

Retardation of skeletal development and cervical abnormalities in transgenic mice expressing a dominant-negative retinoic acid receptor in chondrogenic cells

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ABSTRACT Skeletal formation is a fundamental element of body patterning and is strictly regulated both temporally and spatially by a variety of molecules. Among these, retinoic acid (RA) has been shown to be involved in normal skeletal development. However, its pleiotropic effects have caused difficulty in identifying its crucial target cells and molecular mechanisms for each effect. Development of cartilage primordia is an important process in defining the skeletal structures. To address the role of RA in skeletal formation, we have generated mice expressing a dominant-negative retinoic acid receptor (RAR) in chondrogenic cells by using the type II collagen $\alpha 1$ promoter, and we have analyzed their phenotypes. These mice exhibited small cartilage primordia during development and retarded skeletal formation in both embryonic and postnatal periods. They also showed selective degeneration in their cervical vertebrae combined with homeotic transformations, but not in their extremities. The cervical phenotypes are reminiscent of phenotypes involving homeobox genes. We found that the expression of *Hoxa-4* was indeed reduced in the cartilage primordia of cervical vertebrae of embryonic day 12.5 embryos. These observations demonstrate that endogenous RA acts directly on chondrogenic cells to promote skeletal growth in both embryonic and growing periods, and it regulates the proper formation of cervical vertebrae. Furthermore, RA apparently specifies the identities of the cervical vertebrae through the regulation of homeobox genes in the chondrogenic cells. Great similarities of the phenotypes between our mice and reported RAR knockout mice revealed that chondrogenic cells are a principal RA target during complex cascades of skeletal development.

Retinoic acid (RA), a physiological metabolite of vitamin A, is known to affect skeletal development when applied exogenously. Administration of excess RA to pregnant mothers causes various malformations in their fetuses, such as limb defects, cleft palates, and malformed sternums and vertebrae in rodents (1) and craniofacial malformations in humans (2). In addition to these morphological abnormalities, RA treatment induces various homeotic transformations in the embryonic skeleton, including alteration of the vertebral patterns (3, 4). These observations have led to the prediction that endogenous RA is an important physiological regulator in development. Several lines of supporting evidence have also been obtained from the studies of dietary vitamin A deprivation (VAD) in rodents (5, 6).

RA functions are now known to be mediated by members of the nuclear receptor superfamily that act as ligand-dependent transcription factors (7). Receptors for RA are composed of heterodimers between retinoic acid receptors (RARs) and retinoid X receptors (RXRs). RARs and RXRs are encoded by at least three distinct genes, *RAR α* , *- β* , and *- γ* and *RXR α* , *- β* , and *- γ* , respectively (7, 8). Functions of endogenous RA during development have been addressed by knocking out these receptors. Mice lacking the *RAR γ* gene (9) or *RAR α* and *RAR γ* genes (10) had growth retardation, malformations, and homeotic transformations in their skeletons. These phenotypes have confirmed that endogenous RA is required for normal skeletal development. These results, in turn, have raised the question of what kind of cells and molecules are the RA targets in normal skeletal development.

Skeletons are developed through two distinct processes (11). Flat bones in the skull are generated by intramembranous ossification. On the other hand, most other bones are generated through endochondral ossification: condensed mesenchymal cells first differentiate into chondrocytes to form cartilage primordia, then they are gradually replaced by osteogenic cells and calcified tissues to develop into mature bones. Many of the affected skeletons in VAD mice and RAR knockout mice are apparently made through the latter process. Furthermore, development of chondrocytes and cartilage tissues are regarded as a critical step in outlining the majority of the skeletal structures (12). It is therefore reasonable to postulate that chondrogenic cells, the cells of a chondrocyte lineage, including prechondrocytic mesenchymal cells and mature chondrocytes, are a critical target of endogenous RA during skeletal development and growth.

Homeobox genes are well known genes involved in the specification of body patterning. In mammals, 39 homeobox genes are clustered in four different chromosomal complexes (13). It has been documented that exogenously applied RA alters their segmental expression pattern *in vivo*, concomitant with the appearance of homeotic transformations (3, 4). This strongly suggests that homeobox genes are crucial target molecules of endogenous RA, but the real relationship between endogenous RA and homeobox gene expression during mammalian development has not yet been elucidated.

We have analyzed physiological RA functions during mouse development by expressing a dominant-negative form of RAR, referred to as RAR-E, which effectively blocks RA signaling (14). In the ligand-binding domain, RAR-E contains a point mutation originally identified at the homologous position in

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This paper was submitted directly (Track II) to the *Proceedings* office. Abbreviations: RA, retinoic acid; RAR, retinoic acid receptor; VAD, vitamin A deprivation/deficiency; *En*, embryonic day *n*.

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the thyroid hormone receptor of thyroid hormone-resistant patients (15). We previously generated mice expressing RAR-E in the epidermis and demonstrated the requirement for RA in normal skin development (16). Here, we report the generation of transgenic mice expressing RAR-E in chondrogenic cells and their resultant phenotypes. The significance of the chondrogenic cell-mediated RA functions in skeletal development is discussed through comparing the phenotypes of RAR and homeobox gene knockout mice.

MATERIALS AND METHODS

Generation of Transgenic Mice. The mouse type II collagen $\alpha 1$ chain gene was isolated by screening a mouse genomic library (Stratagene) with a PCR-amplified probe containing its 5' upstream sequence (17). The 2.5-kb promoter region covering the 5' upstream region of the translation start site, the 3.8-kb enhancer region from the first intron, the rabbit β -globin intron, and the polyadenylation site (16–18) were ligated together with β -galactosidase or RAR-E cDNA, as shown in Fig. 1A. Fertilized eggs were recovered from BDF₁ females (C57BL/6 \times DBA/2) crossed with BDF₁ males and microinjected by standard procedures (19). Copy numbers of the transgene were determined by Southern blotting with tail DNA. RAR-E offspring were generated by breeding the founder with C57BL/6 mice. The body weights of the F₂ offspring were followed up to 77 days after birth.

Soft X-Ray Analysis. Mice were anesthetized and subjected to soft x-ray analysis (40 kV, 2.5 mA for 60 sec, HX-100, HITEK, Osaka).

Whole-Mount 5-Bromo-4-chloro-3-indolyl β -D-Galactoside (X-Gal) Staining. Whole-mount X-Gal staining was performed by standard procedure with some minor modifications (20). E12.5 embryos were fixed in buffer containing 1% formaldehyde, 0.2% glutaraldehyde, and 0.02% Nonidet P-40 for 30 min at 4°C and stained with PBS containing 5 mM K₄Fe(CN)₆, 5 mM K₃Fe(CN)₆, and 1 mM X-Gal for 8 h at 30°C.

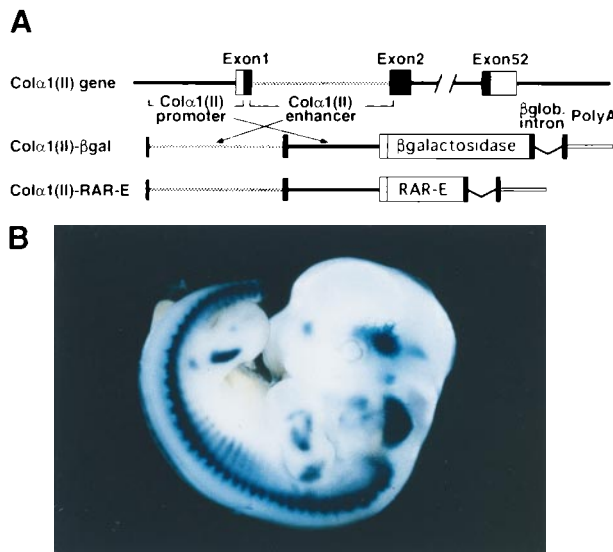


FIG. 1. Structure and expression pattern of transgenes. (A) Schematic structure of each transgene that is under the control of the mouse type II collagen $\alpha 1$ chain gene promoter and enhancer and includes an intron and a polyadenylation site. The transgenes contain either β -galactosidase or a dominant-negative RAR (RAR-E) cDNA. (B) The expression pattern of the β -galactosidase transgene was visualized by 5-bromo-4-chloro-3-indolyl β -D-galactoside (X-Gal) staining. Expression was seen in the cartilage primordia of vertebrae, ribs, long bones, exoccipital bone, and the petrous part of the temporal bone in an embryonic day 12.5 (E12.5) embryo.

Skeletal Staining. E18.5 fetuses were skinned, eviscerated, and fixed in 95% ethanol. Intact skeletons were stained with 150 mg/ml alcian blue 8GX in 75% (vol/vol) ethanol/20% acetic acid for 24–48 h, and excess tissues were removed with 1–2% KOH digestion for 2–3 days. The skeletal preparations were stained with 75 mg/ml alizarin red S in 1% KOH for 24 h and cleared in graded glycerol solutions (21). For adult skeletal

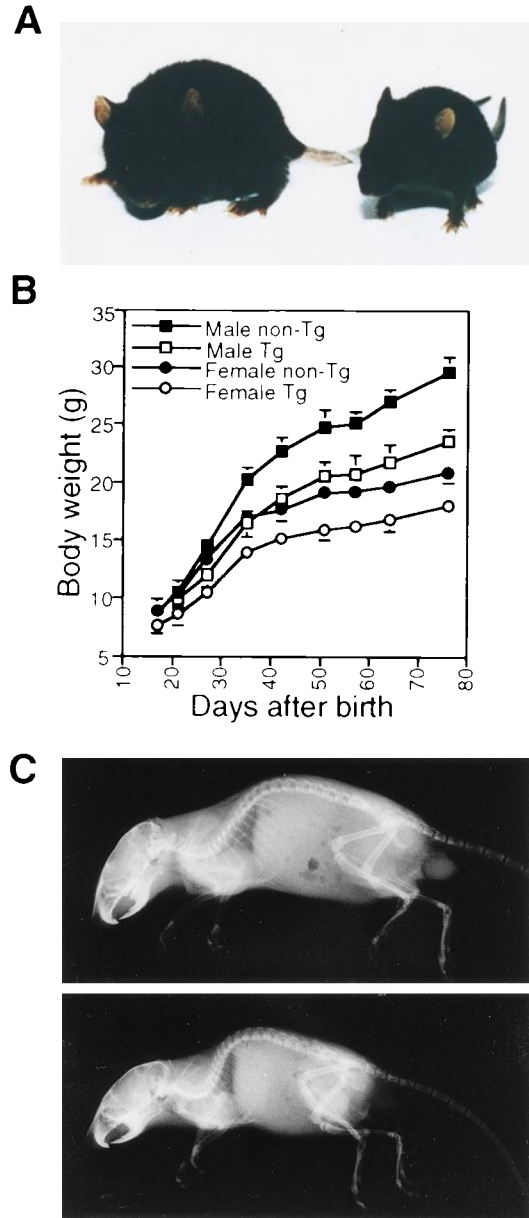


FIG. 2. Retardation of skeletal development in RAR-E mice. (A) Physical appearances of a male founder RAR-E mouse (Right) and its sex-matched nontransgenic littermate (Left). The RAR-E mouse is 24% lighter than the littermate. (B) Growth curves of RAR-E mice and their nontransgenic littermates. On average, transgenic males (\square ; $n = 10$) weighed approximately 19% less than nontransgenic males (\blacksquare ; $n = 12$), and transgenic females (\circ ; $n = 18$) weighed approximately 16% less than nontransgenic females (\bullet ; $n = 25$). Statistical significance was calculated by ANOVA ($P < 0.001$). (C) X-ray photographs of an adult male RAR-E mouse with a conspicuous phenotype, being 22% lighter (Lower) than its sex-matched nontransgenic littermate (Upper). Bones generated through endochondral ossification appear to be preferentially shortened: the lengths of the vertebral column (from C1 to S4) and the femurs of the transgenic mouse were 19–20% shorter than those of the wild-type littermate, whereas the length of the cranium (from frontal to occipital edges) was shortened by less than 5%.

analysis, tissues were digested with 2% KOH for 4–6 days. E14.5 embryos were stained with alcian blue as previously described (22).

Measurement of Bone Size. Bone sizes of the stained skeletons of the E18.5 embryos were measured; vertebral column sizes were compared between the lengths from C1 to S4; limb bone sizes were compared between the lengths along the longer axes; frontal–parietal bone sizes were compared between the lengths from the rostral edges of the frontal bones to the caudal edges of the parietal bones.

In Situ Hybridization. Plasmids containing cDNAs for *Hoxa-3* and *Hoxa-4* were linearized and used as templates for synthesis of digoxigenin-labeled riboprobes (23). E12.5 embryos were fixed with 4% formaldehyde in PBS for 4 h at 4°C, bleached in 5:1 (vol/vol) methanol/H₂O₂ solution for 6 h at room temperature, rehydrated in a graded series of methanol/PBS, and embedded in 2% agarose/PBS gel. Sections 300 μm thick were obtained with a microslicer (DTK-3000, Dosaka EM, Kyoto, Japan). *In situ* hybridization was performed according to the described methods (23, 24).

RESULTS

Generation of Transgenic Mice Expressing RAR-E in Chondrogenic Cells. We utilized a mouse type II collagen α1 chain promoter to express transgenes in chondrogenic cells (Fig. 1A). During embryogenesis, expression of the gene begins in paraxial mesenchymal cells concomitantly with the onset of their differentiation toward chondrocytes around E9.5, and the expression continues to adulthood (25–27). Therefore, type II collagen is a useful marker for cells of the chondrocyte lineage. Strong β-galactosidase expression was specifically observed in cartilage primordia of vertebrae, ribs, and long bones in the E12.5 embryo (Fig. 1B and ref. 28). Four transgenic founders for *RAR-E* were identified among 59 offspring, with transgene copy numbers of 3, 4, 4, and 4 (data not shown). The integrated copy numbers for *RAR-E* were relatively small compared with our previous experiments (16, 29), suggesting the influence of *RAR-E* on mouse viability.

Retarded Skeletal Development and Maturation in the Transgenic Mice. By visual inspection, the *RAR-E* founders appeared to be smaller than their littermates (Fig. 2A). Body weights of their F₂ offspring were monitored for several months. The difference was apparent at birth and continued until adulthood: the body weights of *RAR-E* mice were approximately 17% lower throughout the monitoring periods ($P < 0.001$) (Fig. 2B), indicating that growth retardation in *RAR-E* mice occurs not only during embryonic stages but also postnatally. Consistent with the expression patterns of the transgene, x-ray analysis revealed that the vertebrae and limb bones, generated through endochondral ossification, were more prominently affected than the crania, which are generated mainly through intramembranous ossification (Fig. 2C).

Next, we mated the F₂ offspring to generate *RAR-E* homozygotes from each transgenic line to obtain enhanced phenotypes through a gene-dosage effect. Unexpectedly, all homozygotes died at or shortly after birth (see below). We therefore examined their skeletons at E18.5 (Fig. 3A). As in the adult, skeletons of *RAR-E* embryos were generally shortened,

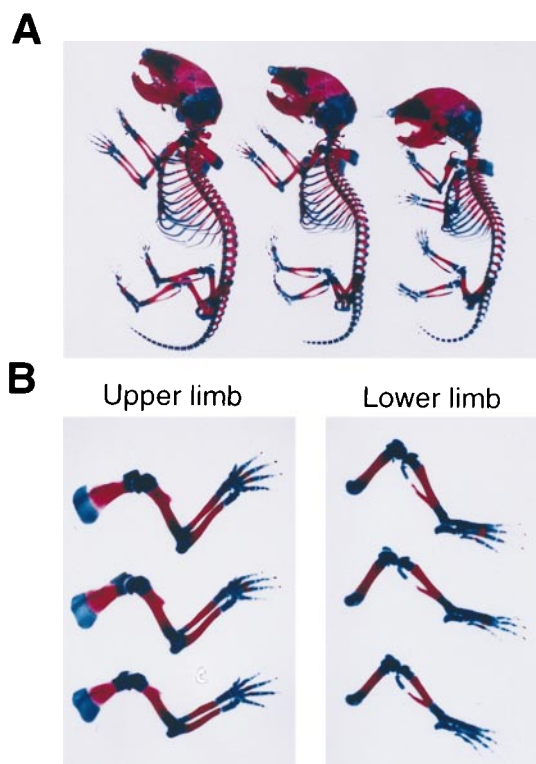


FIG. 3. (A) E18.5 whole skeletons of a nontransgenic mouse (Left), an *RAR-E* heterozygote (Center), and an *RAR-E* homozygote (Right) stained with alcian blue and alizarin red. Skeletons were smaller in transgenic mice in an *RAR-E* gene dosage-dependent manner. (B) E18.5 skeletons of upper and lower limbs from a nontransgenic mouse (Top), an *RAR-E* heterozygote (Middle), and an *RAR-E* homozygote (Bottom). In transgenic mice, the entire limb was decreased in size and bony portions (red) of the scapula, humerus, ulna, radius, femur, tibia, fibula, metacarpal/metatarsal, and phalangeal bones were all shortened in an *RAR-E* gene dosage-dependent manner.

except for skulls. These effects appeared to be transgene-dosage dependent, being more prominent in the homozygote than in the heterozygote: measurement of bone sizes revealed that vertebral columns and limb bones exhibited statistically significant reduction in size in an *RAR-E* dosage-dependent manner, whereas frontal–parietal bone sizes showed no differences (Table 1). Magnified views revealed that all the limb components of *RAR-E* mice were smaller (Fig. 3B). In addition, the ossified regions were narrowed, especially at the distal components (Fig. 3B). Such phenotypes were consistently observed in all four independent lines, with the homozygotes consistently exhibiting more severe phenotypes.

Viability of the Transgenic Mice. Genotypes of offspring from transgenic parents were examined at various stages. They were in accordance with the mendelian rules up to birth: the number of wild types, heterozygotes, and homozygotes were, respectively, 32, 88, and 40 at E12.5; 21, 35, and 20 at E18.5; and 39, 66, and 26 at birth. Therefore, the viability *in utero* was not considerably affected by *RAR-E* in the established transgenic lines. However,

Table 1. Statistical analysis of the bone lengths

Genotype	Relative length, %					
	Vertebral column	Humerus	Ulna	Femur	Tibia	Frontal–parietal
Wild type	100.0 ± 0.6 (8)	100.0 ± 1.0 (8)	100.0 ± 0.9 (8)	100.0 ± 1.0 (9)	100.0 ± 0.9 (9)	100.0 ± 2.5 (8)
Heterozygote	89.2 ± 2.4 (13)*	93.9 ± 2.3 (12)*	94.0 ± 3.0 (12)*	92.3 ± 3.1 (12)*	90.7 ± 3.6 (12)*	100.7 ± 4.9 (12)
Homozygote	79.6 ± 3.2 (8)†	89.2 ± 2.6 (8)†	89.8 ± 4.1 (8)‡	87.6 ± 3.3 (8)†	81.1 ± 5.2 (8)†	101.4 ± 7.9 (8)

Values are expressed as percentages of wild-type (mean ± SD) and the number of samples are indicated in parentheses. Statistical evaluations were made by the Student *t* test between wild types and heterozygotes (*) or between heterozygotes and homozygotes († or ‡): *, $P < 0.01$; †, $P < 0.01$; ‡, $P < 0.05$.

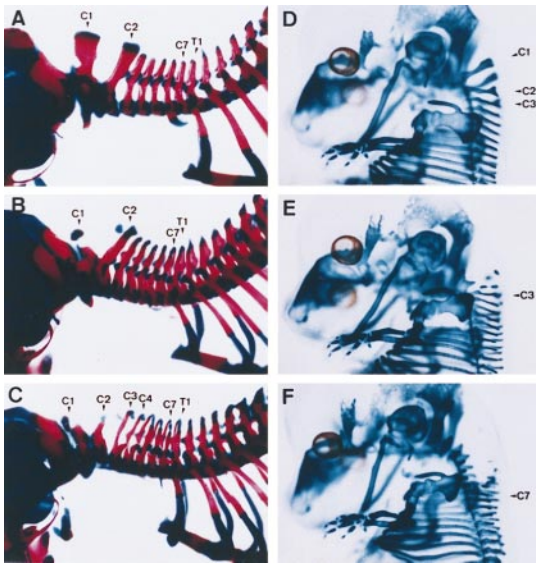


FIG. 4. Malformations of cervical vertebrae in *RAR-E* mice at various developmental stages. (A–C) E18.5 skeletons were stained with alcian blue and alizarin red. (A) Nontransgenic mouse. Neural arches were completely fused dorsally. Note the broad neural arches of C1 and C2. (B) *RAR-E* heterozygote. The cervical vertebral column appeared compressed. C1 dyssymphysis was consistently observed. Neural arch of C2 was considerably hypoplastic. (C) *RAR-E* homozygote. The cervical vertebral column was compressed more severely. Dyssymphysis extended from C1 to C4. (D–F) E14.5 cartilage primordia were stained with alcian blue in nontransgenic (D), *RAR-E* heterozygous (E), and *RAR-E* homozygous (F) embryos. Neural arches from C1 to C2 and from C1 through C6 were hypoplastic in the *RAR-E* heterozygote (E) and the *RAR-E* homozygote (F), respectively. Note the smaller size of the upper limb primordia in an *RAR-E* gene dosage-dependent manner (E and F).

as described above, almost all the homozygotes (24/26) died within several hours after birth, apparently because of respiratory distress. Although the precise cause of death remains to be determined, these results indicate the requirement for proper skeletal development for survival.

Degenerative Malformations of Cervical Vertebrae. In E18.5 *RAR-E* fetuses, the cervical vertebrae were found to be particularly affected (Fig. 4 A–C). Cervical vertebral columns were hypoplastic in a transgene-dosage-dependent manner (Fig. 4 B and C). In addition, their neural arches failed to fuse to the vertebral bodies (dyssymphysis) transgene dosage-dependently: only C1 and C1 to C4 dyssymphyses were observed in the heterozygote and the homozygote, respectively (Fig. 4 B and C). These defects were not observed in thoracic and lumbar vertebrae. Similar defects were observed at E14.5, when chondrogenesis is actively occurring: C1 to C2 and C1 to C6 defects appeared in the heterozygotes and the homozygotes, respectively (Fig. 4 D–F). Note also that at E14.5 the cartilage primordia of the limbs were shortened in an *RAR-E* gene-dosage-dependent manner (Fig. 4 D–F), showing that generalized retardations of skeletal development already occurred at the period of active chondrogenesis.

We next examined the cervix of adult *RAR-E* heterozygotes (Fig. 5), as we could not obtain adult homozygotes. Vertebral deformities were observed through C1 to C7 (Fig. 5 B and C). C1–C2 dyssymphysis frequently occurred. In severely affected lines, the adjacent vertebrae were fused and ossified as a mass (Fig. 5 C, E, and F). Such degenerative changes were not seen in the more caudal vertebrae of T1 and below. In summary, degeneration of skeletons was restricted to the cervical region through developmental stages and adult periods.

Homeotic Transformations in the *RAR-E* Cervical Vertebrae. Analysis of our *RAR-E* mice revealed a significant



FIG. 5. Degeneration and homeotic transformation of cervical vertebrae in adult *RAR-E* mice. Cervical vertebrae of adult *RAR-E* mice (B–F) and an age-matched nontransgenic mouse (A) were stained with alcian blue and alizarin red. (B) Mild phenotypes from one line. Cervical vertebrae from C1 to C7 were compressed but individual vertebrae were not fused. Note the C1 dyssymphysis and T1 to T2 transformation (presence of dorsal process on T1 instead of T2). (C) Severe phenotypes from another line. C1 to C7 vertebrae were deformed and C2 to C5 were fused to each other. C1 and C2 dyssymphyses were observed. (D) C3 to C2 transformation was evidenced by a protruding dorsal process on C3, and C7 to T1 transformation by extension of a rib from the C7 vertebra to the first rib. (E) C7 to C6 transformation is shown by the unilateral shift of the anterior tubercle to C7, which is characteristic of C6. Note that the vertebral bodies from C2 to C5 are fused together. (F) C5 to C6 transformation is shown by the unilateral shift of the anterior tubercle to C5. Note the fusion of the vertebral bodies from C2 to C4. Arrows indicate homeotic transformations.

incidence of homeotic transformations in the cervical vertebrae (Fig. 5 B, D, E, and F, see legend for details). We observed both anterior and posterior transformations; anterior transformations included C3 to C2 and C7 to C6; posterior transformations, C5 to C6, C7 to T1, and T1 to T2 (Table 2). These transformations were observed in independent *RAR-E* lines. No additional transformations were observed in any other parts of the vertebrae or in the digits, or in any of the 34 nontransgenic littermates (data not shown).

Decreased Expression of *Hoxa-4* in the *RAR-E* Cervical Prevertebrae. We noticed that homeotic changes in our mice resembled those reported in *Hoxa-4* knockout mice (30–32). By *in situ* hybridization, we actually found a reduction in *Hoxa-4* expression in the cervical prevertebrae of *RAR-E* homozygous embryos at E12.5 (Fig. 6). In the nontransgenic animal, strong hybridizing signals were observed in the prevertebrae (pv) from pv3 to pv9 and weak signals in pv2 and caudal to pv10 (Fig. 6 A and B). In the *RAR-E* mouse, hybridizing signals in the cervical prevertebrae were significantly reduced (Fig. 6 E and F). The expression boundary

Table 2. Homeotic transformations in *RAR-E* mice

Transformations	Vertebra	Observed	
		%	No.
Anterior	C3 to C2	31	13/42
	C7 to C6	8	4/49
Posterior	C5 to C6	8	4/49
	C7 to T1	18	9/49
	T1 to T2	27	13/49

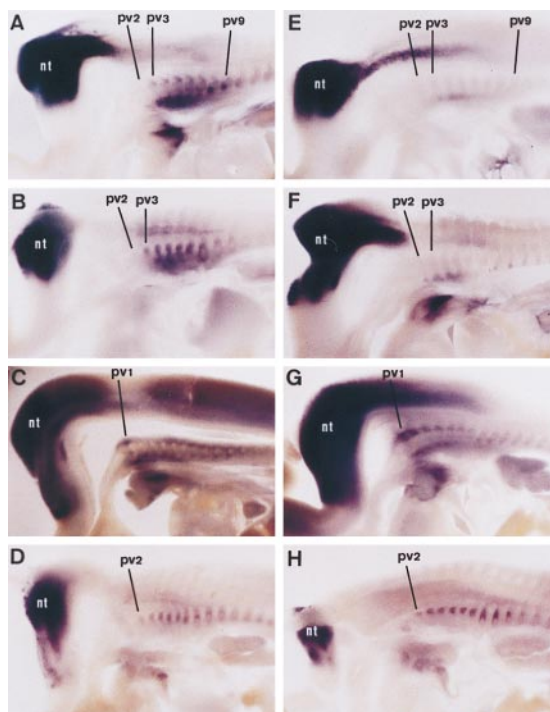


FIG. 6. *In situ* hybridization analysis of *Hoxa-4* and *Hoxa-3* gene expression. *Hoxa-4* (A, B, E, and F) and *Hoxa-3* (C, D, G, and H) expression was detected in E12.5 embryos by using digoxigenin-UTP-labeled riboprobes in nontransgenic (A–D) and *RAR-E* homozygous (E–H) embryos. Sagittal (A and E) and parasagittal (B and F) sections reveal the reduction in the level of *Hoxa-4* expression in the cervical prevertebrae of the *RAR-E* homozygote. *Hoxa-4* expression extends from the second prevertebra down to lower prevertebrae in both nontransgenic and *RAR-E* homozygotes. Note that the *Hoxa-4* expression in the neural tube is indistinguishable in the two mice. In contrast, *Hoxa-3* showed no difference in either the expression level or the boundary, in sagittal (C and G) and parasagittal (D and H) sections: it extends from the first prevertebra to the lower in both mice. pv1, the first prevertebra; pv2, the second prevertebra; pv3, the third prevertebra; pv9, the ninth prevertebra; nt, neural tube.

appeared to stay at pv2 (Fig. 6 E and F) as in nontransgenic mice (Fig. 6 A and B and ref. 33). Note that the expression was not affected in the neural tube, where *RAR-E* was not targeted for expression. In contrast, the *Hoxa-3* expression level was not affected in the same prevertebrae; the expression boundary of *Hoxa-3* was constantly observed at pv1 (Fig. 6 C, D, G, and H, and ref. 34). This observation indicates that the *Hoxa-4* reduction is specific and not the result of suppressive effects of *RAR-E* on the general transcription machinery or the abnormal prevertebra structures. These data strongly indicated that RA specifies identities of the cervical vertebrae through the regulation of homeobox genes in chondrogenic cells.

DISCUSSION

There have long been many unsolved questions regarding the physiological functions of RA, especially in skeletal formation in developmental biology. Accordingly, tremendous efforts have been made to generate mice lacking *RAR* genes to elucidate their functions (9, 10, 35, 36). We have chosen an alternative strategy using dominant-negative *RAR* (*RAR-E*) and have examined the chondrogenic cell-mediated RA functions in skeletal formation. The skeletal formation involves several major types of cells, including chondrogenic cells, osteoblasts, osteoclasts, etc. As RA has been shown to act not only on chondrogenic cells but also on many other cell types (37), we initially supposed that we would obtain a portion of the *RAR* knockout mice phenotypes reflecting RA action on chondrogenic cells. However, a lot more of the *RAR* knockout

phenotypes were reproduced in our mice than expected, revealing that chondrogenic cells are critical RA target cells in skeletal development.

Our mice exhibited generalized growth retardation of the skeletons. Although small skeletons have long been observed in not only *RAR* knockout but also *VAD* mice (6, 9, 10), this phenotype has been estimated to result from the defects and malfunctions of other affected organs. Using *RAR-E*, we were able to assess RA functions in the skeleton by minimizing such secondary effects. The direct effect of RA on skeletal growth is also evident from small cartilage primordia at E14.5 under active chondrogenesis (Fig. 4). Given that hormonal control of embryonic growth has long remained to be elucidated (38), RA could be highlighted as a soluble factor that directly acts on chondrogenic cells and promotes skeletal growth during embryogenesis and also after birth.

Another unexpected replication between *RAR-E* mice and *RAR* knockout mice is the manifestation of lethality. Anatomical and histological examinations of the dead *RAR-E* mice revealed only poorly ventilated lungs; no apparent abnormality was observed in other vital organs (not shown). Such respiratory failure could be caused by a smaller thoracic cage and hypoplastic cartilage lining the respiratory tract, as observed in chondrodysplasia mutant mice (39, 40). Severe cervical hypoplasia, as shown in Fig. 4, may cause fatal cervical dislocations and/or compression of the cervical spinal cord at or after birth. Although the definite causes of death remain to be determined in further analysis, our data indicate that cartilage and skeleton are vital RA targets and that lethality reported in *RAR* knockout mice (10) may be attributed in part to the defect in RA signaling in chondrogenic cells, a finding that was totally unexpected.

Vertebral abnormalities are also replicated phenotypes. An exception is the C2 to C1 transformation (neural arch thickening and an ectopic anterior arch on C2), observed only in partial *RAR* knockout mice (10). However, we do not deny the possibility of C2 to C1 transformation in our mice, because we were unable to fully examine this point due to severe degenerative change as is the case with complete *RAR α* and γ knockout mice (10). Other phenotypes, fusions of the neural arch, dysymphyses in C1 and C2, and homeotic transformations including C7 to C6 and C7 to T1, were commonly observed (9, 10). These results again indicate that chondrogenic cells are a principal RA target during cervical development.

A major difference in the skeletal phenotypes between *RAR* knockout mice and *RAR-E* mice is the absence of front limb deformities in *RAR-E* mice. A possible explanation is that chondrogenic cells might not be the direct RA target for limb patterning. Although RA was proposed as a morphogen that produces positional information in the limb buds (41), subsequent experiments showed that RA itself is not a morphogen but generates a secondary morphogenic signaling molecule(s) that determines the cell identities in the limb-bud mesenchyme (42).

A couple of alternative mechanisms may make it possible to explain the phenotypic differences between *RAR* knockout and *RAR-E* mice. First, *RAR-E* might affect other unknown nuclear receptor signalings whose response elements are highly related to *RARs*'. Second, *RAR-E* expression might cause repression of basal transcription activities through binding to the corepressor (43–45), instead of a failure of the transcriptional activation that occurs in the *RAR* knockout mice. It is difficult to completely rule out these alternative possibilities, but the data that the observed skeletal *RAR-E* phenotypes are mostly, if not all, included in the *RAR* knockout phenotypes are well fit for the idea that additionally observed phenotypes of *RAR* knockout mice are derived from the different cell origins, and therefore are presently consistent with our pre-supposition that tissue-specific expression of *RAR-E* would reveal physiological *RAR* functions in the tissues concerned.

Much experimental evidence has suggested that the expressions of certain homeobox genes are under the regulation of endogenous RA; RAR α - or γ -deficient EC cells showed aberrant expression of homeobox genes *in vitro* (46); RA-responsive elements in the *Hoxb-1* gene are essential for its proper expression *in vivo* (47, 48). In addition to such circumstantial evidence, our data offer strong evidence for a direct link among physiological RA signaling, homeobox gene expression, and body specification in mammalian development. Our RAR-E phenotypes are closest to those of *Hoxa-4* knockout mice; Horan *et al.* (30) reported C3 to C2 transformation and C7 to T1 transformation with penetrance of 71% and 48%, respectively; Kostic and Capecchi (31) reported 100% transformation of C3 to C2. In addition, combined null mutants for *Hoxa-4* paralogues showed C7 to C6 transformation with the penetrance of 85–100% (32). We therefore examined the expression of *Hoxa-4* and observed its reduction in cervical prevertebrae. Identification of a functional RA-responsive element in the 5' upstream regulatory region of human *HOXA-4* gene (49) further supports the idea that *Hoxa-4* is a direct target gene of RA in cervical development. Relatively low penetrance of each transformation in our mice could be explained by the low but detectable level of *Hoxa-4* expression. It is, of course, easily imaginable that homeobox genes other than *Hoxa-4* are also affected in our transgenic mice: some of the transformations, e.g., T1 to T2, were not reported in *Hoxa-4* mutants but were observed in *Hoxa-6* knockout mice (31).

These observations convincingly demonstrate that specification of cervical vertebrae is physiologically determined by RA signaling through chondrogenic cells and that this determination is mediated through the regulation of homeobox gene expression. Furthermore, the present analyses reveal the importance of chondrogenic cells in RAR-mediated body patterning and skeletal development, and they advance our understanding of RA physiology as well as the roles of these cells in body formation.

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