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Developmental Roles of the Retinoic Acid Receptors

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Retinoic acid, one of the principle active metabolites of vitamin A (retinol), is believed to be essential for numerous developmental and physiological processes. Vitamin A deprivation (VAD) during development leads to numerous congenital defects. Previous studies of retinoic acid receptor (RAR) deficient mice failed to reveal any of these VAD-induced defects. This finding suggested that either the RARs are functionally redundant or that they are not critically required during development. In order to address these possibilities, we derived a number of RAR compound mutants. Unlike RAR single mutants, these compound null mutants died either *in utero* or shortly following birth. Histological analysis revealed essentially all of the defects characteristic of fetal VAD. A number of additional malformations, not described in previous VAD studies, were also observed. These included defects of the ocular and salivary glands and their ducts, the skeletal elements of the fore- and hindlimbs, and the cervical region of the axial skeleton. In addition, with the exception of derivatives forming within the first pharyngeal arch, most of the elements derived from mesectoderm emanating from cranial and hindbrain levels were affected. A number of these mutants also exhibited supernumerary cranial skeletal elements characteristics of the reptilian skull. A summary of the defects found in these RAR double mutants is presented.

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INTRODUCTION

Retinoids (compounds with vitamin A activity) play essential roles in vertebrates. Evidence for this was first presented in the early part of this century when it was found that dietary vitamin A deprivation (VAD) results in growth retardation, blindness, sterility and eventual death [1, see ref. 2 for review]. The most characteristic feature of VAD animals is widespread squamous metaplasia of various epithelia (e.g. corneal epithelium, ocular glands, the respiratory tract, urogenital tract and alimentary tract). Further dietary studies showed that vitamin A was also required during development, as fetuses from VAD females exhibited a characteristic spectrum of congenital malformations [3–7]. A partial list of affected organs includes the heart and aortic arches, urogenital tract, respiratory tract, diaphragm and the eye. Addition of retinol to the diet of VAD animals reversed nearly all of these post-partum or congenital malformations, offering further proof that vitamin A was indeed the dietary factor essential for normal development, physiology and homeostasis [6, 8]. Interestingly, the gestational stage of retinol administration was a major determinant as to which VAD-induced congenital malformation was prevented, indicating that vitamin A is required at several distinct stages of development.

The discovery that retinoid excess also caused dysmorphogenesis further implicated retinoids as key developmental factors [9–12]. Retinoic acid (RA), the carboxylic acid derivative of retinol, was found to be a much more potent teratogen than retinol yielding the first clue that RA was the biologically active form of the vitamin. This was further supported by the finding that

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post-partum VAD-induced defects could be prevented or reversed by exogenous RA (with the exception of vision; [8, 13]). However, apart from prevention of aortic arch defects in the quail embryo [14], similar rescue of fetal VAD-induced defects by RA has not been reported.

RA excess induces specific developmental defects, the precise nature depending on the developmental stage of exposure [reviewed in ref. 15]. Given the short half-life of RA *in vivo* [16], a simplistic interpretation of this finding is that these teratogenic effects are reflecting normal retinoid-dependent events. Indeed, based largely on the effects of topical RA administration to the developing chick limb bud [17, see ref. 18 for review], RA has been championed as a morphogen. Although this concept has been challenged [19, 20], the effects of exogenous RA on either development or on tissue culture systems are often correlated with alterations in the level and/or pattern of expression of specific genes, many of which possess retinoic acid response elements (RAREs) in their promoter regions [21–30]. These observations strongly suggest that RA, through its receptors (see below), directly regulates the expression of certain genes essential for normal development.

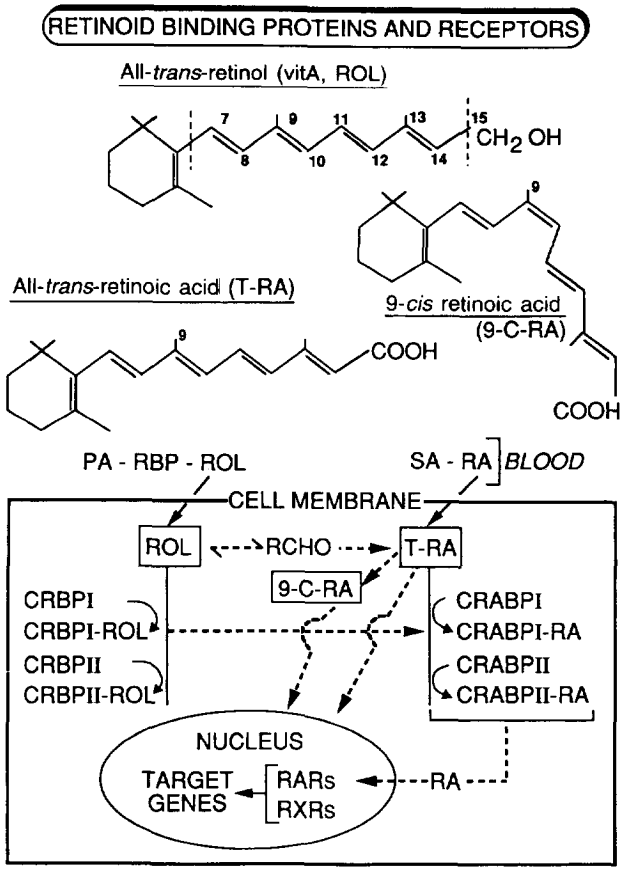

Fig. 1. A schematic representation of the major components of the retinoid-signaling pathway. CRBP, cellular retinol-binding protein; CRABP, cellular retinoic acid-binding protein; RBP, retinol-binding protein; RAR, retinoic acid receptor; RXR, retinoid X receptor.

As illustrated in Fig. 1, two classes of retinoid-binding proteins have been implicated in this signaling pathway. First, the cellular retinoid-binding proteins, which include cellular retinol-binding proteins I and II and cellular retinoic acid-binding proteins I and II. The role of these proteins is uncertain, but they may be involved in retinoid storage, metabolism and the regulation of the free level of biologically active retinoids within a given cell [reviewed in 31]. The second class of retinoid-binding proteins is encoded by two multigene families; the retinoic acid receptors (RARα, β and γ) and the retinoid X receptors (RXRα, β and γ). Both families encode ligand-inducible trans-regulators belonging to the nuclear receptor multigene superfamily [reviewed in 32–37]. RARs can be efficiently activated by low concentrations of two endogenous retinoids, RA or 9-*cis* RA, whereas RXRs are efficiently activated by 9-*cis* RA only [38,39]. Although this observation initially suggested divergent retinoid signaling pathways, more recent studies have shown that most retinoid-responsive genes appear to be regulated by RAR:RXR heterodimers, at least in tissue culture models [40–44; see refs 32–37 for review].

The discovery of murine RAR isoforms (RARα1 and 2, RARβ1–4, RARγ1 and 2; refs 45–48) revealed an additional level of complexity to the retinoid signaling pathway (Fig. 2). These isoforms are derived by differential promoter usage and alternative splicing, and share a common motif with each isoform for a given RAR type diverging only in the 5′ untranslated and A region. Furthermore, the expression of the second of each RAR isoform (i.e. RARα2, β2 and γ2) is modulated by RA through RAREs present in the promoter regions of each of these genes, suggesting a further level of interactive signaling in this transduction pathway.

As for RAR types, interspecies sequence comparisons of each RAR isoform reveals a strong degree of homology, including the N-terminal variant A-region. Since transcriptional activation functions (AFs) have been ascribed to the A/B and E regions of the RARs, and these AFs can function on certain RA-responsive promoters in a differential manner [49, 50], the strong conservation of the A region of the RAR isoforms suggests the retention of critical transcriptional properties. Specific roles for each of the RARs are also implied by the characteristic expression domains for each receptor type both during development and in the adult [51–55; reviewed in 32]. Taken together, the above results suggest that, if RA-dependent transcriptional regulation *in vivo* is dependent on RAR:RXR heterodimers, the pleiotropic effects of retinoids may reside, at least in part, in the regulated expression of different heterodimeric receptor combinations, as well as the availability of free ligand(s) within a given cell [see refs 32, 36 for review].

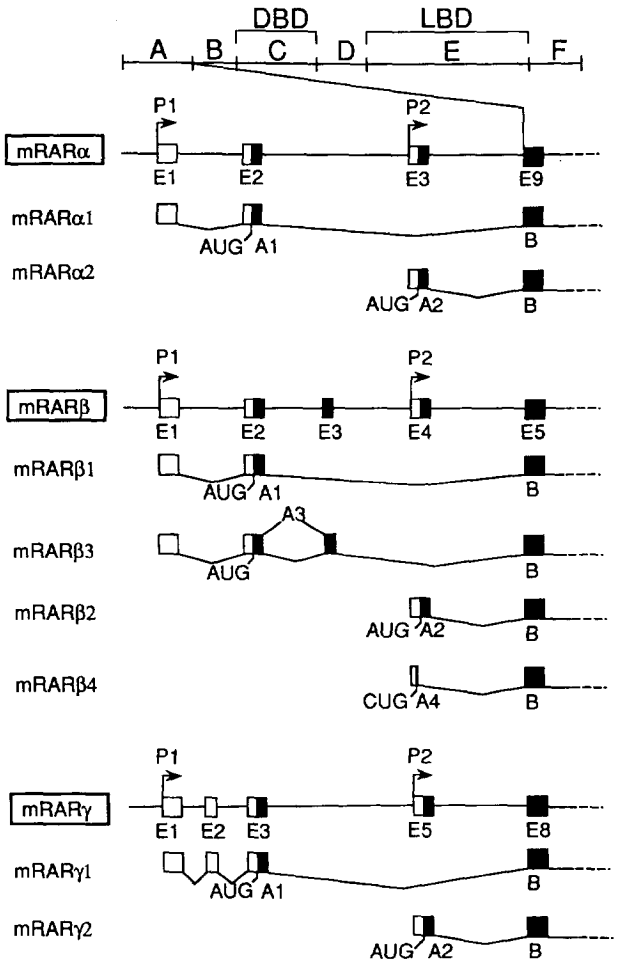

Fig. 2. Representation of the 5′ region of the three mouse RAR genes and their major isoforms. Exons (E) are denoted by boxes and are numbered as in refs 45, 46 and 48. Open boxes denote 5′ untranslated sequences and filled boxes indicate translated sequences. For a given isoform, the numbering A1, A2, A3, A4 and B represent the A1, A2, A3, A4 and B regions of the receptor isoforms. For each receptor, P1 and P2 correspond to the promoters directing the expression of the different isoforms.

RAR ISOFORM-SPECIFIC DISRUPTION

In order to begin to understand the role of each RAR type and isoform, we, and others, have used gene targeting in embryonic stem cells [56] to derive a number of different mouse lines in which a given RAR has been inactivated. Mice null for the RAR isoforms RARα1, β2 or γ2 appear normal, are fertile and are apparently of normal longevity [57–60]. Although subtle changes in expression or post-transcriptional events cannot be ruled out, this lack of a phenotype cannot be explained by compensation through altered expression of the other RARs, as *in situ* analysis and/or RNAase protection studies indicate that there is no gross alteration in the pattern or level of expression of these transcripts. It therefore appears that, despite the circumstantial evidence discussed above, these RAR isoforms do not play a specific, critical, role in the mouse. Since disruption of all isoforms of a given RAR type results in a phenotype (see below), it appears, however, that at least one isoform of that receptor type must be expressed for normal development or physiology. It should also be stressed that the strong conservation of these isoforms during evolution implies a specific function [61]. It is therefore probable that these 'unaffected' mutants are indeed compromised, but that the phenotype is of a subtle nature or does not readily manifest itself in the laboratory environment.

RAR TYPE DISRUPTIONS

In contrast to the lack of a phenotype following disruption of a given RAR isoform, disruption of all isoforms of either RARα or RARγ resulted in phenotypic alterations [58, 59]. Consistent with dietary studies, these mutants recapitulate some of the aspects of postpartum VAD, including a high degree of neonatal mortality, poor weight gain, and male sterility. In the case of RARα null mice, this sterility is caused by degeneration of the testicular germinal epithelium. This is one defect which is difficult to reverse by exogenous RA (but not retinol) administration to VAD males [8, 13, 62], which led to the suggestion that the maintenance of this epithelium may reflect a specific requirement for retinol. The manifestation of this pathology in RARα null animals leaves little doubt that it is in fact RA which is required for the maintenance of this tissue, and that the ability of retinol to reverse this VAD-induced pathology is likely due to a blood–testis barrier which is permeable to retinol, but not RA. RARγ null males were also found to be sterile due to squamous metaplasia of the seminal vesicles and prostate glands, again a feature of postpartum VAD. RARγ mutants also exhibited two congenital malformations, agenesis of the stroma of the Harderian glands and malformations of the axial skeleton, which occurred with variable penetrance and expressivity. The latter defects included several homeotic vertebral transformations, one of which (anterior transformation of the second cervical vertebra to a first vertebral identity) is remarkably similar to vertebral transformations seen in Hoxb-4 deficient mice [63]. This observation supports the growing body of evidence suggesting that RARs directly regulate Hox gene expression during development (see below).

RAR DOUBLE MUTANTS

Although RARα and γ mutants exhibit a subset of postnatal VAD-like abnormalities, none of the RAR single mutants examined to date present any malformations related to the fetal VAD syndrome. This observation suggests that either the RARs are not essential transducers of the retinoid signal as related to these VAD-induced defects, or that there is considerable functional redundancy among the members of this

receptor family. In order to address these possibilities, the single null mutants discussed above were interbred to derive mice lacking different combinations of RARs. For the sake of brevity, only a subset of the defects characterized in these mutants is described in any detail here. A more extensive description of these animals is to be found in the original articles [64, 65].

RAR double mutants and the fetal VAD syndrome

The first indication that the RARs may be functionally redundant came with the observation that all double mutants examined died within, at most, 12 h following delivery by cesarean section at full term. This is in contrast to RAR single mutants which, like wild-type animals, survived for up to 24 h in isolation. In the case of RAR$\alpha\gamma$ mutants (the nomenclature used denotes the genes which are inactivated, thus RAR$\alpha^{-/-}/\gamma^{-/-}$ animals are indicate simply as RAR$\alpha\gamma$ mutants), approx. 50% of the mutant embryos died at variable times during embryogenesis. Subsequent histological analysis showed that, with the exceptions of a shorter ventral retina and pseudohermaphrodism, the congenital malformations characteristic of fetal VAD were completely reproduced among the various RAR compound mutants (Table 1). These findings present convincing evidence that it is RA, acting through the RARs, that is the essential retinoid signal during development.

Non-VAD defects

In addition to the malformations typical of VAD, RAR double mutants also exhibited numerous defects not previously described in such dietary studies (Table 2). This may be due to the fact that inactivation of all RARs (by dietary deprivation) results in embryonic lethality and subsequent resorption of the conceptuses, as reflected by the death *in utero* of 50% of RAR$\alpha\gamma$ mutants. It would appear that the classic pathology associated with fetal VAD reflects a constellation of defects representing non-lethal lesions in RA-dependent processes which are more sensitive to vitamin A deprivation. It is also probable that not all RA-dependent processes have been revealed by these RAR null mutants, since; (a) the role of RARβ (all isoforms) has not yet been determined; and (b) given the apparent redundancy among these receptors, it is likely that simultaneous inactivation of all RARs will be required to reveal the full extent of their function. However, apart from the substantial breeding program that this would entail, it is likely that the resulting triple mutants would yield little additional information as, given the partial embryonic lethality observed for RAR$\alpha\gamma$ mutants, they would probably die during early development. Alternate strategies, using tissue-specific conditional knockouts and restricted expression of dominant-negative RARs, could be employed to further elucidate the roles played by this signaling network.

Table 1. VAD-related defects observed in RAR compound mutants

	RAR mutant					
Defect	$\alpha 1\beta 2$	$\alpha\beta 2$	$\alpha 1\gamma$	$\alpha 1\gamma\alpha 2^{+/-}$	$\alpha\gamma$	$\beta 2\gamma$
Eye						
Coloboma	−	−	−	−	+ +	+
Retrolenticular membrane	+	+ +	−	−	+ +	+ +
Others*	−	−	−	+ / −	+ +	+
Respiratory tract	+ / −	+ +	−	−	−	−
(Lung agenesis or hypoplasia; lack of tracheal–oesophageal septum)						
Heart and aortic arches						
PTA	+ / −	+ +	−	−	+ +	−
Spongy myocardium	−	−	−	−	+	−
Abnormal aortic arch pattern	+ / −	+ +	−	+ / −	+ +	−
Ventricular septal defect	+	+ +	−	+ / −	+ +	−
Kidney and ureter defects						
Renal hypoplasia	+	+	−	+ / −	−	−
Hydronephrosis	+	+	−	−	−	+ / −
Ureter agenesis or ectopia	+	+	−	−	+	−
Genital tract abnormalities†						
Female	+	+	−	−	+	−
Male	−	−	−	+ / −	+	−
Diaphragmatic hernia	−	+ / −	−	−	−	−

*Additional ocular defects included: unfused eyelids, small conjunctival sac, corneal-lenticular stalk, abnormal corneal stroma, abnormal lens fibers, absence of the conjunctiva, cornea, anterior chamber or lens.

†Female: partial or complete agenesis of the uterus, agenesis of the cranial vagina. Male: agenesis or dysplasia of the vas deferens, agenesis of the seminal vesicles.

See refs. [64] and [65] for additional details.

Table 2. Non-VAD-related defects in RAR compound mutants

Defect	RAR mutant					
	$\alpha 1\beta 2$	$\alpha\beta 2$	$\alpha 1\gamma$	$\alpha 1\gamma\alpha 2^{+/-}$	$\alpha\gamma$	$\beta 2\gamma$
Craniofacial defects (Agenesis, dysplasia and ectopias)	−	−	−	+/−	++	−
Hyoid bone defects	+/−	++	−	−	++	−
Laryngeal cartilage defects	+/−	++	+	++	++	+/−
Ectopic cartilage (Semilunar valves, diaphragm, peritoneum)	+	−	−	−	−	+
Thymus, thyroid, parathyroid defects*	+/−	+	−	+/−	+	+/−
Kidney (agenesis, aplasia)	−	−	−	−	+	−
Absence of anal canal	−	+	−	−	−	−
Glandular defects†	−	−	+	+	++	−
Exencephaly	−	−	−	−	+	−
Atavistic skull features	+/−	+	+/−	+	++	−
Vertebral homeotic transformations and malformations	−	+	+	++	+++	+/−
Limb malformations						
Forelimb	−	−	−	−	++	−
Hindlimb	−	−	−	+/−	++	−

*Hypoplasia and/or ectopias.

†Agenesis or shortening of the nasolachrymal, sublingual or submandibular ducts. Agenesis or dysplasia of the Harderian, sublingual or submandibular glands.

Data from refs. [64] and [65].

RARs and limb development

The developing limb bud is under the control of at least two regions; the zone of polarizing activity (ZPA) which specifies the anteroposterior axis, and the apical ectodermal ridge (AER) which directs limb bud outgrowth [see 18 for review]. A role for retinoids in limb patterning was first suggested by the finding that topical application of RA could mimic the effect of the ZPA on anteroposterior limb specification, including altered expression of Hox genes believed to be essential for normal limb morphogenesis [reviewed in 18, 66, 67]. More recently, it was found that RA, together with FGF-4, can fulfill most of the functions of the AER [68]. Although the initial concept of RA as a morphogen has been refuted [19, 20], it is possible that RA may indirectly influence limb patterning by generating a functional ZPA, possibly through the regulation of expression of a secreted protein, sonic hedgehog, which itself has at least some of the properties anticipated for a limb morphogen [69].

Since both RARα and γ transcripts are uniformly expressed in the early stage mouse limb bud [51], we previously speculated that the absence of limb defects in these RAR single null mice was either due to the lack of a role for RA in normal limb bud development or due to functional redundancy between these two receptor types [58, 59]. The latter hypothesis is clearly the case, as limbs from RAR$\alpha\gamma$ double mutants consistently exhibited malformations (Table 3). However, the nature of these defects does not appear to reflect the perturbation of early limb morphogenesis, as all proximal limb skeletal elements (notably the humerus) appeared unaffected. This could be due to compensation by RARβ, since RARβ transcripts have been detected in the flanking mesoderm and in the proximal limb bud in a region which overlaps the ZPA [51, 70]. Furthermore, the AER appeared histologically normal in RAR$\alpha\gamma$ mutants. Again, it is possible that RARβ may fulfill normal RA-dependent processes in the AER, since RAR$\beta 2$ promoter sequences can direct expression to this tissue [70]. Finally, consistent with the lack of a definitive effect on early limb bud patterning, the expression of several markers of ZPA and AER activity, including Hoxd-9 and d-11, MSX-1, BMP-2, FGF4 and sonic hedgehog [67–69, 71–73], appeared unaltered in RAR$\alpha\gamma$ mutant limb buds [64; our unpublished results].

Although RAR$\alpha\gamma$ mutants exhibited two putative digit transformations [see ref. 64 for details], the low frequency and variability of these defects precludes any firm assessment of the role RA plays regarding limb specification. However, RARα and γ are clearly essential for the realization of some forelimb elements. It is noteworthy that malformed elements were found essentially on the preaxial (anterior) portion of the forelimb. In the mouse, as with all tetrapods, limb skeletal elements arise in a proximal-distal fashion through a series of branching and segmentation events arising from prechondrogenic blastemae formed during limb outgrowth [reviewed in 74, 75]. The proximal and distal carpal bones are derived from branching events initiating at the ulna, accounting for the presence of these structures despite the agenesis of the radius observed in some RAR$\alpha\gamma$ mutants (Table 3). The

digital arch, and subsequent digit formation, generally proceeds in a posterior to anterior fashion, with digit 1 and the prepollex forming last. Thus, the agenesis of preaxial structures in RAR$\alpha\gamma$ mutants (i.e. radius, central and D1 carpals, digits 1 and 2 and the prepollex; Table 3) suggests that the final branching event giving rise to these structures was affected. This may be due to insufficient limb mesenchyme, since similar preaxial malformations can be elicited by experimental reduction in the quantity of this mesenchyme [76]. As discussed previously [77], such a deficiency can also allow supernumerary digit anlagen to arise in the limb field, consistent with the finding of one polydactylous RAR$\alpha\gamma$ mutant forelimb (Table 3). In marked contrast to forelimb malformations, RAR$\alpha\gamma$ mutant hindlimbs never exhibited a loss of preaxial skeletal elements, but did display a consistent malformation of a postaxial derivative, the tibia (Table 3). This suggests that either RARα and γ have different roles in fore vs hindlimb development, or that events related to the different time of appearance of the two limbs allow phenotypic rescue of preaxial hindlimb elements. It should be noted that intrinsic differences in the mesenchyme of the fore and hindlimbs have been observed [78], suggesting that these tissues may respond to RA *in vivo* in a different manner. Finally, transgenic mice expressing the Hoxb-8 gene under the control of RARβ2 regulatory sequences exhibit limb malformations restricted to the forelimbs, suggesting either that the Hoxb-8 gene plays different roles in the two limb buds, or that the spatial distribution of the RARs differs in the two limb fields [79].

RARs and axial patterning

We previously reported malformations of the axial skeleton which occur in RAR$\gamma^{-/-}$ offspring [58]. The variable penetrance and expressivity of these defects suggested functional redundancy amongst the RARs. Analysis of double null mutants indicates that this is likely the case, as essentially all of these axial defects were increased in a graded manner with subsequent loss of RARα1 and α2 alleles from the $\gamma^{-/-}$ background (Table 4). It should be noted, however, that concomitant inactivation of all RARα and γ isoforms resulted in severe degeneration of the cervical vertebrae, precluding assessment of most of these transformations [see ref. 64 for details]. RARβ2 also appears to play a role in axial patterning, since RAR$\alpha\beta$2 (but not RARα) mutants displayed a high frequency of anterior transformations of the sixth and seventh vertebrae.

Circumstantial evidence strongly suggests that these vertebral malformations may arise through altered expression of some Hox genes. Results from tissue culture studies indicate that RA may directly

Table 3. Limb malformations in RAR$\alpha\gamma$ double null mutants

		18.5 dpc mutant features						
		1	2	3	4	5	6	Total
Malformation of the scapula	L	+	+	ND	+	+	+	5
	R	+	+	ND	+	+	+	5
Agenesis of the radius	L	·	+				+	2
	R	+			+			2
Malformation of the scapholunatum	L	+	+	+	+	+	+	6
	R	+	+	+	+	+	+	6
Agenesis of the D1 carpal bone	L		+			+	+	3
	R			+	+			2
Agenesis of the central carpal bone	L	+	+		+	+	+	5
	R	+	+	+	+	+	+	6
Prepollex agenic or rudimentary	L	+	+		+	+	+	5
	R	+		+	+	+		4
6 Digits*	L							0
	R					+		1
5 Digits	L	+		+				2
	R		+				+	2
4 Digits†	L		+		+	+		3
	R	+		+	+			3
3 Digits‡	L						+	1
	R							0
Malformation of the tibia	L	+	+	+	+	+	+	6
	R	+	+	+	+	+	+	6

ND; not determined.

*Additional presumptive first digit; †loss of presumptive first or second digit; ‡loss of presumptive first and second digit.

L and R, left and right limb, respectively. 1–6, correspond to the different fetuses which were examined. Total indicates the total number of limbs which exhibited a given malformation. Defects were confined to the forelimb with the exception of malformation of the tibia. Data from ref. [64].

Table 4. Axial skeletal malformations in RAR double mutants

	Genotype and number of 18.5 dpc mutant fetuses examined						
	RARα (21)	RARγ (29)	RARα1γ (16)	RARα1γα2$^{+/-}$ (11)	RARαγ (6)	RARβ2γ (9)	RARαβ2 (10)
Number of mutants with abnormal skeletons	4 (19%)	25 (86%)	16 (100%)	11 (100%)	6 (100%)	7 (78%)	10 (100%)
Abnormalities							
Homeotic transformations							
C2 to C1	1 (5%)	5 (17%)	5 (31%)	7 (64%)	NA	1 (11%)	2 (20%)
C6 to C5	0	4 (14%)	4 (25%)	8 (73%)	NA	0	8 (80%)
C7 to C6	0	4 (14%)	4 (25%)	8 (73%)	NA	0	8 (80%)
C6 to T1	0	0	0	0	2 (33%)	0	0
C7 to T1 or T2	0	0	6 (38%)	3 (27%)	4 (67%)	0	0
Malformations							
C1 malformed	0	2 (7%)	10 63%)	9 (82%)	6 (100%)	5 (56%)	8 (80%)
C2 malformed	3 (14%)	3 (10%)	4 (25%)	5 (45%)	6 (100%)	5 (56%)	7 (70%)
Fusions of cervical neural arches	1 (5%)	5 (17%)	5 (31%)	11 (100%)	6 (100%)	6 (66%)	4 (40%)
Agenesis of cervical neural arches	0	0	0	0	6 (100%)	0	0
Dyssymphysis of cervical neural arches	3 (14%)	0	10 (63%)	10 (91%)	6 (100%)	2 (22%)	4 (40%)

Skeletal malformations were not observed in RARα1 or RARβ2 single mutants nor in RARα1β2 double mutants
NA, not applicable due to vertebral degeneration. Data from ref. [64].

regulate the expression of several of these genes [26, 28, 29]. Furthermore, RAREs have been found in the promoter regions of some of these RA-responsive Hox genes which can recapitulate normal expression either in tissue culture or *in vivo* [23, 27, 30]. Both gain- and loss of Hox function studies clearly illustrate the role of some of these genes in axial patterning [63, 77, 80–86], and several of the homeotic transformations observed in RAR mutants appear identical to those described for some Hox null mutants. Most notable of these are anterior transformation of the second cervical vertebra to a first vertebral identity, seen in Hoxb-4 mutants [63], and Hoxa-5 null animals [82], which display anteriorization of the sixth cervical vertebra and posterior transformation of the seventh cervical vertebra to a fifth cervical and first thoracic identity, respectively. Finally, exposure of mouse embryos to exogenous RA during somite formation and vertebral specification causes vertebral homeotic transformations in a manner that correlates with altered domains of Hox expression [22]. This occurs at a period when RARα and γ are expressed in the presomitic mesoderm [51, 54], consistent with a direct role for these genes in establishing vertebral identity through Hox-mediated events.

Two striking observations regarding the vertebral malformations observed in RAR mutants are: (i) the restriction of the defects to the cervical region; and (ii) the degeneration of the cervical region following disruption of all isoforms of RARα and γ. The confinement of vertebral malformations to the cervical region may reside in the differential response of Hox genes to RA which has been observed in tissue culture models; Hox genes located at the 3′ extremity of each Hox locus respond more rapidly and to a lower concentration of RA than genes located more 5′ [26, 28, 29]. Furthermore, these 3′ Hox paralogues govern morphogenesis of more anterior structures, suggesting that they are preferentially affected in the RAR mutants. In addition, recent work has shown that some Hox genes can act synergistically in directing normal vertebral morphogenesis; concurrent loss of the Hoxa-3 and d-3 paralogues, rather than causing an increase in the frequency or severity of vertebral homeosis, leads to disappearance of the first cervical vertebra [86]. This suggests that the dysmorphogenesis and loss of some aspects of cervical vertebrae seen in RARαγ mutants may be due to concomitant downregulation of expression of several Hox paralogues.

Although these homoeotic transformations argue strongly that the RARs directly regulate the expression of some Hox genes during development, this hypothesis is tempered by several observations. RA administration is capable of 'respecification' of vertebral identities at a later developmental time point than discussed above [87], when RARα and γ transcripts are expressed in sclerotomes [52–54]; this latter event occurs without detectable alterations in Hox expression. It is therefore possible that the vertebral transformations and malformations in RAR mutants occur during this latter period of Hox-independent vertebral morphogenesis. Another line of evidence also argues against direct regulation of Hox expression by the RARs. It has been shown that the RA-inducible Hoxa-1 gene contains a functional RARE [23], and that F9 embryocarcinoma cells lacking RARγ exhibit a much reduced RA induction of this gene [88]. However, with the exception of the lack of structures derived from the otocyst and agenesis of the abducens nerve in RARαγ mutants [64], none of the defects characteristic of Hoxa-1 null mice [89–92] were recapitulated in any RAR mutant. However, it should be noted that more recent studies suggest that RA may regulate only restricted domains of Hox gene

expression [30]. Thus, the vertebral transformations in RAR-deficient mice may reflect the loss of expression of some RA-dependent Hox genes only in the paraxial mesoderm. Such issues must be resolved by extensive *in situ* hybridization analysis. In addition, RARβ (all isoforms) null mutants, alone or intercrossed with the existing mutants, must be examined to further explore the role of RA in regulating Hox gene expression. RARβ may also be essential for the development of many tissues believed to be RA-responsive [for some examples see refs. 24, 25, 93–96] that are not affected in the mutants examined to date (e.g. CNS and neurogenic neural crest derivatives).

RARs and neural crest cells

RAR$\alpha\gamma$ double mutants exhibited malformations of most of the tissues derived from mesenchymal neural crest cells [NCC; Table 5 and see refs. 64 and 65 for additional descriptions]. Given the apparent restriction of RARs to vertebrates [reviewed in 32, 37], together with the appearance of NCC with the emergence of vertebrates [97], it is tempting to speculate that the RARs evolved as factors essential for the realization of mesenchymal NCC-derived structures.

In RAR$\alpha\gamma$ double mutants, mesectodermal derivatives from NCC originating from forebrain and rostral midbrain levels were either agenic or aplastic [Table 5; 98, 99 and references therein]. Furthermore structures derived from mesenchymal NCC emanating from more caudal levels [i.e. rhombomeres 4 and 6 (R4 and R6) and the unsegmented caudal region of the rhombencephalon] which populate the second through sixth pharyngeal arches and the aorticopulmonary septum [98–102] were also malformed or aberrantly localized (Table 5). However, derivatives of mesenchymal NCC emanating from the level of the rostral hindbrain (i.e. R1 and R2) were essentially unaffected in all double mutants. These included the dentary bone, Meckel's cartilage, the malleus and the tympanic bone. Two additional first arch structures, the alisphenoid and incus, exhibited an ectopic process resulting in their fusion (see below) but were otherwise normal.

The lack of malformations of first pharyngeal arch derivatives in RAR$\alpha\gamma$ null fetuses is unlikely due to compensation by RARβ, since RARβ transcripts are weakly expressed in this arch [52–54], nor did RARβ expression appear to be altered in RAR$\alpha\gamma$ mutants [64; our unpublished observations]. Interestingly, NCC emanating from R1/R2 levels and contributing to the first arch is the only mesectodermal population derived from the hindbrain which does not express any Hox gene. Furthermore, both frontonasal and first arch mesenchymal NCC appear to be in a morphogenetic ground state. In the case of first arch NCC, this ground state appears to be modified by the expression of the Hoxa-2 gene product, whereas frontonasal NCC appears to be modified by prolonged proximity to cephalic neuroectoderm [99, 103 and references therein]. These observations suggest that mesenchymal NCC in the morphogenetic ground state does not require RA for its realization. In contrast, modification of this ground state by either neuroectoderm (in the case of fore- and midbrain NCC) or Hox gene expression (in the case of rhombencephalon-derived NCC) appears to result in mesectodermal populations that are RA-dependent.

The dysmorphogenesis of many NCC derivatives in RAR double mutants clearly supports a role for RA in the ontogenesis of these structures. Exposure of vertebrate embryos to exogenous RA at specified developmental stages also has profound effects on many of the same tissues [reviewed in 15]. However, given the short half-life of RA *in vivo*, and the time of emigration of NCC from the neural folds, these teratogenic effects appear to manifest themselves either during or prior to NCC migration [9, 106; reviewed in 15]. In contrast, the defects observed in RAR double mutants suggest a requirement for RA after NCC migration, since RARγ expression has not been observed in premigratory NCC populations, nor in the presumptive fore- or midbrain [53, 55]. Both RARα and γ are, however, highly expressed in the frontonasal mass, periocular mesenchyme and pharyngeal arch mesenchyme after NCC migration [52, 53]. Additional evidence supporting RA-dependence of post-migratory events is the finding that administration of vitamin A to pregnant VAD females is capable of restoring the normal aortic arch pattern and aorticopulmonary septation when administered up to 9.5 or 10.5 dpc (mouse equivalent; [6], respectively, whereas NCC contributing to these structures have completed their migration prior to 9.0 dpc [98, 102; for discussion and further references see 65].

Table 5. Malformations of NCC derivatives in RAR double mutants*

NCC Population	NCC derivatives affected
Forebrain and midbrain levels	Frontal, nasal, ethmoid, incisive, vomer, maxillary, palatine, presphenoid, sphenoid, parietal and interparietal bones, upper incisors, periocular structures (e.g. stroma of cornea and Harderian glands), meninges, retrolenticular mesenchyme, prolabium, eyelids.
Hindbrain (rhombomeres 4,6 and more caudal levels)	Hyoid and styloid bones, stapes, aorticopulmonary septum, tunica media of the aortic arches, thymus, thyroid and parathyroid glands.

*Elements derived in whole or in part from NCC. Data from refs. 64, 65.

Although the basis for the malformations of NCC-derived structures in RAR double mutants is unknown, two events related to these defects have been observed [64, 65]. First, in 10.5 dpc $\alpha\gamma$ mutants, excessive cell death in the mesenchyme of the frontonasal mass has been detected; clearly this could lead to the deficiencies observed in rostral elements of these mutants. Second, aberrant specification of some NCC derivatives has been detected, notably chondrification of the meninges and persistent retrolenticular mesenchyme. Additional cartilaginous ectopias, located in the semilunar cusps, diaphragm and peritoneum of various RAR double mutants, could also be of NCC origin, suggesting either abnormal specification of trunk NCC or abnormal migration or specification of cranial NCC.

RARs and evolution

One of the most intriguing findings stemming from analysis of these double mutants was the observation of supernumerary skeletal elements present in the skull [see 64 for details]. Data from comparative anatomy suggest that these represent atavistic structures [104, 105]. In the first case, a supernumerary pila, fused to the basisphenoid, was found in RARα1γ, RARα1$\gamma\alpha$2$^{+/-}$ and RAR$\alpha\gamma$ mutants caudal to the two pilae normally found in the mouse skull (the pila prooptica and pila metoptica). This supernumerary pila, on the basis of its anatomical relationships, likely corresponds to the pila antotica which is characteristic of reptiles and monotremes (lower mammals). In the second instance, a number of RAR double mutants exhibited a cartilaginous or osseous fusion between the incus and the alisphenoid bone, and an increase in the size of the short process of the incus relative to control animals. It is likely that this fused element corresponds to the pterygoquadrate, or upper jaw, cartilage. Again, this element is characteristic of reptiles [see 64 for details].

The re-emergence of these ancestral traits in RAR double mutants suggests that some RA-dependent events were recruited to modify the skull during the reptilian–mammalian transition. It is surprising that the genetic program responsible for their appearance is still present in the mouse. It is conceivable that additional evolutionary events utilized the retinoid signaling network, an hypothesis that awaits analysis of mice lacking other RAR and RXR genes.

SUMMARY AND CONCLUSIONS

Many common defects were observed amongst different combinations of null mutants ([64, 65]; see also Tables 1 and 2). If one assumes that these malformations arose through identical lesions in cell autonomous events, then the RARs would appear to be functionally equivalent within a given cell type. Requisite transduction of the retinoid signal could then occur if the level of all RARs are above a critical threshold. This model could explain the apparent overlap in defects observed amongst the double mutants. Furthermore, this suggests that the variability in penetrance and expressivity exhibited for many of these malformations may be due to stochastic variations in the levels of the remaining RARs either between contralateral tissues within a mouse (expressivity) or between mice (penetrance). This simplistic, cell autonomous model of RAR action is not likely to be the only scenario; numerous developmental programs, including those proceeding via cell autonomous, inductive and systemic mechanisms, may be altered in these mutants. Retinoids are, in fact, thought to be essential for many of the inductive interactions essential for the ontogenesis of diverse tissues [discussed in 37, see also 65]. It is therefore likely that different combinations of null mutations affect these processes through mechanisticaly different manners, but that the phenotypic outcome is indistinguishable [see 107 for review]. This latter concept is supported by the observation that the patterns of distribution of RARβ and γ transcripts are largely mutually exclusive, yet RAR$\alpha\beta$2 and RAR$\alpha\gamma$ double mutants exhibit a number of seemingly identical malformations.

Comparison of the congenital defects observed in these double mutants also illustrates that the different RARs are clearly required for distinct, although apparently overlapping, developmental events. For example, RAR$\alpha\beta$2 null mutants exhibit defects of the lung, esophagus and trachea that are not seen in RAR$\alpha\gamma$ mutants ([65]; Table 1), suggesting that RARβ2 may play a specific role in the development of these tissues. In a similar fashion, ocular defects are confined essentially to RARγ plus either α or β2 null animals (Table 1; see also [64]), suggesting that RARγ plays a specific role in eye development. Whether these observations are indicative of genetic specificity or whether they are related to the different pattern of expression of the RARs remains to be determined.

These results, together with much work performed over the past 8 years, reveal that the RARs emerged, likely with vertebrates, as members of a transcriptional signaling network essential for the realization of many structures. With the derivation of these compound mutants, we are only beginning to fully characterize the plethora of events controlled by these receptors. Much work must now focus on the precise mechanisms by which normal ontogenesis is controlled by RA. Clearly, these genetically defined RAR null mice will be invaluable tools in furthering this process.

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