0013-7227/00/$03.00/0
Endocrinology

Vol. 141, No. 1
Printed in U.S.A.

Retinoic Acid Is a Potent Regulator of Growth Plate Chondrogenesis

FRANCESCO DE LUCA, JENNIFER A. UYEDA, VERONICA MERICQ, EDNA E. MANCILLA, JACK A. YANOVSKI, KEVIN M. BARNES, MAIJA H. ZILE*, AND JEFFREY BARON

Developmental Endocrinology Branch (F.D.L., J.A.U., V.M., E.E.M., J.A.Y., K.M.B., J.B.), National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20892; and Department of Food Science and Human Nutrition (M.H.Z.), Michigan State University, East Lansing, Michigan 48824-1224

ABSTRACT

Vitamin A deficiency and excess both cause abnormalities in mammalian longitudinal bone growth. Because all-*trans* retinoic acid (RA) is synthesized from vitamin A, we hypothesized that RA regulates growth plate chondrogenesis. Consistent with this hypothesis, a single oral dose of RA reduced the height of the rat proximal tibial growth plate. To determine whether RA acts directly on growth plate, fetal rat metatarsal bones were cultured in the presence of RA. In this system, RA inhibited longitudinal bone growth by three mechanisms: 1) decreased chondrocyte proliferation, (assessed by ^{3}H-thymidine incorporation), particularly in the proliferative zone of the growth plate; 2) decreased matrix synthesis (assessed by $^{35}SO_4$ incorporation into glycosaminoglycans); and 3) decreased cell hypertrophy (determined histologically). The growth-inhibiting effects of RA were completely reversed by a retinoic acid receptor (RAR) antagonist. In the absence of exogenous RA, this antagonist accelerated bone growth, as did an RA-specific neutralizing antibody, suggesting that endogenous RA negatively regulates growth plate chondrogenesis. We conclude that RA, acting through RARs, negatively regulates longitudinal bone growth by inhibiting growth plate chondrocyte proliferation, chondrocyte hypertrophy, and matrix synthesis. (*Endocrinology* **141:** 346–353, 2000)

IN MAMMALS, longitudinal bone growth occurs by a process called endochondral ossification (1), which takes place in the growth plates of the long bones. Chondrocytes secrete cartilage matrix, proliferate, and differentiate into hypertrophic cells (2). In isolation, the resulting chondrogenesis would lead to progressive widening of the growth plate. However, simultaneously the metaphyseal border of the growth plate is invaded by blood vessels and bone cell precursors that remodel the cartilage into bone (3). The net result is longitudinal bone growth.

Retinoids appear to play an important role in mammalian embryonic limb development (4, 5) and in bone growth during fetal and postnatal life. Deficiency of vitamin A, a precursor of all naturally occurring retinoids, inhibits longitudinal bone growth (6). Excess vitamin A (7) or all-*trans* retinoic acid (RA) (8), a potent natural retinoid, causes premature fusion of the epiphyseal growth plate and thus a premature arrest of longitudinal bone growth.

To elucidate the role of retinoids in endochondral bone formation, we first examined the effects of exogenous RA on the growth plate *in vivo*. Then, to distinguish direct effects of RA on the growth plate from indirect effects and to investigate the underlying mechanisms, we examined the effects of RA on cultured fetal rat metatarsal bone rudiments. Unlike isolated cell cultures, this organ culture system preserves the organized histological architecture of the growth plate and thus the intercellular interactions and local microenviroments found *in vivo*.

To determine whether endogenous retinoids play any biological role in growth plate chondrogenesis, we selectively neutralized RA action in the cultured rat metatarsals and assessed the effects on longitudinal bone growth.

Materials and Methods

Animal care

For the *in vivo* study, male Sprague Dawley rats (27–31 days of age) were given a single dose of 300 mg/kg RA suspended in soy bean oil by gavage. Control rats received vehicle alone. The animals (n = 7 per group) were killed at 6, 12, 24, 48, and 96 h after retinoic acid or soybean oil administration. Animal care was in accordance with the Guide for the Care and Use of Laboratory Animals [DHEW Publication (NIH) 85–23, revised 1988]. Animal procedures were approved by the NICHD animal care and use committee.

Organ culture

Metatarsal bone rudiments were dissected from rat embryos (20 dpc) and cultured separately in 24-well culture dishes (9). Each well contained 0.5 ml of MEM (Life Technologies, Inc., Gaithersburg, MD) supplemented with 0.2% BSA (Sigma), 0.3 mg/ml-glutamine (Life Technologies, Inc.), 0.05 mg/ml ascorbic acid (Life Technologies, Inc.), 1 mM sodium glycerophosphate (Sigma), 100 U/ml penicillin, and 100 mg/ml streptomycin (Life Technologies, Inc.).

Bone rudiments were cultured for 2 or 3 days in a humidified incubator with 5% CO_2 in air, at 37 C. The medium was changed daily. For the first sets of experiments, all-*trans* RA (Sigma) and AGN 193109 (donated by Dr. Chandraratna, Allergan, Inc., Irvine, CA) were dissolved in DMSO (Sigma) and further diluted separately into the culture medium (2 μl DMSO/ml medium), to reach the final concentrations of

Received March 19, 1999.
Address all correspondence and requests for reprints to: Francesco De Luca, Department of Pediatrics, University of Maryland School of Medicine, 22 South Greene Street, Room N5E13, Baltimore, Maryland 21201-1595. E-mail: fdeluca@peds.umaryland.edu.
* Supported by a USDA grant and by the Michigan Agricultural Experiment Station.

10, 100, 1000, or 10,000 nM. Control cultures received the same volume of DMSO without RA or AGN 193109.

In the final set of experiments, the bone rudiments were cultured in the presence of a mouse monoclonal antibody (IgG) against RA (ANTI-RA mAb, 200 mg/ml, generated as previously described) or a control mouse monoclonal antibody against *Escherichia coli* β-galactosidase.

Measurement of longitudinal bone growth

The length of each bone rudiment was measured under a dissecting microscope, using an eyepiece micrometer (Carl Zeiss, Thornwood, NY) at 20× magnification (10). Length measurements were performed at 0, 1, 2, and 3 days of culture. Culture medium was briefly removed before each measurement.

Histology

For the *in vivo* study, tibiae from each hind limb were dissected, fixed in phosphate-buffered formalin, decalcified, and paraffin-embedded. 5-μm longitudinal sections were obtained and stained with Masson Trichrome. Slides were viewed with a Leica Corp. microscope, model DMRX. An image of each growth plate was created using a color video camera (model CH-250, Photometric Ltd.) and Oncor Image 2.0, a computer imaging program. The effect of RA on the growth plate was evaluated by measuring the heights (μm) of the whole growth plate, the resting zone, the proliferative zone, and the hypertrophic zone of the growth plate. The magnification was determined by imaging a stage micrometer. All measurements were performed by a single observer blinded to the treatment regimen.

For organ culture studies, metatarsal rudiments were fixed in buffered formalin, embedded in plastic, cut in 5-μm-longitudinal sections and stained with toluidine blue. From each bone rudiment, we obtained three sections parallel to the long axis of the bone and taken 30 microns apart. We then measured the number of hypertrophic cells in both growth plates in each of the three sections, and calculated the average value. Hypertrophic chondrocytes were operatively defined by a height along the longitudinal axis greater than 9 μm (10). All the measurements were performed by a single observer blinded to the treatment regimen.

Enzyme histochemistry

The activity of alkaline phosphatase was localized by enzyme histochemistry. Bone rudiments were embedded in OCT compound (Sakura Finetek, Torrance, CA) and frozen. Ten-micrometer cryostat sections were cut and mounted onto poly-L-lysine-coated slides. Mounted sections were treated at room temperature with 4% formaldehyde in PBS for 10 min, rinsed in PBS, and then placed in 0.1 M triethanolamine-HCl, pH 8.0, for 10 min. Slides were rinsed in DEPC-treated water and then stained for alkaline phosphatase for 3 min at room temperature using the simultaneous-coupling azo dye method (11). In the working solution (0.5% *N,N*-dimethylformamide and Tris buffer, pH 9.1), 0.03% napthol AS phosphate (Sigma) was used as substrate, with 0.1% Fast blue BB salt (Sigma) as the azo dye. Slide were rinsed and then counterstained with 0.25% safranin O (Sigma) for 3 sec, followed by several rinses in distilled water. Sections were then dried and mounted in DAKO Corp. Faramount aqueous mounting medium (DAKO Corp., Carpinteria, CA).

[^{3}H]Thymidine incorporation

Cell proliferation was assessed in the cultured bone rudiments by measuring [^{3}H]thymidine incorporation into the bones (12). [^{3}H]thymidine (Amersham Pharmacia Biotech, specific activity 25 Ci/mmol) was added to the culture medium 3 h before the end of the incubation period, at a concentration of 5 μCi/ml. All rudiments were then washed three times for 10 min with MEM and solubilized with NCS-II Tissue Solubilizer 0.5 N solution (Amersham Pharmacia Biotech). The amount of incorporated isotope was determined by liquid scintillation counting. To analyze the sites of the growth plate where DNA synthesis occurred, we incubated control and RA-treated bones with [^{3}H]thymidine as described above. At the end of the incubation, all bones were fixed in buffered formalin and processed for autoradiography. Autoradiography was performed by dipping the slides in Kodak NTB-2 emulsion (Eastman Kodak Co., Rochester, NY), exposing 1 week, and developing with Kodak D-19 developer. Sections were counterstained with hematoxylin and eosin. The labeling index (number of labeled cells/total cells) was determined separately for the resting zone and for the proliferative zone. Cells in the perichondrium were not included in either analysis. All determinations were made by the same observer blinded to the treatment category.

$^{35}SO_4$ incorporation

As a measure of cartilage matrix synthesis, we assessed ^{35}S-sulfate incorporation into glycosaminoglycans (9). $Na_2{}^{35}SO_4$ (Amersham Pharmacia Biotech, specific activity up to 100 mCi/mmol), at a concentration of 5 μCi/ml, was added to the culture medium 3 h before the end of the 2-day culture period. The rudiments were washed three times for 10 min with Puck's saline solution and then digested in 1.5 ml of MEM with 0.3% papain at 60 C for 16 h. They were then incubated with 0.5 ml of 10% cetyl pyridinium chloride (Sigma) in 0.2 M NaCl at room temperature for 18 h. At the end of the incubation, each precipitate was collected by vacuum filtration through filter paper (Whatman, Clifton, NJ; catalog no. 1001090), washed three times with 1 ml 0.1% cetyl pyridinium chloride in 0.2 M NaCl, and dissolved in 0.5 ml of 23 N formic acid. The amount of radioactivity incorporated into glycosaminoglycans was measured by liquid scintillation.

Statistics

Data were expressed as mean ± SEM. Statistical evaluation was performed by ANOVA and posthoc Fisher's protected least significant difference test.

Results

Effects of RA on the growth plate in vivo

Male rats (27- to 31-day-old) received a single dose of 300 mg/kg RA by gavage. Control rats received vehicle alone (soybean oil). When examined histologically, the growth plate of the RA-treated rats appeared thinner than that of the control rats (Fig. 1). RA caused a significant decrease in the height of the growth plate proliferative zone (26 ± 4 *vs.* 62 ± 5 μm, RA *vs.* control at 48 h, mean ± SEM, $P < 0.001$, Fig. 2A), the hypertrophic zone (38 ± 5 *vs.* 93 ± 3 μm, $P < 0.001$, Fig. 2B) and the overall growth plate (71 ± 8 *vs.* 161 ± 5 μm, $P < 0.001$ (Fig. 2C). These effects were first observed at 48 h. In contrast, the height of the resting zone was not affected by the RA treatment (data not shown).

Effects of RA on chondrogenesis in vitro

Metatarsal bones from 20 dpc rats were cultured in serum-free medium containing 0–1000 nM RA for 2 days. RA caused a concentration-dependent inhibition of longitudinal bone growth (n = 28–34 per group, Fig. 3).

Histological studies were performed on the cultured metatarsals after 48 h of culture. In the growth plate near the ossification center, 100 and 1000 nM RA caused a decrease in the number of hypertrophic chondrocytes per section (24.4% and 6.4% of control respectively, n = 4–8/group, $P < 0.001$). At 1000 nM RA, virtually all the large cells were replaced by small, flat cells (Fig. 4). As assessed by enzyme histochemistry, these flattened cells expressed alkaline phosphatase, a marker for terminally differentiated chondrocytes (Fig. 5).

To determine the site of the growth plate in which chondrocyte proliferation occurred, we performed autoradiography of [^{3}H]thymidine-labeled bones. RA caused a significant decrease of the labeling index only in the proliferative zone

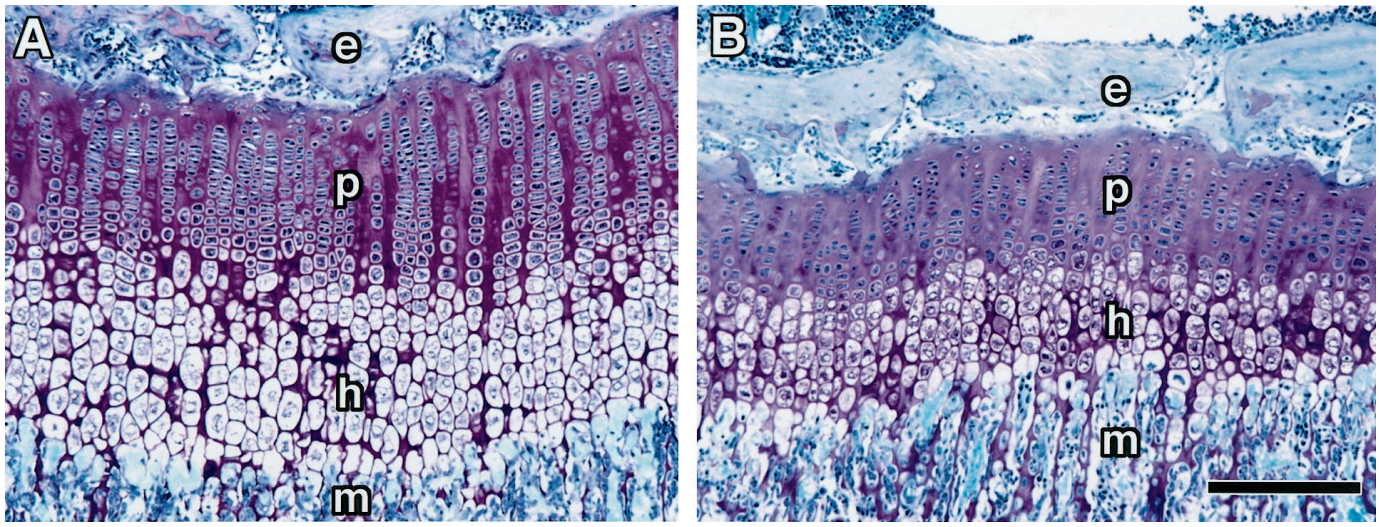

FIG. 1. Representative photomicrographs demonstrating the effects of systemic RA administration on rat growth plate. Male Sprague Dawley rats (27–31 days of age) received a single dose of 300 mg/kg RA (B) or soybean oil vehicle (A) by gavage. After 48 h, proximal tibiae were dissected, fixed, decalcified, and paraffin-embedded. Five-micrometer longitudinal sections were obtained and stained with toluidine *blue*. e, Epiphyseal bone; p, proliferative zone; h, hypertrophic zone; m, metaphyseal bone. *Bar*, 200 μm.

of the growth plate ($P < 0.001$, Fig. 6). The decrease in labeling of proliferative chondrocytes was observed at 100 nM as well as 1000 nM RA. RA at 1000 nM concentration also inhibited total [^{3}H]thymidine incorporation into the metatarsal bones (72.9% of controls, n = 14–16 per group, $P < 0.001$). The effect of RA on cartilage matrix synthesis was assessed by measuring the incorporation of $^{35}SO_4$ into newly synthesized glycosaminoglycans. At 1000 nM RA, glycosaminoglycans synthesis was significantly reduced (53.3% of control, n = 15 per group, $P < 0.001$).

Effects of RA/RARs inhibitors on chondrogenesis

To investigate which class of receptors mediates the inhibitory effects of RA, bone rudiments were cultured with 100 nM RA plus equimolar, 10, or 100-fold molar excess of AGN 193109, a selective RAR-antagonist. Cotreatment with 100-fold molar excess of AGN 193109 not only reversed the RA-mediated inhibition of longitudinal bone growth (n = 8 per group, $P < 0.01$, Fig. 7) but actually produced a growth rate greater than that of controls. Similarly, in metatarsal bones cultured without exogenous RA, AGN 193109 caused a concentration-dependent acceleration in growth rate (n = 31–33 per group, $P < 0.0001$, Fig. 8A).

To determine whether endogenous RA itself regulates longitudinal bone growth, we cultured the bone rudiments in the presence of 200 mg/ml ANTI-RA Ab, a mouse monoclonal antibody highly specific for all-*trans* RA. ANTI-RA-treated metatarsals grew at a significantly greater rate than did control anti-β-galactosidase-Ab-treated metatarsals (n = 31–36 per group, $P < 0.0001$, Fig. 8B).

Discussion

A single dose of RA administered systemically to rats caused a significant reduction of the tibial growth plate height. This effect, first seen 48 h after the administration of RA, resulted from the reduction of both proliferative and hypertrophic zone height. Similar observations have been made by Soeta *et al.* (13) in rats treated with high doses of vitamin A for periods of 1 to 5 days.

While our findings pertain to the acute effects of retinoic acid, several other studies have described the effects of chronic administration of high doses of vitamin A or RA on growth plate morphology (14–18). Microscopic examination revealed a number of degenerative changes, ranging from loss of cartilage matrix to calcified areas in the resting and proliferative zones. Gross lesions included focal thinning followed by premature closure of the growth plate. Early fusion of the epiphyses and growth retardation have also been reported in humans after chronic administration of vitamin A and other retinoids (7, 19–20). Premature fusion could result either from accelerated ossification or from inhibition of chondrogenesis with unchecked ossification. Although the observed reduction of the hypertrophic zone could be explained by either mechanism, the diminution of the proliferative zone suggests an effect on chondrogenesis.

The observed effects of systemic RA could reflect direct actions on the growth plate or indirect actions mediated by metabolic, nutritional, or endocrine factors. To determine whether RA acts directly on the growth plate, we employed a fetal rat metatarsal organ culture system. In this system, RA caused a concentration-dependent inhibition of longitudinal bone growth. This finding, which is consistent with the effects elicited by retinoids *in vivo*, suggests that RA can suppress longitudinal bone growth by a direct, local action on the growth plate.

The inhibition of longitudinal bone growth appeared to be due to multiple mechanisms. First, RA caused a concentration-dependent inhibition of chondrocyte proliferation in the growth plate proliferative zone, as assessed by autoradiography. At the higher concentration (1000 nM), RA also decreased total thymidine incorporation, which is the sum of thymidine incorporation in the resting zone, proliferative zone, ossification center, and perichondrium. Second, RA caused a concentration-dependent inhibition of chondrocyte

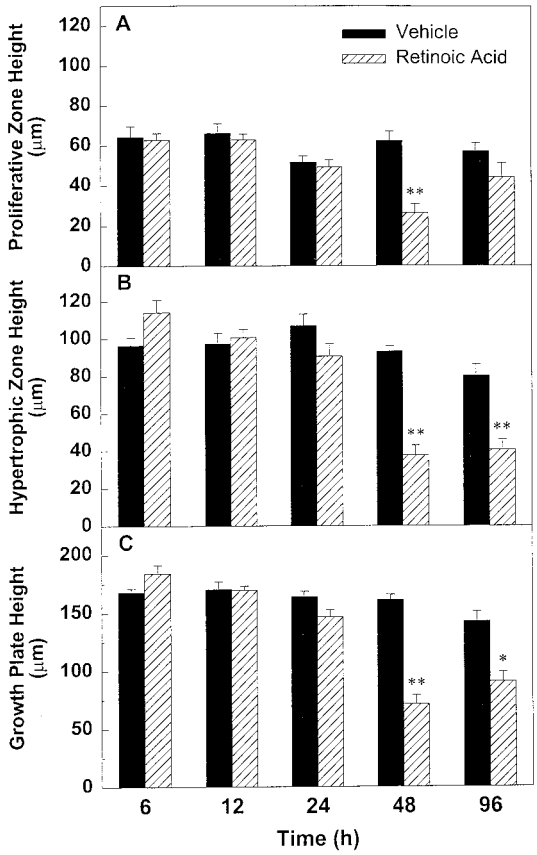

FIG. 2. Effects of systemic RA administration on growth plate height. Male Sprague Dawley rats (27–31 days of age) received a single dose of 300 mg/kg RA or soybean oil vehicle by gavage. The animals (n = 7 per group) were killed at 6, 12, 24, 48, and 96 h after retinoic acid or vehicle administration. Five-micrometer longitudinal sections were obtained as described in Fig. 1. The effect of RA on the growth plate was evaluated by measuring the heights (μm) of the proliferative zone (A), the hypertrophic zone (B), and the whole growth plate (C). All measurements were performed by a single observer blinded to the treatment regimen. *, $P < 0.01$ *vs.* vehicle. **, $P < 0.001$ *vs.* vehicle.

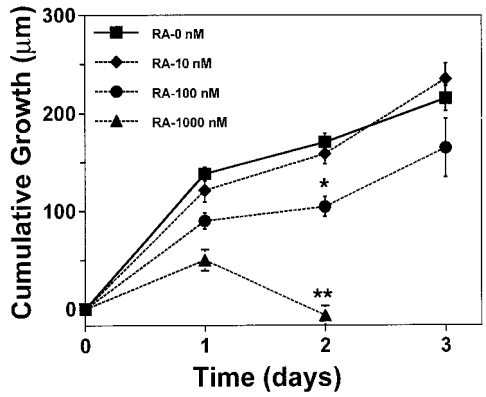

FIG. 3. Effects of RA on longitudinal bone growth (mean ± SEM). Fetal rat metatarsals (20 dpc, n = 28–34/group) were cultured for 3 days in serum-free medium containing 0–1000 nM RA. The length of the bones was measured daily using an eyepiece micrometer in a dissecting microscope. By the third day of culture, metatarsals incubated with 1000 nM RA had become curved, preventing accurate measurement of linear growth. *, $P < 0.001$ (*vs.* 0 nM RA and *vs.* 1000 nM RA). **, $P < 0.001$ (*vs.* 0 nM RA and *vs.* 100 nM RA).

hypertrophy. In the RA-treated bones, cells adjacent to the ossification center expressed alkaline phosphatase, a marker for terminally differentiated chondrocytes, but did not show the normal enlargement in height (the dimension parallel to the longitudinal axis of the bone). This cellular enlargement is thought to be critical for longitudinal bone growth (21, 22). Third, the highest concentration of RA had a negative effect on cartilage matrix synthesis, as assessed by $Na_2{}^{35}SO_4$ incorporation into glycosaminoglycans. Thus, 100 nM RA inhibited chondrocyte proliferation and chondrocyte hypertrophy but did not affect matrix synthesis. Because this RA concentration is close to those found in the developing limb bud and in serum, our observations suggest that endogenous RA may physiologically inhibit longitudinal bone growth by decreasing growth plate chondrocyte proliferation and chondrocyte hypertrophy. The inhibition of $Na_2{}^{35}SO_4$ incorporation into glycosaminoglycans occurred only at the highest RA concentration. Thus, regulation of proteoglycan synthesis may not represent a physiological effect of RA but may still represent one of the pharmacological mechanisms by which retinoid toxicity causes premature closure of the growth plate.

Previous *in vitro* studies of RA effects on chondrocytes have primarily used isolated cell cultures. In one study, inhibition of matrix synthesis by RA was reported (23), consistent with our findings in organ culture. Some cell culture studies suggest that RA inhibits proliferation (24), whereas others suggest that it stimulates proliferation (23, 25). Similar conflicting results have been reported concerning the effects on chondrocyte differentiation (25, 26). These discrepancies may be due in part to the loss of normal architecture and the resulting loss of cell-cell and cell-matrix interactions that occur in isolated cell culture. Consequently, the behavior of dispersed cells may differ from that of cells in the intact growth plate and may be more dependent on the precise conditions such as time in culture, confluence, and growth factors in the medium. Therefore, our findings in organ culture may better reflect the *in vivo* effects of RA on longitudinal bone growth. In fact, our findings in organ culture are consistent with and provide a possible explanation for the observed effects of RA *in vivo*, including: 1) the rapid reduction in growth plate height observed in the current study; and 2) the disappearance of the cartilage growth plate reported by others.

To investigate the role of retinoic acid receptors, we used AGN 193109 (27), a selective antagonist of RARs (28). Incubation of rat metatarsals with RA and AGN 193109 resulted in reversal of the RA-mediated inhibition of longitudinal bone growth. These results suggest that the inhibitory effects of RA are mediated by the activation of RARs. However, our findings cannot exclude the possibility that RXR receptors may also play some role in longitudinal bone growth. However, the lack of skeletal malformations in RXR-α, RXR-β, and RXR-γ null mice suggests a less critical role for RXR receptors during bone growth and development compared with RAR receptors (29, 30). To determine whether the effects elicited by exogenous RA reflect the physiological effects of endogenous retinoids in the growth plate, fetal rat metatarsals were

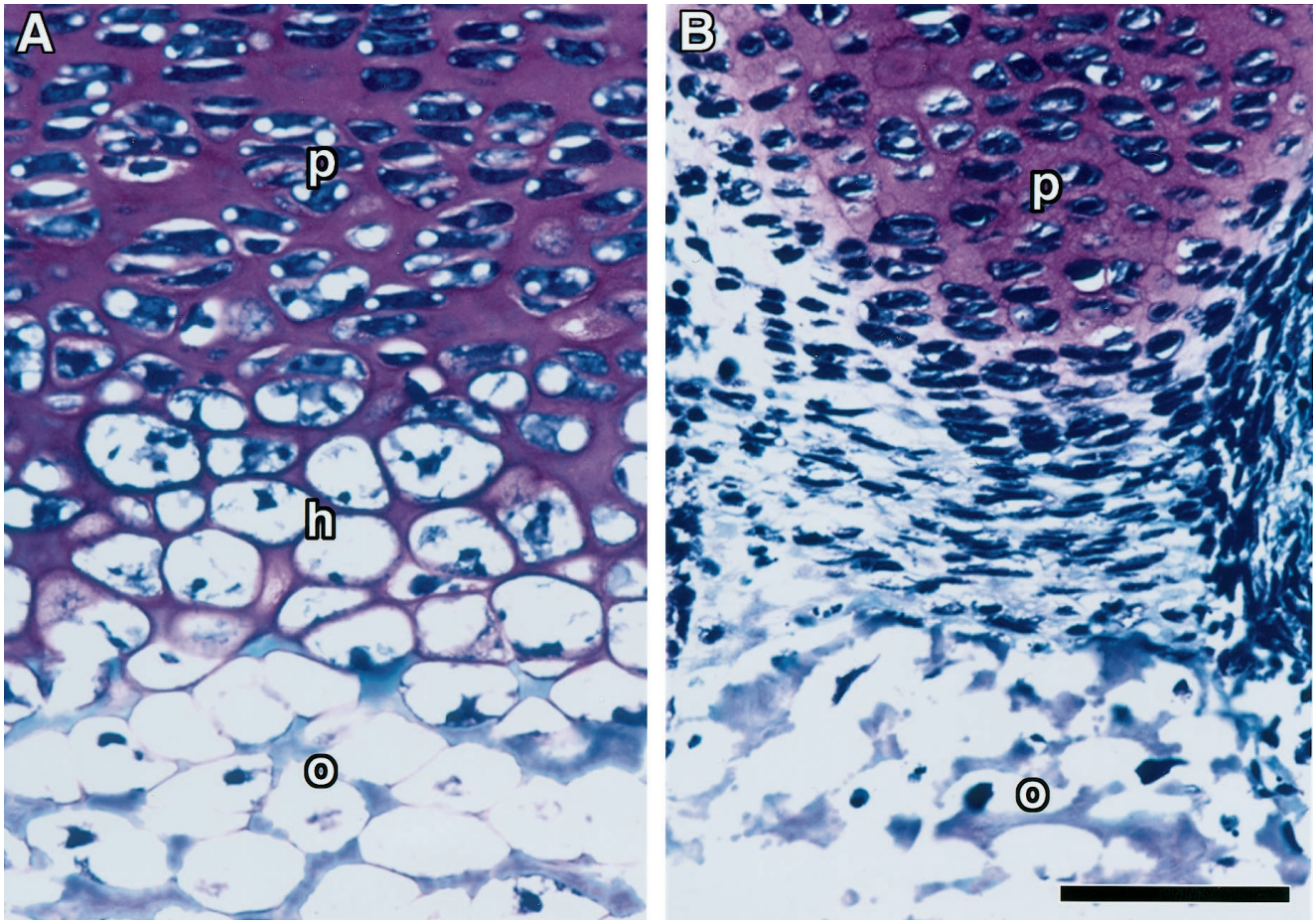

FIG. 4. Representative photomicrographs demonstrating the effects of RA on cultured fetal rat metatarsals. Metatarsal bones from 20-dpc rat fetuses were cultured for 2 days in serum-free medium without RA (A) or with 1000 nM RA (B). After routine histological processing, bones were embedded in plastic, and 5-μm longitudinal sections were obtained and stained with toluidine *blue*. p, Proliferative zone; h, hypertrophic zone; o, primary center of ossification. *Bar*, 50 μm.

cultured in the presence of AGN 193109 without any exogenous RA. This RAR antagonist alone produced a significant, concentration-dependent increase in longitudinal growth. This finding suggests a physiological inhibitory role for RARs in the growth plate.

The stimulatory effect of AGN 193109 does not necessarily imply a role for all-*trans* RA. Other natural retinoids can activate RARs. Furthermore, in cultured human keratinocytes, AGN 193109 may act as an inverse agonist, inhibiting activity of unliganded RARs (31). Therefore, to define the specific role of endogenous retinoic acid in the growth plate, we used a neutralizing mouse monoclonal antibody directed against RA (32). This antibody cross-reacts with a derivative of all-*trans*-retinoic acid (all-*trans*-3,4-didehydroretinoic acid), but it has very low cross-reactivity with other natural retinoids including 9-cis-retinoic acid (0.4–4%) retinol (1.1–5%) and retinal (4%) (33). Bone rudiments cultured in the presence of this antibody grew significantly more rapidly that did bones cultured with a control mouse monoclonal antibody. This finding suggests that all-*trans* endogenous RA physiologically restrains growth plate chondrogenesis by acting as an autocrine/paracrine factor.

Our studies in organ culture suggest that blocking endogenous retinoid activity stimulates bone growth. In previous studies, the effects of vitamin A deficiency on bone growth have been conflicting. Consistent with our findings, Havivi and Tal reported increased long bone growth in vitamin-A deficient chicks (34). Conversely, others have observed either decreased (6, 35) or unaffected (36, 37) growth plate chondrogenesis and longitudinal bone growth. Comparison of these *in vivo* studies of vitamin A deficiency to our *in vitro* studies blocking retinoid action is difficult. Vitamin A deficiency may not only act directly on the growth plate but also may indirectly affect growth by systemic mechanisms. For example, retinoids appear to be required for GH secretion (38, 39), and for thyroid hormone secretion and action (40, 41).

Other studies also suggest a physiological role for endogenous retinoids in skeletal growth and development. In mice, concomitant disruption of RAR-α and RAR-γ receptors by homologous recombination affects limb development, causing agenesis and/or malformations of the scapulae, radii, ulnae, and digits (4). Growth retardation in both embryonic and postnatal periods has been observed in mice expressing a dominant-negative RAR in chondrogenic cells (42). Unlike ours, these data would suggest a stimulatory role for RARs

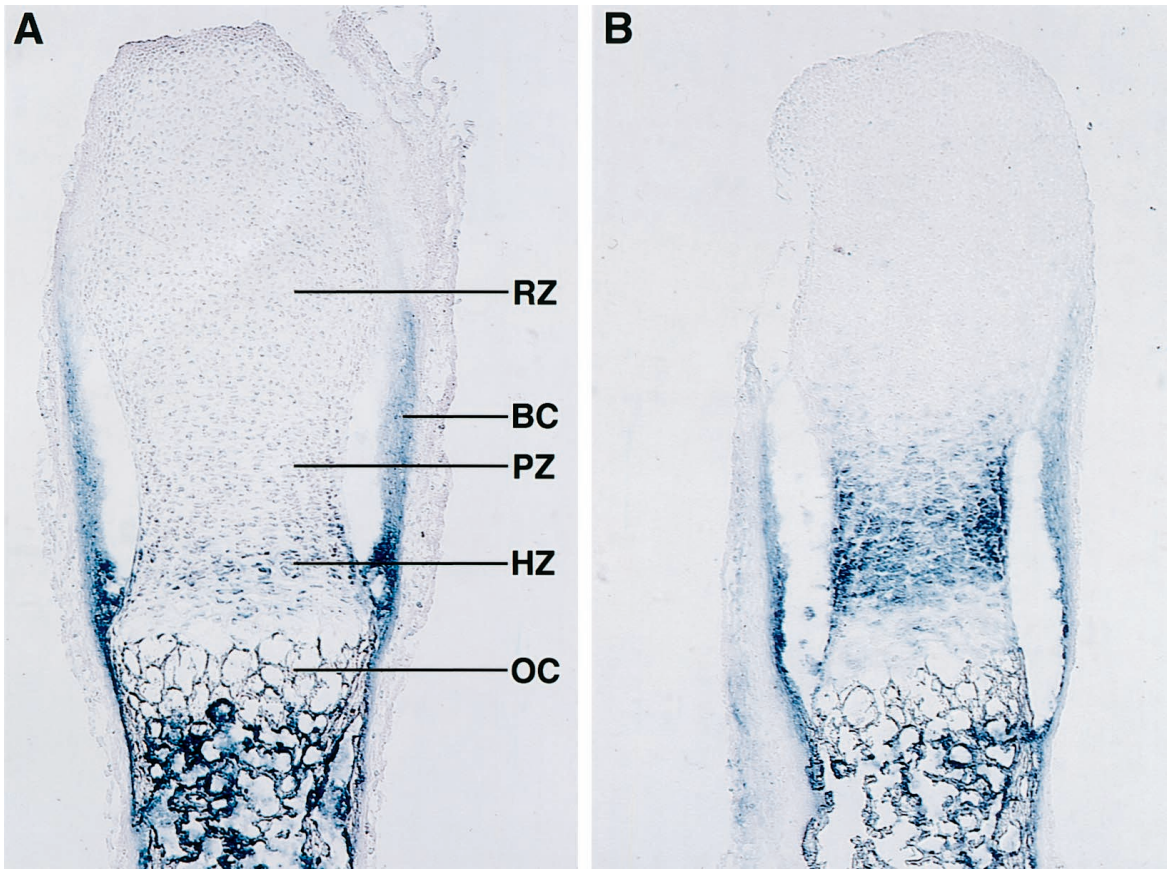

FIG. 5. Representative photomicrographs demonstrating the effects of RA on alkaline phosphatase activity. Fetal rat metatarsals were cultured for 2 days in serum-free medium without RA (A) or with 1000 nM RA (B). Samples were embedded in OCT compound, and 10-μm cryostat sections were cut and mounted onto poly-L-lysine-coated slides. Enzyme histochemistry was performed using the simultaneous-coupling azo dye method. Alkaline phosphatase activity produces *blue* stain. Separation of the perichondrium/bone collar from the interior of the bone rudiments is an artifact that occurs in frozen sections. RZ, Resting zone; BC, bone collar; PZ, proliferative zone; HZ, hypertrophic zone; OC, ossification center.

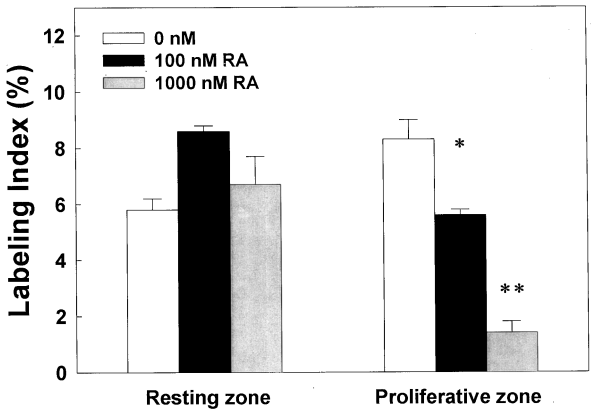

FIG. 6. [^{3}H]thymidine labeling indices (mean ± SEM). Fetal rat metatarsals (n = 8–10/group) were cultured for 2 days in serum-free medium containing 0, 100, or 1000 nM RA, labeled with [^{3}H]thymidine, and prepared for autoradiography as described in Fig. 7. Labeling index (number of labeled cells per total cells) was determined by a single observer blinded to the treatment group. *, $P < 0.05$ (*vs.* 0 nM RA). **, $P < 0.0001$ (*vs.* 0 nM RA); $P < 0.01$ (*vs.* 100 nM RA).

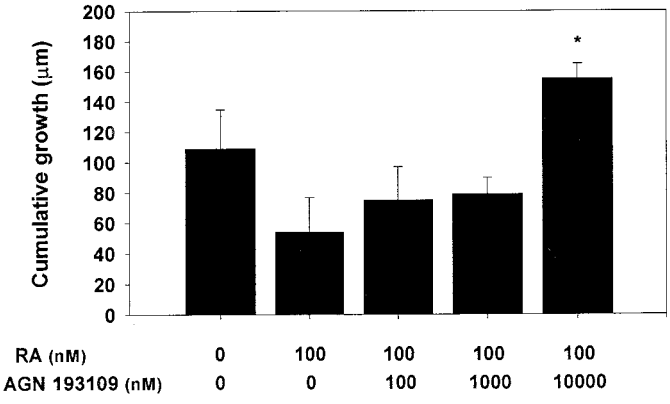

FIG. 7. Antagonistic effect of AGN 193109 on RA-induced inhibition of longitudinal bone growth. Fetal rat metatarsals (n = 8/group) were cultured for 3 days in serum-free medium containing 0 or 100 nM RA and 0–10,000 nM AGN 193109, a selective RAR-antagonist. The length of the bones was measured daily using an eyepiece micrometer in a dissecting microscope. *, $P < 0.01$ (*vs.* 100 nM RA).

in skeletal growth. However, in these previous experiments the inactivation of RARs beginning early in embryonic life altered the formation of the skeletal structures. Thus, the observed growth retardation may reflect the effects on early skeletal development rather than the role of RARs on longitudinal bone growth after skeletal formation.

In conclusion, our data suggest that endogenous retinoic acid, acting through RARs, serves to negatively regulate growth plate chondrogenesis by decreasing chondrocyte

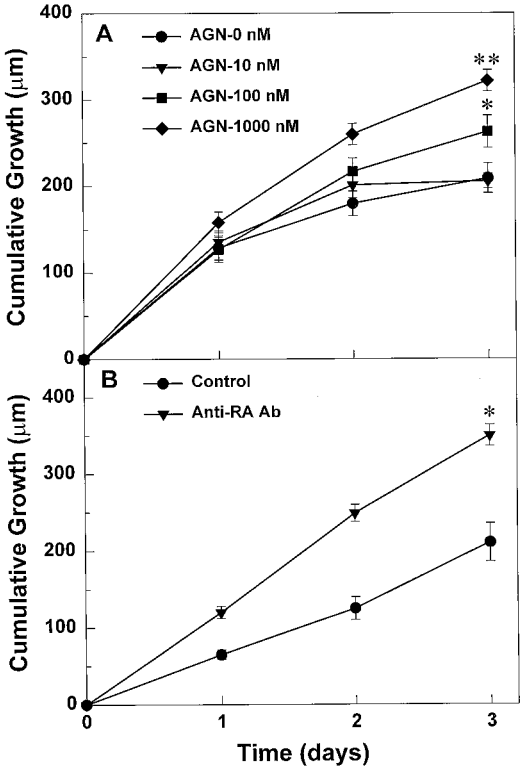

FIG. 8. Effects of AGN 193109 and Anti-RA Ab on longitudinal bone growth (mean ± SEM). A, Fetal rat metatarsals (n = 31–33/group) were cultured for 2 days in serum-free medium containing 0–1000 nM AGN 193109 in the absence of retinoic acid. The length of the bones was measured daily using an eyepiece micrometer in a dissecting microscope. *, $P < 0.05$ (*vs.* 0 nM AGN 193109). **, $P < 0.001$ (*vs.* 0 nM AGN 193109) and $P < 0.01$ (*vs.* 100 nM AGN 193109). B, Fetal rat metatarsals (n = 31–36/group) were cultured for 3 days in serum-free medium containing 200 mg/ml of either ANTI-RA Ab (a neutralizing monoclonal antibody highly specific for RA) or a control monoclonal antibody against *Escherichia coli* β-galactosidase. The length of the bones was measured daily using an eyepiece micrometer in a dissecting microscope. *, $P < 0.0001$ (*vs.* control).

proliferation and chondrocyte hypertrophy. In pharmacological concentrations, retinoic acid also inhibits cartilage matrix production.

Our findings raise the possibility that some growth disorders may be caused by abnormalities of retinoid function in the growth plate and that manipulation of the retinoid/RAR system may provide new approaches for the treatment of some forms of growth failure.

Acknowledgments

We thank Dr. Roshantha A. S. Chandraratna and Allergan, Inc. for providing AGN 193109.

References

1. **Hunziker EB** 1994 Mechanism of longitudinal bone growth and its regulation by growth plate chondrocytes. Microsc Res Tech 28:505–519
2. **Farnum CE, Wilsman NJ** 1987 Morphologic stages of the terminal hypertrophic chondrocytes. Anat Rec 219:221–232
3. **Aharinejad S, Marks SC, Bock P, Mackay CA, Larson EK, Tahamtani A, Mason-Savas A, Firbas W** 1995 Microvascular pattern in the metaphysis during bone growth. Anat Rec 242:111–122
4. **Lohnes D, Mark M, Mendelsohn C, Dolle' P, Dierich A, Gorry P, Gasmuller A, Chambon P** 1994 Function of the retinoic acid receptors (RARs) during development. (I) craniofacial and skeletal abnormalities in RAR double mutants. Development. 120:2723–2748
5. **Means AL, Gudas LJ** 1995 The roles of retinoids in vertebrate development. Annu Rev Biochem 64:201–233
6. **Wolbach SB** 1947 Vitamin-A deficiency and excess in relation to skeletal growth. J Bone Joint Surg 29:171–192
7. **Pease CN** 1962 Focal retardation and arrestment of growth of bones due to vitamin A intoxication. J Am Med Assoc 182:980–985
8. **Standeven AM, Davies PJA, Chandraratna RAS, Mader DR, Johnson AT, Thomazy VA** 1996 Retinoid-induced piphyseal closure in guinea pigs. Fundam Appl Toxicol 34:91–98
9. **Bagi C, Burger EH** 1989 Mechanical stimulation by intermittent compression stimulates sulfate incorporation and matrix mineralization in fetal mouse long-bone rudiments under serum-free conditions. Calcif Tissue Int 45:342–347
10. **Mancilla EE, De Luca F, Uyeda JA, Czerwiec FS, Baron J** 1998 Effects of fibroblast growth factor-2 on longitudinal bone growth. Endocrinology 139:2900–2904
11. **Burstone MS** 1960 Histochemical observations on enzymatic processes in bone and teeth. Ann NY Acad Sci 85:431–444
12. **Bagi CM, Miller SC** 1992 Dose-related effects of 1,25-dihydroxivitamin D_3 on growth, modeling, and morphology of fetal rat metatarsals cultured in serum-free medium. J Bone Miner Res 7:29–40
13. **Soeta S, Mori R, Kodaka T, Naito Y, Taniguchi K** 1999 Immunohistochemical observations on the initial disorders of the epiphyseal growth plate in rats induced by high doses of vitamin A. J Vet Med Sci 61:233–238
14. **Keene AT, Farndon FF, Tucker MJ, Willars GB, Ward SJ, Turton JA** 1991 All-trans retinoic acid (TRA) induces premature closure of the long bone epiphyses in the guinea pig. Hum Exp Toxicol 10:503–504
15. **Dhem A, Goret-Nicaise M** 1984 Effects of retinoic acid on rat bone. Fundam Chem Toxicol 22:199–206
16. **Kodaka T, Takaki H, Soeta S, Mori R, Naito Y** 1998 Disappearance of epiphyseal growth plates in rats with hypervitaminosis A. J Vet Med Sci 60:815–821
17. **Woodard JC, Donovan GA, Fisher LW** 1997 Pathogenesis of vitamin (A and D)-induced premature growth-plate closure in calves. Bone 21:171–182
18. **Clark L** 1970 The effect of excess vitamin A on longbone growth in kittens. J Comp Pathol 80:625–634
19. **Prendiville J, Bingham EA, Burrows D** 1986 Premature epiphyseal closure—a complication of etretinate therapy in children. J Am Acad Dermatol 15:1259–1262
20. **Marini JC, Hill S, Zasloff MA** 1988 Dense metaphyseal bands and growth arrest associated with isotretinoin therapy. Am J Dis Child 142:316–318
21. **Breur GJ, VanEnkevort BA, Farnum CE, Wilsman NJ** 1991 Linear relationship between the volume of hypertrophic chondrocytes and the rate of longitudinal bone growth in growth plates. J Orthop Res 9:348–359
22. **Noonan KJ, Hunziker EB, Nessler J, Buckwalter JA** 1998 Changes in cell, matrix compartment, and fibrillar collagen volumes between growth-plate zones. J Orthop Res 16:500–508
23. **Enomoto M., Pan H, Suzuki F, Takigawa M** 1990 Physiological role of vitamin A in growth cartilage cells: low concentrations of retinoic acid strongly promote the proliferation of rabbit costal growth cartilage cells in culture. J Biochem 107:743–748
24. **Hagiwara H, Inoue A, Nakajo S, Nakaya K, Kojima S, Hirose S** 1996 Inhibition of proliferation of chondrocytes by specific receptors in response to retinoids. Biochem Biophys Res Commun 222:220–224
25. **Takishita Y, Hiraiwa K, Nagayaku M** 1990 Effect of retinoic acid on proliferation and differentiation of cultured chondrocytes in terminal differentiation. J Biochem 107:592–596
26. **Iwamoto M, Shapiro IM, Yagami K, Boskey AL, Leboy PS, Adams SL, Pacifici M** 1993 Retinoic acid induces rapid mineralization and expression of mineralization-related genes in chondrocytes. Exp Cell Res 207:413–420
27. **Johnson AT, Klein ES, Gillett SJ, Wang L, Song TK, Pino ME, Chandraratna RA** 1995 Synthesis and characterization of a highly potent and effective antagonist of retinoic acid receptors. J Med Chem 38:4764–4767
28. **Giguere V** 1994 Retinoic acid receptors and cellular retinoid binding proteins: complex interplay in retinoid signaling. Endocr Rev 15:61–79
29. **Krezel W, Dupe V, Mark M, Dierich A, Kastner P, Chambon P** 1996 RXR gamma null mice are apparently normal and compound RXR-α +/−/RXR β−/−/RXR-gamma−/− mutant mice are viable. Proc Natl Acad Sci USA 93:9010–9014
30. **Wendling O, Chambon P, Mark M** 1999 Retinoid X receptors are essential for early mouse development and placentogenesis. Proc Natl Acad Sci USA 96:547–551
31. **Klein ES, Pino ME, Johnson AT, Davies PJA, Nagpal S, Thacher SM, Krasinsky G, Chandraratna RA** 1996 Identification and functional separation of retinoic acid receptor neutral antagonists and inverse agonists. J Biol Chem 271:22692–22696
32. **Zhou H-R, Abouzied MM, Zile MH** 1991 Production of a hybridoma cell line secreting retinoic acid-specific monoclonal antibody. J Immunol Methods 138:211–223
33. **Twal W, Roze L, Zile MH** 1995 Anti-retinoic acid monoclonal antibody lo-

calizes all-trans retinoic acid in target cells and blocks normal development in early quail embryo. Dev Biol 168:225–234
34. **Havivi E, Tal R** 1974 Effect of vitamin A deficiency on several bone parameters in chicks. Int J Vitam Nutr Res 44:8–13
35. **Conne P, Franquin JC, Baume LJ** 1970 The influence of vitamin A on condylar, epiphyseal and articular cartilages in the rat. Int J Vitam Res 40:465–469
36. **Howell JM, Thompson JN** 1967 Observations of the lesions in vitamin A deficient adult fowls with particular references to changes in bone and central nervous system. Br J Exp Pathol 48:450–454
37. **Gallina AM, Helmboldt CF, Frier HI, Nielsen SW, Eaton HD** 1970 Bone growth in hypovitaminotic A calf. J Nutr 100:129–141
38. **Evain-Brion, D, Porquet D, Therond P, Fjellestad-Paulsen A, Grenecge MO, Francois L, Czernichow P** 1994 Vitamin A deficiency and nocturnal growth hormone secretion in short children. Lancet 343:87–88
39. **Djakoure C, Guibourdenche J, Porquet D, Pagesy P, Peillon F, Li JY, Evain-Brion D** 1996 Vitamin A and retinoic acid stimulate within minutes cAMP release and growth hormone secretion in human pituitary cells. J Clin Endocrinol Metab 81:3123–3126.
40. **Nockels CF, Ewing DL, Phetteplace H, Ritacco KA, Mero KN** 1984 Hypothyroidism: an early sign of vitamin A deficiency in chickens. J Nutr 114:1733–1736
41. **Higueret P, Pailler I, Garcin H** 1989 Vitamin A deficiency and tri-iodothyronine action at the cellular level in the rat. J Endocrinol 121:75–79
42. **Yamaguchi M, Nakamoto M, Honda H, Nakagawa T, Fujita H, Nakamura T, Hirai H, Narumiya S, Kakizuza A** 1998 Retardation of skeletal development and cervical abnormalities in transgenic mice expressing a dominant-negative retinoic acid receptor in chondrogenic cells. Proc Natl Acad Sci USA 95:7491–7496
