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Suppression of retinoic acid receptors may contribute to embryonic skeleton hypoplasia in maternal rats with chronic vitamin A deficiency

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Abstract

Vitamin A (VA) is essential for embryonic development and the retinoic acid receptors (RARs) are crucial in mediating the diverse actions of VA in embryogenesis. However, the association between RARs and teratogenicity on skeleton growth and development of vitamin A deficiency (VAD) is not clear. In this present study, weaning female Sprague–Dawley rats were fed purified diets containing graded levels of VA (0, 0.4, 4 IU/g diet) for 70 days before mating, and some of them were supplemented with VA (10 IU/g diet) through pregnancy. Embryos were recovered at embryonic day 19.5 (E19.5) for the analysis of skeleton growth and development and the E12.5 embryos were collected for analysis of select mRNA of *RARα, RARβ, RARγ, Hoxa2, Hoxa5* and *Hoxa9*. Normal gene expressions and morphogenesis were observed in all embryos from group fed 4 IU/g diet. The embryos from group fed VA-free diet showed a comprehensive suppression of all the genes and general fetal resorption. The embryos from group fed 0.4 IU/g diet exhibited a moderate down-regulation on *RARβ, RARγ, Hoxa2* and *Hoxa5*, and the E19.5 fetuses displayed a series of skeletal hypoplasia. The VA supplement groups fed 10 IU/g diet displayed normal gene expressions and morphologic appearances. These findings suggested that the suppression of RARs resulted from VAD could disturb the proper expression of *homeobox* genes, which might, at least in part, contribute to the embryonic skeletal hypoplasia due to maternal rats with chronic VAD.

Keywords: Vitamin A deficiency; Retinoic acid receptor; *Hox* genes; Skeletal development; Rat; Embryogenesis

1. Introduction

Vitamin A (VA) and its biological derivatives (the retinoids) are of essential importance to normal embryonic growth and development [1,2]. Early in the 1940s, Warkany et al. [3–5] had found a large array of congenital malformation in embryos isolated from VA-deficient (VAD) pregnant rats and categorized these defects as symptoms of the VAD syndrome, which involves ocular, genitourinary tract, kidneys, diaphragm, lung, aortic arch and heart. Subsequently, White et al. [6–8] reported additional abnormalities involved in the craniofacial region, postotic cranial nerves, pharyngeal arches, otic vesicle, cardinal vein and sinuatrial valve. The homozygous null mutant mice of retinaldehyde dehydrogenases-2 (RALDH2), an important dehydrogenase in the generation of retinoic acid (RA), displayed severe embryonic abnormalities including lack of heart looping and chamber morphogenesis, incomplete neural tube closure, shortening of the trunk region and absence of limb buds, all of which are similar to the VAD phenotype [9].

It is now widely accepted that the effects of VA as an important morphogen in embryogenesis are modulated primarily by the heterodimers of the RA receptors (RARs) and the retinoid X receptors [10–13]. Compound mutant mice lacking multiple RAR isoforms died before or shortly after birth and displayed severe malformations of many organs and tissues, which, in many ways, mirror the defects seen both in RALDH2 mull mutant mice and at RA-insufficient rat embryos [9]. Data from in situ hybridization studies show that, in the process of embryogenesis, different RAR subtypes are widely expressed in its unique spatiotemporal patterns along the entire anterior/posterior (A/P) axis, which confirms an action mode, under

Abbreviations: A/P, anterior/posterior; AD, diet free of vitamin A; AM, diet marginally deficient of vitamin A; AN, control diet; AS, diet supplement with vitamin A; CRBP, cellular retinoid binding proteins; E12.5/19.5, embryonic day 12.5/19.5; *Hox* gene, *homeobox* genes; RA, retinoic acid; RARs, retinoic acid receptors; RARE, retinoic acid response element; VA, vitamin A; VAD, vitamin A deficiency/deficient.

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which VA and its derivatives regulate a large network of gene activation processes via binding the nuclear receptors [10–13].

Homeobox genes (*Hox* genes) are widely present in eukaryotes genome and contain a highly conserved DNA sequence encoding a homeodomain that functions as part of a transcription factor to bind DNA and specify a positional identity along the embryonic axes [14]. In vertebrates, the 39 *Hox* genes are organized into four different chromosomal clusters, known as the *Hox* A, B, C and D clusters and oriented in the same 5′ to 3′ transcriptional direction. During embryonic development, *Hox* genes display an intriguing colinearity between their location along the cluster and their temporal and spatial expression: genes at the 3′ end of each cluster are expressed earlier in development and in more anterior regions of the embryo, while the genes at the 5′ end of the clusters are expressed at later times and in posterior regions [15].

There has been much research work focusing on the relationship between the retinoids and the expression of *Hox* genes in embryonic pattern development [16]. Interestingly, the congenital abnormality arising from RAR compound null mutant embryos is similar to the effect of loss-of-function mutations of *Hox* genes [17]. Furthermore, RA response elements (RAREs) have been identified in the flanking regions of several *Hox* genes, including *Hoxa1, Hoxb1, Hoxa4,* and *Hoxd4* both in vitro and in vivo [18–24], which manifests that at least some *Hox* genes are directly involved as the RA target genes during embryonic development. More directly, both the deficiency and the excess of VA have been shown to influence the spatiotemporal expression pattern of *Hox* genes resulting in various developmental defects [8,9]. In a study involving the distribution of RARs and cellular retinoid binding proteins (CRBP), it was shown that the CRBP functions as controllers to regulate the concentration of RA by interacting with the RAR distribution pattern. However, the synergistic effects of VAD on the expression of *RARs* and *Hox* genes have been poorly investigated.

In the present study, we aimed to find out the action mode of RARs and detect the response of *Hox* genes on cluster A in embryos in different VA statuses. In addition, we hoped to imitate the marginal VAD problem in human population via the establishment of a marginally VAD animal model and to seek the molecular mechanisms for early supplement on maintaining normal development and growth of the offspring.

2. Materials and methods

2.1. Diets

Rats were fed a casein-based purified experimental diet modified according to the American Institute of Nutrition 93 Growth Purified Diet and previous description [25,26]. The diet compositions differed only in VA contents: VA completely deficient diet (0 IU/g diet, AD), VA marginally deficient diet (0.4 IU/g diet, AM), VA normal diet (4 IU/g diet, AN) and VA supplement diet (10 IU/g diet, AS). The retinol acetate was provided as the VA source, and the content of VA in each diet was shown in Table 1.

2.2. Rats

Animal procedures were approved by Tongji Medical College Council on Animal Care Committee of Huazhong University of Science and Technology, China, and animals were cared for according to the Guiding Principles in the Care and Use of Animals. Fifty weanling female Sprague–Dawley rat (60–65 g, obtained from Sino-British Sippr/BK Laboratory animal, Shanghai) were housed in stainless steel cages with automatically controlled temperature (22±1°C), relative humidity (55±5%) and a light/dark cycle of 12 h. Both diet and water were available ad libitum. Twenty-eight animals were fed with AD diet, 16 animals were fed with AM diet and 6 animals were fed with AN diet.

Table 1
The VA content in each diet and the diet treatment of each group

	AN	AD	DS	AM	MS
Before E0.5 (IU/g diet)	4	0	0	0.4	0.4
Pregnancy (E0.5-E19.5) (IU/g diet)	4	0	10	0.4	10

Table 2
Primers used for real-time PCR

Gene	Sequence (5′ to 3′)		Product size (bp)
RARα	Forward	tcagtgccatctgcctcatct	177
	Reverse	tgatactccgaaggtccgtgat	
RARβ	Forward	gaaagcccaccaggaaacc	158
	Reverse	gcttggcgaactccacgat	
RARγ	Forward	ctgaccctgaaccgaaccca	143
	Reverse	ccacagatgaggcagatagcac	
Hoxa2	Forward	agcataagaggcaaacccagt	85
	Reverse	tcctccactttgtccgaatc	
Hoxa5	Forward	agaggtcatcaggcaggatttac	164
	Reverse	gcggtcgtttgtgcgtctat	
Hoxa9	Forward	cgcaggatgaaaatgaagaaga	139
	Reverse	gagaggggagagaaagggaga	
β-Actin	Forward	catcactatcggcaatgagc	156
	Reverse	gacagcactgtgttggcata	

After 70 days, the female rats were mated with normal males, and the noon when a vaginal plug was detected was designated embryonic day 0.5 (E0.5). The rats on the AD diet were randomly subdivided into two groups: a subset was shifted to AS diet started at E0.5 (DS group), and the others continued to be fed with AD diet (AD group). Similarly, the rats on AM diet were randomly subdivided into two groups: a subset was shifted to AS diet started at E0.5 (MS group) and the others continued to be fed with AM diet (AM group). At this stage, there were five groups of rats in our experiments, namely, the AN (control group), the AD, the DS, the AM and the MS groups. The groups and diet treatments were shown in Table 1. The experiment was continued for another 19 days (the pregnancy for maternal rats). All the maternal rats were killed by cervical dislocation at E12.5 or E19.5. E12.5 embryos were rapidly separated, immediately frozen in liquid nitrogen and then stored at −80°C until analysis. Survival and morphology were detected from the fetuses collected at E19.5. Maternal blood was collected to determine VA status.

2.3. Plasma retinol analysis

Blood was collected by decapitation, and plasma was separated by centrifugation at 3000 rpm for 10 min and frozen at −80°C until analysis. Total retinol was extraction into hexane after saponification and determined by reversed-phase high-performance liquid chromatographic (LCQ Deca xp; Finnigan, US) using an aqueous mobile phase (methanol/acetonitrile/ammonium acetate, 75:12.5:12.5) at the speed of 0.2 ml/min and ultraviolet detection at 325 nm. Diluted sample were injected into a ZORBAX SB-C18 reversed-phase column (150 mm×2.1, 5 μm; Agilent, US).

2.4. Reverse transcription by real-time polymerase chain reaction

Total RNA of the E12.5 embryos was isolated using Trizol (Invitrogen) as directed by the manufacturer's instruction of the kit. Using the RT system (Promega, Madison, WI), we synthesized cDNA from 3 μg at 37°C for 60 min followed by 95°C for 5 min. Real-time polymerase chain reaction (PCR) was performed with SYBR® Premix Ex TaqTM (Takara Bio, Dalian, China), using the ABI 7900HT real-time thermocycler (Applied Biosystems, Forster, CA), and the cycle conditions were 95°C for 10 s and 40 cycles of 95 for 5 s and 60°C for 30 s. The dissociation curve of each gene was performed and analyzed using the ABI 7900HT software, and the result confirmed the product specificity. Each sample was analyzed two or three times and normalized to β-actin. The primers for the genes were listed in Table 2.

2.5. Western bolt analysis

The extraction nuclear protein from E12.5 embryos was performed based on published method. The embryonic tissues were firstly homogenized in phosphate buffer solution (PBS), and the collected cells were suspended in 600 μl cold hypotonic buffer (1.5 mM $MgCl_2$, 10 mM KCl, 1 mM dithiothreitol, 10 mM HEPES, pH 7.9, 0.4 phenylmethanesulfonyl fluoride) containing protease inhibitor cocktail (Amresco Inc, USA). The supernatant was then removed by centrifuge at 10,000×*g* for 20 min, and the resultant nuclear pellet was resuspended in 100 μl nuclear extraction buffer [1.5 mM $MgCl_2$, 0.42 M NaCl, 0.2 mM EDTA, 0.1 mM EGTA, 20 mM HEPES, 25% (v/v) glycerol] containing the protease inhibitor cocktail. After incubation on ice for 20 min, the nuclear suspension was centrifuged at 20,000×*g* for 5 min to collect supernatant as nuclear protein. The nuclear protein (50 μg) was separated by 12.5% sodium dodecyl sulfate polyacrylamide gel and then transferred onto a nitrocellulose membrane (Bio-Rad, Munich, Germany). The membranes were blocked with 5% bovine serum albumin (BSA) overnight and incubated with mouse RARα monoclonal antibody (1:500) at room temperature for 60 min, followed by incubation with antimouse IgG conjugated with peroxidase (Sigma, St. Louis, MO) at 1:10,000 dilution. After washing, the immunoreactive bands were detected by means of Amersham ECLTM Detection Kit (GE Healthcare Life Sciences, Little Chalfont, Bucks, UK) and quantified with a Biometra

Table 3
Body weight and plasma retinol concentration of maternal rats[a,b]

Group	Body weight (g)			Plasma retinol (μmol/L)		
	1st day	35th day	70th day	70th day	E12.5	E19.5
AN	60.60±3.55	165.79±19.90	236.93±21.12	1.62±0.43	1.54±0.27	1.49±0.24
AD	65.20±2.95	150.63±22.28 *	185.80±27.00 **	0.07±0.04 **	0.05±0.03 **	0.03±0.02 **
DS					1.35±0.33	1.27±0.25
AM	63.08±2.65	168.63±13.43	229.32±15.64	0.65±0.11 *	0.49±0.07 *	0.37±0.06 *
MS					1.52±0.38	1.41±0.19

[a] Values are means±S.E., n=6 to 14.
[b] The data of body weight and the plasma retinol were represented in accordance with the diets, so the body weight and plasma retinol on 70th day of AD and DS groups and AM and MS groups were the same.
* P<.05.
** P<.01, compared with AN group.

densitometer (Biometra, Göttingen, German). The blots were incubated with mouse monoclonal antibodies to RARβ (1:1000), RARγ (1:1000) and TBP (TATA binding protein, 1:2000, as control), respectively. The monoclonal antibodies to RARs and TBP were purchased form Abcam, Cambridge, Cambs, UK.

2.6. Analysis embryonic survival and morphogenesis at E19.5

At E19.5, we examined the survival and growth and development of fetuses. The fetuses were fixed in 4% paraformaldehyde or 95% ethanol after the measurement of their growth indices (body weight, body length and tail length). The fetuses fixed in 4% paraformaldehyde were analyzed for gross external morphology, whereas the ones fixed in 95% ethanol were analyzed for skeletal morphology by using Alcian blue and alizarin red, which stain cartilage and ossified tissue, respectively. The skeleton morphology was examined according to the atlas previously described [27].

2.7. Statistical analysis

SPSS 12.0 software package was used for statistical analysis. Data were presented as means±S.E. Statistical comparisons of measurement data were made using one-way analysis of variance and posttested using the least significant difference (LSD) test, while numeration data were using χ^2 test and Fisher's exact probability test. The results of real-time PCR were analyzed with the $2^{-\Delta\Delta Ct}$ method as previously described [28]. Significance of differences was accepted at P<.05 or P<.01.

3. Results

3.1. Effects of VAD on maternal rats

During the 70 days of depletion of VA and pregnancy, the VA intake was measured: the rats on AD diet received no VA, the rats on AM diet ingested 6.6±0.5 IU per rat per day, whereas those receiving AN diet consumed 65±2.7 IU per rat per day, the maternal rats on AS diet during pregnancy ingested 170±9.7 IU per rat per day. Before mating, the plasma retinol concentration was approximate to 0.07 μmol/L (a value generally accepted to be a standard for defining VAD in rat) [29], and the data were under the standard during pregnancy. The rats fed with AM were defined as marginally VAD as determined by plasma retinol concentration <0.7 μmol/L, a value frequently used to define marginal VAD in rat [6]. Accordingly, the rats' body weight of AN and AM had no difference and increased steadily, whereas the rats fed with AD diet were obviously delayed in growth since the statistical difference in data appeared in the fifth week and the growth retardation continued in the following weeks (Table 3). Meanwhile, rats on AD diet exhibited an array of VAD symptoms, as appetite loss, loss of whiskers, patches of fur, ocular porphyrin deposits and depression in response to external stimuli. With the supplement of VA starting at E0.5, the plasma retinol of DS and MS groups resumed to normal level while the data of AD and MD groups kept decreasing.

3.2. Embryonic survival, growth and development at E19.5

All of the fetuses in the AD group maintained on AD diet were completely resorbed at E19.5 (Table 4), even early at E12.5 (data not shown). In contrast, when AD maternal rats were supplemented with VA diet at E0.5, the majority of fetuses were alive, whereas the number of dead and resorbed fetuses still differed from AN and MS groups. We measured the growth indices for the live fetuses. The body weight and body length of the AM fetuses were significantly lower than those of the other three groups.

3.3. Skeleton morphology at E19.5

Embryonic morphology was normal at E19.5 in all fetuses from AN group. Likewise, fetuses from the maternal rats fed AS diet were morphologically normal despite some malformation, which had no statistical difference compared with the control group (Table 5). Furthermore, none of the VA supplement fetuses exhibited abnormalities, which are characteristic of VA excess toxicity [30]. In contrast, all the embryos from the AM group showed the retardation of ossification and the reduced size of skeleton extending from axial to appendicular skeleton. In the cranium region, the nasal turbinates and frontal and parietal bones were hypoplastic at a high penetrance of 100%, and half of the fetuses were found missing or with underdeveloped hyoid. The more attractive malformations were the one- to two-vertebral anteriorization that extends throughout the axial skeleton (Table 5, Fig. 2). Fetuses in AN or VA supplement (DS and MS) groups displayed the normal pattern of axial skeleton: 7 cervical, 13 thoracic and 6 lumbar vertebrae, and 4 sacral vertebrae attached to pelvis. The AM fetuses showed a sweep of anteriorization in cervical vertebrae: the rostral curvature of basioccipital bone became flat or curved caudally, even fused with the den of C1; the posterior cervical vertebrae gained more identity of anterior vertebrae, including C2 to C1, C3 to C2, C4 to C2/3, C7 to C6. Similar to the cervical region, the thoracic vertebrae exhibited the gain of anterior identity, and the evidences were seen in T3 to T2

Table 4
Effects of VAD on survival and growth indices of E19.5 embryos[a]

Group	n	Live embryos[b]				Dead and resorbed embryos		
		n	Body weight (g)	Body length (cm)	Tail length (cm)	n	Dead	Resorbed
AN	77	76	2.36±0.16	2.80±0.16	1.12±0.06	1	0	1
AD	61	0	–	–	–	61 **	0	61 **
DS	63	59	2.31±0.31	2.78±0.19	1.15±0.08	6 *	2	4
AM	91	57	2.05±0.24 *	2.58±0.20 *	1.06±0.08	34 **	25	9
MS	85	82	2.33±0.36	2.78±0.21	1.09±0.10	3	2	1

[a] Values are means±S.E., n=6 to 14.
[b] There were no data in AD group for all the embryos were resorbed.
* P<.05.
** P<.01, compared with AN group.

Table 5
Skeleton abnormalities in E19.5 fetuses from AM group (percentage of total)

Group	AN	AM	DS	MS
No. of fetuses/no. of litters	10/3	12/4	10/3	11/4
Cranial and cervical regions				
Hypoplastic nasal turbinates	–	100%	10%	–
Hypoplastic frontal and parietal	–	100%	–	–
Hyoid underdevelopment/missing	–	50%	–	–
Basioccipital flat or curved caudally	–	58%	–	–
C1 — dens of C1 fused with basioccipital	–	8%	–	–
C2 — neural arch abnormal/fused	–	33%	–	–
C2 — ventral lamina C1 like	–	33%	–	–
C3 to C2 conversion	–	17%	–	–
Prominent neural arch on C3	–	17%	–	–
C4 to C3/C2 conversion	–	8%	–	–
Tuberculum anterior located on C6	100%	67%	100%	100%
C7 to C6 (tuberculum anterior located on C7)	–	33%	–	–
Thoracic and lumbar regions				
First full rib on v8/T1	100%	100%	100%	100%
Reduced size and/or hypoplastic ribs	–	100%	–	–
Long and cranially directed neural spine on v9/T2	100%	33%	100%	100%
T2 to T1 (long and cranially directed neural spine missing)	–	67%	–	–
T3 to T2 (Long and cranially directed neural spine on T3)	–	17%	–	–
Six to seven ribs attached to the sternum	100%	58%	100%	100%
Four to five ribs attached to the sternum	–	33%	–	–
Normal sternum, 4–6 sternebrae	100%	–	90%	100%
Malformed sternum (0–1 sternebrae)	–	100%	–	–
Split sternum, fused only anterior	–	50%	–	–
Hammer-like flat spine on v17/T10	100%	33%	100%	100%
T10 to T9 (dorsal process curved caudally)	–	67%	–	–
T11 to T10 (dorsal process curved hammer-like flat)	–	33%	–	–
T12 to T10 (dorsal process curved hammer-like flat)	–	17%	–	–
Last full rib on v20/T13	100%	75%	100%	100%
L1 to T (gain of partial rib at v21)	–	25%	–	–
S1 to L (v27 lumbar in identity)	–	8%	–	–
Four vertebrae attached at pelvis	100%	67%	100%	100%
Three vertebrae attached at pelvis	–	17%	10%	–
Limbs				
Retardation of bone ossified	–	100%	–	–
Reduced or interrupted acromion	–	50%	–	–
Reduced size of limbs	–	100%	–	–

C, cervical; T, thoracic; v, vertebra; L, lumbar. "–" indicates 0% incidence.

(long and cranially directed neural spine on T3), T10 to T9 (dorsal process curved caudally), T11 to T10 (dorsal process curved hammer-like flat) and T12 to T10 (dorsal process curved hammer-like flat). This sweep of anteriorization was continued in the sacral region, where the 27th vertebra became a lumbar characteristic by the addition of an extranumerary rib.

3.4. Effects of VAD on RARs mRNA and protein expression in E12.5 embryos

Retinoic acid receptors play important roles in mediating biological effects of VA on embryonic development. The effect of VAD on *RARs* expression at mRNA and protein levels was therefore examined. As shown in Fig. 3. we observed a dose-dependent effect of the maternal VA intake on the expression of *RARs* at mRNA and protein levels. Vitamin A-free diet markedly suppressed the expression levels of all three *RARs* mRNA and protein in E12.5 embryos. However, in the AM group, the expression of *RARα* was relatively promoted; yet, the expressions of *RARβ* and *RARγ* remained down-regulated both at mRNA and protein levels. The suppressions of *RARβ* and *RARγ* were diminished in embryos from DS and MS groups, which were fed with AS diet throughout pregnancy. Interestingly, the *RARα* gene was most abundant in embryos from all the five groups compared with the *RARβ* and *RARγ*.

3.5. Effects of VAD on relative expression levels of Hox genes in E12.5 embryos

Real-time reverse transcriptase (RT)-PCR was used to quantitate gene expression of three *Hox* genes (*Hoxa2, Hoxa5, Hoxa9*) scattered on cluster A in E12.5 embryos, and the relative expression levels for each gene were shown in Fig. 1. The embryos from AD group had much lower mRNA levels of all the genes relative to AN group. In contrast, the embryos from AM group exhibited moderately decreased gene expression of *Hoxa2* and *Hoxa5*, which displayed significantly lower mRNA levels relative to AN group. The genes expression of the two VA supplement groups had no difference relative to AN group. Likewise, the *Hoxa2*, which was close to 3′ end of cluster A, had higher levels of relative expression than the *Hoxa9*, which was near the more 5′ end of the cluster.

4. Discussion

Embryonic pattern formation is controlled by intricate interactions of developmental genes, which provide positional and functional cues to cells' migration to the appropriate destinations and generate the proper structures [16]. The retinoids function as important morphogens, and they can affect the expression of *Hox* genes in both cell lines and embryonic tissues via combinations of nuclear retinoid receptors acting as ligand-inducible transcription factors. Previous studies have shown that deficient and excess VA could disturb the normal spatial and temporal expression of *Hox* genes. In the present study, we found that a VA-insufficient environment also repressed the expressions of the *RARs*, which constituted a more complicated molecular mechanism of the teratogenic effects in embryos of VAD. The quantitative analysis of *RARs* expressions in the E12.5 embryo tissue exhibited a dose-depend effect of the maternal VA intake on the expression of *RARs* at E12.5 embryos: the higher plasma retinol concentration (within the normal level) of maternal rats, the higher expression levels of *RARs* in embryos tissues. Using in situ hybridization, previous researches have demonstrated that RARα was almost ubiquitous in embryo at all stages, whereas the distribution of RARβ and RARγ were distinctive during embryo development [10–13]. Nevertheless, single knockouts mutants of RARs exhibited few congenital malformations in embryos, whereas compound mutants of RARs displayed serious birth defects, which suggested a high degree of functional redundancy among these receptors [9]. Noteworthy, the normal level of expression of *RARα* and less expressed *RARβ* and *RARγ* of embryos from AM group was reminiscence to compound mutants of RARβ and RARγ and indicated distinct responses of *RARs* to different levels of VAD. At

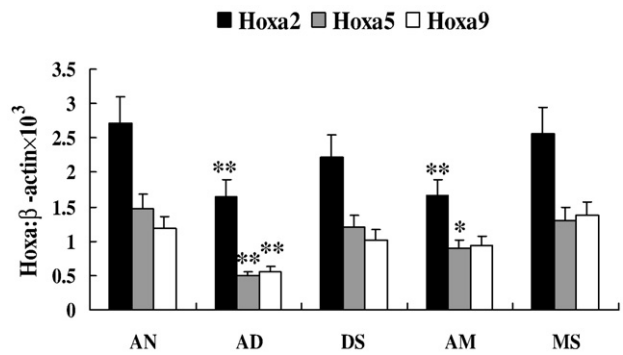

Fig. 1. Effects of VAD on relative expression levels of *Hox* genes in E12.5 embryos. Real-time RT-PCR was used to measure *Hoxa2, Hoxa5* and *Hoxa9* mRNA levels in E12.5 embryos tissue from five groups. Bars represent mean±S.E. for two to three replicates. Significance levels for each gene are $^{*}P<.05$, $^{**}P<.01$, compared with AN group.

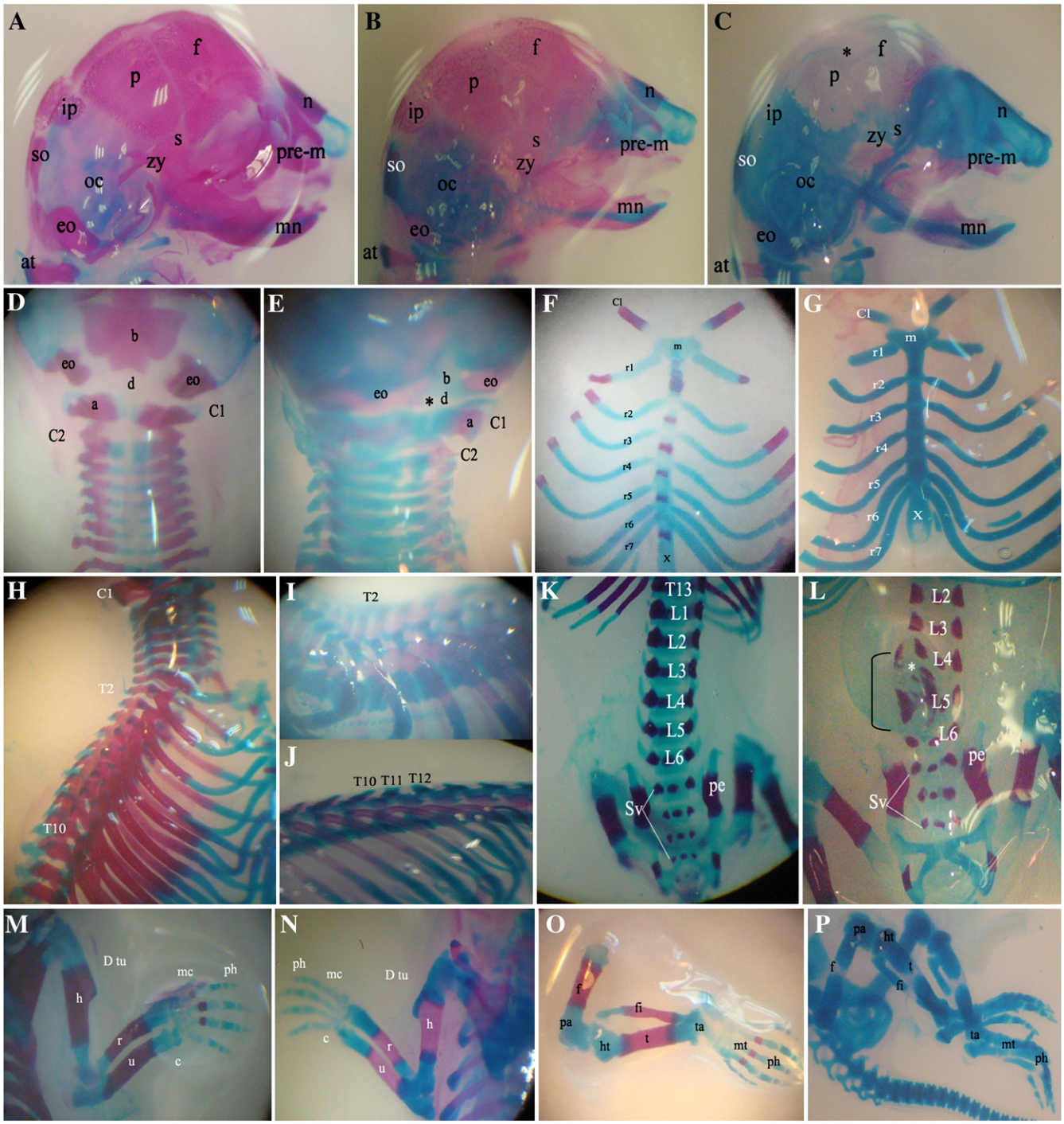

Fig. 2. Comparison of skeleton development in control (AN, DS, MS) and AM E19.5 fetuses. Cranium (A, B, C). (A) Lateral view of an control fetuses showing normal development of cranial elements: nasal (n), premaxilla (pre-m), mandible (mn), frontal (f), parietal (p), squamosal (a), zygomatic (zy), interparietal (ip), otic capsule (oc), supraoccipital (so), exoccipital (eo), atlas (at). (B, C) Group AM fetuses showing marked hypoplasia of cranium. Note the loss of frontal and parietal bones in panel C (asterisk). Craniocervical region (D, E). (D) Ventral view of craniocervical structure of a control embryo showing normal development of exoccipital (eo), basioccipital (b), atlas (a, the first cervical vertebrae, C1), the second cervical vertebrae (C2). (E) An embryo from AM group showing caudal curvature of the dens (d, asterisk). Sternum (F, G). (F) Ventral view of an embryo from control group showing normal development of the clavicle (cl), manubrium (m), xiphoid process (x), ribs (r, numbers indicate vertebra number) and six ossified points on the sternum. (G) An E19.5 fetus showing sternum with lost ossified point. Cervical and thoracic regions (H, I, J). (H) Lateral view of a control fetus showing normal skeleton elements of cervical and thoracic regions. The T2 is characterized by a long and cranially directed neural spine; T10 shows a typical hammer-like spine. (I) An AM fetus showing the loss of identity of T2 and (I) anteriorization of T11 to T10 (shift of hammer-like spine from T10 to T11). Lumbar and sacral regions (K, L). (K) Ventral view of an control fetus showing normal lumbar and sacral elements. (L) Ventral view of an AM fetus showing marked distortion of L4, L5, L6 (bracket). Limbs (M, N, O, P). An fetus of control group depicting normal forelimb (M) structure [humerus (h), radius (r), ulna (u), deltoid tuberosity (D tu), carpal bones (c), metacarpus (mc), phalanges (ph)] and hind limb (O) elements [femur (f), patella (pa), head of tibia (ht), fibula (fi), tibia (t), tarsus (ta), metatarsus (mt), phalanges (ph)]. Compared with a normal fetus, the AM fetus depicts moderately dysostosis of forelimb (N) and hind limb (P).

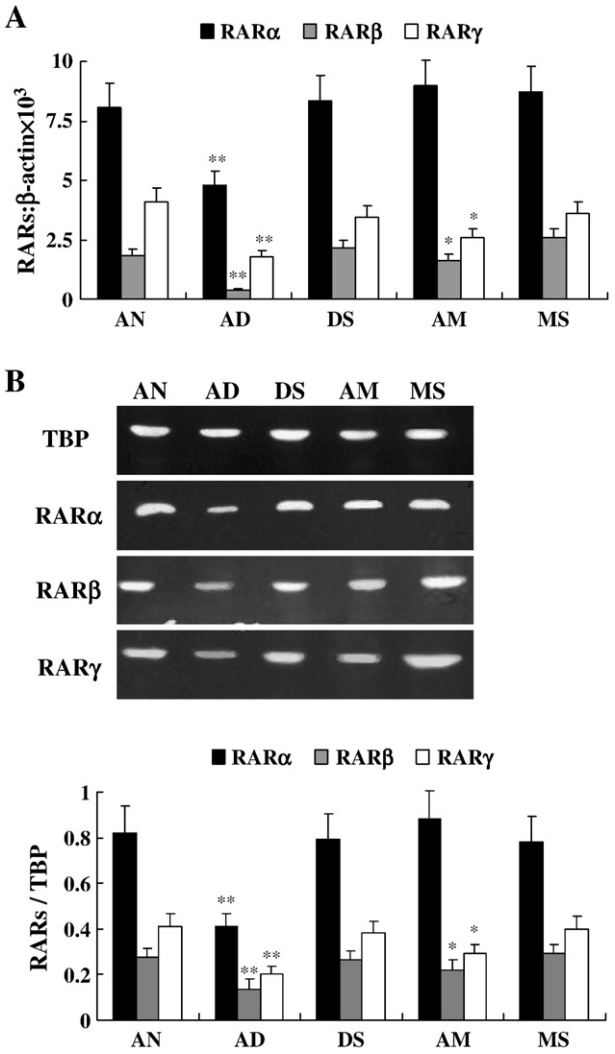

Fig. 3. Effects of VAD on *RARs* mRNA and protein expression in E12.5 embryos. (A) Representative real-time RT-PCR analysis of *RARα*, *RARβ*, *RARγ* in E12.5 embryos. The bars represent mean±S.E. for two to three replicates. $^{*}P<.05$, $^{**}P<.01$, compared with AN group. (B) Representative Western blot analysis of *RARα*, *RARβ* and *RARγ* in E12.5 embryos. The bars represent mean±S.E. for two to three replicates. $^{*}P<.05$, $^{**}P<.01$, compared with AN group.

E19.5, skeletal malformations were diverse and extensive through A/P axis, indicating important roles of *RARβ* and *RARγ* in embryonic skeletal development. These discoveries of *RARs* expression in VAD animal model might be a meaningful supplementary of the ligand–receptor molecular mechanism of VA signaling in embryogenesis. The VAD decreased the quantity of ligand and suppressed the expressions of *RARs*, and the cascade effects could facilitate the teratogenicity of VAD on the skeleton development which might even lead to the death of the embryos. Similar study has been carried out in quail embryos, and it was addressed that the expression of *RARα2* and *RARβ2* was severely diminished. However, the administration of RA to VAD embryos at or before the 4/5 somite stage rescued the expressions of *RARα2* and *RARβ2* and restored normal development. After neurulation, the expressions of all retinoid receptors in the VAD quail embryo became independent of VA status and were similar to that of the normal [31]. Our results essentially agreed with the previous study. However, we recognized that the *RARβ* and *RARγ* expressions were significantly different in both AD and AM groups relative to AN group.

Another important finding in this study was that *Hox* genes were obviously repressed, and the sensitivity to VA of each gene on cluster A was relevant to their location from the 3′ end to 5′ end. The *Hoxa2* has been shown to be expressed in the developing mouse neural tube and the paraxial mesoderm and in the neural crest cells of the second and third branchial arch. In *Hoxa2* null mutants, the hyoid skeleton is replaced by a duplicated set of mandibular and middle ear structures [32–35]. In our study, the hyoid was detected underdeveloped or missing. *Hoxa5* was reported to correct specification of the cervical and upper thoracic region of the skeleton [36]. The loss of *Hoxa5* function perturbs axial specification in the third cervical (C3) and the second thoracic vertebrae (T2). Anterior transformations of C3, C4 and C6, posteriorization of C7 and T1 and dorsal process on T2 are observed in *Hoxa5* mutants. Most of the skeleton deformations mentioned above were detected in a majority of embryos from AM group. *Hoxa9* is involved in patterning the thoracolumbar region [37] and plays an essential role in limbs [38–40]. The E19.5 fetuses from AM group displayed skeletal malformations with respect to the abnormities, which Fromental–Ramain has reported, including the supernumerary ribs on L1, and anterior homeosis of lumbar and sacral vertebrae. It has been approved that *Hoxa1* and *Hoxa4* which are the upstream genes of *Hoxa2* and *Hoxa5* respectively, have RAREs in the flanking regions. The sensitivity of *Hoxa2* and *Hoxa5* might owe to the neighborhood effects [41]. Kaiser et al. [42] has reported an anteriorization throughout the axial skeleton result from early VA deficiency, and he indicated that the posterior shift expression in the mesodermal expression of *Hoxa9* was associated with the anteriorization in thoracic and lumbar region. In our study, the anterior homeotic shift was detected in the same region, although the penetrance and severity were moderately lower. However, the expression levels of *Hoxa9* seemed equivalent to the normal expression level, and we attributed the disagreement to the limitation of evaluation procedures. In addition, late VAD fetuses displayed both anteriorization of cervical regions and novel posteriorization events at the thoracic and sacral levels of the skeleton [43], however we did not discover the posteriorization in our study.

Vitamin A deficiency is a major public health problem worldwide, which has contributed substantially to the health threat among young children and women of reproductive age in developing countries. The low level of maternal VA status, which lacks enough attention, primarily results from the concealment of characteristic and dramatic clinical signs, putting the next generation at a high risk [44]. The situation was confirmed in the present study, especially on the marginal deficiency of VA group (0.4 IU/g diet). The rats fed with AM diet exhibited no hypoplasia or clinical signs of VAD before mating. However, the marginal deficient VA environment was reflected in their dead, malformed and dysplastic embryos. Fortunately, the supplement of sufficient VA (10 IU/g diet) at early pregnancy displayed improved outcomes. The lethality and malformation occurrence of the offspring from supplement groups decreased significantly, and the growth indices became normal. In addition, the rats fed on AD and AM diets had a longer estrous cycle and lower conceptual quotient, indicating a possible alteration in hormonal balance at severe levels of VAD. The impaired reproductivity of the AD and AM maternal groups was in compliance with the data derived from the animal models with maternal VAD in pregnancy, which led to placental dysfunction, fetal loss and congenital malformations [45]. It would be expected to contribute to higher rates of neonatal and infant morbidity and mortality.

In summary, in this study, we demonstrated a synergistic suppression effect of *RARs* and *Hox* genes coexpression by VAD: the deficiency of maternal VA status did not only decrease the quantity of ligand but also down-regulated the expressions of the receptors (RARs), which suppressed the expressions of target genes, the *Hox* genes. The expressions of *RARβ* and *RARγ* in E12.5 embryo exhibited

higher levels of sensitivity to deficient status of maternal VA. In addition, the three *Hox* genes from 3′ to 5′ end on cluster A showed a nonuniform sensitivity to VA: the Hox genes on cluster A also showed a gradient in abundance and gradual sensibility from 3′ end to the 5′ end; the genes near the 3′ end displayed a more vulnerable response to lower VA status, which emphasized the role of VA as an important signaling molecule to control early development of embryos and the essentiality of early supplement of VA.

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