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DECLARAÇÃO

Em adendo ao Certificado 035/10/CEUA, aprovado em 13.04.10, e por solicitação da Profa. Dra.**Cecilia Helena de Azevedo Gouveia Ferreira**, coordenadora da linha de Pesquisa, autorizo a inclusão dos alunos ***Marcos Vinícius da Silva e Manuela Miranda*** ao Projeto de Pesquisa "*Avaliação do hormônio tireoideano na estrutura e fisiologia óssea de camundongos com inativação dos genes dos adrenoceptores Alfa 2ª e Alfa2C*", uma vez que se trata de utilização da mesma espécie animal e de métodos experimentais similares ao Projeto.

Lembramos que a validade da sua licença para uso de animais para fins científicos vai até **12.04.2013**, havendo interesse, a licença poderá ser renovada mediante preenchimento de solicitação, conforme modelo constante no site **icb.usp.br.**

São Paulo, 21 de março de 2013.

Prof. Dr. Wothan Tavares de Lima
Coordenador da CEUA
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"Avaliação do hormônio tireoideano na estrutura e fisiologia óssea de camundongos com inativação dos genes dos adrenoceptores Alfa 2A e Alfa2C "

Prezada Professora,

Informo que a sua licença para uso de animais em experimentação, constante no protocolo em epígrafe, **foi prorrogada até 13.04.2016.**

Reitero que havendo alteração de metodologia e inserção de novos alunos ao projeto de pesquisa vinculado à referida licença a CEUA/ICB deverá ser informada.

Cordialmente,

Prof. Dr. WOTHAN TAVARES DE LIMA
Coordenador - CEUA-ICB/ /USP

Ilma.Sra.
Profa Dra. CECÍLIA HELENA DE AZEVEDO G. FERREIRA
Departamento de Anatomia
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DEDICATION

I would like to dedicate my thesis to my mother, grandfather, sister and brother for all the affection and support to get here.

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RESUMO

Rodrigues MM. Estudo da interação do Hormônio Tireoideano com o sistema α2 adrenérgico no crescimento ósseo endocondral: uma avaliação em cultura de órgão**.** [Tese (Doutorado em Ciências Morfofuncionais)]. São Paulo: Instituto de Ciências Biomédicas, Universidade de São Paulo; 2017.

Sabe-se que o hormônio tireoideano (HT) é essencial para o crescimento e desenvolvimento ósseos. No entanto, os mecanismos pelos quais o HT regula esses processos são pouco entendidos. Recentemente, o sistema nervoso simpático (SNS) foi identificado como um potente regulador do metabolismo ósseo. Estudos do nosso grupo mostraram que o HT interage com o SNS para regular a massa e estrutura ósseas, e que essa interação envolve a sinalização adrenoceptora α2 (α2-AR). Também identificamos a presença de todos os subtipos de adrenoceptores α2 adrenérgicos, o α2A- , α2B- e α2C-AR, na lâmina epifiseal (LE) de camundongos. Além disso, observamos que camundongos com inativação gênica isolada do α2A-AR e α2C-AR (α2A-AR$^{-/-}$ e α2C-AR$^{-/-}$) apresentam LEs desorganizadas, ossos longos mais curtos, e atraso na ossificação endocondral. Estudos in vivo revelaram, que as LEs de animais α2A-AR$^{-/-}$ e α2C-AR$^{-/-}$ respondem de forma diferente (do que as LEs de animais selvagens) ao excesso e deficiência de HT, o que sugere fortemente que o HT também interage com o SNS para regular o crescimento e o desenvolvimento ósseos. Através de um sistema de cultura de órgãos de ossos longos, o presente estudo teve como objetivo investigar se o HT interage com o SNS diretamente no esqueleto, para regular o crescimento linear ósseo, e se os receptores α2 adrenérgicos estão envolvidos nessa interação. Assim sendo, avaliamos, in vitro, o crescimento linear ósseo de tíbias derivadas de embriões de camundongos (com 15 dias de vida intrauterina) selvagens (WT) e α2C-AR$^{-/-}$ (KO) durante 6 dias. Vimos que as tíbias KO apresentam um menor crescimento longitudinal quando comparadas às tíbias WT, e que o tratamento com 10^{-8} M de triiodotironina (T3) diminuiu significativamente o crescimento longitudinal das tíbias WT, o que não foi visto nas tíbias KO. Vimos, ainda, que o tratamento com UK 14,304 (UK), um agonista α_2 não seletivo, induziu o crescimento longitudinal somente nas tíbias KO. A expressão de genes relacionados com a diferenciação terminal de condrócitos (Col X, IGF-1, Wnt-4 e Runx2) mostrou-se aumentada nas tíbias KO (quando comparada à expressão nas tíbias WT). O tratamento com T3, como esperado, estimulou a expressão desses genes nas tíbias WT, porém diminui a expressão nas amostras KO, chamando a atenção para a importância desses receptores na modulação das ações do T3. Observamos, ainda, que a ativação local dos α_2-ARs com o UK bloqueia a expressão desses genes relacionados à diferenciação dos condrócitos, além de bloquear os efeitos positivos do T3 (UK+T3) na expressão desses genes. Esses achados mostram que os receptores α2 adrenérgicos atuam diretamente no esqueleto para controlar a diferenciação terminal dos condrócitos e, portanto, o crescimento longitudinal ósseo, além de permitirem uma interação com a via de sinalização do HT para controlar esses processos. Em conclusão, este estudo mostra que HT interage com o SNS, localmente no esqueleto, via sinalização α2 adrenérgica, para modular o crescimento linear ósseo.

Palavras chave: Crescimento Ósseo. Hormônio Tireoideano. Sistema Nervoso Simpático.

ABSTRACT

Rodrigues MM. Thyroid Hormone interaction with the sympathetic nervous system, via α_2 adrenoceptor signaling, to regulate endochondral bone growth: and in vitro evaluation. [Ph.D. Thesis (Morphofunctional Sciences)]. São Paulo: Instituto de Ciências Biomédicas, Universidade de São Paulo; 2017.

It is well known that thyroid hormone (TH) is essential for normal bone growth and development. However, the mechanisms by which TH regulates these processes are poorly understood. Recently, the sympathetic nervous system (SNS) was identified as a potent regulator of bone metabolism. In vivo studies by our group have shown that TH interacts with the SNS to regulate bone mass and structure, and that this interaction involves α_2 adrenoceptor (α_2-AR) signaling. We have also identified the presence of α_{2A}-, α_{2B}-, and α_{2C}-AR subtypes in the epiphyseal growth plate (EGP) of mice. In addition, we have found that mice with isolated gene deletion of α_{2A}-AR and α_{2C}-AR (α_{2A}-AR$^{-/-}$ and α_{2C}-AR$^{-/-}$) show a disorganized EGP, shorter long bones and a delay in endochondral ossification (EO). In vivo studies revealed that the EGP of α_{2A}-AR$^{-/-}$ and α_{2C}-AR$^{-/-}$ animals respond differently (than those of wild-type animals), to TH excess and deficiency, which strongly suggests that TH also interacts with the SNS to regulate bone growth and development. Through a long bone organ culture system, the present study had the goal of investigating if TH interacts with the SNS directly in the skeleton, to regulate the longitudinal bone growth and if α_2-AR is involved in this process. Therefore, we evaluated the linear bone growth of tibias derived from 15.5-day-old WT and α_{2C}-AR$^{-/-}$ mouse embryos (E15.5) for 6 days. We have seen that the KO tibias showed a lower longitudinal growth when compared to WT tibias, and that treatment with 10-8 M triiodothyronine (T3) significantly decreased the longitudinal growth of the WT tibias, which was not seen in the KO tibias. We found that the treatment with UK 14.304 (UK), a non-selective α2-agonist, induced the longitudinal growth only of the KO tibias. The expression of genes related to the terminal differentiation of chondrocytes (Col X, IGF-1, Wnt-4 and Runx2) was shown to be increased in the KO tibias (when compared to the expression in WT tibias). Treatment with T3, as expected, stimulated the expression of these genes in WT tibias, but decreased the expression in KO samples, highlighting the importance of these receptors in the modulation of T3 actions. We observed that the local α_2-AR activation by UK blocked the expression of these chondrocyte differentiation-related genes, in addition to blocking the positive effects of T3 (UK + T3) in the expression of these genes. These findings show that α2 adrenoceptors act directly in the skeleton, to control the terminal differentiation of chondrocytes and, therefore, the longitudinal bone growth, in addition to allow an interaction with the TH signaling pathway to control these processes. In conclusion, this study shows that TH interacts with the SNS, locally in the skeleton, via α2 adrenergic signaling, to modulate the longitudinal bone growth.

Keywords: Longitudinal Bone Growth. Thyroid Hormone. Sympathetic Nervous System.

ABREVIATIONS

AC- adenyl cyclase

ANS- autonomic nervous system

APC- adenomatous polyposis coli

BMD- bone mineral density

BV/TV- trabecular bone volume

cAMP- cyclic 3’5’ adenosine

CNS- central nervous system

Col I- Collagen type I

Col II- Collagen type II

Col X- Collagen type X

D1- iodothyronine deiodinases type 1

D2- iodothyronine deiodinases type 2

D3- iodothyronine deiodinases type 3

Dsh- disheveled

ECM- extracellular matrix

EGP- epiphyseal growth plate

FGF23- *fibroblast growth factor* 23

Frizzled- 7-transmembrane domain-spanning frizzled receptor

GH- growth hormone

GHR- GH receptor

GP- growth plate

GSK3- glycogen synthase kinase 3

HBM- high bone mass

HC- hipetrophic chondrocytes

HZ- hypertrophic zone

IGF-I- insulin-like growth factor 1

Ihh- indian hedgehog

KO- *Knockout*

LBG- longitudinal bone growth

LRP5/6- low-density lipoprotein receptor-related protein 5 and 6

MAPK- mitogen-activated protein kinase

NE- norepinephrine

PC- proliferative chondrocytes

PNS- parasympathetic nervous system

POC- primary ossification center

PTH- Parathyroid hormone

PTHrP- parathyroid hormone–related protein

PZ- proliferating zone

RANK-L- receptor activator of nuclear factor Kappa B

RZ- resting zone

SNS- sympathetic nervous system

T3- triiodotironine

T3r- reverse T3

T4- tetraiodotironine

Tb.N- trabecular number

Tb.Sp- trabecular separation

Tb.Th- trabecular thickness

TCF/LEF- T cell transcription factor/ lymphoid enhancer factor

TH- thyroid hormone

TREs- thyroid hormone responsive elements

TRs- nuclear receptors

VEGF- vascular endothelial growth factor

WT- wild-type

α2A/α2C-AR$^{-/-}$- gene deletion of α_{2A} e α_{2C} adrenergic receptors

α2A-AR$^{-/-}$- gene deletion of α_{2A} adrenergic receptor

α2A-AR- α_{2A} adrenergic receptor

α2C-AR$^{-/-}$- gene deletion of α_{2C} adrenergic receptor

α2C-AR- α_{2C} adrenergic receptor

β2-AR$^{-/-}$- gene deletion of β_2 adrenergic receptor

β2-AR- β_2 adrenergic receptor

µCT- micro tomography

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SUMARY

1 INTRODUCTION

1.1 - Skeletal development

Skeletal development stands for a number of skeletal-related processes that follow from the early days of pregnancy until the skeleton has attained complete development in late puberty. These processes include (i) skeletogenesis, which involves recruitment, migration and condensation of precursor cells to the future skeletal sites, tailed by their differentiation to form the skeletal tissues; (ii) skeletal patterning, which determines bone number and shape; (iii) skeletal growth in length and width, which depends on proliferation, survival and differentiation of chondrogenic and/or osteogenic cells; (iv) and maturation of the skeleton, determined mainly by ossification of a primordial skeleton (1, 2).

Skeletogenesis and bone maturation occur through two processes, endochondral and intramembranous ossification. In both processes, groups of mesenchymal cells aggregate (mesenchymal condensation) and initiate a characteristic genetic program that leads to bone formation. In intramembranous ossification, cells in the mesenchymal condensations differentiate directly into osteoblasts and, consequently, there is bone formation. In endochondral ossification, there is an intermediate stage where a cartilaginous template is formed to be replaced by bone. Thus, the cells in the mesenchymal condensations differentiate into chondrocytes, which differs in a population of centrally located proliferating chondrocytes expressing collagen type II (Col II), and in a population of chondrocytes located more peripherally, which express collagen type I (Col I) (2), thus forming a cartilaginous template.

In response to several signals, the chondrocytes in the central region of the cartilaginous template stop proliferating, change their genetic program to suffer hypertrophy and to synthesize collagen type X (Col X) and other proteins and factors characteristic of hypertrophic chondrocytes (HC), composing a centrally located hypertrophic region in the cartilaginous mold (3). These HCs then signals to the adjacent perichondrial cells to differentiate into osteoblasts, inducing the formation of a bone collar and periosteum around the cartilaginous template. At the same time, HCs mediate the mineralization of the surrounding

extracellular matrix (ECM), release vascular endothelial growth factor (VEGF), which induces angiogenesis, and undergo apoptosis leaving gaps in the cartilaginous matrix. These gaps are invaded by blood capillaries, leading to the vascularization of the avascular cartilaginous mold. With this vascularization, pre-osteoblastic cells invade the hypertrophic region, secrete Col I in the ECM and mediate the formation of bone tissue within the cartilaginous mold, composing the primary spongiosa (consisting of osteo-cartilaginous spicules), which constitutes a primary ossification center (POC). HCs also secrete receptor activator of nuclear factor kappa B ligand (RANKL), a factor that induces osteoclastogenesis, contributing to the recruitment of osteoclast cells into the cartilaginous template (4). Osteoclasts, in turn, digest the matrix synthesized by HCs.

As the ossification process proceeds, the ossification center expands toward the bone ends. In the case of the cartilaginous molds of the long bones, the primary ossification center occurs in the diaphysis and expands towards the epiphyses (5). In the epiphyses, rounded chondrocytes continue to proliferate, and those near the primary ossification center flatten and form columns of proliferating chondrocytes (PCs). These cells stop proliferating and differentiate into HCs, which are located even closer to the primary ossification center. Subsequently, secondary ossification centers are formed in the epiphysis.

The rounded chondrocytes in the center of the ends of the cartilaginous mold (future bone epiphyses) stop dividing, differentiate into HCs, and lead the formation of the secondary ossification centers. Cartilage is maintained at the ends of the bone epiphysis (articular cartilage) and between the primary and secondary ossification centers, establishing an epiphyseal growth plate (EGP), which contains the longitudinal bone growth machinery.

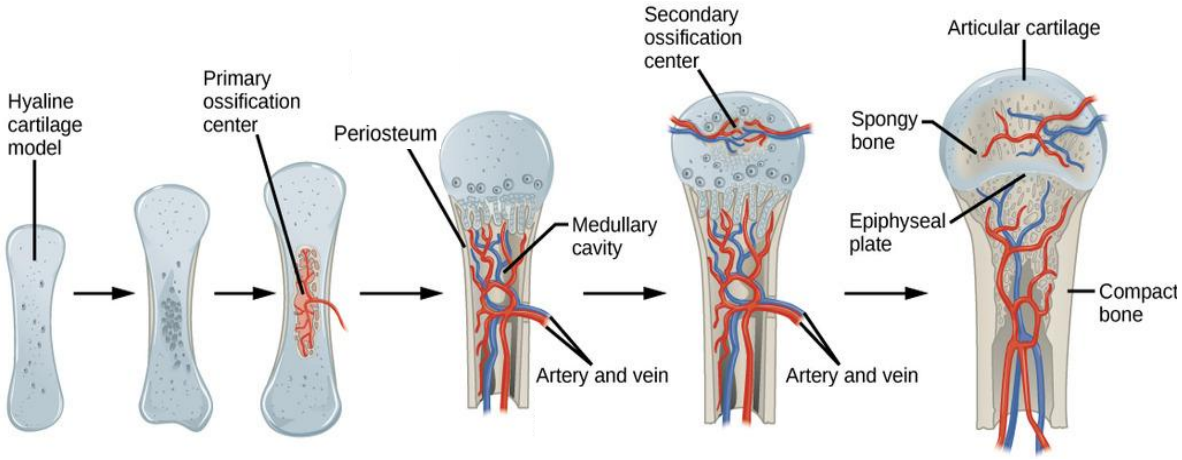

Figure 1 – Schematic diagram of endochondral ossification. (Boundless Biology – Types of Skeletal System, page 2).

The mammalian EGP is composed of three well defined main zones that follow an elegant developmental program: reserve (RZ), proliferative (PZ) and hypertrophic (HZ) zones (6). The RZ, the one closest to the epiphysis, consists of undifferentiated progenitor cells, the reserve or resting chondrocytes (RC). These cells are rounded, relatively small and are dispersed in an ECM rich in type II collagen and proteoglycans. These cells differentiate into proliferating chondrocytes (PC), which are disk-shaped (flattened) cells organized in columns parallel to the longitudinal axis of the long bones (7), composing the PZ. The PCs secrete mainly Col II, the main component of the PZ ECM of the PZ, and differentiate into HCs.

It is worth mentioning that the PZ plays a crucial role in endochondral ossification and linear bone growth, since it is an active site of cell replication in the EGP (8). When the PC divides, the two daughter cells align to the longitudinal axis of the bone (9). This spatial organization directs bone growth and is thus responsible for the elongated shape of various endochondral bones. The HCs also assume a columnar organization in the HZ. These cells, as well as in the primary and secondary ossification centers, secrete Col X and VEGF, among other proteins and factors, and command the endochondral ossification process. Thus, the linear bone growth occurs in the EGP by deposition of cartilage in the epiphysis-diaphysis direction and endochondral ossification of this cartilage in the diaphysis-epiphysis direction (7). Therefore, growth is determined by chondrocyte proliferation, ECM synthesis and chondrocyte

hypertrophy in the EGP. In adolescence, ossification overcomes the deposition of cartilage, resulting in the total ossification of the EGP, which ceases linear bone growth by age of 18 years in women and 21 years in men.

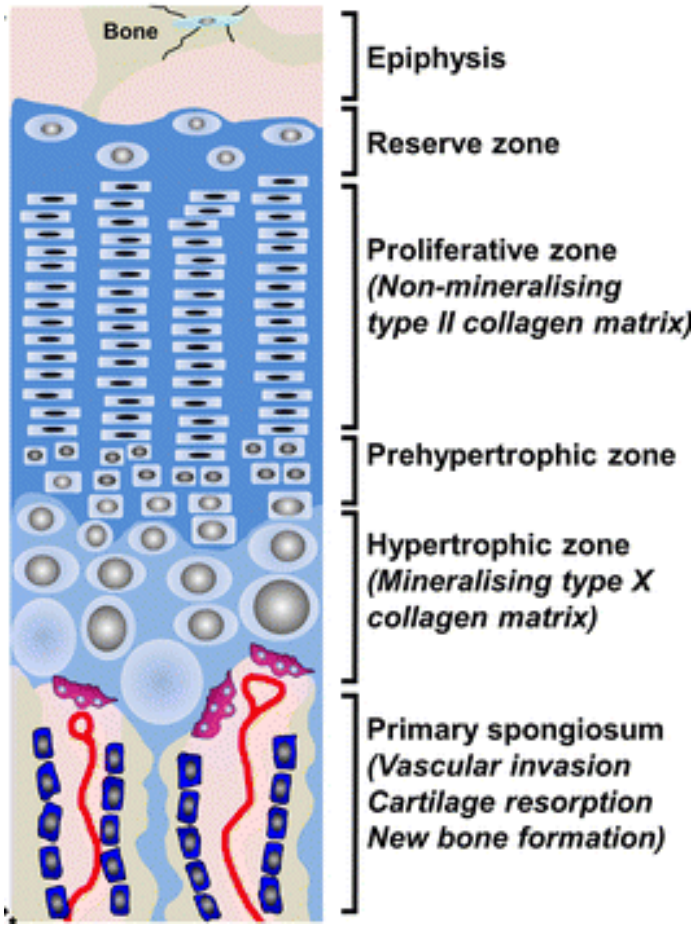

Figure 2 – Schematic representation of the growth plate, showing chondrocytes proliferation, differentiation and apoptosis (Modified from Williams GR, 2017 (10)).

The mechanisms of regulation of endochondral ossification and linear bone growth, including regulation of proliferation, organization, differentiation and apoptosis of chondrocytes, as well as subsequent vascular invasion are still poorly understood. It is known, however, that alterations of these processes can result in numerous diseases such as chondrodysplasias and other developmental disorders, demonstrating the need of a better understanding of the mechanisms, pathways and factors that regulate endochondral ossification and linear bone growth.

1.2 Thyroid hormone

The main secretory products of the thyroid gland are iodothyronines, compounds that result from binding of two molecules of iodinated tyrosine. In mammals, about 60-90% of thyroid production is 3,5,3',5'-tetraiodothyronine (thyroxine or T4), 10-40% is 3,5,3'-triiodothyronine (T3) and less of which 1% is represented by other biologically inactive iodothyronines. T4 basically functions as a prohormone and is converted to the active form of TH, T3, by the action of cellular enzymes, the iodothyronine deiodinases. This conversion occurs in the thyroid gland itself and, mainly, in the target tissues of TH (11). Three types of iodothyronine deiodinases have been identified in mammalian tissue, type I, II and III (D1, D2 and D3, respectively). D1 and D2 convert T4 to T3, therefore, they activate TH. D1 also converts T4 to reverse T3 (rT3; 3,3 ', 5'-T3), and rT3 and T3 to T2 (3,3'-T2). Considering that both rT3 and T2 are inactive, D1 is capable of both activating and inactivating iodothyronines (12). D3 catalyzes the degradation of T4 to rT3, and T3 to T2. Studies of our group showed that the three enzymes are expressed in the skeleton of neonates and young mice, which highlights the importance of these enzymes in pre and postnatal skeletal development (13). The actions of TH are primarily the result of T3 interaction with its nuclear receptors (TRs), which are T3-inducible transcription factors. These receptors bind to specific regions of the target genes, the TH responsive elements (TREs), and modify gene expression, increasing or decreasing the rate of gene transcription. There are four classical isoforms of TR encoded by two genes (14), THRA and THRB, located in humans on chromosomes 17 and 3, respectively. THRB encodes TRβ1 and TRβ2, while THRA encodes TRα1 and TRα2. This latter isoform does not bind to T3 and functions, at least in vitro, as an antagonist of TRα1 and TRβ1 (15). TRα1, TRα2 and TRβ1 are expressed in the major skeletal cells, osteoblasts, osteoclasts and chondrocytes (16, 17). A series of studies has shown that TRα1 is the predominant isoform in the bone tissue (18, 19) and the main TR isoform to mediate TH actions in the skeleton. However, a body of studies showed that TRβ1 also mediates important effects of T3 on osteoblastic cells of rats and mice (20), and especially, in the skeleton of developing rats (21).

1.2.1 Thyroid Hormone and Bone Development.

Classical studies of more than 40 years have identified TH as a potent regulator of skeletal growth and maturation. TH deficiency in the child is often associated with severe delay in bone maturation, short stature, and mechanical failure of the EGP of both hips (22). In animal models of hypothyroidism, T3 deficiency results in delayed intramembranous and endochondral ossification, together with important alterations in EGP, such as reduction in thickness, disorganization of PC columns, and an important deficiency of the differentiation of PCs into HCs. In addition, hypothyroidism during development significantly limits the acquisition of peak bone mass (21). On the other hand, the increase in TH concentrations may result in accelerated skeletal maturation, premature closure of EGPs and a subsequent decrease in linear bone growth (23).

TH, along with growth hormone (GH), insulin-like growth factors, glucocorticoids, estrogens and androgens represent the main systemic factors that influence skeletal growth and maturation. The first four, during childhood and the last two, during adolescence (24). Before adolescence, TH is considered the main prerequisite for normal skeletal maturation. One of the mechanisms through which TH stimulates bone development is indirectly, increasing the synthesis and secretion of GH and IGF-1 (25), factors that have direct effects on bone tissue. GH and T3 interact to promote longitudinal bone growth and bone maturation. The crosstalk between T3 and GH action pathways occurs at various levels, including the effect of T3 on the regulation of GH gene transcription (26), and the stimulation of IGF-1 expression in vivo and in vitro (27, 28). Thus, in hypothyroidism, there is a reduction in the serum and tissue levels of GH and IGF-1, which promotes important bone changes. However, there is evidence that TH acts directly in EGP chondrocytes through mechanisms independent of GH.

It has been shown, for example, that GH alone is not able to induce the organization of the columns of chondrocytes, neither to promote the differentiation of PCs in HCs in the EGP of hypothyroid rats (29, 30). Robson et al. (31) have shown that, in primary cultures of the rat tibiae, T3 acts directly inhibiting the proliferation of PCs, while promoting their differentiation into HCs. The presence of TRs in chondrocytes of humans (16), rats (32) and mice

support a direct effect of T3 on skeletal development. In rodents, TRα1, TRα2 and TRβ1 isoforms were identified in the reserve, proliferative, and prehypertrophic zones and in the primary spongiosa of the EGP, but were not detected in the HZ (31).

In humans, gene and protein expression of TRα1, TRα2 and TRβ1 were identified in reserve, proliferating and hypertrophic chondrocytes of endochondral ossification sites, such as osteophytes, heterotopic bone or osteoclastomas (16, 33). Our group showed that treatment of hypothyroid rats with GC-1, a TRβ selective agonist, does not normalize serum IGF-1 levels or IGF-1 expression in the EGP, and does not rescue the growth plate columnar organization, However, GC-1 was able to normalize the differentiation of HCs, the expression of type X collagen and the thickness of the EGP in hypothyroid rats (21). In addition, we showed that GC-1 has positive, though modest, effects on bone growth, bone mass acquisition, and endonchondral ossification. These findings strongly suggest that TH modulates skeletal development independently of the GH/IGF-1 axis and that TRβ mediates some essential effects of T3 on bone.

Some years ago, two signaling proteins, Indian hedgehog (Ihh) and parathyroid hormone-related protein (PTHrP), were identified as crucial for the regulation of growth plate chondrocyte differentiation (34). Ihh, produced by the prehypertrophic zone of the EGP, stimulates PTHrP production in the perichondrium. PTHrP acts on the PZ, inhibiting cell differentiation and thus maintaining a proliferating state. This negative feedback system has the opposite effect of T3. Interestingly, in rats, it was demonstrated an increase in PTHrP mRNA expression in hypothyroidism and a reduction of PTHrP receptor (PTHrP-R) in thyrotoxicosis (35). These effects correlate with the epiphysial dysgenesis and growth retardation seen in hypothyroidism, and with the accelerated growth rate seen in thyrotoxicosis, suggesting that important physiological actions of T3 on the EGP are mediated through the Ihh/PTHrP-R feedback system.

In the last decades, the identification of mutations in fibroblast growth factor (FGF) receptors has defined essential roles of the FGF signaling pathway in bone development. For example, there is evidence that the FGF pathway negatively regulates proliferation of EGP chondrocytes (5). Studies show that

T3 stimulates the expression of FGF receptors (FGFR-1 and FGFR-3) and increases the effect of FGF2 on the mitogen-activated protein kinase (MAPK) signaling pathway in ATDC5 chondrogenic cells, osteoblasts and osteocytes (36). In addition, FGFR-3 expression has been shown to be normal in TRβ KO mice, but markedly reduced in the EGP of TRα KO mice, which have a phenotype similar to that of hypothyroidism (37). Therefore, these studies suggest that T3 inhibitory effects on the proliferation of EGP chondrocytes are mediated by the FGF pathway and via TRα.

More recent evidence shows that TH also regulates the terminal differentiation of epyphiseal chondrocytes by modulating the Wnt/β-catenin signaling pathway (38). The Wnt (consists of a family of proteins) signaling pathway plays an important role in the development and maintenance of various organs and tissues, including bone. Although Wnt proteins signal through various pathways to regulate cell growth, differentiation, function, and death, the Wnt/β-catenin signaling pathway, also known as canonical, appears to be the most important for bone biology (39). If Wnt proteins are not expressed or the binding to their receptors is inhibited, β-catenin degradation is facilitated through their interaction with a protein complex consisting of APC (adenomatous polyposis coli), axin and GSK3 (glycogen synthase kinase 3). APC and axin act as supporting proteins, allowing GSK3 to bind and phosphorylate β-catenin, thereby marking it for ubiquitin/proteasome degradation. Activation of the Wnt/β-catenin pathway occurs by binding of Wnt proteins to their Frizzled (7-transmembrane domain-spanning frizzled receptors) and LRP5/6 (low-density lipoprotein receptor-related protein 5 and 6) receptors. This binding inhibits the activity of GSK3, through mechanisms involving axin, Frat-1 and Dsh (disheveled) proteins, causing accumulation of β-catenin, which in turn is translocated to the nucleus, where it binds to TCF/LEF (T cell transcription factor / lymphoid enhancer factor) and activates transcription of Wnt responsive genes (39).

Recently, members of the Wnt family have been recognized as key regulators of chondrocyte proliferation and differentiation during limb development. Inactivation of β-catenin in chondrocytes of mouse embryos produces a dwarfing phenotype with decreased chondrocyte proliferation, and delayed differentiation of HCs and endochondral ossification (40).

Overexpression of Wnt-8c or β-catenin induces terminal differentiation of chicken chondrocytes (40) and positively regulates gene expression of Runx2 (an essential gene for osteoblastic differentiation), through the activation of the TCF/LEF binding sites in the promoter of Runx2 (41). Overexpression of ICAT (β-catenin and TCF inhibitor) in mouse cartilage impairs long bone growth, with a delay in chondrocyte differentiation and a delay in the appearance of the secondary ossification centers.

Wang et al. (42) have shown that TH stimulates Wnt-4 expression and Wnt/β-catenin signaling in the EGP of rats. In addition, they have shown that Wnt antagonists, Frzb/sFRP3 and Dkk1, inhibit T3 activation of the Wnt/β-catenin pathway, besides inhibiting the T3 effects on the terminal maturation of growth plate chondrocytes. These findings strongly suggest that TH regulates terminal differentiation of EGP chondrocytes, at least in part, through the modulation of the Wnt/β-catenin pathway. More recently, the same group demonstrated that TH interactions with the Wnt/β-catenin pathway, to regulate differentiation of EGP chondrocytes, are modulated by the IGF-1/IGF-1R signaling pathway (42). Wang et al (2010)(43) showed that IGF-1/IGF-1R induces Wnt-4 expression and β-catenin activation in EGP chondrocytes. Besides, they showed that IGF-1/IGF-1R has prominent effects on chondrocyte proliferation and modest effects on terminal chondrocyte differentiation, both of which were shown to be partially inhibited by the Wnt antagonists sFRP3 and Dkk1 (Wang et al., 2010 (43)). Interestingly, this study also showed that T3 positively regulated proliferation in addition to induce Wnt-4 expression, β-catenin activation, and terminal differentiation of growth plate chondrocytes, but these effects were partially prevented by the IGF-1R inhibitor picropodophyllin (Wang et al., 2010) (43). These data indicate that the interactions between TH and Wnt/β-catenin signaling pathways to regulate growth plate chondrocyte proliferation and terminal differentiation involve T3 actions on IGF-1/IGF1R pathways (Wang et al., 2010 (43)).

In summary, it is known that TH, independently of the GH/IGF-1 axis, plays an essential role in endochondral ossification, in the columnar organization of PCs, and in the differentiation of HCs. Due to these effects, TH is essential for normal bone growth and maturation. To date, T3 has been shown to promote some of its effects on skeletal development by modulating

the GH/IGF-I axis; the Ihh/PTHrP-R pathway, FGFs and Wnt/β-catenin. However, a number of T3 mechanisms of action in skeletal development remain to be identified and studied. The identification and study of these mechanisms will contribute to the understanding of how T3 promotes bone maturation and linear bone growth.

1.3 The sympathetic nervous system

The Autonomic Nervous System (ANS) is the efferent component of the Visceral Nervous System, and is divided into Sympathetic Nervous System (SNS) and Parasympathetic Nervous System (PNS). The vast majority of post-glanglionic sympathetic fibers have noradrenaline (NE) as the neurotransmitter and are therefore adrenergic (44). The adrenergic system is an essential regulator of neural, endocrine, cardiovascular, vegetative and metabolic functions. Endogenous catecholamines, adrenaline and NE, transmit their biological signals via G protein-coupled receptors to regulate a variety of cellular functions (45). These receptors are called adrenergic receptors, and it is through them that the SNS performs its actions on the target organs. Most of these receptors are in the plasma membrane of neurons and in neural and non-neural target cells. In the target cells of this system, there are α-adrenergic and/or β-adrenergic receptors. There are 9 subtypes of adrenergic receptors, which are subdivided into α1 (α1A, α1B and α1D) (46), α2 (α2A, α2B and α2C) and β (β1, β2 and β3) (47). All nine subtypes of adrenergic receptors are activated by adrenaline and NE. All β-adrenergic receptor isoforms stimulate adenylate cyclase (AC) and, thus, induce cyclic 3',5'-adenosine (cAMP) synthesis (48), which is the second messenger of NE actions mediated by β -adrenergic antagonists. On the other hand, α2 receptors inhibit AC and, therefore, decrease cAMP formation, antagonizing the β-adrenergic receptor-mediated actions.

α2-adrenergic receptors (α2-ARs) are located in the presynaptic membranes of adrenergic neurons (sympathetic endings), where they act as autoreceptors (an autoreceptor is a type of receptor present in the presynaptic membranes of nerve cells, which is only sensitive to the neurotransmitters or hormones released by the neuron on which it is situated), negatively regulating the exocytosis of NE from the sympathetic nerves as part of a negative

feedback loop (49). α2-ARs are also located in non-adrenergic neurons, where they act as heteroceptors (a heteroceptor is sensitive to neurotransmitters and hormones that are not released by the cell on which it is located), modulating the release of other neurotransmitters or hormones, such as serotonin (Mongeau et al, 1998 (50)), GABA (Jackisch et al, 1999 (51)), dopamine (Milan et al, 2000 (52)) and GHRH (53). α2-ARs, acting as autoreceptors and heteroceptors, have been described in the central and peripheral nervous systems (54)(Gilsbach, 2011). In addition to these presynaptic neuronal receptors, α2-ARs have been also identified in many non-neuronal cell types, including blood vessels, myometrium, spleen, adipose tissue and pancreatic islets (46), where they have essential roles, for example, regulating blood pressure and insulin release.

The in vivo activation of α2-ARs by the endogenous (adrenaline and NE) or synthetic agonists, such as brimonidine, clonidine, dexmedetomidine, moxonidine, medetomidine and rilmenidine (Cambridge, 1981; Kallio et al., 1989; Bylund et al., 1994; Fairbanks et al., 2009), causes diverse effects, including hypotension, bradycardia, analgesia, hypothermia, sedation, hypnosis and anesthetic-sparing (54).

The role of each α2-AR isoform is not clear yet, because of the lack of drugs with sufficient isoform-selectivity. On the other hand, studies in mice with inactivation of individual α2-AR subtypes have contributed to dissociate the role of each isoform, revealing that only a few functions are mediated by a single subtype. These studies showed that α2B-AR has an important role in the development of the placenta (55, 56) and the lung (57), besides regulating vascular tone (58) and mediating some analgesic effects (59). α2C-AR was identified as the major feedback receptor of adrenaline release from chromaffin cells of the adrenal medulla (60), in addition to cooperated with α2A-AR in the presynaptic inhibition of NE release. On the other hand, α2A-AR was identified as the main receptor acting to inhibit the release of NE from sympathetic nerves. It is noteworthy that the majority of the physiological and pharmacological actions of α2-AR activation seems to be mediated by α2A-AR subtype (54, 61). Some of these α2A-AR-mediated actions include insulin release by the pancreatic islets (62), the facilitation of working memory by the prefrontal cortex (63), hypotension, bradycardia and modulation of the

baroreflex sensitivity (MacMillan et al., 1996; Lakhlani et al., 1997; Niederhoffer et al., 2004), in addition to sedation and hypnosis (63).

The study of a transgenic mouse model that does not express α2C-AR globally but expresses α2A-AR only in adrenergic neurons (autoreceptors) (64) confirmed the role of α2A-AR as the main autoreceptor to control NE release from the sympathetic nerve terminals and to control spontaneous locomotor activity at night-time (64). More importantly, further studies of this mouse model revealed that the hypotensive effects (bradycardia, hypotension and baroreceptor sensitivity) of α2-AR agonists are primarily mediated by α2A-heteroceptors, whereas α2A-autoreceptor has only a minor role in mediating these effects. In addition, sedation, anesthetic sparing, hypothermia and analgesia were also shown to be primarily mediated by α2A-heteroceptors. Therefore, most of the known effects α2-AR activation are mediated by α2A-heteroceptors.

α1 receptors act mainly on the regulation of vascular tone and cardiac development, besides participating in behavior modulation. These α1 receptors are located in the CNS, right atrium, ventricle, liver, spleen, ileum, parotid glands, nasal mucosa, bladder, urethra, cavernous body and prostate glands and veins of various tissues (46, 65).

1.3.1 Sympathetic nervous system actions in the skeleton

An important finding of the recent years is that bone remodeling is also under control of the central nervous system (CNS), with the SNS acting as the peripheral effector via β_2-adrenoceptors (β_2-AR) expressed in osteoblasts(66, 67). It was shown that the SNS inhibits bone formation and stimulates bone resorption (68, 69). Therefore, the SNS activation has osteopenic effects.

The role of the SNS in bone remodeling was supported by a high bone mass (HBM) phenotype in mouse models of low sympathetic activity: Ob/Ob mice, which are deficient in leptin; and mice with gene inactivation of dopamine β- hydroxylase ($DBH^{-/-}$), the rate-limiting enzyme responsible for the synthesis of catecholamines (69). These mouse models, however, have endocrine dysfunctions including hypercorticism, hyperinsulinemia and hypogonadism, which can interfere with the SNS effects on bone tissue. A more specific role of β_2-AR in bone mass was shown by the analyzes of animals with gene

inactivation of this receptor, the β2-AR$^{-/-}$ mice, which have no endocrine or metabolic abnormalities. These animals present normal body weight, but also exhibit a HBM phenotype, due to an increase in bone formation and decrease in bone resorption (70). In addition, these mice are resistant to bone loss induced by ovariectomy.

Subsequently, a study of our group analyzed the skeletal phenotype of a mouse model with chronic sympathetic hyperactivity (47), due to the double gene deletion of α2A and α2C adrenergic receptors (α2A/α2C-AR$^{-/-}$). As discussed before in this introduction (section 3.1), α_2-ARs are presynaptic autoreceptors that negatively regulate the release of catecholamines, especially norepinephrine (NE). Therefore, activation of these receptors in the brain stem decreases the sympathetic tone, which results in decreased heart rate and blood pressure(48). These effects are enhanced by stimulation of α_2-ARs in sympathetic nerve terminals. Hein et al (71) showed that simultaneous blockade of α2A-AR and α2C-AR in mice leads to a chronic elevation of sympathetic tone.

These knockout (KO) mice have high mortality and decreased heart function after 4 months of age (71). As expected, we observed that α2A/α2C-AR$^{-/-}$ mice present high plasma levels of NE and cardiac hypertrophy, which confirms the hyperactivity of the SNS. Considering the evidence that the activation of the SNS has negative effects on bone mass, our expectation was that α2A/α2C-AR$^{-/-}$ mice would present an osteopenic skeleton. Surprisingly, we found that these animals show a widespread phenotype of HBM, a better quality in bone microarchitecture and greater bone resistance to mechanical stress (46). By immunohistochemistry, we identified α2A-AR, α2B-AR and α2C-AR expression in osteoblasts, osteocytes and osteoclasts. In addition, these receptors were also detected in the EGP, specifically in chondrocytes of the reserve and hypertrophic zones, and in hypertrophic chondrocytes of the secondary ossification centers. The fact that α2A/α2C-AR$^{-/-}$ animals present HBM phenotype, even with an increase in sympathetic tone and normal β_2-AR expression, shows that β_2-AR is not the only adrenoceptor involved in bone metabolism control, and that α2A-AR and/or α2C-AR may play an important role in mediating the actions of the SNS in bone mass regulation. Interestingly, we found that mice with single inactivation of α2C-AR (α2C-AR$^{-/-}$) present lower

trabecular bone volume (BV/TV) and number (Tb.N), and increased trabecular separation (Tb.Sp) in the femur compared with WT mice; which was accompanied by decreased bone strength in the femur and tibia. The contrary was found in the vertebra, where α2C-AR$^{-/-}$ mice show higher BV/TV, Tb.N and trabecular thickness (Tb.Th), and decreased Tb.Sp, compared with WT animals. These findings suggest that α2C-AR subtype mediates the effects of the SNS in the bone in a skeletal site-dependent manner (72).

The presence of α_2 adrenergic receptors in the EGP and HC of the secondary ossification centers raised the hypothesize that the SNS also has a role in the longitudinal bone growth and skeleton ossification. Corroborating this hypothesis, recent studies from our laboratory showed that adult mice with isolated α2A-AR or α2C-AR gene inactivation present shorter femurs and tibias (FAPESP Projects number: 2010/06409-0, 2010/50068-2 and 2010/04911-10). Further studies of our group (FAPESP Project number 2013/02247-3 and 2012/11858-3) showed that the EGP morphology of α2A-AR$^{-/-}$ and α2C-AR$^{-/-}$ animals present several modifications, when compared with WT mice. The growth plate of α2C-AR$^{-/-}$ animals showed a structural disorganization in the columns of PCs and a significant increase (30%) in the number of HCs. The single KO mice for α2A-AR (α2A-AR$^{-/-}$) showed a more evident disorganization in the PZ than α2C-AR$^{-/-}$ animals. On the other hand, α2A-AR$^{-/-}$ mice showed a reduction in the number of HCs. Altogether, these findings support the hypothesis that SNS regulates the morphology and physiology of the EGP, as well as the longitudinal bone growth. Also, they suggest that the SNS actions in these processes are, at least partially, modulated by α_2 adrenoceptors. On the other hand, these findings raise an important question: Do these SNS actions take place directly in the skeleton or are they indirect, i.e. depend on the SNS action in other tissues, organs and systems?

1.4 Evidence that thyroid hormone interacts with the SNS, via α_2 adrenergic receptors, to regulate bone mass and bone metabolism.

Thyroid Hormone (TH) modulates bone remodeling and bone mass, besides controlling bone development. Triiodothyronine (T3) stimulates bone formation and resorption by regulating the activity of osteoblasts and

osteoclasts (73). TH excess stimulates more osteoclastic than osteoblastic activity, which results in increased calcemia and decreased bone mass.

An important characteristic of TH, but poorly understood, is its interaction with the SNS. It is well known that a synergism between catecholamine and TH is required for the maximum thermogenesis, lipolysis, glyconeogenesis and glycogenolisis (74). Previous studies have shown that patients with hyperthyroidism have increased sympathetic tone (75).

Considering that TH excess causes bone loss and the SNS activation also has a negative effect on bone mass, we hypothesized that TH also interacts with the SNS to regulate bone metabolism and, consequently, bone mass. One evidence of this possible interaction is the fact that treatment of hyperthyroid patients with propanolol (a β-adrenergic antagonist) corrects the thyrotoxicosis-induced hypercalcemia (76). Furthermore, patients with hypothyroidism treated with propanolol showed a decrease in the urinary excretion of hydroxyproline, a biochemical marker for bone resorption (77).

To investigate this hypothesis, 40 day- old WT and α2A/α2C-AR$^{-/-}$ female mice were treated with approximately 10 times the physiological dose of T3 (10xT3 μ=3.5 g/100g BW/day) or saline during 12 weeks (78). As expected, T3 treatment resulted in a generalized decrease in the bone mineral density (BMD) of WT mice (determined by DEXA), followed by deleterious effects on the trabecular and cortical bone microstructural parameters (determined by μCT) of the femur and vertebra. In addition, this treatment also had deleterious effects on the biomechanical properties (maximum load, ultimate load, and stiffness) of the femur. Surprisingly, α2A/α2C-AR$^{-/-}$ mice showed to be resistant to most of these T3-induced negative effects. Interestingly, the mRNA expression of osteoprotegerin (OPG), a protein that limits osteoclast activity, was upregulated and downregulated by T3 in the bone of KO and WT animals, respectively. β1-AR mRNA expression and IGF-I serum levels, which exert bone anabolic effects, were increased by T3 treatment only in α2A/α2C-AR$^{-/-}$ mice. In vitro, T3 inhibited the cell growth of calvarias-derived osteoblasts isolated from WT mice, but this effect was abolished or reverted in cells isolated from KO mice. Altogether, these findings support the hypothesis of a TH-SNS interaction to control bone mass and structure of young adult mice and suggests that this interaction may involve α2-AR signaling. Mice with single inactivation of α2C-AR

(α2C-AR$^{-/-}$) also showed resistance to the detrimental effects of thyrotoxicosis on bone mass, architecture, and biomechanical properties in the femur and tibia, suggesting that thyrotoxicosis depends on α2C-AR signaling to promote bone loss, which sustains the hypothesis of a TH-SNS interaction to modulate bone remodeling and structure.

As previously mentioned, studies from our laboratory (FAPESP Project numbers: 2010/06409-0, 2010/04911-10 and 2010/50068-2) showed that adult mice with isolated α2A-AR or α2C-AR inactivation present shorter femurs and tibias. More importantly, these studies have shown that treatment for 30 or 90 days with supraphysiological doses of T3 impairs the linear growth of the femur and tibia of WT mice, but not of α2A-AR$^{-/-}$ or α2C-AR$^{-/-}$ mice. These findings, along with the fact that the expression of these receptors was detected in the EGP (46), strongly suggest an interaction of TH with the SNS via α2A-AR$^{-/-}$ and / or α2C-AR$^{-/-}$ to control longitudinal bone growth.

To confirm this hypothesis, we performed a detailed evaluation of the effects of TH deficiency and excess on the EGP of α2A-AR$^{-/-}$ and α2C-AR$^{-/-}$ mice (FAPESP project numbers 2013/02247-3 and 2012/11858-3). As expected, in WT mice, hypothyroidism (hypo) promoted significant reductions on bone longitudinal length, disorganization of the PZ and a reduced number of HC. Surprisingly, in α2C-AR$^{-/-}$ animals, the effects of hypo were quite different than those observed in WT animals. The euthyroid α2C-AR$^{-/-}$ mice presented a disorganized PZ and an increase in the number of HC. In α2C-AR$^{-/-}$ animals, hypo was able to revert, at least partially, the disorganization of the PZ, and resulted in an increase in the number of HC. Hyperthyroidism (hyper) led to an increase in the number of HC in WT animals and a reduction in α2C-AR$^{-/-}$ animals. The EGP responses of α2A-AR$^{-/-}$ mice to hypo and hyper status were also surprising. Hypo had no effect on the EGP of α2A-AR$^{-/-}$ animals, on the other hand, hyper led to an increase in the RZ thickness, which was not seen in WT animals. These findings demonstrate that the SNS regulates the EGP morphology and the longitudinal bone growth. They also show that the integrity of the SNS, especially of the α_2 adrenergic pathways, is necessary for TH to promote its normal actions in the growth plate.

It is important to consider, however, that the studies described above were conducted *in vivo* with global KO models of α_2 adrenoceptors (α2A/α2C-

$AR^{-/-}$, α2A-$AR^{-/-}$ and α2C-$AR^{-/-}$ mice), which provide important information, but, at the same time, present limitations. An important limitation of *in vivo* studies, using global KOs, is the difficulty of discriminating direct effects (local) or the role of certain genes. Therefore, the findings described above do not allow us to elucidate whether (i) the actions of the SNS are direct (skeletal); (ii) if α_2 adrenergic receptors mediate the SNS actions locally in the skeleton; (ii) if the TH-SNS interaction occurs locally in the skeleton; and finally, (iv) if α_2 adrenergic receptors mediate local interaction of TH with the SNS.

In vitro studies using bone organ cultures and primary cultures of chondrocytes, derived from bone epiphyses, are valuable tools to investigate these questions.

1.5 Clinical relevance of the SNS actions, via α_2 adrenergic Receptors, in the Skeleton.

Since the skeletal effects of α_2-AR activation are only now been identified, there is no information in the literature regarding positive or adverse effects of α_2-AR agonists in the skeleton. On the other hand, α_2-AR agonists have been in clinical use for decades, to manage a variety of therapeutic conditions (59, 79). These agonists have been primarily used in the treatment of hypertension, because of the well-known hypotensive effects of α_2-AR activation (bradycardia, hypotension, and baroreceptor sensitivity), however, their side effects, including sedation, drowsiness and dry mouth, have limited their prescription to special hypertension conditions (80, 81).

Other important applications occur particularly in the field of anesthesia and pain management, due to the capacity of α_2-AR agonists to enhance the effects of traditional anesthetics. Thus, these agonists have been used as sedatives and to reduce the need for inhalative anesthetics during surgical or invasive procedures (82). They have also been used to lower the sympathetic tone during cardiac surgery (83). Studies have shown that premedication with α_2-AR agonists prevents postoperative cardiovascular complications (84) and lowers mortality (85). On the other hand, limiting side effects for these indications are the profound bradycardic and hypotensive effects of α_2-AR activation. Another use of α_2-AR agonists is its intravenous administration after

induction of anesthesia to reduce the incidence, severity, and duration of postoperative shivering (86).

Clinical trials have tested the use of moxonidine, one of the α_2-AR agonists, to treat chronic heart failure. This drug was effective to lower plasma catecholamine in patients with chronic heart failure (87), however, it caused adverse effects and even increased the mortality in chronic heart failure. Some of these side effects are probably triggered by α2-heteroreceptor activity, which includes augmentation of vagal tonus, a crucial factor for the hypotensive and bradycardic responses, and for the increased baroreceptor sensitivity (88, 89). Considering these negative results (90), the use of α2-AR agonists for the treatment of cardiovascular diseases is very limited.

In the ophthalmic practice, α2-AR agonists are used to prevent intra-ocular hypertension during laser surgery of the eye and as a daily medication for the treatment of glaucoma. Of note, the chronical use of these agonists has been proved to be useful for the treatment of chronic pain, including neuropathic pain in cancer patients; and for the treatment of muscle spasticity, opiate and alcohol withdrawal, and for some behavior disorders, such as insomnia, Tourette syndrome, tics, and attention-deficit hyperactivity disorder (ADHD) (59).

It is noteworthy that most of these disorders may be present in childhood and adolescence, and that the chronic treatment of these disorders with α2-AR agonists seems to be increasing. ADHD, for example, is one of the most common behavior disorders in children (3-5 % of all children), that persist into adolescence and adulthood in most of the cases. ADHD is marked by a pattern of inattention, hyperactivity, and impulsivity, which may result in learning disabilities and poor school performance. The pharmacological treatment of ADHD normally uses central nervous system stimulants, such as d-amphetamine and methylphenidate. These drugs increase the ability to focus and decrease the hyperactivity in most patients. However, some individuals (10 to 30%) are unresponsive to these drugs or do not stand their side-effects. Alternative treatments for these patients include α2-AR agonists, such as clonidine (91). Many trials have supported the effectiveness of clonidine in the treatment of ADHD, as a monotherapy and specially as an adjuvant to stimulant-based therapy (92). Its combined use with methylphenidate has also

been shown to be effective to treat sleep disturbances, aggressive behavior and tics associated to ADHD (93, 94), allowing the doses of stimulant to be reduced. As expected, the safety of this therapy has focused on the known side effects of α2-AR activation, especially the cardiovascular effects.

Considering the emerging roles of α2-AR signaling to control bone mass and linear bone growth, skeletal side effects of chronic therapies with α2-AR agonists should be considered, especially during childhood and adolescence. Therefore, the elucidation of the roles and mechanisms of action of α2-AR signaling in the skeletal are extremely relevant at this point.

2 GOALS

2.1 General goals:

1. To investigate whether α_2 adrenoceptors have local (skeletal) actions in linear bone growth and bone development;
2. To investigate whether TH interacts with the SNS locally in the skeleton, via α_2 adrenoceptor signaling, to regulate linear bone growth and development.

2.1.1 Specific goals:

1. To investigate, in a bone organ culture system, if tibias derived from WT mice and mice with single inactivation of α2C-AR (α2C-AR$^{-/-}$) are responsive to UK14,304 (UK), a specific α_2 adrenoceptor agonist, regarding to:

1.1 The longitudinal bone growth;

1.2 The endochondral ossification;

1.3 The EGP morphology;

1.4 The expression of genes related to the differentiation of chondrocytes in the EGP.

2. To investigate if the effects of T3 in all parameters listed in Specific Goal 1 are modified by the local absence of α2C-AR (α2C-AR$^{-/-}$) or by the simultaneous treatment with UK.
3. To investigate, in primary cultures of chondrocytes derived from WT and α2C-AR$^{-/-}$ mice, the expression of TRs and α_2-ARs.

3 MATERIALS AND METHODS

3.1 Animal maintenance and manipulation

A cohort of mice with global gene inactivation of α2C adrenoceptor isoform (α2C-AR$^{-/-}$ in a C57BL/6J background) (71) and their wild-type (WT) controls were studied. The KO animals were provided by Dr. Patrícia C. Brum, Associate Professor of the School of Physical Education and Sport, University of São Paulo. The animals were kept under controlled conditions of light and temperature (12 hours light/dark cycle with constant ambient temperature of approximately 25 °C), with ad libitum access to food and water. The experimental procedures involving the animals were performed in accordance with the guidelines of the Ethics Committee on Animal Use (CEUA) of the Institute of Biomedical Sciences of the University of São Paulo which approved all the procedures of this study (protocol number 035/10 and 039/2016).

For breeding, two females and one male were placed in a cage for 12 hours. At the end of this period, the presence of a vaginal plug was considered as day zero of embryonic life (E0). The pregnant mice underwent euthanasia and the fetuses were obtained on E15 (15 days of embryonic life). For a postnatal study, newborns underwent euthanasia on the day of birth (P0).

3.1 Bone organ culture (Long bones)

Immediately after euthanasia, E15 or P0 tibias were dissected, using a stereomicroscope (Leica Microsystems S6E, Heerbrugg, Switzerland), in solution of Puck Salina A (PSA), composed of (KCL, NaCl, NaHCO3 and Glucose). Then, the bones were cultured in serum-free medium, composed of αMEM supplemented with 0.05 mg/ml ascorbic acid (Sigma-Aldrich, St. Louis, MO), 1mM β- glycerophosphate (Sigma-Aldrich), 0.2% BSA (Sigma-Aldrich), 100 U/ml penicillin and 100µg streptomycin (Gibco) as previously described (95). The tibias were individually cultured in 24-well plates containing 1ml of medium/well. Depending on the experiment, the bones were cultured for six or twelve days and the medium was changed every other day. Cultures were

treated with 10^{-8}M T3 (Sigma-Aldrich) and/or with 10^{-5}M UK (UK 14,304; Sigma-Aldrich), an α2-AR agonist. Treatment with T3, UK or a combination of both drugs started on the first day of culture (day 1).

3.2 Longitudinal bone growth

Cultured tibias were photographed, using the tablet system BLC-250 Digital Camera CMOS 2.5 (Bestcope International Limited, Beijing, China), coupled to a steriomicroscope (Leica Microsystems S6E). Bone length was measured using the Image-Pro Plus software (Media Cybernetics, Silver Spring, MD, USA). Accordingly, to Fig. 1A, the total length (TL) was determined by measuring the distance between the ends of the two epiphyses; Bone lengths were measured on day 1 (baseline), just before adding T3 and/or UK treatment, and on the last day of treatment (final measurement).

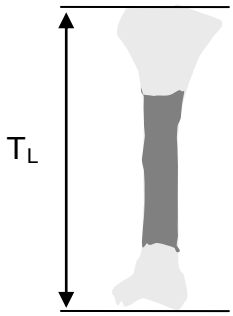

Figure 3 – Longitudinal Bone Growth Measurements. T_L, total length.

3.3 Epiphyseal growth plate histology and morphology.

The tibias were fixed in a solution of 4% paraformaldehyde at room temperature for 24 hours. Subsequently, samples were dehydrated in a battery of ascending alcohols (10 minutes in each concentration of 70%, 80% and 95% for 10 minutes, and 3 times for 10 minutes, in 100%,). The samples were then diaphanized in xylene (3 times for 10 minutes) and soaked in Paraffin (Merck, Darmstadt, Germany) at 60 °C (3 times for 30 minutes). Longitudinal cuts of 5 µm thickness were prepared and stained with Safranin O (64). The sections were photographed using a BLC-250 DigitalCameral 2.5 CMOS (Bestcope International Limited, Beijing, China), coupled to an Eclipse E-600 microscope (Nikon, Dusseldorf, Germany), and the morphometry of the proximal ephiphysis of the tibia was performed using the Image- Cybernetics (Silver Spring, MD,

USA) software. The thickness of the following regions was determined: epiphysial zone or reserve zone (RZ), characterized by the presence of reserve chondrocytes (RC), which are small round chondrocytes, dispersed in the cartilaginous matrix; proliferative zone (PZ), characterized by the presence of proliferanting chondrocytes (PC), which are flat cells, distributed in columns parallel to the longitudinal axis of the bone; and hypertrophic zone (HZ), characterized by the presence of hypertrophyc chondrocytes (HC), defined by a diameter greater than or equal to 9 μm, which form another columnar layer (zone), adjacent to the PZ and to the calcified region of the bone (primary ossification center), as shown in Fig. 2.

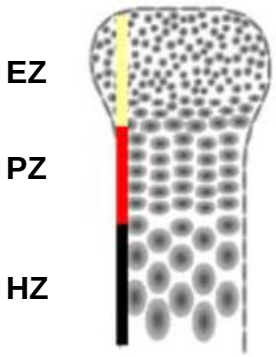

Figure 4 – Scheme of epiphysis zone that was measured. EZ, epiphysis zone; PZ, proliferating zone; HZ, hypertrophic zone (modified from Leijten et al, 2012 (65)).

3.4 Primary culture of epiphyseal chondrocytes

Tibias, femurs, radios and ulnas were dissected from E15 WT or KO embryos in culture medium (α-MEM, 1% BSA, 0.1% L-glutamine, and 0.1% penicillin/streptomycin), pooled together and left overnight in a tissue culture incubator. On the following day, medium was removed, bones were washed off in PBS and then treated with trypsin-EDTA for 15 min at 37 °C. Then, bones were digested for 2 hour in 2 mg/ml collagenase P (Roche Applied Science) in Dulbecco's modified Eagle's medium (DMEM - Invitrogen) with 10% fetal bovine serum (FBS) at 37 °C. The reaction was inactivated by the addition of primary cell culture medium (60% F-12, 40% DMEM, 10% FBS, 0.1% penicillin/streptomycin and 0.1% L-glutamine). Cells were centrifuged for 5 min at 1000 rpm, at room temperature, and pellets were re-suspended in cell culture medium, counted and plated at a density of 1.10^4 cells/well in a 24-well dish.

After 24 h in culture, medium was replaced and treated with 10^{-8} M T3 (Sigma Chemical Co).

3.5 Real time PCR

The distal and proximal epiphysis of both tibias of each animal were dissected using a stereomicroscope (Leica Microsystems S6E, Heerbrugg, Switzerland), pooled in microtubes, and immediately frozen. Total RNA was extracted using TRIZOL reagent (Invitrogen), following the manufacturer protocol, and later treated with DNAse I (Fermentas, Hanover, MD, USA). Total RNA from primary cultures of chondrocytes was also extracted using Trizol. All Ct values were normalized using GAPDH as an internal control (). Relative gene expression quantification was assessed by the DDCt method (Livak, 1997). The final values are reported as fold induction relative to the expression of the control, with the mean control value being arbitrarily set to 1.

Table – Primers sequence for Real Time PCR

Runx2/Cbfa1	F: 5'-TTTAGGGCGCATTCCTCATC-3' R: 5'-GAGGGCCGTGGGTTCT-3'
Col10a1	F: 5'-GATCATGGAGCTCACGGAAAA-3' R: 5'-CCGTTCGATTCCGCATTG-3'
IGF-1	F: 5'-GCTATGGCTCCAGCATTCG-3' R: 5'-AGATCACAGCTCCGGAAGCA-3'
Sox9	F:5'-GGAGCTCGAAACTGACTGGAA-3' R:5'-GAGGCGAATTGGAGAGGAGGA-3'
Wnt4	F: 5'- AACCGGCGCTGGAACTG-3' R: 5'-GGTCCCTTGTGTCACCACCTT-3'
TRα	F: 5'- GTGACTGACCTCCGCATGAT-3' R: 5'-ATCCTCAAAGACCTCCAGGAA-3'
TRβ	F: 5'- GCAGACTTCCCCACACCTT-3' R: 5'-ACAGGTGATGCAGCGATAGT-3'
α2A-AR	F: 5'- CGCAGGCCATCGAGTACAA-3' R: 5'-GATGACCCACACGGTGACAA-3'
α2B-AR	F: 5'- TCCCTCTGGGAGGCAAGTG-3' R: 5'-GGCCAGGATTCCAGACCATT-3'
α2C-AR	F: 5'-CATGGGCGTGTTCGTACTGT-3' R: 5'-CAGGCCTCACGGCAGATG-3'
18s	F: 5'-AGTCCCTGCCCTTTGTACACA-3'

	R: 5’-GATCCGAGGGCCTCACTAAAC-3’

F = forward e R = reverse

3.6 Statistical analysis

All data collected were from at least three independent trials. Data were expressed as mean ± SD. Statistical analysis was performed using two-way analysis of variance (ANOVA), followed by the Tukey's post-hoc test, using the GraphPad Prism 6.0 (GraphPad software, San Diego, CA). For all tests, $p<0.05$ was considered statiscally significant.

4 RESULTS

4.1 Bone growth

To evaluate linear bone growth in organ culture, E15 WT and KO tibias were treated with 10^{-8} M T3, 10^{-5} M UK (an α_2-AR agonist), or with T3 combined with UK for 6 days (Fig. 5A). As we can see, the linear growth rate is 16% lower in KO tibias when compared with WT tibias, which points out the relevance of skeletal (local) α2C adrenoceptor signaling in the modulation of linear bone growth. As expected, T3 significantly reduced the linear bone growth in WT tibias, but not in KO bones, showing that the lack of α2C-AR isoform in the skeleton alters the skeletal response to T3, which supports a local interaction between TH and the α_2-AR signaling to control bone growth. The activation of α_2-ARs with UK had no growth effect in WT tibias, but increased linear bone growth in KO bones, showing that the net result of the mutual activation of α2A-AR and α_B-AR isoforms is linear bone growth, reinforcing a local action of α_2-ARs to modulate this process. Interestingly, when T3 and UK treatments were combined, UK disturbed the negative effect of T3 in the linear bone growth of the WT tibias. On the other hand, T3 decreased the positive effect of UK in the linear growth of the KO tibias (in 12%, $p<0.01$). We also evaluated bone growth in neonatal tibias derived from WT and KO mice treated with the same doses of T3 for 12 days. In this postnatal model, α2C-AR$^{-/-}$ tibias also showed a decreased rate of linear growth when compared with WT tibias, and the

treatment with T3 again decreased growth in the WT tibias, but not in the KO tibias.

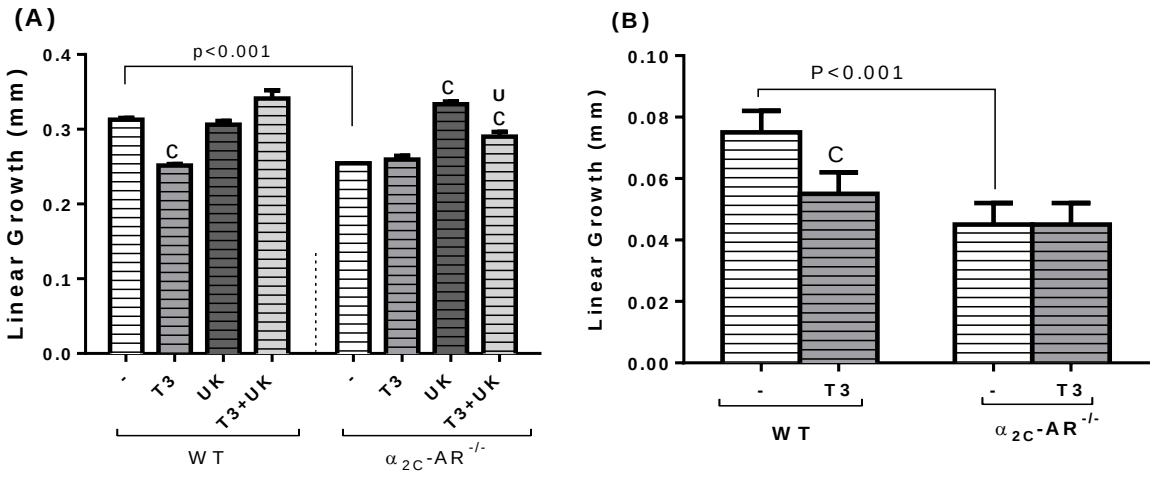

Figure 5 - Linear Bone Growth of Tibia. E15 and P0 tibias from WT (B6) and α2C-AR$^{-/-}$ mice were maintained in organ culture for 6 or 12 days. The tibias were treated with 10^{-8} M T3, 10^{-6} M UK and with T3+UK. (A) Growth of E15 tibias for 6 days. (B) Growth of P0 tibias for 12 days. Significance between the groups was determined by 2-way ANOVA, followed by Turkey's test. Values are expressed by means ± SD (n=6/group). C = p<0.001 vs Controls; U =. p<0.01 vs UK-treated.

4.2 Histology

The morphometry of the proximal epiphysis of the tibia (Fig. 6) showed that α2C-AR$^{-/-}$ samples presented a significant increase in the thickness of the main epiphyseal zones, RZ, PZ and HZ (14%, 26% and 57%, respectively), which was more evident in the HZ (Fig. 6C), suggesting that α2C-AR signaling has a general inhibitory effect in epiphyseal chondrocytes, but mainly in HC differentiation. We also observed that treatment with UK decreased the thickness of all epiphyseal zones analyzed in both WT and KO samples, except for the RZ of WT epiphyses (not affect by UK treatment). These findings suggest that not only α2C adrenoceptor subtype but also α2A and/or α2B subtypes have/has inhibitory effects in epiphyseal chondrocytes. Treatment with T3 decreased the thickness of RZ (20%) and PZ (26%) in both WT and KO epiphyses (Fig. 6A-B). As expected, T3 increased the thickness of the HZ in WT epiphyses (34%), but, in contrast, decreased HZ thickness (27%) in KO epiphyses (Fig. 6C), suggesting that TH interacts with the α2C-AR signaling to promote HC differentiation. It is noteworthy that UK blocked the positive effect of T3 in the HC thickness, which reinforces the idea of an interaction between

TH and α2-AR signaling to control HC differentiation. We next analyzed the ratio between the proliferative and hypertrophic zones (PZ/HZ), which reflects the differentiation of PC into HC (the lower the ratio, the higher the differentiation). Fig 6D shows that α2C-AR$^{-/-}$ epiphyses present a decreased PZ/HZ (51%), which indicates an increase in chondrocyte terminal differentiation, supporting the idea that α2C-AR limits HC differentiation. As expected, T3 treatment decreased the ratio in WT epiphyses (47%), but increased it in KO epiphyses (77%, vs untreated KO), which shows again an important interaction between TH and α2C-AR signaling to control HC differentiation. UK treatment increased the PZ/HZ ration both in WT and KO epiphyses (15 and 112%, respectively), and blocked the T3 effect on the PZ/HZ ratio in WT but not in KO epiphyses, pointing out α2C-AR as the main α2C adrenoceptor subtype to mediate TH actions in HC differentiation.

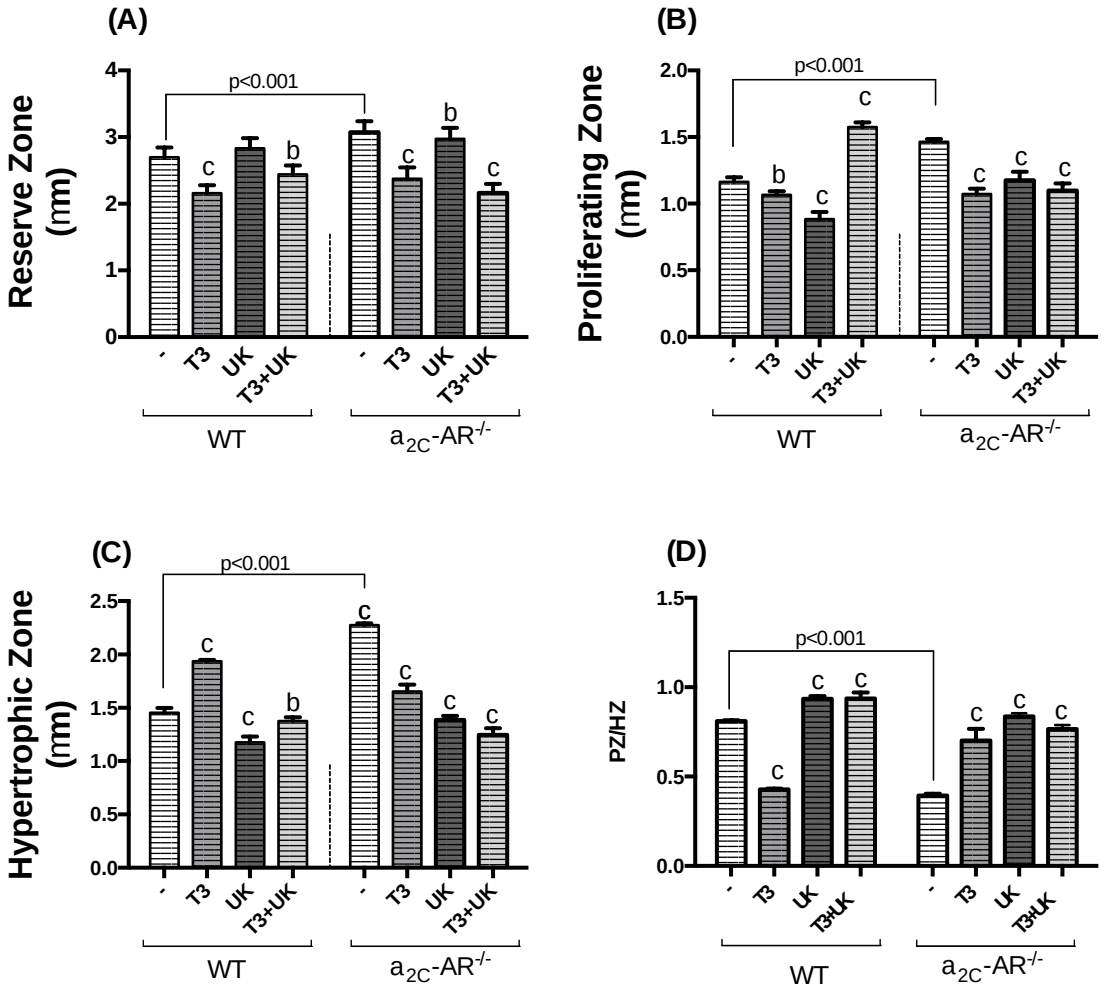

Figure 6 - **Morphometric analysis of the Proximal Epiphysis of the Tibia from E15.5 mice**. Tibias were maintained in Organ Culture and treated with 10^{-8}M T3, and 10^{-5} M UK. (**A) Reserve zone (RZ) (B) Proliferating zone (PZ)**. **(C) Hypertrophic zone (HZ)**. **(D) Ratio**

between the proliferative and hypertrophic zones (PZ/HZ). Significance between the groups was determined by 2-way ANOVA followed by Turkey's test. Values are expressed by means ± SD (n=6/group). b=p<0.01 and c=p<0. 001. vs Control.

4.3 Gene expression in epiphyseal chondrocytes

Considering that one of the most prominent actions of TH on the growth plate is the induction of chondrocyte terminal differentiation, we evaluated the gene expression of Collagen Type X (Col X), a HC marker. As shown in Fig. 7, KO epiphyses had a 3.2-fold increase in Col X expression, suggesting that α2C-AR signaling has an overall negative effect in terminal differentiation of chondrocytes. As expected, T3 treatment increased Col X gene expression (3.6-fold) in WT epiphyses, but, surprisingly, it decreased Col X expression (2.4-fold) in KO epiphyses, showing that the lack of α2C-AR modifies the response of chondrocytes to T3, which also indicates a local interaction between these two systems to control the chondrocytes terminal differentiation. We found that UK alone promoted a very slight but significant increase in Col X mRNA expression (10%) in WT epiphyses, while decreased (20%) it in KO epiphyses, suggesting that the different α_2 adrenoceptor subtypes may have antagonistic actions to control HC differentiation. The combined treatment of UK with T3 showed that UK decreases the effect of T3 in the Col X gene expression in WT epiphyses, but not in KO epiphyses, suggesting that α2C-AR signaling is important for T3 actions in the skeleton.

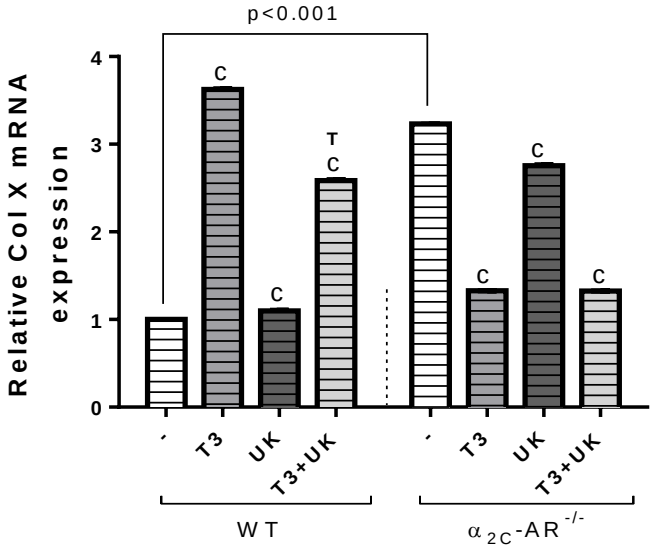

Figure 7 – Col X mRNA expression in the epiphyses of the tibia. Tibias derived from E15 WT (B6) and α2C-AR$^{-/-}$ mice were maintained in organ culture for 6 days. The tibias were

treated with 10^{-8} M T3, 10^{-6} M UK (an α_2-AR agonist), and with T3+UK. Significance between the groups was determined by 2-way ANOVA followed by Turkey's test. Values are expressed by means ± SD (n=6/group). C = $p<0.001$ vs the respective controls (untreated WT or KO); T = $p<0.05$ vs. T3-treated.

To investigate possible pathways that may be involved in the interaction of TH with the SNS to regulate the linear bone growth, we analyzed the expression of genes involved in the signaling pathways of chondrogenesis and chondrocyte differentiation.

Initially, we evaluated the expression of Sox9, a chondrogenic factor, whose expression should be decreased to allow the terminal differentiation of chondrocytes (96). We found an increase of 27% in Sox9 gene expression in α2C-AR$^{-/-}$ epiphyses, suggesting that α2C adrenoceptor has a negative effect in Sox9 expression and in chondrogenesis. As expected, T3 decreased Sox9 mRNA expression in WT and KO epiphyses (35% and 36%, respectively). The activation of α_2 adrenoceptors with UK decreased Sox9 mRNA expression as much as T3 (35%) in WT epiphyses, but increased it (42%) in KO epiphyses, which supports the idea that α2C-AR subtype activation decreases Sox9 expression, and that α2A-AR and/or α2B-AR subtypes have opposite effects in the regulation of Sox9 expression and chondrogenesis. The combination of T3 with UK further decreased Sox9 mRNA expression in WT epiphyses (70% vs untreated epiphyses), but it did not modify the effect of T3 alone in the KO epiphyses.

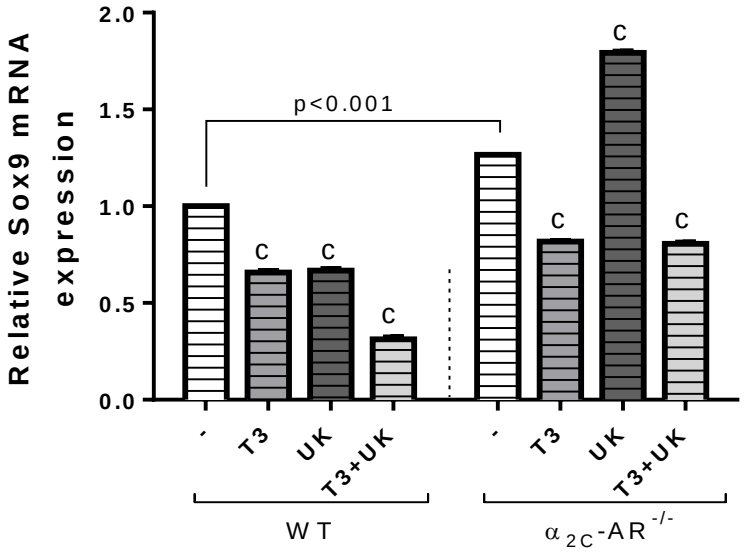

Figure 8 – Sox9 mRNA expression in the epiphyses of the tibia. Tibias derived from E15 WT (B6) and α2C-AR$^{-/-}$ mice were maintained in organ culture for 6 days. The tibias were treated with 10^{-8} M T3, 10^{-6} M UK (an α_2-AR agonist), and with T3+UK. Significance between the groups was determined by 2-way ANOVA followed by Turkey's test. Values are expressed by means ± SD (n=6/group). C = p<0.001 vs the respective controls (untreated WT or KO).

Considering that T3 has important interactions with the GH/IGF-1 signaling pathway, we also evaluate the mRNA expression of IGF-1. The KO epiphyses presented an increase in IGF-1 gene expression (60%), showing that α2C adrenoceptor signaling has an inhibitory effect on IGF-1 mRNA expression. Treatment with T3, as expected, increased IGF-1 mRNA expression in WT and KO epiphyses (147% and 41%, respectively), however this effect was more important in the WT epiphyses, suggesting that the α2C adrenoceptor signaling is important for the T3 effect in IGF-1 mRNA expression. UK treatment decreased gene expression of IGF-1 in WT and KO epiphyses (37% and 45%, respectively), suggesting that α2A-AR and/or α2B-AR also mediate(s) a negative effect on IGF-1 mRNA expression. Finally, UK did not modify the effect of T3 in IGF-1 mRNA expression both in WT and KO epiphysis.

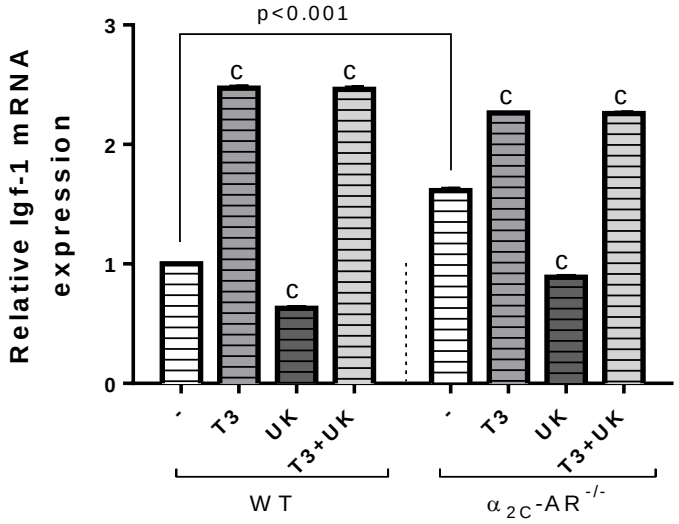

Figure 9 – IGF-1 mRNA expression in the epiphyses of the tibia. Tibias derived from E15 WT (B6) and α2C-AR$^{-/-}$ mice were maintained in organ culture for 6 days. The tibias were treated with 10^{-8} M T3, 10^{-6} M UK (an α_2-AR agonist), and with T3+UK. Significance between the groups was determined by 2-way ANOVA followed by Turkey's test. Values are expressed by means ± SD (n=6/group). C = p<0.001 vs the respective controls (untreated WT or KO).

Next, we analyzed the mRNA expression of Wnt-4, a factor involved in the regulation of chondrocyte terminal differentiation, which signaling pathway has been shown to be modulated by TH (38). The α2C-AR$^{-/-}$ epiphyses presented a 70% increase in the mRNA expression of Wnt-4, when compared with WT epiphysis (Fig. 10A), showing that α2C-AR has a negative effect in Wnt-4 expression. Corroborating previous studies, T3 treatment promoted a 3.9-fold increase in the mRNA expression of Wnt-4 in WT epiphyses, but decreased Wnt-4 mRNA expression in KO epiphyses by 30%, suggesting that α2C-AR adrenoceptor signaling is important for the positive effect of T3 in Wnt-4 mRNA expression. Treatment with UK alone showed that the activation of α_2-AR signaling decreases Wnt-4 expression both in WT and KO epiphyses (32% and 34%, respectively). Interestingly, in WT epiphyses, activation of α_2-AR signaling by UK treatment blocked the positive effect of T3 (T3+UK) on Wnt-4 expression; in addition, it decreased the mRNA expression of Wnt-4 by 77%, when compared with control epiphyses, an effect also observed in the KO epiphyses, where T3+UK resulted in a 72% reduction in Wnt-4 expression when compared with untreated KO epiphyses. Altogether, these findings suggest that the TH interaction with the α_2-AR signaling pathway to control chondrocyte terminal differentiation involves the Wnt-4 signaling pathway.

We next analyzed the mRNA expression of Runx2, an important transcription factor, positively regulated by the Wnt-4 signaling pathway, that drives the expression of genes related to the chondrocyte terminal differentiation. As shown in Fig. 10B, KO epiphyses presented a 2.3-fold increase in the mRNA expression of Runx2, showing again an inhibitory role of α2C-AR signaling in chondrocytes differentiation. Treatment with T3 slightly increased the mRNA expression of Runx2 (5%) in WT epiphyses, but decreased it (5%) in KO epiphyses, showing that α2C adrenoceptor signaling is important to control the T3 actions in chondrocyte terminal differentiation. Treatment with UK alone decreased Runx2 mRNA expression in WT mice (14%), but increased it in KO mice (13%), suggesting that α2C-AR is the main α_2 isoform to inhibit Runx2 expression. The combination of UK with T3 treatment significantly decreased Runx2 mRNA expression in WT and KO epiphyses, by 50% and 64%, respectively.

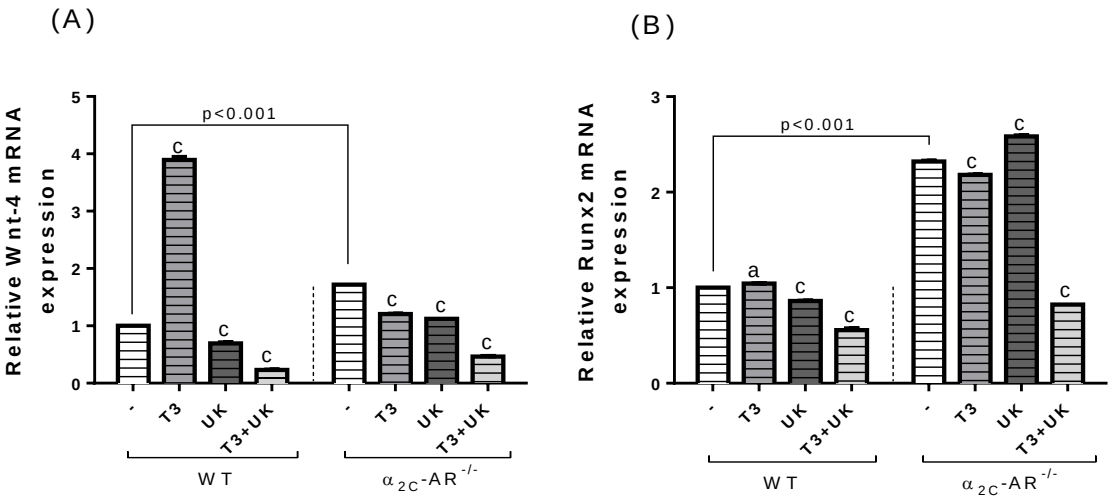

Figure 10 –Wnt-4 and Runx2 mRNA expression in the epiphyses of the tibia. Tibias derived from E15 WT (B6) and α2C-AR$^{-/-}$ mice were maintained in organ culture for 6 days. The tibias were treated with 10^{-8} M T3, 10^{-6} M UK (an α_2-AR agonist), or with T3+UK. **(A)** Gene expression of Wnt-4. **(B)** Gene expression of Runx2. Significance between the groups was determined by 2-way ANOVA followed by Turkey's test. Values are expressed by means ± SD (n=6/group). a= 0.05 and C = p<0.001 vs the respective controls (untreated WT or KO).

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We also analyzed the gene expression of all α2-AR subtypes in the tibial epiphyses. We found that the gene inactivation of α2C-AR lead to a compensatory increase in the mRNA expression of both α2A-AR (1.8-fold) and α2B-AR (2.3-fold) (Fig. 11A-B). Interestingly, T3 treatment increased α2A-AR mRNA expression (Fig 11A) both $_{in}$ WT and KO epiphyses (1.9- and 1.4-fold,

respectively). On the other hand, T3 decreased α2C-AR mRNA expression in 36% in WT epiphyses (Fig. 11C). Treatment with T3 had no effect in the mRNA expression of α2B-AR in WT epiphyses, but decreased it by 20% in KO epiphyses (Fig. 11B).

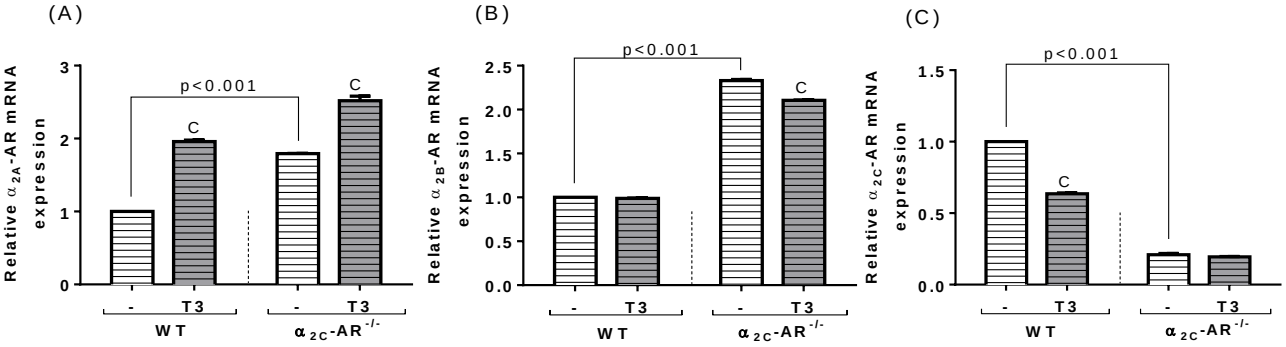

Figure 11 – Gene expression of α2-AR isoforms in the epiphyses of the tibia. Tibias derived from E15 WT (B6) and α2C-AR$^{-/-}$ mice were maintained in organ culture for 6 days. The tibias were treated with 10^{-8} M T3. **(A)** Gene expression of α2A-AR. (B) Gene expression of α2B-AR. **(C)** Gene expression of α2C-AR. Significance between the groups was determined by 2-way ANOVA followed by Turkey's test. Values are expressed by means ± SD (n=6/group). C = p<0.001 vs the respective controls (untreated WT or KO).

To better check the effect of TH on the expression of α2-AR subtypes, we analyzed their time-course expression in primary cultures of epiphyseal chondrocytes treated with T3. Corroborating the organ culture results, T3 treatment significantly increased α2A-AR mRNA expression in WT and α2C-AR$^{-/-}$ chondrocytes, in a range of 10 to 72% and 23 to 74%, respectively. This positive effect increased as the time of treatment increased, and was slightly more intense in the KO cells (Fig. 12A and D). Once more corroborating the organ culture results, T3 significantly decreased the mRNA expression of α2C-AR subtype in WT cells, in a range of 51 to 70% (Fig. 12C). In these primary cultures, T3 showed to also have a negative effect on α2B-AR mRNA expression in WT cells, which was not observed in the organ cultures, where a negative effect had only been observed in the KO epiphyses (Fig. 11B). T3 significantly decreased α2B-AR mRNA expression after 24 and 72 hours, by 91 % and 63%, respectively. A less intense, but significate negative effect of T3 on α2B-AR mRNA expression was observed in the KO cells, at 24 h and 48 h (14% and 17%, respectively). These findings show that TH modulate α_2-AR

expression in chondrocytes and suggest that a local interaction between TH and the α_2 adrenoceptor system occur at the at receptor level.

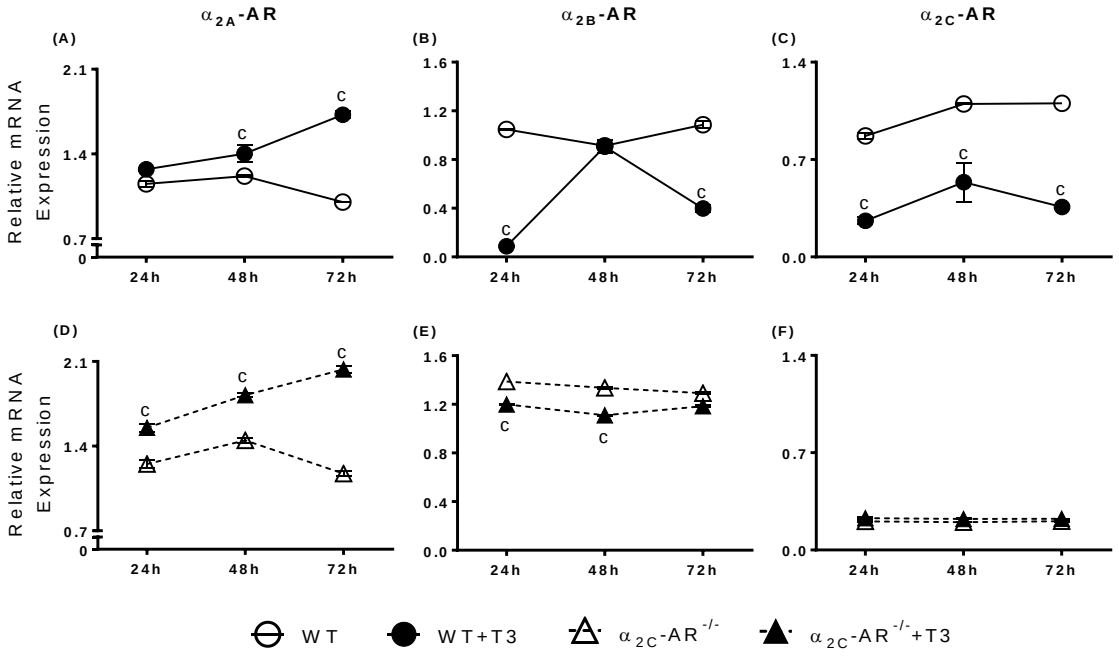

Figure 12 – Effect of TH on gene expression of α2-AR isoforms in primary cultures of epiphyseal chondrocytes. Primary cultures of chondrocytes derived from E15 WT (B6) and α2C-AR$^{-/-}$ tibial epiphyses were treated with 10^{-8} M T3 for 24 hours, 48 hours and 72 hours. **(A-C)** Primary cultures of WT chondrocytes. (D-F) Primary cultures of α2C-AR$^{-/-}$. (A and D) Gene expression of α2A-AR. **(B and E)** Gene expression of α2B-AR. **(C and F)** Gene expression of α2C-AR. Significance between the groups was determined by 2-way ANOVA followed by Turkey's test. Values are expressed by means ± SD (n=6/group). C = p<0.001 vs untreated cells.

Finally, we evaluated the expression of thyroid receptors (TRs) in α2C-AR$^{-/-}$ epiphyses and the response of TRα and TRβ mRNA expressions to the activation of α_2-AR signaling, by UK treatment. We found that the KO epiphyses presented a decrease of 54% in the mRNA expression of TRα (Fig. 13A), suggesting that α2C adrenoceptor subtype has a stimulatory effect on TRα expression. Treatment with T3 promoted an 82% increase in gene expression of TRα in WT epiphyses, but promoted a much lower increase in TRα expression (11%) in KO epiphyses. The α2-AR activation by UK treatment (Fig 13A) increased gene expression of TRα in both WT and KO epiphyses, however this effect was higher in WT epiphyses (84% in WT vs 55% in KO). Interestingly, the combination of T3 with UK, increased the expression of TRα mRNA in WT epiphyses by 88%, but promoted a striking decrease of 81% in

the expression of TRα gene in KO epiphyses, which supports the hypothesis that α2C-AR may have a role in the modulation of TRα expression. In contrast to TRα, the inactivation of α2C-AR gene (α2C-AR$^{-/-}$ epiphyses) resulted in a significant increase in TRβ mRNA expression (74%) (Fig 9B); in addition, UK treatment decreased TRβ expression in WT and KO epiphyses (by 76% and 84%, respectively), suggesting that α2 adrenoceptor signaling downregulates TRβ mRNA expression. Accordingly, to previous studies (20), treatment with T3 increased TRβ mRNA expression, which was observed both in WT and KO epiphyses (99% and 34%, respectively). Interestingly, α2-AR activation by UK, blocked the positive effect of T3 (T3+UK) on TR mRNA expression in WT and KO epiphyses. In addition, T3+UK decreased the gene expression of TRβ in both WT and KO epiphyses (60% and 84%, respectively).

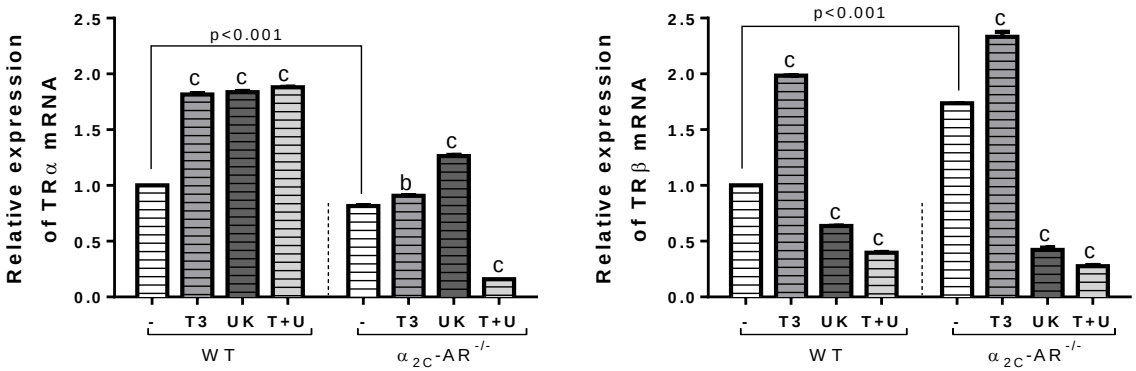

Figure 13 – Gene expression of Thyroid Hormone Receptor (TR) isoforms in the epiphyses of the tibia. Tibias derived from E15 WT (B6) and α_{2C}-AR$^{-/-}$ mice were maintained in organ culture for 6 days. The tibias were treated with 10^{-8} M T3. **(A)** Gene expression of TRα. (B) Gene expression of TRβ. Significance between the groups was determined by 2-way ANOVA followed by Turkey's test. Values are expressed by means ± SD (n=6/group). b= 0.01 and c = p<0.001 vs the respective controls (untreated WT or KO).

5 DISCUSSION AND CONCLUSION

There is increasing evidence that the SNS controls cartilage homeostasis (97), besides controlling bone remodeling (98). The presence of all known isoforms of α2 adrenoceptors in the growth plate of mice raised the hypothesis that α2-AR activation could control bone development, in addition to a role in bone remodeling (46). This hypothesis was supported by the evaluation of mice with isolated gene inactivation of α2A or α2C adrenoceptors (α2A-AR$^{-/-}$ and

α2C-AR$^{-/-}$ mice, respectively (108, 109) These animals present shorter femurs and tibias, and important alterations in their growth plates. In both mouse models, the growth plates present important columnar disorganization. A detailed evaluation showed that there is a significant decrease in the number of HC in the growth plates of α2A-AR$^{-/-}$ mice, and a striking increase in the number of HCs in the growth plates of α2C-AR$^{-/-}$ mice, suggesting that α2A-AR favors chondrocyte terminal differentiation, whereas α2C-AR would have an antagonistic effect, inhibiting HC differentiation. Altogether, these findings further support a role of α_2-AR signaling in skeletal development.

Subsequent in vivo studies showed that linear bone growth and the growth plate morphology were differently affected by TH deficiency and excess in α2A-AR$^{-/-}$ or α2C-AR$^{-/-}$.mice, when compared to WT mice (108, 109). Linear bone growth was somehow resistant to the deleterious effects of hypothyroidism (HYPO) and hyperthyroidism (HYPER) in these animals. Besides, whereas HYPO significantly decreased the number of growth plate HCs in WT mice, it had no effect in the number of HCs in α2A-AR$^{-/-}$ mice (Silva, 2016), and promoted a lower decrease of HCs in the growth plates of α2C-AR$^{-/-}$ mice (109). These findings show that TH interacts with the SNS, via α_2-AR signaling, to regulate EGP morphology and linear bone growth.

In the present study, our goal was to investigate if α_2-AR signaling acts locally in the skeleton to control bone development, and to investigate if there is a local (skeletal) interaction of TH with the α_2-AR signaling pathways to control the morphology of the EGP and the linear bone growth. To address these issues, we established a bone organ culture system, in order to isolate the bone elongation machinery (EGP) in vitro, in addition to separate local factors from systemic factors (hormones, toxins, etc.).

For more than 50 years, organ culture systems have been used to study the biology of bone and cartilage (99). In the recent years, this technique has been widely used primarily to study bone growth and development (100), because it is an experimental model that provides, in certain aspects, a condition closer to the in vivo model. An important advantage of this technique is the maintenance of the morphological and spatial organization of tissues and cells, and, thus, the natural tissue-tissue, cell-cell, and cell-MEC interactions are maintained, which allows cellular and tissue responses, to a particular stimulus,

to occur in a way that more closely mimics the in vivo responses. Organ cultures, therefore, contain heterogeneous cellular populations coexisting in their virtually natural milieu. Thus, in bone organ culture systems, the different types of cells, present in the bone, cartilaginous, and bone marrow tissues, can interact (autocrine and paracrine interactions) similarly to what occurs in vivo. Moreover, since bone and cartilaginous tissues have a predominance of MEC relative to the number of cells, the organ culture systems also allow the study of cell-MEC interactions. However, like most of the techniques, bone organ cultures present limitations. One is the lack of blood supply, which limits the angiogenesis, a crucial process for skeletal development. Moreover, these cultures cannot be maintained for long periods of time (more than one month), since bone begins to deteriorate, presenting cellular necrosis and tissue dissolution (100). Nevertheless, this method presents more advantages than disadvantages.

We, therefore, isolated tibias from E15 (15-day-old embryos) WT and α2C-AR$^{-/-}$ mice, and maintained them in organ culture for 6 days. The tibias were treated with T3, with the nonselective α2 adrenoceptor agonist UK 14.304 (UK), or with the combination of both agents (T3+UK). Corroborating the in vivo studies (Miranda-Rodrigues, 2015), KO tibias presented a 16% lower linear growth, when compared with WT samples, which strongly support a local role of α2C-AR signaling to modulate bone elongation. In addition, α2A and/or α2B adrenoceptors seem to have positive effects on linear growth, since the activation of α_2 adrenoceptor signaling by UK increased bone elongation in α2C-AR$^{-/-}$ tibias, which reinforces the relevance of skeletal α_2-AR signaling to modulate bone development.

To evaluate a possible local interaction of TH with the α_2-AR signaling to control bone growth, the tibia organ cultures were treated with a supraphysiological dose of T3 (10^{-8} M), to guarantee TR saturation and skeletal responses. As predicted, the high dose of T3 significantly reduced bone elongation in the WT tibias, but not in the α2C-AR$^{-/-}$ tibias, which shows that α2C-AR signaling is important for the normal effects of T3 on bone elongation, and supports a local interaction between TH and the α_2-AR signaling to control this process. It is noteworthy that UK combined with T3 (T3+UK) blocked the

negative effect of T3 on bone elongation in WT tibias, which further sustain a skeletal interaction between TH and the α_2-AR signaling to control the linear bone growth.

Interestingly, the thickness of the RZ, PZ and HZ of the proximal epiphyses of the tibia were increased in the KO epiphyses, when compared with the WT epiphyses, which once again corroborates the in vivo results (109). These findings suggest that α2C-AR signaling has an inhibitory effect in the proliferation of the reserve and proliferating chondrocytes, in addition to an inhibitory effect in chondrocyte terminal differentiation (Fig. I). In vivo, the highest increase in thickness was observed in the RZ (60% vs. WT) (109), whereas, in vitro, the highest increase was observed in the HZ (57% vs. WT), suggesting that local α2C-AR signaling preferentially inhibits HC differentiation. The fact that UK decreased the thickness of all epiphyseal zones in both WT and KO samples (except the RZ of WT epiphyses) suggests that α2A and/or α2B signaling also have/has local inhibitory effects in the proliferation and differentiation of epiphyseal chondrocytes. T3 treatment decreased the thickness of the reserve and proliferative zones in both WT and KO epiphyses, and increased the HZ in WT epiphyses, which corroborates findings in primary cultures of chondrocytes derived from rat tibia, where T3 decreased the proliferation of PCs and increased the differentiation of HCs (31). Surprisingly, T3 decreased HC differentiation (determined by a decrease in HZ thickness and an increase in the PZ/HZ ratio) in the KO epiphysis, showing that T3 promotes HC differentiation in an α2C-AR-dependent way, reinforcing a local interaction between TH and the α2-AR signaling pathway. Further supporting this interaction, UK blocked the positive effect of T3 (T3+UK) in HC differentiation in WT samples (Fig. I).

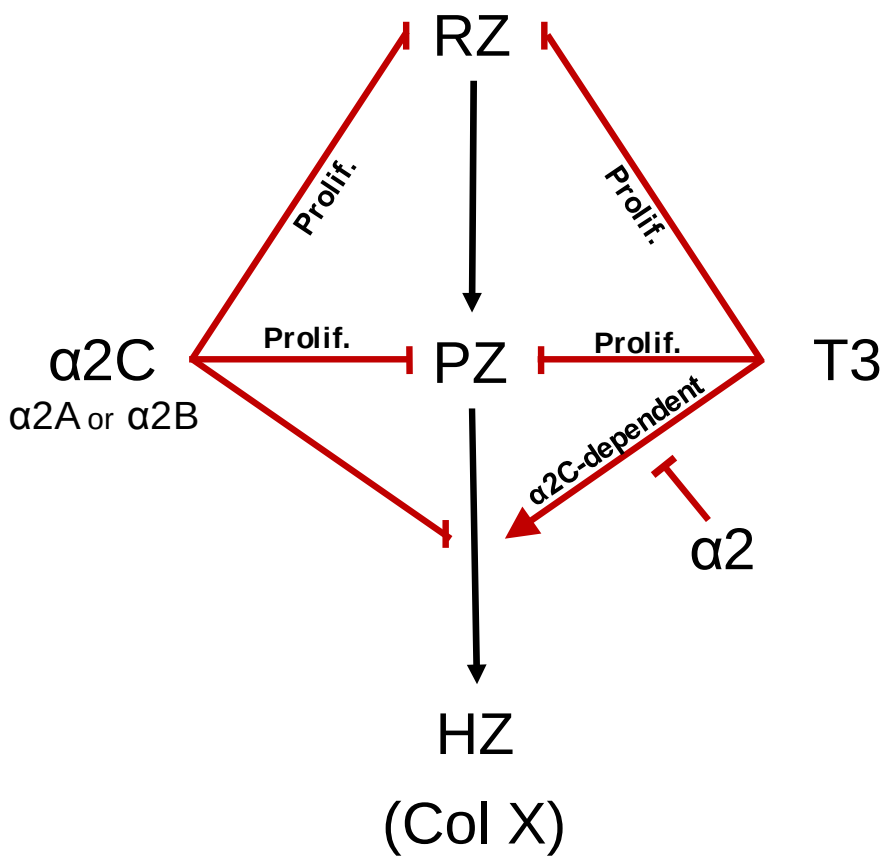

Figure 14 - Squematic representation of α2 adrenoceptor (α2A, α2B or α2C) actions in the chondrocyte zones of tibial epiphyses. Reserve, proliferative and hypertrophic zones (RZ, PZ, and HZ, respectively).

The gene expression of Col X, a marker of HCs, somewhat matches the morphological findings regarding chondrocyte terminal differentiation. The lack of α2C adrenoceptor signaling (α2C-AR$^{-/-}$) resulted in a significant increase in Col X expression (3.2-fold); T3 treatment, as anticipated, increased Col X mRNA expression (3.6-fold) in WT epiphyses, but decreased it (2.4-fold) in KO epiphyses; while UK attenuated the T3-induced effect in Col X expression in WT samples, revealing once more that local α2-AR signaling is important for local actions of T3 in the skeleton, especially those that favor chondrocyte terminal differentiation.

To investigate possible pathways that may be involved in the local interaction of TH with the α2 adrenoceptor signaling pathways, to control growth plate morphology and function, we analyzed the expression of genes related to signaling pathways that were previously shown to be associated with the actions of TH in growth plate chondrocytes. Initially, we evaluated the expression of SRY-related high-mobility group-box gene 9 (Sox9), a transcription factor that has a key role in chondrogenesis. Sox9 is expressed in

all chondroprogenitor cells and differentiated chondrocytes, except HCs (101). Sox9 induces chondrogenic differentiation of mesenchymal stem cells, and, therefore, induces expression of genes encoding chondrocyte specific extracellular matrix proteins, such as Col II and aggrecan (101). On the other hand; this transcription factor inhibits chondrocyte terminal differentiation, preventing transition of PCs into HCs (102). In pellet cultures of chondrocytes derived from mouse ribs, T4 significantly downregulated the mRNA expression of Sox9 and induced chondrocyte terminal differentiation, suggesting that HC differentiation involves TH modulation of Sox9 expression (103). Corroborating these findings, T3 decreased Sox9 mRNA expression (Fig. II), but this effect was observed both in WT and α2C-AR$^{-/-}$ epiphyses, suggesting that Sox9 pathway is not a mechanism of interaction between TH and α2C-AR signaling. UK also decreased Sox9 expression in WT epiphyses, and its combination with T3 (T3+UK) had an additive effect on Sox9 suppression, which was not observed in KO mice. Interestingly, α2C-AR inactivation resulted in increased expression of Sox9, whereas UK increased Sox9 expression in KO epiphyses, suggesting that α2C is the adrenoceptor subtype that inhibits Sox9 expression (Fig. II). The increased Sox9 mRNA expression in α2C-AR$^{-/-}$ epiphyses may explain the increased thickness of the reserve and proliferating zones, but not the increased HC differentiation. On the other hand, the increased Sox9 expression in KO epiphyses may result in an increased number of cells available to differentiate into HCs.

We next investigated a possible contribution of the IGF-1 signaling pathway in the skeletal interaction of TH with the SNS to control bone development. IGF-1 induces clonal expansion of PCs and chondrocyte hypertrophy, in both autocrine- and paracrine-manners (104, 105). TH directly stimulates the expression of IGF-1 in osteoblasts and chondrocytes (27, 28, 106). A TRE was identified in intron 1 of IGF-1 gene of mice, and in vitro studies showed that T3-TRα1-TRE interactions, but not T3-TRβ1-TRE interactions, result in IGF-1 transcription in osteoblasts, confirming the functionality of this TRE (107). TH is, therefore, one of the key regulators of the levels of IGF-1 in the skeleton, including growth plate chondrocytes. Therefore, some of the TH effects on bone development are certainly mediated by IGF-1. The lack of α2C-AR resulted in a 60% increase in IGF-1 mRNA expression in the tibial

epiphyses, whereas UK decreased gene expression of IGF-1 in WT and KO epiphyses, suggesting that not only α2C-AR, but also α_{2A}-AR and/or α2B-AR, have a negative effect on IGF-1 mRNA expression (Fig. II). This inhibitory effect of α2-AR signaling in IGF-1 expression partially explains the increased HC differentiation in α2C-AR$^{-/-}$ epiphyses. As expected, T3 treatment increased IGF-1 mRNA expression in WT epiphyses, which was also observed in the KO epiphyses, however this effect was more important in the WT epiphyses, suggesting that the α2C adrenoceptor signaling may have a slight importance for the T3 induction of IGF-1 mRNA expression (Fig. II). UK did not modify the effect of T3 (T3+UK) in IGF-1 mRNA expression, and vice-versa, both in WT and KO epiphysis, which shows that the IGF-1 signaling is not a key pathway for the interaction between TH and α2C-AR signaling to control skeletal development.

We also analyzed Wnt-4 and Runx2 mRNA expression to investigated if Wnt/β-catenin signaling pathway could represent a *via* of interaction between TH and α2-AR signaling to regulate EGP morphology and linear bone growth. Recently, members of the Wnt family have been recognized as key regulators of chondrocyte proliferation and differentiation (41), and to positively regulate gene expression of Runx2, an essential gene for osteoblast and chondrocyte differentiation (40). Wang et al. (38) have shown that TH stimulates Wnt-4 expression and that Wnt antagonists, Frzb/sFRP3 and Dkk1, inhibit T3 activation of the Wnt/β-catenin pathway, besides inhibiting the T3 effects on the terminal maturation of growth plate chondrocytes. These findings strongly suggest that TH regulates terminal differentiation of EGP chondrocytes, at least in part, through the modulation of the Wnt/β-catenin pathway. More recently, the same group demonstrated that TH interactions with the Wnt/β-catenin pathway, to regulate differentiation of EGP chondrocytes, are modulated by the IGF-1/IGF-1R signaling pathway (42). Wang et al 2010 (43) showed that IGF-1/IGF-1R induces Wnt-4 expression and β-catenin activation in EGP chondrocytes. This study also showed that the terminal differentiation of growth plate chondrocytes was partially prevented by the IGF-1R inhibitor picropodophyllin (43), indicating that the interactions between TH and Wnt/β-catenin signaling pathways to regulate growth plate chondrocyte proliferation and terminal

differentiation involve T3 actions on IGF-1/IGF1R pathways (43). The inactivation of α2C-AR signaling (α2C-AR$^{-/-}$) in the tibial epiphysis resulted in a 70% increase in the mRNA expression of Wnt-4 (when compared with WT epiphyses), and treatment with UK alone decreased Wnt-4 expression both in WT and KO epiphyses (32% and 34%, respectively), showing that activation of not only α2C-AR, but also α2A-AR and/or α2B-AR, have an inhibitory effect on Wnt-4 expression (Fig. II). Corroborating the literature, T3 treatment increased the mRNA expression of Wnt-4 in WT epiphyses (by 3-9-fold), but decreased it in KO epiphyses (by 30%), suggesting that α2C-AR adrenoceptor signaling is important for the T3 induction of Wnt-4 mRNA expression. Interestingly, UK completely blocked the T3 (T3+UK) induction of Wnt-4 mRNA in WT epiphysis (Fig. II) and, in fact, decreased Wnt-4 mRNA in both WT and KO epiphyses, which mirrors the decreased HC differentiation and Col X expression in the T3+UK-treated epiphyses. Overall, these findings show an inhibitory role of α_2-AR signaling in Wnt-4 pathway, and reveal this pathway as a *via* of interaction between TH and the α_2-AR signaling pathway to control chondrocyte terminal differentiation (Fig. II). Following the increased Wnt-4 mRNA expression, Runx2 expression was also increased in α2C-AR$^{-/-}$ epiphyses, which probably explains the increases in Col X mRNA expression, HZ thickness, and HC differentiation (↓PZ/HZ). α2C-AR seems to be the main α2-AR isoform to inhibit Runx2 expression, since treatment with UK alone decreased Runx2 mRNA expression in WT mice (14%), but increased it in KO mice (13%). Interestingly the combination of UK with T3 treatment (T3+UK) significantly decreased Runx2 mRNA expression in WT and KO epiphyses, which probably tails the decreases in Wnt-4 expression and drives the decreases in Col X expression and HC differentiation, which supports the Wnt-4/Runx2 pathway as an important via of cross-talk between TH and α2C-AR signaling.

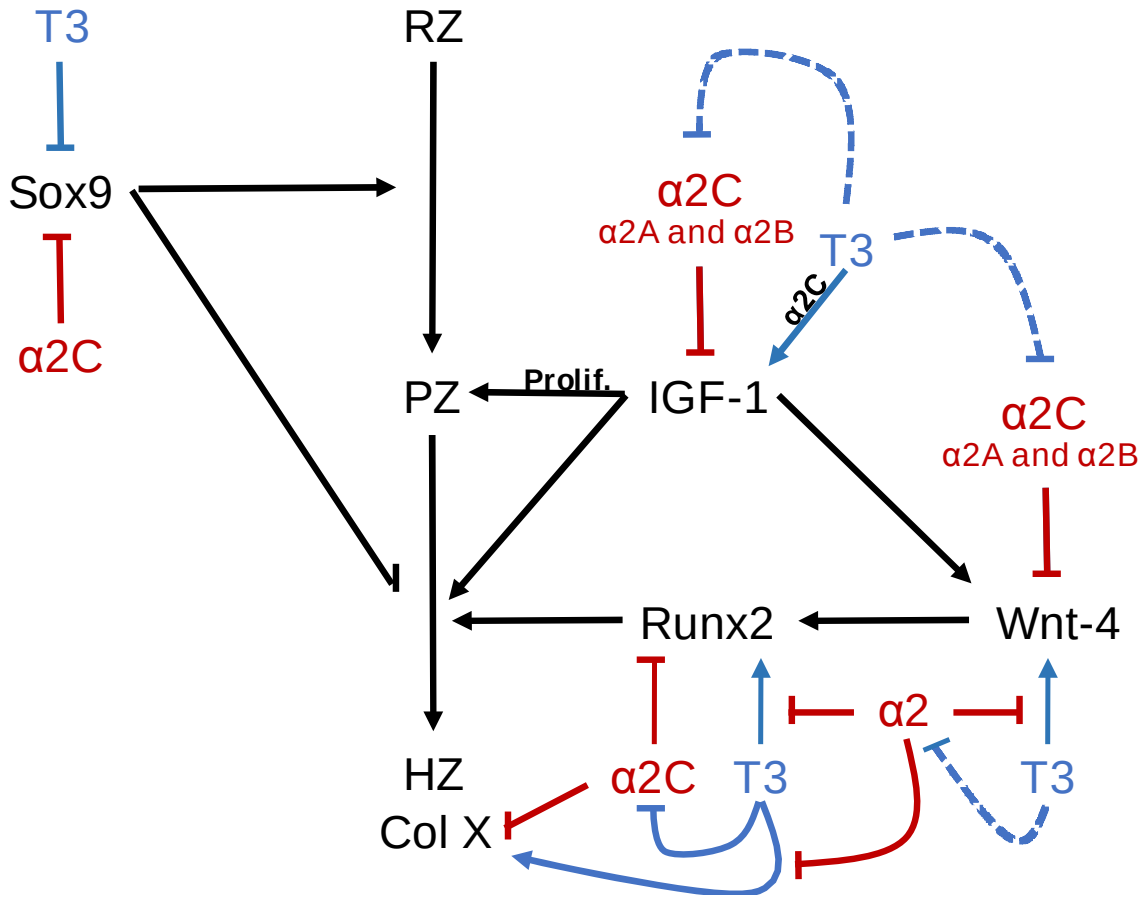

Figure 15 - Squematic representation of the antagonistic actions of α2 adrenoceptors (α2A, α2B or α2C) and T3 in Sox9, IGF-1 and Wnt-4/Runx2 Pathways in Epiphyseal Zones (RZ, PZ, and HZ, respectively).

The analysis of the gene expression of all α2-AR subtypes in the tibial epiphyses and in primary cultures of chondrocytes derived from long bone epiphyses showed a compensatory increase in both α2A-AR and α2B-AR mRNAs, suggesting that α2C-AR may have an inhibitory effect over the expression of these α2 adrenoceptor subtypes (Fig. III). More importantly, this analysis showed that TH upregulates α2A-AR mRNA expression and downregulates α2B-AR and α2C-AR expression, which may represent an important mechanism to control HC differentiation (Fig. III). The in vivo studies showed a decreased number of HC in α2A-AR$^{-/-}$ growth plates (Silva 2016), suggesting that α2A-AR isoform has a positive effect on HC differentiation. On the other hand, the in vivo findings (Miranda-Rodrigues, 2014), combined with the results of the present study (in vitro/organ culture) show an increased HZ in α2C-AR$^{-/-}$ epiphyses, suggesting that α2C-AR has an inhibitory role on HC differentiation. Considering these observations, we propose that one of the

mechanisms (Fig. IV) through which TH promotes chondrocyte terminal differentiation is decreasing α2C-AR mRNA expression, inhibiting, therefore, a HC differentiation-suppressive signaling pathway. At the same time, TH increases α2A-AR expression, to favor HC differentiation. It seems, therefore, that a local interaction between TH and the α_2 adrenoceptor system occur at the receptor expression level.

In this sense, we also evaluated the expression of TRs in the tibial epiphyses, which revealed a reciprocal regulation between TR and α2-AR subtypes signaling (Fig. III). These studies suggest that α2C-AR signaling stimulates TRα expression and decreases TRβ expression. On the other hand, α2A-AR and α2B-AR signaling seem to increase TRα expression and to inhibit TRβ expression. Confirming previous findings (20), T3 increased both TRα and TRβ expression (82% and 99%, respectively) in WT epiphyses, but these effects were attenuated in α2C-AR$^{-/-}$ epiphyses, especially the T3-induction of TRα expression, suggesting that α2C-AR is important for this induction. UK significantly blocked the T3 induction of TRβ expression, mainly in KO epiphyses, which supports the inhibitory action of a general α2-AR activation on TRβ mRNA expression. This reciprocal regulation may be important to fine tune HC chondrocyte differentiation.

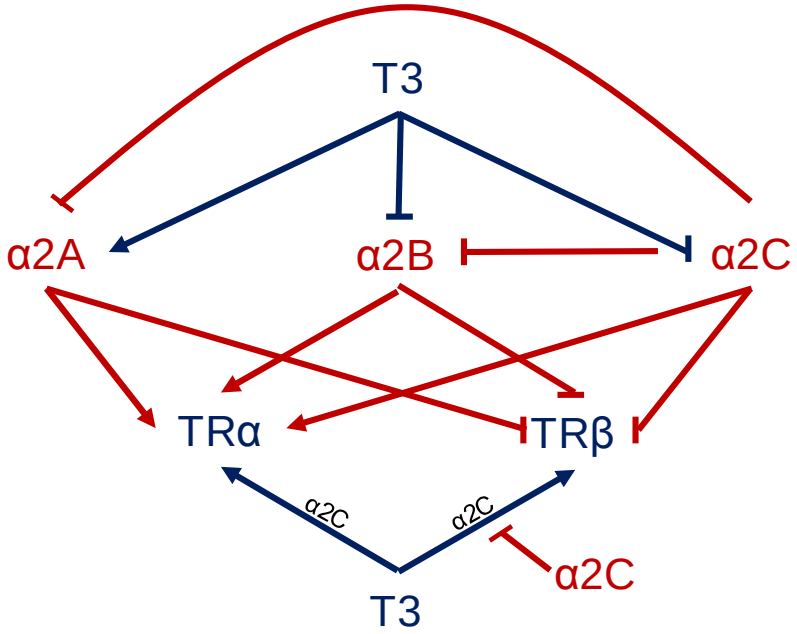

Figure 16 - Squematic representation of the cross-talk among TR isoforms and α2-AR isoforms.

In summary, these in vitro studies revealed that α2C-AR signaling has primarily an inhibitory and direct effect on chondrogenesis, chondrocyte proliferation and terminal chondrocyte differentiation, through the inhibition of key signaling pathways, such as Sox9, IGF-1 and Wnt-4/Runx2 pathways. It explains why gene inactivation of α2C-AR leads to epiphyses with increased thickness in all zones of chondrocytes (RZ, PZ and HZ). Apart from the effects on Sox9 expression, all the other effects of α2C-AR activation are opposite to the T3 effects to promote HC differentiation (Fig. II). Therefore, while T3 favors terminal chondrocyte differentiation, α2-AR signaling inhibits this process. Accordingly, T3 increases IGF-1, Wnt-4, Runx-2 and Col X mRNA expression, while α2C-AR activation seems to decrease these factors (Fig. II). Interestingly, practically all effects of T3 were attenuated or blocked by the simultaneous treatment with UK in WT epiphyses; or were inverted or absent in α2C-AR$^{-/-}$ epiphyses, revealing local interaction between TH and α2-AR signaling to control bone development, especially HC differentiation. In this regard, we identified the Wnt-4/Runx2 pathway as an important via of cross-talk between TH and α2C-AR signaling to modulate chondrocyte differentiation and bone elongation. In addition, we could observe that TH directly modulates the expression of α2-AR subtypes in epiphyseal chondrocytes, increasing α2A-AR and decreasing α2C-AR and α2B-AR mRNA expression (Fig. III). On the other hand, α2-AR signaling also modulates TR expression. TRα seems to be upregulated, whereas TRβ seems to be downregulated by α2-AR signaling. In addition, α2-AR activation or inactivation modifies the T3-induction of TRs. All these effects reveal a reciprocal regulation between TR and α2-AR subtypes (Fig. III), which may be important to modulate bone development processes, especially HC differentiation.

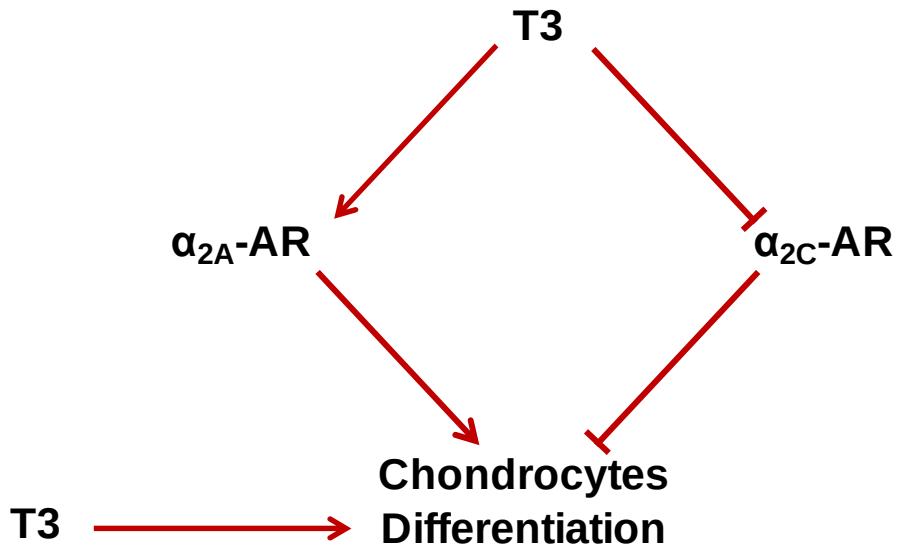

Figure 17 - Squematic representation of a proposed mechanism of action of TH to control HC chondrocyte differentiation, modulating the expression of α2-AR receptors.

In conclusion, the present study shows that α2-AR signaling has local actions in epiphyseal chondrocytes to modulate the growth plate morphology and the linear bone growth. In addition, this study shows that TH interacts with the SNS, locally in the skeleton, via α2-AR signaling, to regulate bone development.

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