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LOMA LINDA UNIVERSITY
School of Medicine
in conjunction with the
Faculty of Graduate Studies

Retinoic Acid Regulation of Thyroid Hormone Action in Bone Cells

by

Anjali Babbar

A Thesis submitted in partial satisfaction
of the requirements for the degree of
Master of Science in Microbiology and Molecular Genetics

March 2014

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Each person whose signature appears below certifies that this dissertation in his/her opinion is adequate, in scope and quality, as a dissertation for the degree Master of Science.

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ABBREVIATIONS

TH	Thyroid hormone
RA	Retinoic acid
ALP	Alkaline phosphatase
OC	Osteocalcin
RUNX2	Runt-related transcription factor 2
TSH	Thyroid stimulating hormone
TRE	Thyroid response element
RXR	Retinoid X receptor

ABSTRACT OF THE THESIS

Retinoic Acid Regulation of Thyroid Hormone Action in Bone Cells

by

Anjali Babbar

Master of Science, Graduate Program in Microbiology and Molecular Genetics
Loma Linda University, March 2014
Dr. Subburaman Mohan

Retinoic acid and thyroid hormone are known to play key roles in the regulation of endochondral ossification. However, the issue of whether these two hormones interact with each other to regulate bone functions remains to be established. We investigated how thyroid hormone and retinoic acid interact to regulate cells involved in endochondral bone formation. We demonstrate that thyroid hormone treatment stimulates differentiation of ATDC5 chondrocytes and promotes formation of mineralized nodules while retinoic acid treatment at high dose inhibits chondrocyte differentiation and formation of mineralized nodules. Furthermore, thyroid hormone induced mineralized nodule formation is inhibited by co-treatment with retinoic acid in ATDC5 cells. Dose response studies in ATDC5 cells showed that retinoic acid treatment caused a biphasic effect on ALP and osterix mRNA levels. At low dose, it increased expression levels and at high dose it decreased the expression levels of ALP and osterix genes. Since thyroid hormone action in the nucleus is known to be mediated via binding of thyroid hormone receptors either as homodimer or heterodimer with retinoic acid receptors to regulatory regions of target genes, our findings raise the possibility that the presence of retinoic acid may interfere with the differentiation promoting actions of thyroid hormone in chondrocytes by influencing nuclear proteins that are recruited to the thyroid hormone

receptor complex. In conclusion, our findings suggest that the effect of retinoic acid treatment appears to be complex involving both stimulation and inhibition of differentiation depending on dose as well as differentiation status of cells.

CHAPTER ONE

INTRODUCTION

Bone is a dynamic organ which undergoes continuous remodeling throughout life. Bone remodeling is a process in which osteoblasts form new bone and osteoclasts resorb the bone. This process is essential for maintaining the required bone mass to provide mechanical support to the body. An increase in bone mass occurs during childhood and adolescence by the process of endochondral bone formation (1). Bone mass attains its maximum peak during adulthood, where there is a balance in the activity of osteoblasts and osteoclasts. In normal conditions, the bone mass is sustained by tight coupling between the osteoblasts and osteoclasts. Numerous factors like diet, exercise and endocrine hormones influence bone growth and development. Endocrine hormones regulate the communication between osteoblasts and osteoclasts (2).

Ossification of Bone

The process of ossification involves two different mechanisms - intramembranous ossification and endochondral ossification. Intramembranous ossification is a process where bone cells are directly formed from mesenchymal cells. This type of ossification is responsible for the development of flat bones in the body like clavicle, scapula and bones of the skull. The other process of ossification which involves the development of long bones of the body is known as endochondral ossification. It is a mechanism which involves formation of a cartilaginous model through condensation of mesenchymal cells

which later is gradually replaced by bone. Endochondral ossification occurs at two different sites in long bones i.e. primary (diaphysis) and secondary (epiphysis) (3). Bone development starts at the primary center of ossification and the secondary center is ossified later. Between the two centers of ossification lies the epiphyseal cartilage which is responsible for the growth of long bones. The epiphyseal cartilage consists of various layers of chondrocytes. The basal layer which is away from the ossification front is the zone of resting chondrocytes. Adjacent to the basal layer is the zone of proliferation where rounded cells gradually become flattened as they pack into multicellular clusters. Following proliferation, chondrocytes pass through a transition stage of pre- hypertrophic chondrocytes where they undergo maturation and secrete extracellular matrix and then die. As the chondrocytes die, this region is invaded by blood vessels and macrophages which clear up the dying chondrocytes and precursors of osteoblasts start laying down the bone.

Bone Cells

Osteoblasts are specialized mesenchymal cells which are responsible for bone formation. Depending on the signaling pathway, mesenchymal stem cells can differentiate into cells such as fibroblasts, chondrocytes, myoblasts or adipocytes (4). Once osteoblasts differentiate they undergo proliferation and then deposit extracellular matrix and bring about matrix maturation and mineralization. Expression levels of various differentiation markers such as alkaline phosphatase (ALP), collagen type1, osteopontin and osteocalcin are used to study osteoblast differentiation. RUNX2 acts as a main switch to initiate osteoblast differentiation and it regulates the expression level of

the above stated genes (5). Osterix which is downsteam of Runx2 is another key transcription factor for osteoblast development (6).

Chondrocytes are mesenchymal derived cells whose function is to produce the extracellular matrix of cartilage. This matrix includes aggrecans and type II collagen and other proteins. Chondrocytes are responsible for both appositional and interstitial growth of cartilage and are involved in endochondrial bone formation (7). In endochondral bone formation, differentiated chondrocytes within cartilage undergo controlled proliferation and hypertrophy. They form distinct zones along the longitudinal axis during the process of bone elongation. Collagen type II is expressed by proliferating chondrocytes and hypertrophic chondrocytes express collagen X. Indian hedgehog is expressed by prehypertrophic chondrocytes and provides a signal to adjacent perichondrium to stimulate osteoblast differentiation (8).

Regulators of Endochondral Ossification

The chondrocytes of the growth cartilage are under the influence of both systemic factors and local factors (9). The systemic factors include growth hormone, thyroid hormone (T3), glucocorticoids and vitamins. The local factors include insulin like growth factor, indian hedgehog, parathyroid hormone related peptide, bone morphogenic proteins and fibroblast growth factors. Among the various regulators of endochondral ossification, the present study concentrates on thyroid hormone and how it regulates endochondral ossification.

Thyroid Hormone

Bone development is dependent on coordinated actions of genetic, nutritional, endocrine and environmental factors. Thyroid hormone (T3) is considered essential for growth and development in children. Thyroid hormone is secreted by the thyroid gland under the control of thyroid stimulating hormone (TSH) released by the pituitary. Hypothyroidism is caused by a deficiency of thyroid hormone or by a mutation in the thyroid hormone receptor that can lead to growth arrest, a slowing of bone formation or epiphyseal dysgenesis in children (10).Thyrotoxicosis, a condition that leads to hyperthyroidism in growing children, results in accelerated bone growth, premature closing of the growth plate and short stature.

The actions of thyroid hormone are mediated by thyroid hormone nuclear receptors. The thyroid receptors act as hormone activated transcription factors which modulate gene expression (11). These receptors are expressed as TRα and TRβ; each of these exhibits in two isoforms- TRα1, TRα2, TRβ1, and TRβ2. TRα and TRβ possess related T3 and DNA binding domains but separate amino terminals (12). Thyroid receptors are usually expressed in the reserve, proliferative and pre hypertrophic zones of the epiphyseal plate and in osteoblasts of primary spongiosum (13,14).

Thyroid hormone receptors have an amino terminal domain, a central DNA binding domain and a carboxy terminal ligand binding domain. The DNA binding domain is present in the central part of thyroid hormone receptor which consists of two zinc fingers formed by four cysteine residues. Thyroid hormone receptors bind to a small segment of DNA known as the thyroid response element (TRE). The binding of thyroid hormone receptor to TRE can be in the form of homodimers, i.e., with the other thyroid

hormone receptor, or in the form of heterodimers with the retinoid X receptor (RXR). The heterodimers are known to increase the DNA binding and transcription process (15). The ligand binding domain binds to thyroid hormone and is also responsible for dimerization, transactivation and basal repression by ligand free thyroid hormone receptor. Thyroid hormone penetrates the nucleus where it binds to the thyroid hormone receptors which may already be bound to TRE located in the promoter regions of target genes. Ligand- bound thyroid hormone receptor complex which is bound to the TRE is crucial for the repression or activation of the target genes involved. Various cotransfection studies have revealed that unliganded thyroid hormone receptors can repress the transcription of positively regulated TREs (16, 17). In the ligand- free state, the transactivation domain of the T3 free receptor, heterodimerized with RXR, adopts a conformation that encourages the assembly of transcriptional corepressor factors. The formation of the corepressor complex has histone deacetylase activity which is associated with the formation of compact chromatin, which represses transcription of affected genes (18). On the contrary, in the ligand bound state, the binding of T3 to its receptor stimulates a conformational change in the receptor that makes it incapable of binding to the corepressor complex, but capable of binding to a group of coactivator proteins (19). The coactivator protein complex promotes histone transacetylase activity which exposes the chromatin and activates transcription of linked genes.

Retinoic Acid

Retinoic acid (RA) is a fat soluble molecule which is synthesized from vitamin A. Retinoic acid is essential for vision, immune responses, bone growth, reproduction and hematopoiesis (20). Production of retinoic acid in the body does not happen at all stages

of development but instead follows a spatiotemporal pattern (21). Deficiency or excess of retinoic acid has been considered as teratogenic due to anteroposterior patterning defects (22). Retinoic acid enters the nucleus and functions as a ligand for two families of nuclear receptors that bind to DNA and regulate transcription. The receptors of retinoic acid are RAR (RARα, RARβ, RARɣ) and RAX (RAXα, RAXβ, RAXɣ). RAR receptors usually bind to all-trans retinoic acid, which is the most abundant form of retinoic acid in body, and RAX binds to 9-cis-retinoic acid (23). 9-cis-retinoic acid is normally undetectable in the body except when there is an excess of vitamin A (24). Retinoic acid signaling is initiated by the binding of retinoic acid to the retinoic acid receptor RAXs, which forms a stable heterodimer with RARs, vitamin D receptor and thyroid hormone receptor. The complex formed by binding of RAX with the various receptors in turn binds to the retinoic acid response elements in the regulatory regions of target genes (22).

Retinoic acid is known to both promote and inhibit endochondral ossification but how it modulates the thyroid hormone action in bone cells is not known (25, 26, 27, 28, 29). The present study is therefore focused on understanding if and how thyroid hormone and retinoic acid interact to regulate cells involved in endochondral bone formation.

Hypothesis

Retinoic acid modulates thyroid hormone effect in bone cells.

Specific Aims

1. To determine if retinoic acid influences thyroid hormone's effect on differentiation of chondrocytes and osteoblasts.

2. To evaluate the effect of thyroid hormone and retinoic acid on transcription factors which are critical for chondrocyte and osteoblast differentiation.

CHAPTER TWO

MATERIALS AND METHODS

Cell Culture

ATDC5 Cells

The ATDC5 cell line is a murine chondrogenic cell line which was obtained from Ardent, San Diego. This cell line is widely used to study chondrocyte growth and differentiation. ATDC5 cells were maintained in DMEM/F12 media containing 5% fetal bovine serum. The cells were kept at 37^0 in a humidified atmosphere of 5% CO_2 for the culture period.

Primary cells

The primary chondrocyte cells were isolated from ribs of mice aged 3-7 days. The rib cages of mice were isolated and digested in collagenase for one hour in a shaker at 37^0 to separate and clean the ribs. The ribs were then washed with PBS and digested again with collagenase for four hours in a shaker to isolate chondrocytes. After incubation, the cells were filtered and cultured in ascorbic acid- free α-MEM with 10% fetal bovine serum. When cells were 70%-80% confluent they were passed and plated again for the experiments. The primary osteoblasts were isolated from calvariae of mice aged 3-7 days. The mice were decapitated and calvaria was isolated and cut into four pieces. The calvarial pieces were treated with digestion buffer (collagenase and hyaluronidase mixture) and kept in a shaker at 37^0 for 15 minutes. After incubation, they

were washed with PBS and filtered in a strainer. The fragments of calvaria were collected from the strainer and again treated with digestion buffer in a shaker for one hour and then washed with PBS. Filtrate was centrifuged and cultured in ascorbic- acid free α-MEM with 10% fetal bovine serum. When cells were 70%-80% confluent they were passed and plated again for the experiments.

In-Vitro Bone Formation Assay

Alizarin Red Staining

ATDC5 cells were plated in six well plates to determine the degree of bone mineralization in culture. Initially the cells were grown in proliferating media until they are 80-90% confluent. The proliferating media constitutes DMEM/F12 media containing 5% fetal bovine serum + penicillin streptomycin. When cells became 90% confluent, we shifted the cells to differentiation media. The control differentiation media contains DMEM/F12 + 5% fetal bovine serum + penicillin streptomycin + β-glycerol phosphate (10μM) and the experimental differentiation media contains DMEM/F12 + 5% fetal bovine serum + penicillin streptomycin + β-glycerol phosphate (10μM) + ascorbic acid (50μg/ml). Cultured cells were treated with retinoic acid (100nM), thyroid hormone (10ng/ml) and a combination of retinoic acid and thyroid hormone for 25 days. Fresh differentiation medium was changed every three days. Cells were observed closely after 20 days as a layer of cells might start to lift around the edges. At 25 days, the cells were washed with PBS after carefully removing the media and fixed with 100% methanol for 1 hour. After fixing, the cells were stained with alizarin red stain and incubated at room temperature for 10 min. Alizarin red stain was removed and nodules were washed with de-ionized water and agitated slowly repeatedly until the background appeared clear and

nodules were obvious. The quantification of the nodules was done as percent of area stained by using Image J software.

ALP Staining

Alkaline phosphatase staining was performed on day 7 in ATDC5 cells treated with retinoic acid (100nM), thyroid hormone (10ng/ml) and their combination. The cells were washed with PBS and fixed in 100% methanol for 20 min at the 7th day of treatment. The methanol was removed and cells were stained in ALP staining solution (substrate-diazonium solution) and incubated for 30 min at 37^0 incubator. ALP positive cells (red) were identified under the microscope.

RNA Isolation and Gene Expression

RNA was extracted from the cells by Trizol, chloroform and RNeasy mini kit (Qiagen, Valencia, CA).The culture dishes were washed with PBS after removing the media. Trizol was added to the culture dish to lyse the cells and cell scrapper was used to remove the cell lysate from the dish plates. The cell lysate was collected in a micro-centrifuge tube and chloroform was added to the cell lysate and incubated for 2-3 min. The cell lysate was centrifuged at 4^0 for 15 min. Following centrifugation, the mixture separated into a lower pink phase, a chloroform layer, an interphase layer and a colorless upper aqueous layer. RNA located in the aqueous layer was carefully removed without disturbing the interphase and added to a new tube. RNA was precipitated from the aqueous phase by adding 70% ethanol. The samples were vortexed and transferred to columns of the RNeasy mini kit. The samples were then washed with wash buffers I and

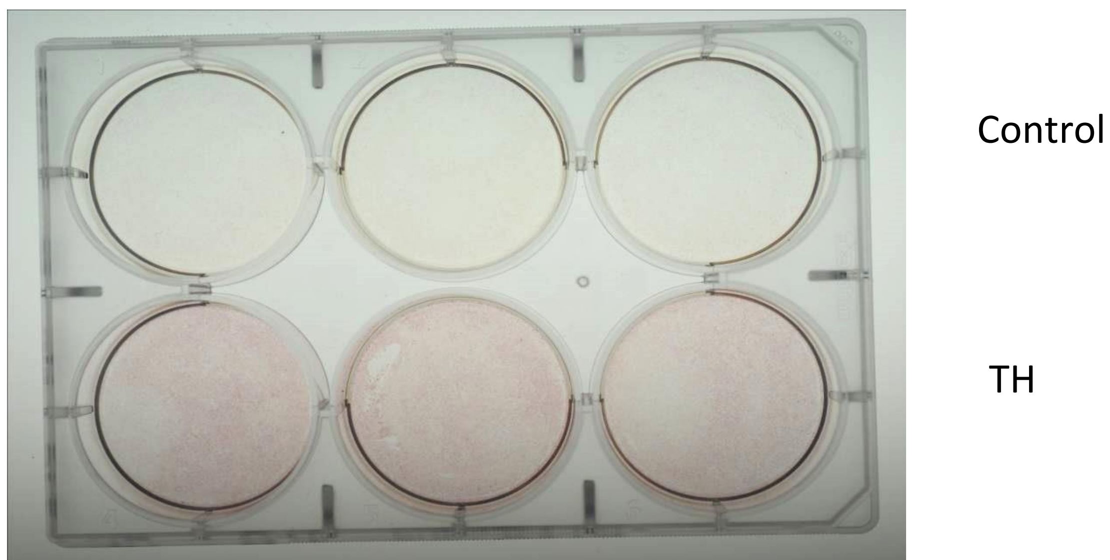
II. After washing the columns were transferred to RNA collecting tubes and 25 uL of DEPC water was added on the membrane of the column. Tubes were centrifuged for 1 min and RNA was collected. RNA was quantified using NanoDrop spectrophotometer. Reverse transcription was done using Superscript (Invitrogen, Carlsbad, CA), to produce cDNA. Quantitative real time PCR was performed to examine the expression of osterix, osteocalcin, ALP, RUNX2 and SOX9 genes. The PPIA gene was used as an internal control in PCR reaction. ΔCT values were obtained by subtracting CT value for control gene from CT value for the gene of interest.

CHAPTER THREE

RESULTS

Effect of TH and/or RA on Alkaline Phosphatase Staining in ATDC5 Chondrocytes

Initially to estimate the level of chondrogenic differentiation, alkaline phosphatase staining was performed in ATDC5 cells on the seventh day of treatments with various factors. In vehicle treated control cells, a moderate level of alkaline phosphatase staining was observed. The intensity of staining was increased in cultures treated with TH (Fig. 1a). The positive effect of TH on alkaline phosphatase staining was abolished by co-treatment with retinoic acid (Fig. 1b).



Control

TH

Fig. 1a. Alkaline phosphatase staining in ATDC5 cells treated with Control and TH for 7 days.

Fig. 1b. Alkaline phosphatase staining in ATDC5 cells treated with RA and TH + RA for 7 days.

Effect of TH and/or RA on Formation of Mineralized Nodules in ATDC5 Cells

In order to determine whether TH and/or RA influences mineralization in ATDC5 chondrocytes, ATDC5 cells were treated with 10ng/ml of TH and/or 100nM of RA. Alizarin red staining of mineralized nodules after 25 days of treatment with TH and/or RA revealed that TH caused a significant increase in % of mineralized area (Fig 2a). On the contrary, RA decreased % mineralized area both in vehicle and TH treated cultures (Fig 2b).

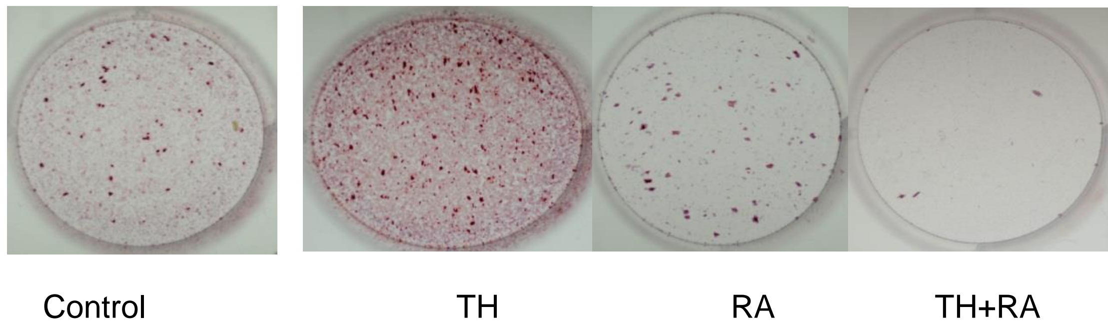


Fig. 2a. Effect of TH and/ or RA on formation of mineralized nodules in ATDC5 cells.

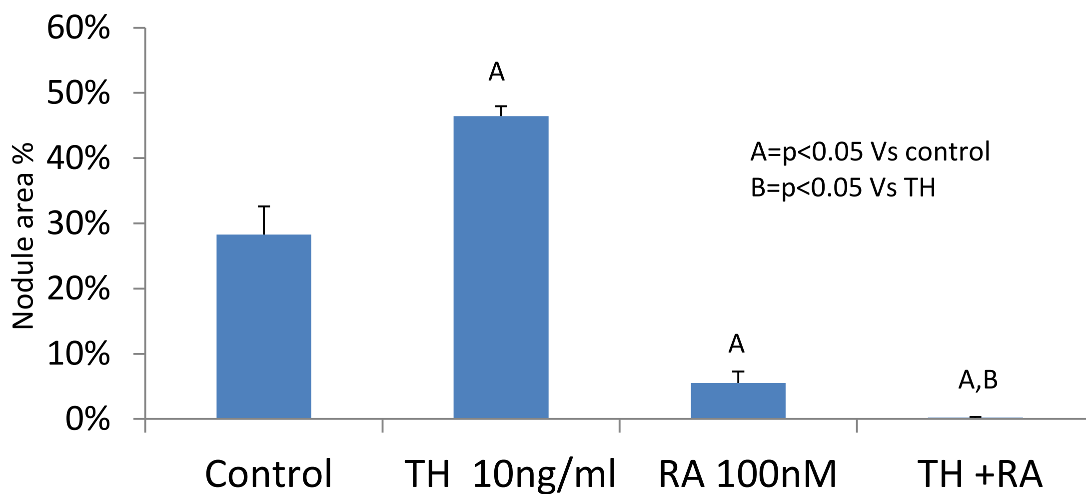


Fig. 2b. Quantitation of mineralized nodule area in ATDC5 cells treated with TH, RA, and TH+RA.

Effect of TH and/or RA on Osteocalcin mRNA Levels

To determine if TH and RA regulate the expression levels of osteocalcin, a key marker of differentiated osteoblasts, ATDC5 chondrocytes were treated with TH and/or RA. We observed a significant increase in the expression of osteocalcin mRNA by TH

hormone treatment. However, TH effect on osteocalcin expression was increased by treatment with RA (Fig. 3.)

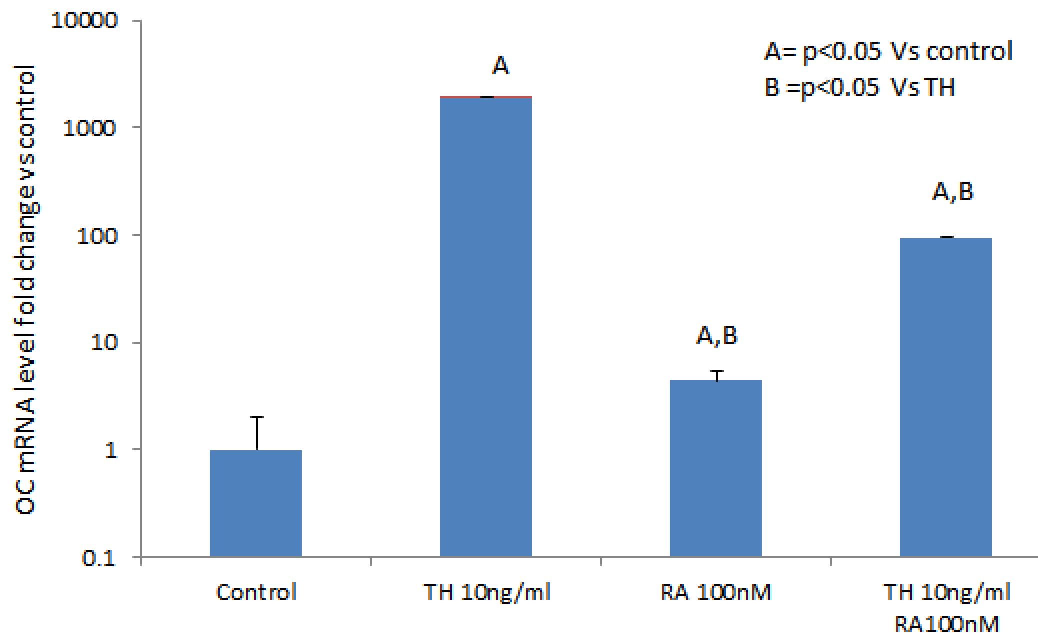


Fig. 3. Effect of TH and/ or RA on osteocalcin mRNA levels.

Dose-dependent Effect of RA on TH-induced ALP mRNA

In order to investigate dose effect of RA on the TH induced increase in ALP mRNA levels, ATDC5 cells were plated and treated with different concentrations of RA (0.01nM- 10nM) with and without TH (1ng/ml) for 24 hours. RA treatment caused a biphasic effect on ALP mRNA levels with an increase at low dose and a decrease at high dose (Fig. 4). The TH-induced increase in ALP mRNA was decreased by RA treatment in a dose dependent manner (Fig. 4).

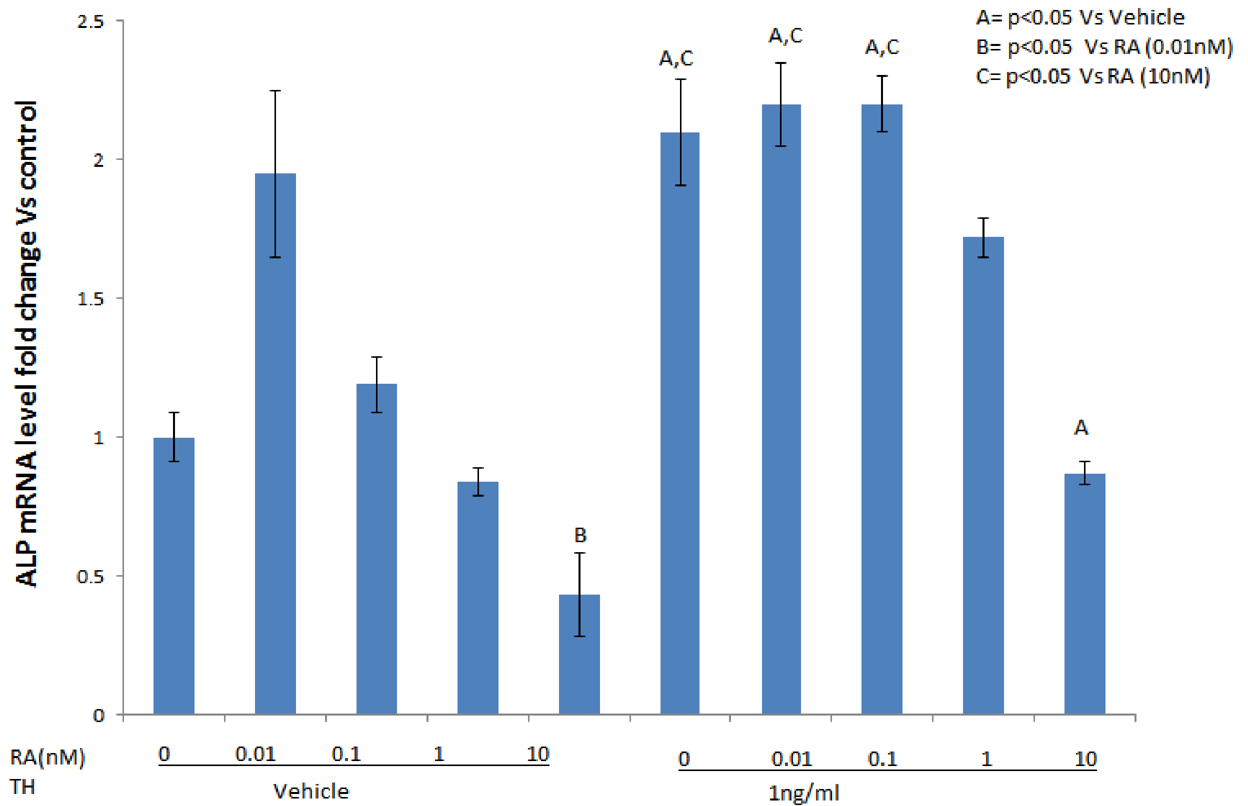


Fig. 4. Dose response effect of RA and TH with RA on ALP mRNA levels in ATDC5 cells.

Dose-dependent Effect of Retinoic Acid on TH-induced Osterix mRNA Levels

ATDC5 chondrocytes showed an increase of 2.2 fold in osterix expression upon treatment of RA at 0.01nM as compared to vehicle treatment (Fig 5). However, this increase in osterix expression by retinoic acid was not seen at high doses. TH treatment alone caused a significant increase in osterix mRNA level which was further increased by RA at low dose but not at high dose (Fig 5).

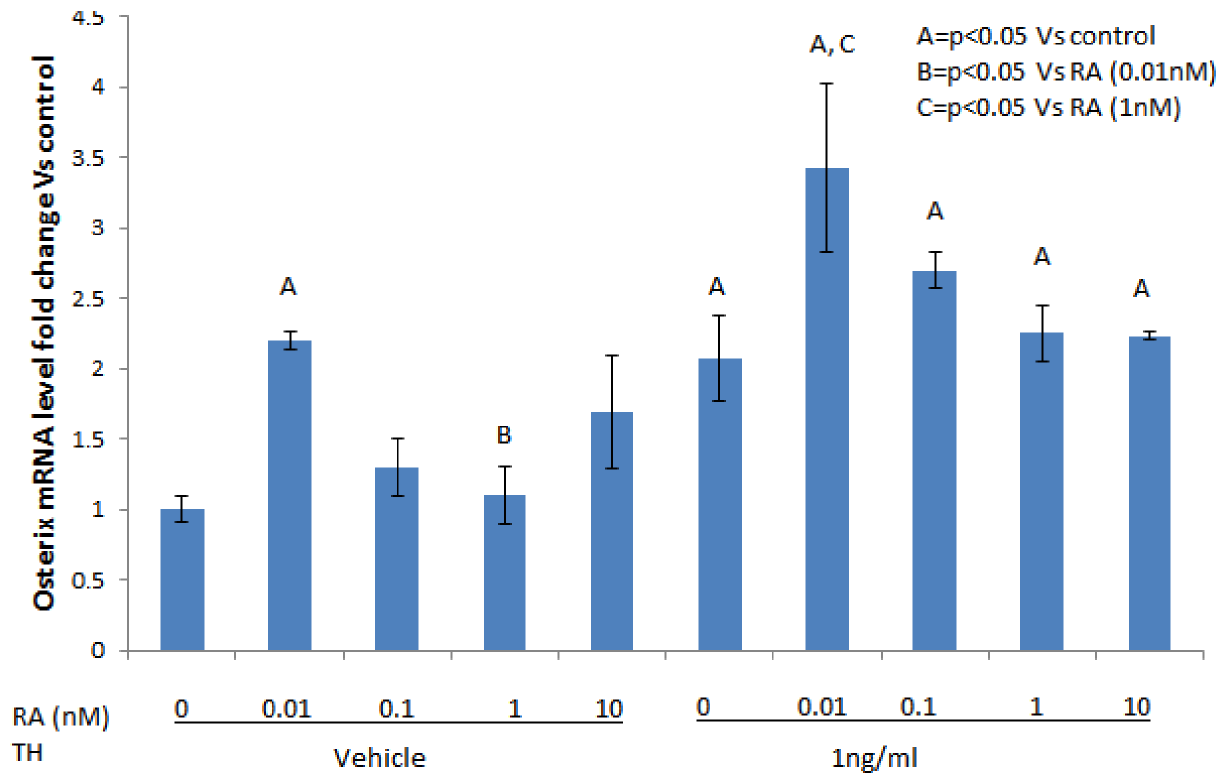


Fig. 5. Dose response effect of RA and TH with RA on osterix mRNA levels in ATDC5 cells.

Effect of TH and/or RA on Osterix, ALP, Osteocalcin and SOX9 mRNA Levels in Primary Chondrocytes

To determine if TH and/or retinoic acid produced similar effects on gene expression in primary chondrocytes, we evaluated the effects of these treatments using untransformed normal primary chondrocytes. TH caused a significant increase in osterix expression. However, RA treatment did not significantly change TH's effect on osterix expression (Fig 6a).

Both TH and RA caused similar increases in ALP mRNA levels. Their combination, however, resulted in greater induction than that observed with individual treatments (Fig 6b).

Treatment with TH or RA increased osteocalcin mRNA levels. RA did not significantly alter TH effects on osteocalcin mRNA levels (Fig 6c). SOX9 mRNA levels displayed an increased expression with TH treatment. However, RA alone and in combination with thyroid hormone treatment did not increase SOX9 expression levels (Fig 6d).

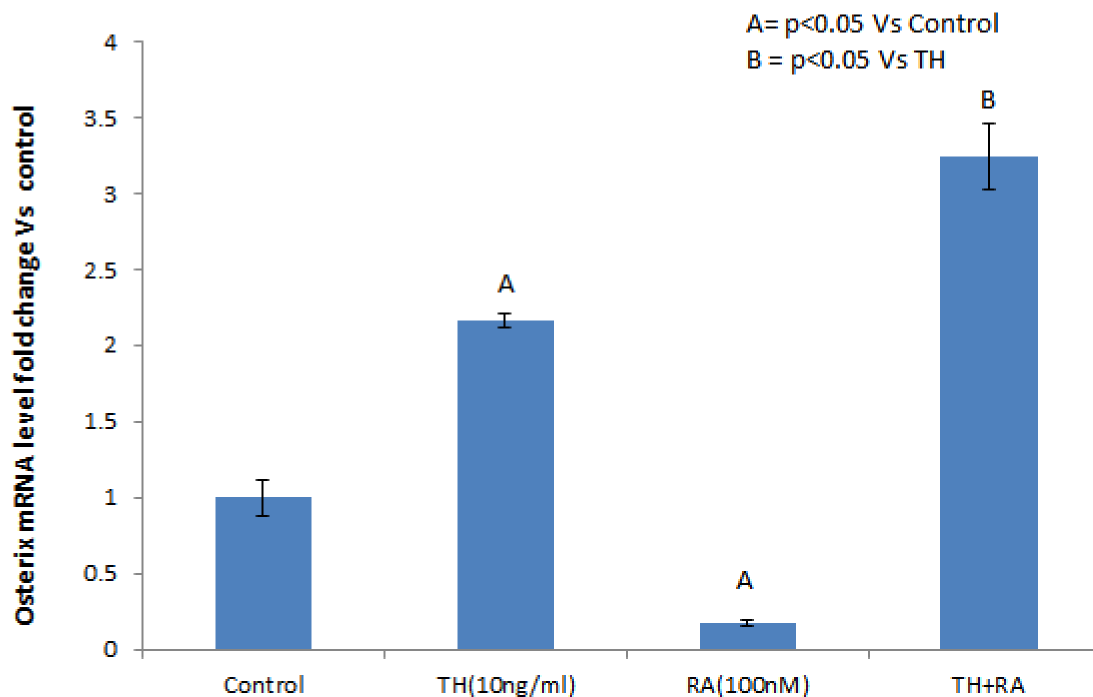


Fig. 6a. Effect of TH and/ or RA on osterix mRNA levels in primary chondrocytes.

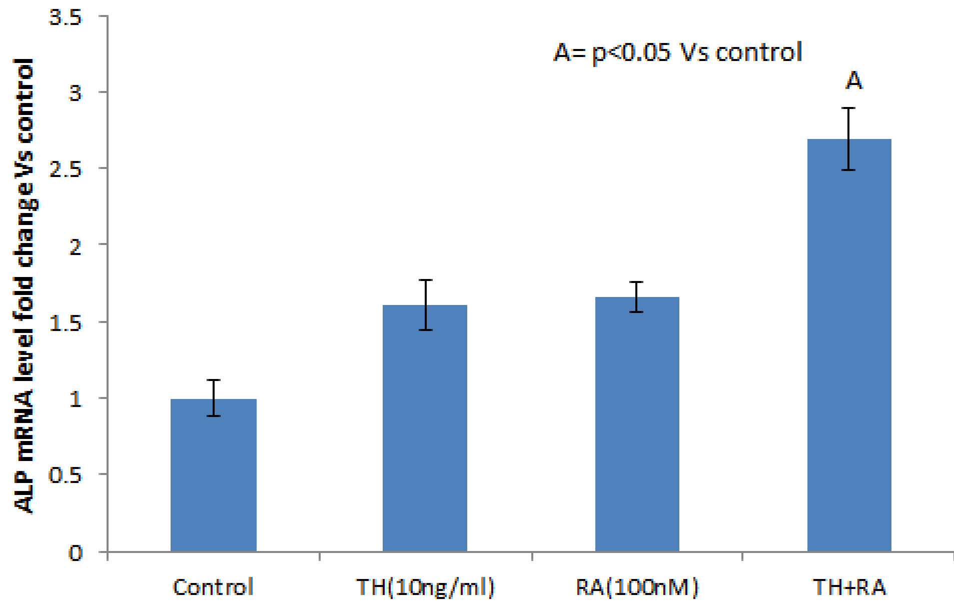


Fig. 6b. Effect of TH and/ or RA on ALP mRNA levels in primary chondrocytes.

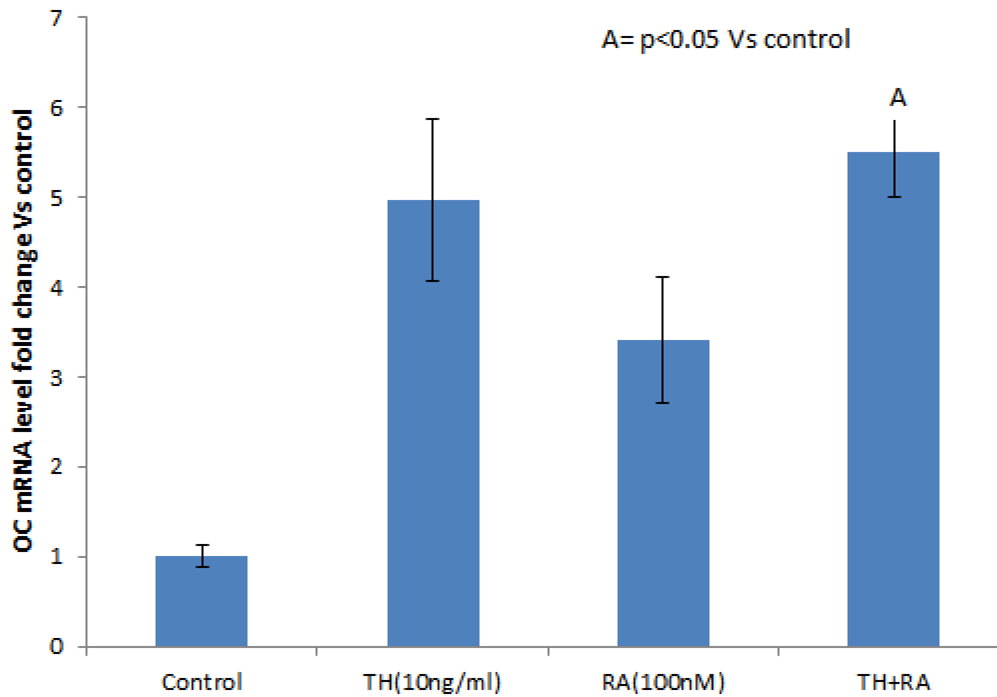


Fig. 6c. Effect of TH and/ or RA on osteocalcin mRNA levels in primary chondrocytes.

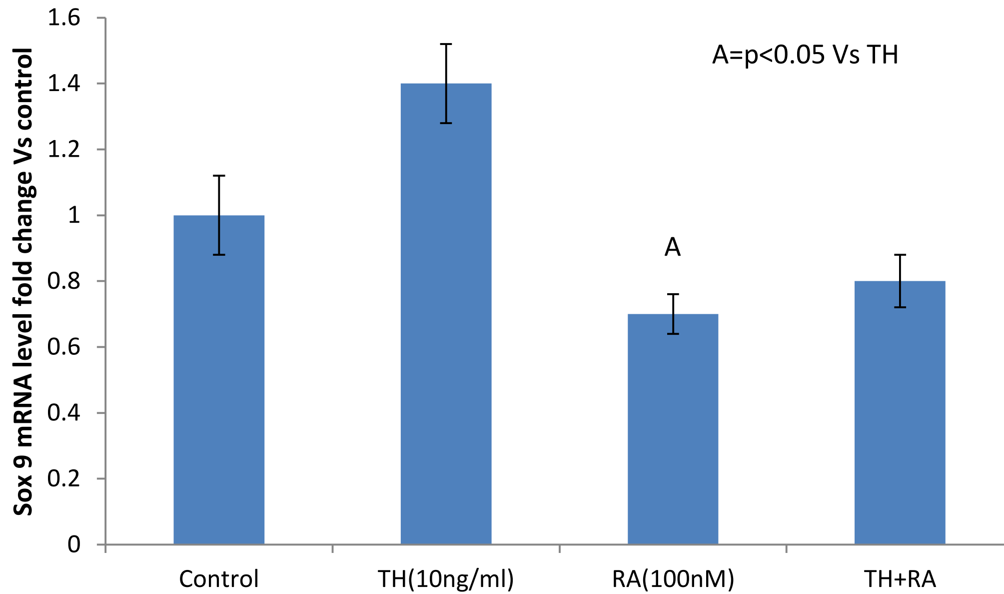


Fig. 6d. Effect of TH and/ or RA on SOX9 mRNA levels in primary chondrocytes.

Effect of TH and/or RA in Osterix and ALP mRNA Levels in Primary Osteoblasts

Untransformed primary osteoblasts derived from calvariae of 3 day old mice were cultured and treated with TH, RA and their combination for 24 hrs. Treatment with TH and RA caused an increase in osterix and ALP expression levels (Fig 7a and 7b). RA did not suppress the TH induced increase in osterix and ALP mRNA levels (Fig 7a and 7b)

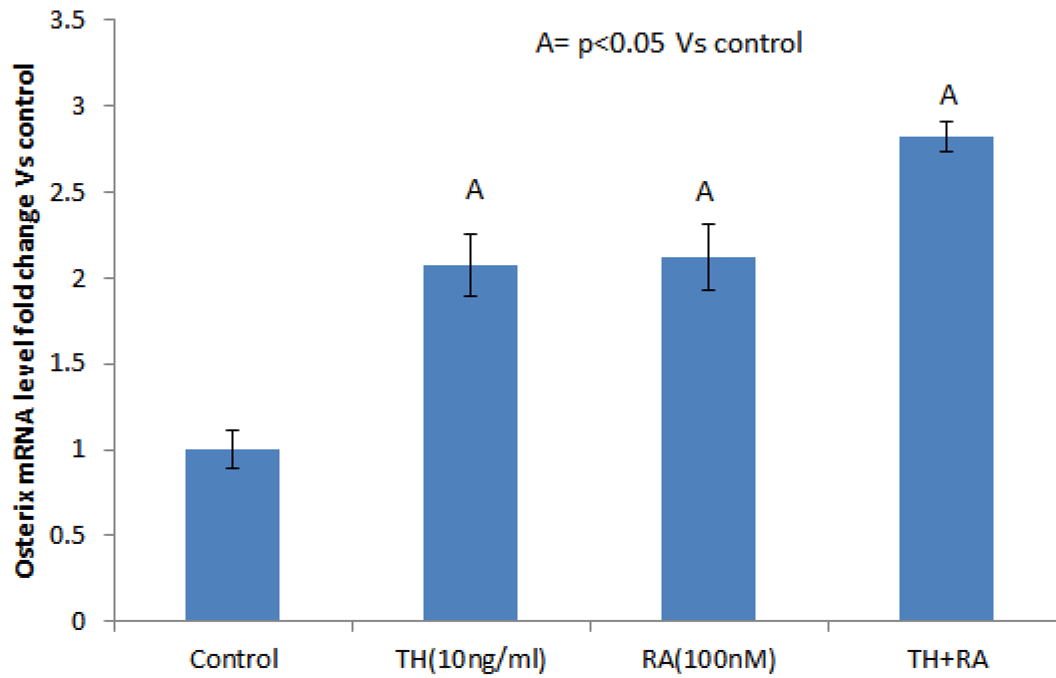


Fig. 7a. Effect of TH and/ or RA on osterix mRNA levels in primary osteoblasts.

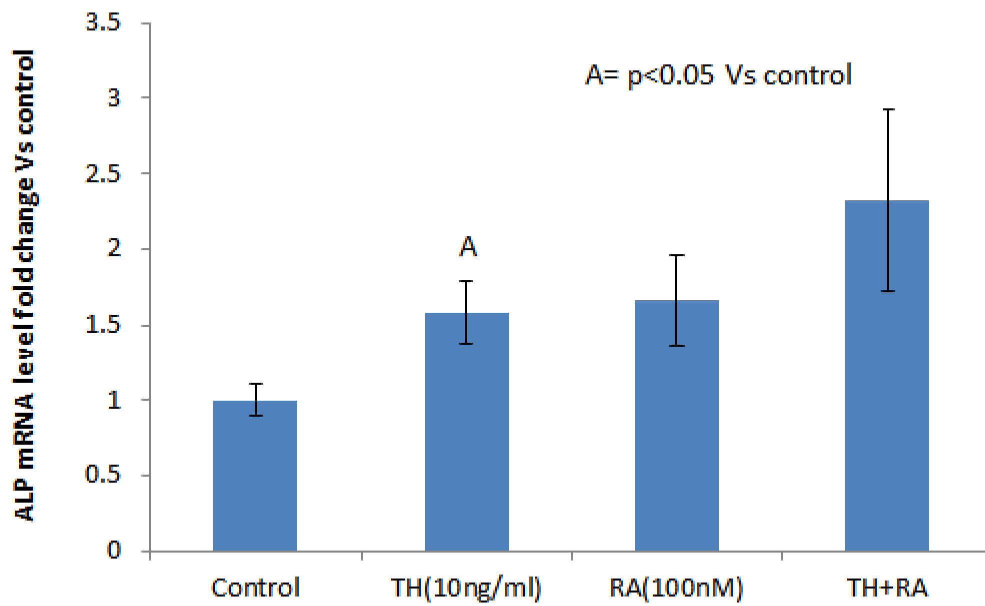


Fig. 7b. Effect of TH and/ or RA on ALP mRNA levels in primary osteoblasts.

CHAPTER FOUR

DISCUSSION

Vitamin A is required to maintain healthy vision, teeth, strong immune system, cell growth and differentiation. It is usually an essential component found in multivitamin supplements, cod liver oil, eggs and fortified foods. Deficiency and excess of Vitamin A are known to cause profound effects on bones. Early studies to observe the function of Vitamin A on the developing skeleton demonstrated that it acts to control the location and activity of osteoblasts, thus determining the shape and texture of bones (30). The excess of Vitamin A has been implicated to produce bone fractures in both in vivo and in vitro studies (30, 31). Many studies have associated an excess of retinol with old age hip fractures, especially in postmenopausal women (32). This effect of hypervitaminosis A has been shown to increase the size and number of osteoclasts thus leading to bone resorption, deterioration of cartilage in rats and spontaneous fractures (33, 34).

Our study indicates that treatment of thyroid hormone stimulates differentiation of ATDC5 chondrocytes and promotes formation of mineralized nodules. On the contrary, retinoic acid treatment decreased formation of the mineralized area both in vehicle and thyroid hormone treated cultures. Retinoic acid has been shown to exhibit a similar decrease in formation of mineralized nodule in periodontal ligament cells (35). Thus, retinoic acid at high dose blocks the thyroid hormone effects on cell differentiation.
One of the key findings of this study is that the retinoic acid effect on chondrocyte differentiation is dose-dependent and biphasic. Dose response experiments showed that at

low doses, retinoic acid exerts anabolic actions by increasing the expression levels ALP and osterix genes. Accordingly, a previous study showed that low doses of retinoic acid increased expression levels of ALP, osteocalcin and osteopontin genes (28). Retinoic acid at high doses inhibited chondroycte differentiation as reflected by ALP staining as well as expression levels of chondrocyte differentiation markers. Consistent with our findings, retinoic acid at high doses caused inhibition of longitudinal bone growth by causing premature epiphyseal closure (25, 26).

Thyroid hormone treatment induces increases in ALP, osterix and osteocalcin mRNA levels of ATDC5 cells, primary chondrocytes and primary osteoblasts cells. Our findings on the positive effects of thyroid hormone in bone cells are consistent with published reports demonstrating increased ALP activity and osteocalcin mRNA levels in thyroid hormone treated cultures (36, 37). Our study also disclosed that the treatment of retinoic acid at high dose inhibited thyroid hormone effects in ATDC5 cells. There was a significant decrease in the expression of mRNA levels of ALP, osterix osteocalcin genes when thyroid hormone treatment was given along with retinoic acid at high dose. In terms of potential explanation for retinoic acid modulation of thyroid hormone action, it is known that thyroid hormone receptors interact with retinoic acid receptors to form heterodimers at the thyroid hormone response element of target genes. Thus, retinoic acid binding to retinoic acid receptor might alter the interaction of retinoic acid receptor with thyroid hormone receptor and thereby influence the type of nuclear proteins recruited to the transcription complex of target genes such ALP, osterix and osteocalcin genes.

Surprisingly, retinoic acid treatment did not block the thyroid hormone effect on expression levels of ALP, osteocalcin and osterix in primary cultures of chondrocytes. Similarly, the differentiation promoting effects of thyroid hormone were not inhibited by retinoic acid in primary cultures of osteoblasts. One can offer a number of potential explanations for the observed differences in retinoic acid action in ATDC5 versus primary chondrocytes. In this regard, it is known that the actions of hormones and growth factors on target cells are dependent on stage of differentiation. Thus, the effect of retinoic acid on thyroid hormone action may be specific for a certain stage of development in bone cells, and primary chondrocytes and ATDC5 cells represent different stages in the chondrocyte differentiation pathway. Another possibility is that the nature of thyroid hormone and retinoic acid receptors expressed in the two cell types may be different. In any case, our findings are consistent with the known *in vivo* actions of retinoic acid that have been shown to vary depending on developmental stage, concentration and time (38, 39, 40).

Another explanation for the dissimilarity in the behavior of ATDC5 cells and primary cells might be the origin of these two cells types. ATDC5 cells are pure chondrocytes whereas primary osteoblasts and chondrocytes are derived from the ribs of mice. The cells from different origins and isolation methods have their own characteristics. ATDC5 cells represent more cell homogeneity whereas the primary cultures contain connective tissue, fibroblasts and adipocytes, thus suggesting that these other cell types may influence retinoic acid’s effects on bone cells.

Our findings cast a new light on the effect of retinoic acid on thyroid hormone action in bone cells. Our study demonstrated that retinoic acid treatment inhibits the

function of thyroid hormone by reducing mineralized nodule formation and suppressing the expression of ALP, osterix and osteocalcin genes. Our observation in the present study raises some questions regarding how the retinoic acid is interfering with the coactivators at the thyroid response element complex of ALP, osterix and osteocalcin genes. To evaluate the effect of retinoic acid on thyroid hormone action, future studies should examine the molecular pathway by which retinoic acid might interfere with recruitment of co-activators at the thyroid response element complex. Organ culture experiments using long limbs of mice/rat can be performed with thyroid hormone and/or retinoic acid treatments to study how retinoic acid influences chondrocyte differentiation and endochondral bone formation. *In- vivo* studies in mice involving administrating thyroid hormone with increasing doses of retinoic acid can provide us with answers as to how retinoic acid regulates thyroid hormone's effect on endochondral ossification.

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