupials, but is present in placental mammals as well as in their reptilian ancestors (71). Although its absence in *Rar*-null mutants cannot be interpreted as an atavistic trait, it supports the possibility that changes in the temporal or spatial patterns of expression of *Rar* genes has provided a general mechanism for modifying the number and shape of individual cranial skeletal elements during vertebrate evolution. Interestingly, such a function has also been assigned to members of the BMP family (reviewed in 72–74). Thus, BMPs, which can elicit ectopic bone formation, possibly by promoting the entry of multipotent stem cells into the chondrogenic pathway, and whose loss-of-function mutations result in the disruption of specific subsets of skeletal elements, could mediate effects of RA on cranial skeletal patterning.

The persistent and hyperplastic vitreous body (PHPV) seen in *Rarb*-null mutants (Figure 2*i*) is comparable to a normal vascular and pigmented projection from the optic disk found in some reptiles, the pecten oculi, which is thought to function in the nutrition of the retina (75). The shortening of the ventral retina observed in *Rarb/g*-null mutants might also be interpreted as a modification toward an ancestral condition because comparative embryology of the retina shows that the ventral retinal field increases in size as one ascends the vertebrate scale from fishes to amphibia, reptiles, and mammals (75). Thus, RARβ and RARγ might have been instrumental in the expansion of the ventral retinal field during vertebrate evolution.

RXRα Is the Main RXR Isotype Involved in Embryogenesis

Rxra-null mutants all display a hypoplasia of the compact layer of the ventricular myocardium (Table 2), which appears to be the main cause of mutant death occurring by cardiac failure around E14.5 (60, 76–78). That a similar myocardium defect is observed in VAD and *Rar*-null fetuses (6, 39) suggests that RXRα is involved in the transduction of the RA signal required for myocardial growth. This requirement is unlikely to be cell autonomous, as overexpressing RXRα in cardiomyocytes by means of trangenesis does not prevent the *Rxra*-null mutation-induced hypoplasia of the ventricular myocardium (79). Recent data further indicate that *Rxra* expression in the epicardium is required for triggering a paracrine signal necessary for myocardial growth (80, 81).

Approximately one third of the *Rxra*-null mutants lack the conotruncal septum, which normally divides the embryonic heart outflow tract (or conotruncus) into the intracardiac portions of the aorta and the pulmonary trunk (76). Interestingly, deficiencies of this septum represent both a classical VAD defect in rodents and a leading cause of human congenital heart defects, ranging from high interventricular septal defects to double outlet right ventricle (DORV). In *Rxra*-null mutants, the

agenesis of the conotruncal septum appears secondary to an enhanced rate of cell death in the mesenchymal cells of the conotruncal ridges and the parietal conotruncal cardiomyocytes, therefore indicating that RXRα is required for the transduction of the RA signal that controls apoptosis in the conotruncal segment of the embryonic heart (36).

In addition to heart defects, all *Rxra*-null fetuses display a characteristic ocular syndrome associating a persistent and hyperplastic vitreous body (PHPV), closer eyelid folds, a thickened ventral portion of the corneal stroma, a ventral rotation of the lens, an agenesis of the sclera, and a shortening of the ventral retina (Table 2 and Figure 2*b*) (76). As similar defects are present in VAD fetuses and in *Rarb/g*-null mutants (Table 2 and Figure 2*k*) (6, 31), RXRα appears to play an essential role in the transduction of the RA signals required for several ocular morphogenetic processes, notably the formation of the ventral retinal field.

Importantly, the fact that mice lacking both RXRβ and RXRγ (*Rxrb/g*-null mutants) do not display any obvious morphogenetic defects, even when additionally lacking one allele of *Rxra*, clearly indicates that RXRα is functionally the most important RXR during morphogenesis of the embryo proper (82).

The AF-1-Containing A/B Domain and the Ligand-Dependent AF-2 of RXRα Are Differentially Involved in Development

The role played by RXRs as either active or silent heterodimerization partners in the transcription of target genes, inferred from in vitro studies, has been a controversial issue (see What Have We Learned About Retinoid Receptor Functions from In Vitro Studies, above, and Conclusions and Perspectives, below). To determine the transcriptional role of RXRα in vivo, mouse mutants were engineered that express truncated RXRα proteins lacking (*a*) the N-terminal AF-1-containing A/B region (*Rxra*^{af1o} mutants; 83), (*b*) the AF-2 AD core-containing helix 12 located at the C-terminus of the E region (*Rxra*^{af2o} mutants; 32), and (*c*) both AF-1 and AF-2 (*Rxra*^{afo} mutants; 84).

The *Rxra*^{af2o} mutants display the myocardium hypoplasia and the ocular syndrome that are hallmarks of the *Rxra*-null phenotype (Figure 2*b*; 76), but at low frequency (32). This may reflect a functional compensation by RXRβ as (*a*) the frequency of the myocardium hypoplasia increases from 5% in *Rxra*^{af2o} mutants to 50% upon the additional inactivation of *Rxrb* (which, on its own, has no effect; 85), and (*b*) the frequency of the ocular syndrome increases from approximately 15% in *Rxra*^{af2o} mutants to 100% upon further inactivation of *Rxrb* (32). The full penetrance of the *Rxra*-null ocular syndrome observed in *Rxra*^{af2o}*/Rxrb*-null mutants (32), as well as in *Rxra*^{afo} mutants (84), supports the view that the AF-2 of RXRα (and thus possibly a RXR agonistic ligand) is indispensable for ocular morphogenesis. On the other hand, the rare or modest penetrance of the myocardium hypoplasia in *Rxra*^{af2o} (5%), *Rxra*^{af2o}*/Rxrb*-null (50%), and *Rxra*^{af2o}*/Rxrb/g*-null mutants (50%) suggests that the transcriptional activity of RXRα becomes necessary for myocardial growth only in unfavorable genetic backgrounds. Accordingly,

we recently found that the heart histology is normal in 80% of *Rxra*^{afo} fetuses (84), indicating that a transcriptionally silent RXRα can promote myocardial growth.

Involution of the primary vitreous body represents the developmental process which likely requires the highest concentration of RA-liganded retinoid receptors because VAD (6), *Rarb* ablation (31), *Rxra* ablation (76), deletion of RXRα AF-2 (32), and deletion of RXRα AF-1 and AF-2 (84) almost consistently yield a PHPV (Figure 2). Apart from an occasional PHPV, the *Rxra*^{af1o} mutants never display any of the *Rxra*-null developmental defects (83). This near-absence of defects does not reflect a functional compensation by RXRβ or RXRγ, as *Rxra*^{af1o}*/Rxrb*-null and *Rxra*^{af1o}*/Rxrb/g*-null mutants display no other developmental defect than a PHPV (Figure 2*e*). However, the frequency of this PHPV increases from 10% in *Rxra*^{af1o} mutants to 100% in *Rxra*^{af1o}*/Rxrb/g*-null mutants (83). Altogether, these observations indicate that involution of the primary vitreous body requires RXRα AF-1 and AF-2, whereas the other RA-dependent ocular morphogenetic events require RXRα AF-2 only, as they normally take place in the absence of the RXRα AF-1-containing A/B domain.

Thus, these data support the view that the activation functions of RXRα are differentially required for eye morphogenesis and that they can be dispensable for heart development. They also indicate that, owing to functional redundancy, the role played by each activation function can be revealed only in genetic backgrounds impaired for RA-signaling (or under RA-insufficiency conditions). The assumption that the frequent requirement of RXRα AF-2 for developmental events reflects the binding of an agonistic ligand raises the question of the possible existence and nature of a physiological RXR ligand(s) in vivo. The fact that 9-cis RA is undetectable in rodent embryos (86, 87) makes it doubtful that this RXR physiological ligand could be 9-cis RA (see also What Have We Learned About Retinoid Receptor Functions from In Vitro Studies?, above).

Interestingly, the RXRα AF-1-containing A/B region has a unique role in the RA-dependent disappearance of the interdigital mesenchyme. The first evidence implicating RA in the involution of the interdigital mesenchyme was provided by organ culture experiments using whole limb in a RA-deprived medium (88). Subsequently, it was shown that mice lacking both alleles of either *Rara* or *Rarg*, as well as mice heterozygous for the *Rxra*-null mutation, occasionally exhibit mild forms of interdigital webbing (i.e., soft tissue syndactyly) (Table 1) (31, 38, 76, 89). Surprisingly, this defect was absent in *Rarb*-null mutants, even though *Rarb* is strongly and specifically expressed in the interdigital necrotic zones (INZs) (31). However, disruption of one (or both) allele(s) of *Rarb* in a *Rarg*-null genetic background consistently yields interdigital webbing (31). The persistence of the fetal interdigital mesenchyme responsible for digit webbing is caused by a marked decrease in programmed cell death, as well as by an increase of cell proliferation in the mutant INZs (59). As *Rarb* and *Rarg* are not coexpressed in the INZs, interdigital mesenchyme involution must involve paracrine interactions between this mesenchyme, which expresses *Rarb*, and either the cartilaginous blastema of the digits or the surface epidermis, which both express *Rarg* (discussed in 59).

The RXRα AF1-containing A/B region is indispensable for the function of RXRα/RARβ and/or RXRα/RARγ heterodimers involved in interdigital mesenchyme involution because the majority of *Rxra*^{af1o} mutants and all *Rxra*^{af1o}*/Rxrb/g*-null mutants display a soft tissue syndactyly (83). In contrast, *Rxra*^{af2o} and *Rxra*^{af2o}*/Rxrb/g*-null mutants never display this defect (32), indicating a specific requirement of the RXRα AF1-containing A/B region in the involution of the interdigital mesenchyme. Interestingly, phosphorylation of RXRα at a specific serine residue located in the A domain is necessary for the antiproliferative response of F9 teratocarcinoma cells to RA (15, 90). Phosphorylation of the RXRα A domain may therefore play an important function in the cascade of molecular events that, in vivo, leads to the normal disappearance of the interdigital mesenchyme.

Retinoic Acid Signals Are Transduced by Specific RXRα/RAR Heterodimers During Development

Compound mutants, in which a null mutation of a given RAR isotype is associated either with a *Rxra*-null, a *Rxra*^{af1o}, or a *Rxra*^{af2o} mutation, altogether recapitulate the abnormalities exhibited by *Rar*-null mutants (Table 4 and Figure 2) (30, 32, 76, 83). This synergism between *Rar* and *Rxra* loss-of-function mutations supports the conclusion that RXRα/RARα, RXRα/RARβ, and RXRα/RARγ heterodimers are the functional units transducing RA signals during embryogenesis. Moreover, that *Rxrb/g*-null mutants develop normally indicates that RXRα is functionally the most important RXR isotype during development (83). This conclusion is further supported by a lack of synergism, during embryogenesis, between *Rara*-, *Rarb*-, or *Rarg*-null mutations and *Rxrb*- or *Rxrg*-null mutations (30).

Comparing the severity and penetrance of a given abnormality between various mutants led to the identification of heterodimers preferentially involved in transducing RA signals in a given developmental process. For instance, myocardial hypoplasia is found in none of the *Rxra*^{af1o} mutants and in 5% of the *Rxra*^{af2o} mutants. It is observed in 45% of the *Rxra*^{af1o}*/Rara*-null and in 80% of the *Rxra*^{af2o}*/Rara*-null mutants, but in none of the *Rxra*^{af1o}*/Rarb*-null or *Rxra*^{af1o}*/Rarg*-null mutants and in only 20% of the *Rxra*^{af2o}*/Rarb*-null or *Rxra*^{af2o}*/Rarg*-null mutants (32, 83). Together, these genetic data indicate that RXRα/RARα heterodimers are preferentially transducing the RA signal acting on myocardial growth.

Similarly, although all three RARs are expressed in developing ocular structures (91), several lines of evidence indicate the involvement of RXRα/RARβ and RXRα/RARγ but not of RXRα/RARα heterodimers during eye morphogenesis. First, there is a strong synergism between *Rxra*- and *Rarb*- or *Rarg*-null mutations that is manifested by a marked increase in the severity of the *Rxra*-null ocular defects, whereas no synergism is observed with the *Rara*-null mutation (Table 4 and Figure 2*c* and *d*) (30). Second, there is also a strong synergism between the *Rarb*- or *Rarg*-null mutations and ablation of either the AF-1-containing A/B domain or the AF-2 of RXRα for the generation of ocular defects (Table 4 and Figure 2*g* and *h*) (32, 83). In fact, apart from the PHPV discussed above, the characteristic *Rxra*-null ocular syndrome is never observed in *Rxra*^{af1o} mutants, and it is found

TABLE 4 Evidence that RXRα and RAR act synergistically on embryonic development. Similar congenital defects absent (or very rare) in *Rxra*-null, *Rxra^{af1o}*, *Rxra^{af2o}*, and *Rara-*, *Rarb-*, *Rarg*-null mutants are observed in compound *Rxra/Rara-*, *Rxra/Rarb-*, and *Rxra/Rarg*-null mutants (Xα/Aα, Xα/Aβ, and Xα/Aγ); in compound *Rxra^{af2o}/Rara-*, *Rxra^{af2o}/Rarb-*, and *Rxra^{af2o}/Rarg*-null mutants (Xα*af*2o/Aα, Xα*af*2o/Aβ, and Xα*af*2o/Aγ); in compound *Rxra^{af1o}/Rara-*, *Rxra^{af1o}/Rarb-*, and *Rxra^{af1o}/Rarg*-null mutants (Xα*af1*o/Aα, Xα*af1*o/Aβ, and Xα*af1*o/Aγ), as well as in compound *Rara/b-*, *Rara/g-*, and *Rarb/g*-null mutants (Aα/Aβ Aα/Aγ and Aβ/Aγ). From References 30, 32, 76, 83

	Genotypes of *Rxr/Rar* and *Rar/Rar* compound mutants showing similar defects		
Abnormalities	**Compound *Rxra/Rar*-null mutants**	**Compound *Rar*-null mutants**	**Compound *Rxra^{af1o}* or *Rxra^{af2o}/Rar*-null mutants**
Ocular defects (VAD)			
• Severe shortening or agenesis of the ventral retina	Xα/Aβ, Xα/A$\gamma^{+/-}$#, Xα/Aγ#	Aβ/Aγ#	Xα*af*2o/Aβ, Xα*af*2o/Aγ, Xα*af1*o/Aβ
Respiratory system defects (VAD)			
• Lung hypoplasia	Xα/Aα^*	Aα/Aβ#	Xα*af*2o/Aα#, Xα*af*2o/Aβ, Xα*af1*o/Aα
• Agenesis of the esophagotracheal septum	Xα/Aα^*	Aα/Aβ#	Xα*af*2o/Aα, Xα*af*2o/Aβ, Xα*af1*o/Aα
Heart outflow tract defects (VAD)			
• Persistent truncus arteriosus	Xα/A$\alpha^{+/-}$, Xα/Aα#, Xα/Aβ, Xα/Aγ	Aα/Aβ#, Aα/Aγ#	Xα*af*2o/Aα, Xα*af1*o/Aα
• Abnormal arteries derived from aortic arches	Xα/A$\alpha^{+/-}$, Xα/Aα^*, Xα/Aβ, Xα/Aγ	Aα/Aβ#, Aα/Aγ#	Xα*af*2o/Aα, Xα*af*2o/Aβ, X*af1*o/Aα
Urogenital system defects (VAD)			
• Kidney hypoplasia	Xα/Aα#	Aα/Aβ#	Xα*af*2o/Aα, Xα*af1*o/Aα,
• Complete agenesis of Müllerian ducts	Xα/Aα#	Aα/Aβ#	Xα*af1*o/Aα
Hypoplasia of the sub-maxillary gland	Xα/Aγ#	Aα/Aγ#	Xα*af*2o/Aγ#
Skeletal defects			
• Multiple Cranio-facial defects	Xα/Aγ	Aα/Aγ#	Xα*af*2o/Aγ#
• Limb defects	Xα/Aγ	Aα/Aγ#	Xα*af*2o/Aγ

*:This abnormality is present in a majority of the mutants.

#: This abnormality is fully penetrant.

VAD: These abnormalities belong to the fetal vitamin A deficiency syndrome (6).

in less than 15% of the *Rxra*^{af2o} mutants. On the other hand, this ocular syndrome is observed in 100% of the *Rxra*^{af1o}*/Rarb*-null, *Rxra*^{af1o}*/Rarg*-null, *Rxra*^{af2o}*/Rarb*-null, and *Rxra*^{af2o}*/Rarg*-null mutants, whereas it is absent in all *Rxra*^{af1o}*/Rara*-null and *Rxra*^{af2o}*/Rara*-null mutants (32, 83). Altogether, these genetic data indicate that RXRα/RARβ and RXRα/RARγ are the heterodimers that are instrumental to ocular morphogenesis.

PHARMACOLOGICAL AND SOMATIC MUTAGENESIS APPROACHES PROVIDE CLUES ON RAR-CONTROLLED MECHANISMS OPERATING IN THE HEAD REGION DURING DEVELOPMENT

The Endoderm of Branchial Arches Is the Target of RA Action Mediated by RARα and/or RARβ

Rara/b-null mutants, analyzed at fetal stages of gestation, display the complete set of defects that can be generated in the chick by surgical ablation of postotic neural crest cells (NCC), namely thymus and parathyroid gland ageneses or ectopias, aberrant patterning of cephalic arteries, absence of pulmonary arteries and aorticopulmonary septum (31, 39, 52, and references therein). These defects are also present in the CATCH22 syndrome, which is an archetype of human neurocristopathy (i.e., congenital malformations of NCC-derived structures). These and other observations led to the proposal that cranial NCC giving rise to mesenchymal derivatives (i.e., the mesectodermal cells) are major targets of RA action (reviewed in 92). Unexpectedly, *Rara/b*-null mutants analyzed at embryonic stages of gestation display very small caudal branchial arches (BAs) but do not show NCC alterations (53). BAs are transient bulges of the embryonic head and neck, partially filled with NCC, and separated from one another by evaginations of the endoderm, the pharyngeal pouches. Caudal BAs and pouches give rise to the adult organs affected in the aforementioned NCC ablation experiments. As the BA defects observed in *Rara/b*-null embryos are less severe than those displayed by embryos lacking RALDH2 (i.e., embryos devoid of RA; 41), they do not reflect a complete block in RA signal transduction. To analyze NCC migration, as well as formation of BA and pharyngeal pouches, in a situation where the degree of the block in RA signal transduction could be precisely controlled, a culture system was designed in which wild-type embryos are exposed at selected times to the panRAR antagonist BMS493 (48).

Treatment with BMS493 induces a lack of caudal BAs and pharyngeal pouches and slightly disturbs the paths of postotic NCC migration without affecting the amount of NCC. Moreover, and most interestingly, this treatment inhibits caudal BA development only during a narrow window of time, which does not correspond to the period of postotic NCC migration. Thus, contrary to what was expected from the set of abnormalities displayed by *Rara/b*-null fetuses (53), migrating NCC destined to the caudal BAs do not represent primary targets of RA action. On the

other hand, BMS493-induced alterations in endodermal expression of patterning genes and of genes encoding peptides involved in paracrine signaling pathways indicate that RA signaling (*a*) is required to specify the pharyngeal endoderm and (*b*) may provide a permissive environment for NCC migration through secretion of specific paracrine factors by the pharyngeal endoderm (49, 92). These data also raise the possibility that genes responsible for the human CATCH22 syndrome are actually expressed in the endoderm, under the control of RA during the fourth and fifth weeks of gestation.

RARs Act on Top of a Genetic Cascade Controlling Hindbrain Segmentation

The embryonic hindbrain is transiently divided into segments (rhombomeres), of which seven (R1 to R7) are visible in mammals. Although early and transient, hindbrain segmentation is instrumental to organize adult structures such as cranial nerves. The hindbrain of *Rara/g*-null embryos shows a posterior expansion of R3 and R4 markers, but fails to express *kreisler*, a specific marker of R5 and R6 (49). In contrast, the neuroectodermal territory corresponding to R5 and R6 is markedly enlarged in *Rara/b*-null embryos (53). Treating E7.0 wild-type embryos with the panRAR antagonist BMS493 duplicates the abnormal hindbrain phenotype of *Rara/g*-null embryos, whereas BMS493 administration started at E8.0 results in a *Rara/b*-null-like phenotype (48). Therefore, the distinct phenotypes in *Rara/b- and Rara/g*-null embryos are related to RA actions during different windows of time. At E7.5 (when RA synthesis begins in the embryo; 41), RARγ and RARα transduce a signal required to specify the R5/R6 territory. At E8.0, RARβ and RARα mediate a local increase in RA signaling in the posterior portion of the hindbrain to control the position of the R6 caudal boundary, thus allowing the next caudal rhombomere, R7, to be specified (48, 58, 92).

That the expression domains of several important hindbrain patterning genes are altered in *Rara/b-* and *Rara/g*-null embryos provides evidence that RA acts on top of the genetic hierarchy controlling hindbrain patterning (49). Moreover, generation of a graded embryonic block in RA signal transduction by varying the concentrations of the panRAR antagonist in the cultures demonstrates that individual rhombomeres are specified by distinct thresholds of RA signaling and supports the view that RA acts as a posteriorizing signal for the patterning of the embryonic hindbrain (49, 58, 93, and references therein). Threshold levels of RA signaling can be set up through modulations of the expression levels of RAR or of RA-synthesizing and catabolizing enzymes (RALDHs and CYP26s, respectively) (42, 94–96).

Retinoic Acid-Dependent Eye Morphogenesis Is Orchestrated by the Neural Crest

As mentioned above, *Rarb/g-*, *Rxra/Rarb-,* and *Rxra/Rarg*-null mutants, but not *Rxra/Rara*-null mutants, display similar, albeit much more severe, ocular defects

than *Rxra*-null mutants, indicating that RXRα/RARβ and RXRα/RARγ heterodimers are instrumental to the morphogenesis of eye structures, including the ventral retina (Tables 2–4; Figure 2). Both *Rarb* and *Rarg* are expressed in the periocular mesenchyme (POM), but not in the retina (31, 91). Therefore, RA signaling in the POM (a NCC-derived tissue) may be instrumental to the morphogenesis of the retina (a neuroectoderm-derived tissue). Using a somatic mutagenesis approach (reviewed in 97), we have recently demonstrated that selective excision of both *Rarb* and *Rarg* genes in POM precursor cells (*Rarb/g*$^{NCC-/-}$ mutants) recapitulates the eye defects generated upon germline ablation of the same receptor genes (Figure 2*k* and *l*). In POM cells, RXRα/RARβ and RXRα/RARγ heterodimers appear to control the extent of cell death involved in POM remodeling and the expression of *Foxc1* and *Pitx2* genes (98), which play crucial roles in the development of the anterior eye segment in mice and humans (99). Interestingly, the POM does not express any RA-synthesizing enzymes (RALDHs), and is therefore unable to synthesize RA. Instead the neural retina, the retinal pigment epithelium and the corneal ectoderm express RALDH1 and RALDH3 (98, and references therein). The fact that in mutants lacking both RALDH1 and RALDH3 (*a*) the activity of a RA-sensitive reporter transgene is abolished in the POM and (*b*) the ocular defects that are generated recapitulate those observed in *Rarb/g*-null mutants establishes that these two RA-synthesizing enzymes provide the RA required to activate RXRα/RARβ and RXRα/RARγ heterodimers in the POM. Therefore, in the developing eye, RA acts as a paracrine signal: It is synthesized by epithelial compartments (i.e., the retina, retinal pigment epithelium, and corneal ectoderm) but exerts its effect on the mesenchymal compartment (i.e., the POM). Conversely, the mesenchymal compartment appears to respond to the RA signal by synthesizing a yet unknown paracrine factor required for the growth of the ventral retina (98).

PHYSIOLOGICAL FUNCTIONS OF RETINOIDS CANNOT BE EXTRAPOLATED FROM THEIR TERATOGENECITY

Retinoid-Induced Teratogenesis Is Receptor Mediated

RA is a potent teratogen that, at pharmacological concentrations, can induce congenital defects in all vertebrate species, as well as in certain invertebrates. The nature and spectrum of RA-induced malformations depend on the dose and on the developmental stage at the time of exposure (100–102). Evidence that most of the teratogenic effects of retinoids are receptor-mediated has been inferred from (*a*) the comparison of the potency of synthetic retinoids to induce congenital defects with their ability to activate their cognate receptors and (*b*) from RA treatment of genetically modified animals overexpressing wild-type and dominant negative (dn) retinoid receptors, or carrying null mutations at *Rar* or *Rxr* loci. Synthetic

retinoids that are unable to activate RARs are nonteratogenic (103), whereas each RAR isotype-selective agonistic retinoid produces a distinct spectrum of congenital defects (104, 105). Overexpression of *Rarg* in *Xenopus* embryos potentiates the RA-induced loss of cranial structures, whereas expression of dn RARs triggers resistance to RA-induced malformations (106–108). Along the same lines, both *Rarg*- and *Rxra*-null mouse embryos are resistant to several RA-induced malformations (38, 109–111), whereas the genetic ablation of *Rarg* can restore the viability of mice lacking the RA-degrading enzyme CYP26A1 (112). However, the teratogenic effects resulting from administration of exogenous RA to embryos do not necessarily reflect the physiological roles of the retinoid receptors. This is clearly the case for the RA excess-induced lumbosacral truncation that is mediated by RARγ, which is dispensable for the normal development of the lumbosacral vertebrae (38).

In humans, oral intake of Accutane (13-cis RA) during gastrulation and early organogenesis (weeks 2–5 postconception) results in a spectrum of congenital malformations collectively referred to as the retinoic acid embryopathy (RAE) (113). It has been assumed that the BA defects observed in RA-exposed embryos at E8.0 and E9.0 in the mouse (i.e., the equivalent of weeks 4–5 postconception in humans), can account for alterations displayed in newborns (114, 115). Fusion and hypoplasia of the first two BAs, a hallmark of RAE, is generated in cultured E8.0 mouse embryos treated with BMS453, a synthetic compound exhibiting RARβ-selective agonistic properties. In contrast, no BA defects are observed following treatment with the synthetic retinoids BMS753 and BMS961 exhibiting RARα- and RARγ-selective agonistic properties, respectively (116). The BMS453-induced BA defects are potentiated in the presence of the panRXR agonist BMS649, which is not teratogenic on its own, and are accompanied by ectopic expression of *Rarb* and of several other direct RA-target genes in the morphologically altered region. On the other hand, BA defects and ectopic expression of RA-target genes cannot be induced in *Rarb*-null embryos treated with BMS453 (116). These data indicate that the craniofacial abnormalities characteristic of RAE are caused by an ectopic activation of RXR/RARβ heterodimers in which the ligand-dependent activity of RXR is subordinated to that of RARβ. Several other studies also indicate that a variety of RA-induced developmental defects are mediated through increased activation of RXR/RAR heterodimers (117, 118). However, the possibility that pharmacological concentrations of RA may cause cytotoxic effects by altering the structure of biological membranes cannot be excluded (119, and references therein).

Finally, it should be stressed that biological retinoids that are known to be teratogenic through their binding to RARs are not necessarily involved in physiological RA signaling during development. For example the RA metabolite 4-oxo-RA, which is produced in vivo by CYP26 enzymes (120, and references therein), is a potent teratogen (121, 122). However, genetic evidence indicates that CYP26-synthesized RA metabolites are not required for embryonic development (123).

The Teratogenic Effect of Retinoids on a Developing Structure Is Not Concomitant with the Physiological Effects of Endogenous Retinoic Acid

Several lines of evidence indicate that RA can be teratogenic at stages where it is normally absent from the embryo, and therefore cannot play any role in normal development at these stages. Indeed, the most sensitive period for the induction of malformations by retinoids in the mouse embryo corresponds to the presomitic stage of development (i.e., E6.5–E7.0), prior to the onset of embryonic RA synthesis in the embryo itself (i.e., at E7.5; 41). During this window of time, the doses of RA required to induce exencephaly and severe ocular abnormalities are approximately 50- to100-fold lower than those commonly required to observe teratogenic effects at later developmental stages (124). Along the same lines, exogenous RA can cause posterior limb duplications when administered at E5 (125, 126) and can affect in vitro the development of the blastocyst (127).

Different Mechanisms Underlie the Generation of Similar Malformations by Retinoic Acid Excess or Deficiency

Numerous malformations generated upon silencing RA-dependent pathways resemble those induced by excess RA. For instance, a CATCH22-like spectrum of defects is recapitulated in (*a*) *Rara/b*-null mutants (31, 39), (*b*) mutants bearing a hypomorphic allele for RALDH2 (128), and (*c*) mouse fetuses exposed to excess vitamin A (129). Such similarities have popularized the idea that both RA excess and RA deficiency generate defects through identical mechanisms. However, contrary to this common belief, the spectrum of malformations induced in the embryo by RA in excess and by defective RA signaling are far from being identical. There are many examples of congenital abnormalities generated upon administration of either RAR synthetic agonistic ligands or excess RA that are not duplicated in mice suffering from dietary-, pharmacologically, and genetically induced blocks in RA signaling (e.g., caudal regression syndrome, spina bifida, posterior limb duplications, undescended testis, fusions of teeth) (118,130–132). With respect to the early development of the oropharyngeal region, RA in excess and RA deficiency clearly do not disrupt identical mechanisms, as (*a*) exposure to excess RA at E8.0 yields a hypoplasia of the first two BAs (116), whereas antagonizing the RA signal induces hypoplasia of BAs 3 to 6, without altering the development of BAs 1 and 2 (48); (*b*) blocking RA signaling after E8.0 does not affect the formation of BA 3 (48), whereas hypoplasia of BA 3 is now induced by RA excess given at E9.5 (129); (*c*) RA excess, but not RA deficiency, alters RA signaling in the endoderm lining the first two BAs (48, 116); and (*d*) the deleterious effects of RA excess on the first two BA formations are specifically mediated by RARβ (116), whereas deleting *Rarb* does not affect BA development (31). These examples clearly indicate that similar malformations observed in situations of RA excess and deficiency are generated through disrupting distinct cellular and molecular mechanisms, and

they further support the view that the physiological functions of RA cannot be extrapolated from results of teratogenic studies using RA in excess.

CONCLUSIONS AND PERSPECTIVES

The phenotypic analyses of mutants lacking retinoid receptors have provided compelling evidence that RA is in fact the metabolite of vitamin A that is active during early embryogenesis and organogenesis, as well as postnatally. This conclusion was subsequently strengthened by the demonstration that RA, synthesized by the retinaldehyde dehydrogenases (RALDH1, RALDH2, and RALDH3), acts as an indispensable developmental hormone (41–47, 98, 128, 133). Furthermore, the results from genetic and pharmacological studies conducted in the mouse have conclusively established that (*a*) the molecular mechanisms underlying transduction of the RA signal by retinoid receptors that were suggested from in vitro studies (see What Have We Learned About Retinoid Receptor Functions from In Vitro Studies?, above) are instrumental to retinoid signaling under physiological conditions; (*b*) the teratogenic effects resulting from administration of exogenous RA to embryos do not reflect the physiological role of endogenous RA in the corresponding developmental processes.

RXRα/RAR heterodimers are clearly the main functional units transducing RA signals during development, and specific heterodimers are involved in given developmental processes. This strongly supports the initial proposal that the pleiotropic effects of RA reflect sophisticated combinatorial mechanisms, through which multiple RXR/RAR heterodimers differentially transduce retinoid signals to selectively control the expression of numerous sets of RA target genes (13). Second, in vivo the RXR partner can be either transcriptionally active (thus acting in synergy with its RAR partner) or inactive within RXR/RAR heterodimers, depending on the developmental event under consideration. Third, the transcriptional activity of RXR is subordinated to ligand binding to the RAR partner in vivo (116, 118, 134), as is the case in cultured cells in vitro (15, 135, 136), and when RXRα is transcriptionally active, either one or both activation functions (AF-1 and AF-2) can be involved, their activity depending on the nature of the RA-controlled developmental event (32, 83).

The genetic studies summarized in this review have revealed an extensive functional redundancy within the members of each family (RARs or RXRs), although each of these members appears to individually exert at least one specific physiological function. Because the members of each family share a common ancestor (29), such a redundancy is not surprising. However, it raises the question as to whether redundancy is physiologically relevant or artifactually generated when a given receptor is missing (15, 137, 138). In this respect, note that the existence of two fully redundant genes is, in an evolutionary sense, unlikely (139, 140). It is also noteworthy that the occurrence of a given morphological defect in *Rar* double-null mutants when contrasted with its absence in *Rar* single-null mutants should not

be taken as definite proof of a cell-autonomous functional redundancy. Another possible explanation could imply the action of distinct RARs in different tissues, which may independently direct the making of a given structure. Such a possibility may apply to the case of the interdigital soft tissue, which involutes normally in all *Rarb*- and in almost all *Rarg*-null mutants but persists in all *Rarb/g*-null mutants yielding webbed digits (Tables 1 and 3; see The AF-1-Containing A/B Domain and the Ligand-Dependent AF-2 of RXRα Are Differentially Involved in Development, above). In this instance, a functional cell-autonomous redundancy between RARβ and RARγ is hardly conceivable, as *Rarb* and *Rarg* exhibit nonoverlapping expression patterns in the limbs (31).

Importantly, there is much less functional redundancy in RXRα/RAR compound mutants (Tables 2 to 4 and Figure 2). As RXRα/RAR heterodimers are the functional transducing units, the easiest way to interpret these observations is to postulate that redundancy occurs only when a single partner of the physiological heterodimer is missing (30, 32, 83). In other words, the activity of the alternative heterodimer may still be above the functional threshold level when either the RXR or the RAR partner of the physiological heterodimer is missing, but not when both are deleted. According to this interpretation, the selective involvement of a given RAR or RXR could be revealed only under conditions where the functional threshold level is not reached. This would notably account for the observations that the role of the RXRα AF-1 or AF-2 cannot be fully revealed, unless the activity of the physiological heterodimer is altered by the additional ablation of either the RAR partner or the redundant RXR isotypes (32, 83). Thus, any conditions that would lower the activity of RXR/RAR heterodimers (for instance, a decreased availability of RA) may reduce or abrogate functional redundancy. As the supplies in vitamin A, the precursor of RA, could be more limiting in animals living in the wild than those living in the context of an animal facility, the functional redundancies between RAR and RXR may therefore be much less prominent in natural environments.

Quite surprisingly, RXR loss-of-function mutants do not display defects in morphogenesis other than those observed in the fetal VAD syndrome or upon ablation of the RA-synthesizing enzymes. This suggests that RXRs are involved in morphogenesis solely through their heterodimerization with RARs. Accordingly, mice harboring null mutations for the other NRs dimerizing with RXRs and for which a ligand is known (i.e., PPARα, PPARβ, TRα, TRβ, VDR, LXRα, LXRβ, FXR, PXR, CAR) do not display morphological abnormalities (141–149), with the exception of PPARγ-null mice (150). In this latter case, the embryonic heart defect is clearly secondary to a severe placental hypoplasia (150). Thus, among the multiple hormone-like signals that RXR/NR heterodimers can integrate, RA appears to be the most crucial, if not the only, one involved in morphogenesis of the embryo proper.

The genetic approach summarized in this review has provided valuable insights on the functions of RA receptors during development. However, this strategy has intrinsic limitations, which are mostly due to the introduction of the mutations in the

germ line. First, the effect of a germline mutation may be functionally compensated for during development, thus precluding the appearance of a defect. On the other hand, the mutation can be lethal in utero (e.g., the *Rxra* knockout and the RAR compound null mutants), thus preventing analysis of the functions of the gene at postnatal stages. The mutation can also arrest the development of a given organ at an early stage, thus precluding further analysis of gene functions at a later stage. Moreover, introducing mutations in germline makes it difficult to distinguish cell-autonomous from non-cell autonomous functions of genes belonging to families, such as RARs and RXRs, that are involved in pleiotropic signaling pathways. In many instances, these limitations may actually prevent the determination of the function of a given gene product in a defined cell-type/tissue and/or at a given time of the animal life. This is obviously the case for RARs and RXRs.

To overcome all these limitations, strategies for spatio-temporally controlled somatic mutagenesis of RARs and RXRs in mice have been designed that are based on the cell-type-specific expression of an inducible form of the Cre recombinase (called Cre-ER^{T} and Cre-ER^{T2}; reviewed in 97, 151). Using this innovative approach, the selective ablations of *Rxra* and both *Rara* and *Rarg* genes in keratinocytes of adult mice has shown that RXRα has key roles (*a*) in hair cycling, probably in heterodimer with VDR, (*b*) in the homeostatic maintenance of epidermal keratinocytes in heterodimer with NRs other than RARs (152, 153), and (*c*) in pharmacologically RA-induced proliferation of epidermal keratinocytes in heterodimer with RARγ through controlling the production of a paracrine signal in the suprabasal layers (154). The same approach allowed us to demonstrate that in adult mice, RXRα (*a*) is involved in preadipocyte differentiation, adipogenesis, and survival of mature adipocytes in heterodimer with PPARγ (155, 156), and (*b*) plays important cell-autonomous functions in liver regeneration and in mechanisms controlling the lifespan of hepatocytes (157). These examples show that the combined use of transgenic mice expressing chimeric tamoxifen-inducible Cre recombinases in specific cell types and of mouse lines harboring loxP-flanked conditional alleles for RAR and RXR genes will provide invaluable models to elucidate the postnatal functions of retinoid receptors.

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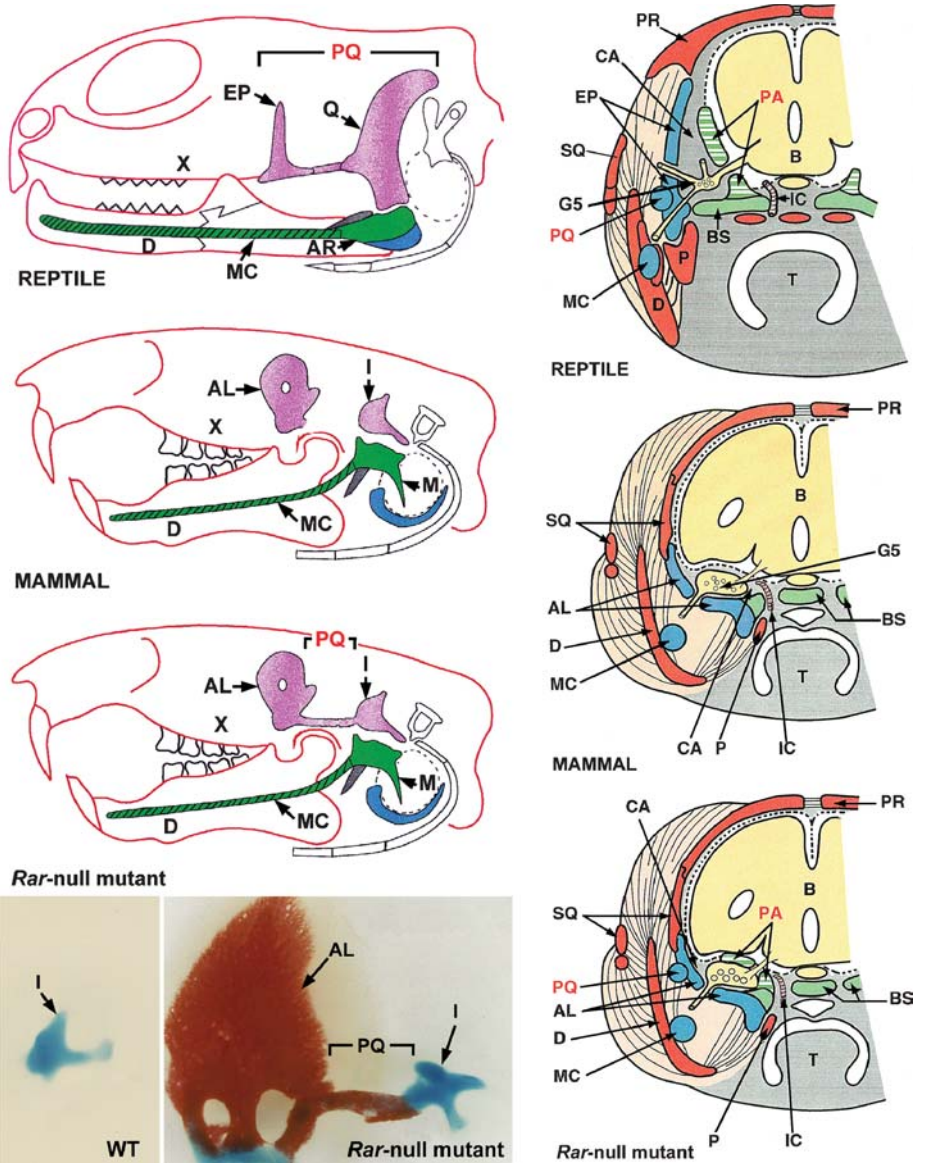

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Figure 1 *Rar*-null mutant mice develop atavistic skeletal elements. Left panel, top: Schematic representations of reptilian, normal mammalian, and *Rar*-null mutant jaws. In reptilian fetuses, the quadrate (Q) and epipterygoid (EP) bones develop from a single cartilaginous anlage, the pterygoquadrate (or maxillary) cartilage (PQ), whereas the mammalian homologs of these two bones, the alisphenoid (AL) and incus (I), always chondrify separately. The connection between the rostral and caudal derivatives of the pterygoquadrate cartilage (i.e., epipterygoid/alisphenoid and quadrate/incus, respectively) was lost at some stage of the reptilian–mammalian transition. Skeletal elements belonging to the reptilian upper jaw and to their (normal and mutant) mammalian derivatives are in pink, whereas their counterparts in lower jaws are in green, blue, and grey. Left panel, bottom: Photographs of an incus (I) from a wild-type (WT) mouse and of an incus from a *Rar*-null mutant mouse with its abnormal connection to the alispenoid bone (AL) via an ossified pterygoquadrate cartilage (PQ). Right panel: Schematic representations of frontal sections through the head of a reptile, of a normal mammal, and of a Rar–null mutant mouse. The reptilian pila antotica (PA) forms a medial wall to the cavum epiptericum (CA), an extracranial space that lodges the trigeminal ganglion (G5). The pila antotica was lost during the reptilian–mammalian transition, presumably to accommodate the expansion of the brain. Green and blue on the right panel represent cartilages or endochondral bones, and orange represents membrane bones. Abbreviations: AL, alisphenoid bone; AR, articular bone; B, brain; BS, basisphenoid bone; CA, cavum epiptericum; D, dentary bone; G5, trigeminal ganglion; I, incus; IC, internal carotid; MC, Meckel's cartilage; P, pterygoid bone; PA, pila antotica; PQ, pterygoquadrate cartilage; PR, parietal bone; SQ, squamosal bone; T, tongue; X, maxillary bone. For further details see References 31, 37, and 52.

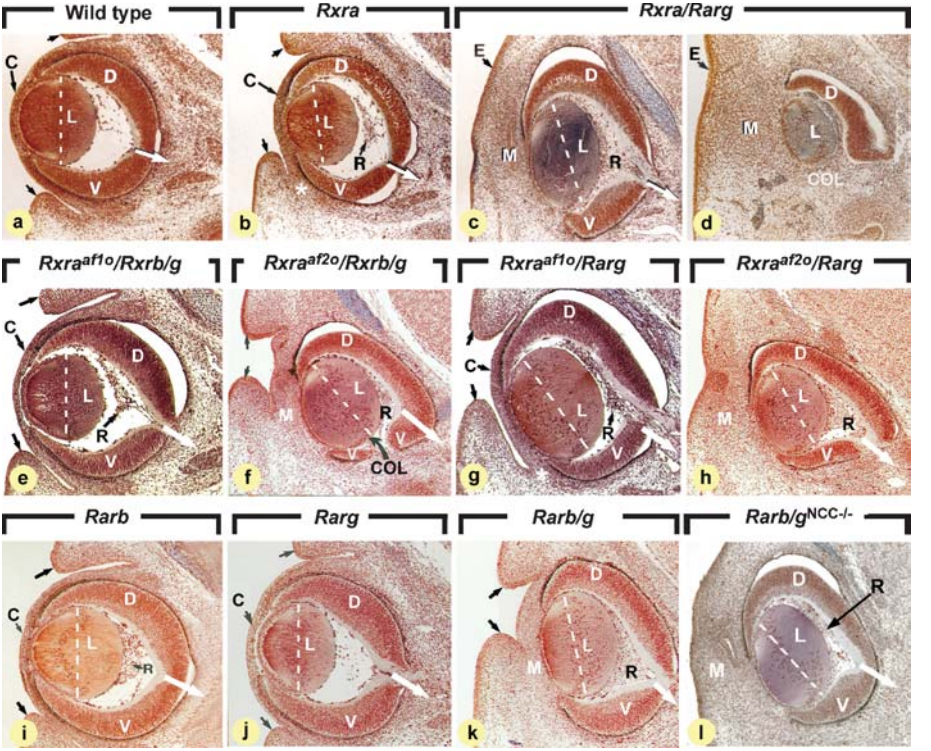

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Figure 2 Effects of *Rar* and *Rxra* loss-of-function mutations on eye morphogenesis at E14.5. (*a*) Histological section through a wild-type eye. (*j*) Eyes from *Rarg*-null mutants appear histologically normal, and (*i*) eyes from *Rarb*-null mutants and from (*e*) *Rxra*^{af1o}*/Rxrb/g*-null mutants display no other defect than a persistent and hyperplastic vitreous body (R, retrolenticular membrane). (*b*) In contrast, all fetuses lacking *Rxra* show a characteristic ocular syndrome associating a persistent and hyperplastic vitreous body (R), closer eyelid folds (*small black arrows*), thickened ventral portion of the corneal stroma (*white asterisk*), ventral rotation of the lens (L and *dotted line*), and a shorter ventral retina (V). (*f*) A severe form of the *Rxra*-null ocular syndrome is observed in mutants lacking all RXR AF-2 transcriptional activities (*Rxra*^{af2o}*/Rxrb/g*-null mutants) thereby suggesting the requirement of an RXR ligand for eye morphogenesis. (*c*, *d*, *g*, *h*) RXRα acts on eye morphogenesis in the form of heterodimers with RARγ (and with RARβ, not illustrated), as there is a strong synergism between *Rxra* loss-of-function mutations and *Rarg* inactivations that is manifested by a marked increase in the severity of the Rxra-null ocular defects in (*c*, *d*) *Rxra/Rarg*-null, (*g*) *Rxra*^{af1o}*/Rarg*-null, and (*h*) *Rxra*^{af2o}*/Rarg*-null fetuses. This synergism also supports the view that the activation functions of RXRα are required for normal eye development, and that, due to functional redundancy, the role played by RARαAF-1 and AF-2 can be revealed only in certain impaired genetic backgrounds, and perhaps in RA insufficiency conditions. (*k*) The severe form of the *Rxra*-null ocular syndrome observed in mutants lacking both Rarb and *Rarg* (*Rarb/g*-null mutants) indicates that these receptors are functionally redundant for eye morphogenesis. (*l*) This functional redundancy appears to be cell autonomous as the ablation of the *Rarb* and *Rarg* genes only in neural crest cells (NCC) duplicates this ocular phenotype (*Rarb/g*$^{NCC-/-}$ mutants). C, cornea; COL, retinal coloboma; D, dorsal portion of the retina; L, lens; M, abnormal mesenchyme partially replacing the corneal stroma and the eyelids; R, retrolenticular membrane; V, ventral portion of the retina. The large white arrow indicates the position of the optic nerve exit point. The small black arrows point to the eyelid folds. The white asterisks indicate the thickened ventral portion of the corneal stroma. For further details see References 31, 32, 76, 83, and 98.

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