Thyroid hormone-stimulated differentiation of primary rib chondrocytes *in vitro* requires thyroid hormone receptor β

Bndicte Rabier^{1,2}, Allan J Williams³, Frederic Mallein-Gerin⁴, Graham R Williams³ and O Chassande^{1,2}

¹INSERM U577-Biomatriaux et Rparation Tissulaire, Universit Bordeaux 2 Victor Segalen, Zone Nord, Btiment 4A, 2me tage, 33076 Bordeaux Cedex, France

²Universit Victor Segalen Bordeaux 2, Bordeaux, France

³Molecular Endocrinology Group, Division of Medicine, Faculty of Medicine, MRC Clinical Sciences Centre, Imperial College London, Hammersmith Hospital, Du Cane Road, London W12 0NN, UK

⁴Laboratoire de Biologie et Ingnierie du Cartilage, Institut de Biologie et Chimie des Protines, UMR 5086 CNRS/UCBLyon 1, 7 passage du Vercors, 69367 Lyon Cedex 07, France

(Requests for offprints should be addressed to O Chassande; Email: chassande@bordeaux.inserm.fr)

Abstract

The active thyroid hormone, triiodothyronine (T_3), binds to thyroid hormone receptors (TR) and plays an essential role in the control of chondrocyte proliferation and differentiation. Hypo- and hyperthyroidism alter the structure of growth plate cartilage and modify chondrocyte gene expression *in vivo*, whilst TR mutations or deletions in mice result in altered growth plate architecture. Nevertheless, the particular roles of individual TR isoforms in mediating T_3 action in chondrocytes have not been studied and are difficult to determine *in vivo* because of complex cellular and molecular interactions that regulate growth plate maturation. Therefore, we studied the effects of TR α and TR β on chondrocyte growth and

differentiation in primary cultures of neonatal rib chondrocytes isolated from TR α - and TR β -deficient mice. T_3 decreased proliferation but accelerated differentiation of rib chondrocytes from wild-type mice. T_3 treatment resulted in similar effects in TR α -deficient chondrocytes, but in TR β -deficient chondrocytes, all T_3 responses were abrogated. Furthermore, T_3 increased TR β 1 expression in wild-type and TR α -deficient chondrocytes. These data indicate that T_3 -stimulated differentiation of primary rib chondrocytes *in vitro* requires TR β and suggest that the TR β 1 isoform mediates important T_3 actions in mouse rib chondrocytes.

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Introduction

Triiodothyronine (T_3) plays a key role in bone growth and maturation (Bassett & Williams 2003). Hypothyroidism results in growth retardation, epiphyseal dysgenesis and delayed bone mineralization. In contrast, thyrotoxicosis accelerates growth and advances bone age, but causes short stature due to early closure of the growth plates. Longitudinal growth results from proliferation of growth plate chondrocytes, differentiation of proliferating chondrocytes and enlargement of hypertrophic chondrocytes, which increase their volume by tenfold during maturation. Hypertrophic chondrocytes also play a key role in the control of bone vascularization and thus regulate endochondral ossification (Gerber & Ferrara 2000, Goldring *et al.* 2006).

Both hypothyroidism and thyrotoxicosis alter the structure and organization of the growth plate *in vivo* (Lewinson *et al.* 1994, Stevens *et al.* 2000, Freitas *et al.* 2005). Hypothyroidism increases the width of the proliferative zone but reduces the size of the hypertrophic zone. In contrast, thyrotoxicosis results in a reduced width of both proliferative and hypertrophic zones. Nevertheless, the mechanisms by which altered thyroid status

regulates the growth plate remain unclear. *In vivo*, thyroid hormones regulate expression of parathyroid hormone related peptide (PTHrP) and PTHrP receptors, which control the pace of chondrocyte proliferation during endochondral ossification (Stevens *et al.* 2000). In organ cultures, thyroxine (T_4) is a potent stimulator of chondrocyte differentiation (Wakita *et al.* 1998, Miura *et al.* 2002), whilst *in vitro* T_3 decreases the growth of chondrocyte colonies and inhibits cell proliferation (Bohme *et al.* 1992, Ohlsson *et al.* 1992, Robson *et al.* 2000, Okubo & Reddi 2003). Many investigators have also demonstrated a positive role for T_3 in regulating terminal differentiation of chondrocytes *in vitro* in several species using different culture systems (Ohlsson *et al.* 1992, Quarto *et al.* 1992, Ballock & Reddi 1994, Bohme *et al.* 1995, Alini *et al.* 1996, Leboy *et al.* 1997, Ishikawa *et al.* 1998, Okubo & Reddi 2003). Mechanistic studies have revealed that thyroid hormone (TH) stimulates expression of p21, a cyclin D1 inhibitor, in rat epiphyseal chondrocytes *in vitro* (Ballock *et al.* 2000) and inhibits the expression of Sox-9, a transcription factor that maintains chondrocytes in an undifferentiated state, in mouse rib chondrocytes (Okubo & Reddi 2003). Furthermore, T_3 stimulates fibroblast growth factor (FGF) receptor expression

in ATDC5 cells and enhances FGF signalling in these cells and also in primary mouse rib chondrocytes (Barnard *et al.* 2005). However, precisely how these responses are involved in the T₃ control of chondrocyte proliferation and differentiation has not been determined.

T₃ actions are mediated by nuclear T₃ receptors (TR) (Yen *et al.* 2006), which act as ligand-controlled transcription factors. TR α 1 is encoded by the *TR α* gene, which also encodes TR α 2, TR $\Delta\alpha$ 1 and TR $\Delta\alpha$ 2 isoforms that do not bind T₃ and whose function is largely unknown. TR α 1 and TR α 2 are expressed in chondrocytes and osteoblasts. TR β 1, TR β 2 and TR β 3 are encoded by the *TR β* gene. TR β 1 is expressed in chondrocytes and osteoblasts. Mice which lack TR α 1 and TR α 2 display abnormal growth plate architecture and impaired long bone mineralization during post-natal growth (Gauthier *et al.* 2001) associated with reduced FGFR1 and FGFR3 expression in osteoblasts and chondrocytes (Stevens *et al.* 2003, Barnard *et al.* 2005). Introduction of a point mutation into the *TR α* gene results in the expression of a dominant-negative TR α protein that inhibits the function of wild-type TRs and causes a similar, but more severe, skeletal phenotype than that observed in TR α -deficient mice (O'Shea *et al.* 2005). In contrast, mice with the homologous mutation in TR β have elevated TH levels, advanced bone age and short stature with a reduced width of the growth plates affecting both the proliferative and the hypertrophic zones. These features have been interpreted to result from elevated TH levels acting via intact TR α 1 in bone (O'Shea *et al.* 2003). Nevertheless, a specific role for TR β in chondrocytes has also been suggested by studies of the TR β selective agonist GC-1 (Freitas *et al.* 2005). Hypothyroid rats exhibited disorganized chondrocytes columns, reduced hypertrophic chondrocyte differentiation and impaired mineralization. These abnormalities were all rescued by the administration of T₃, whereas the TR β -selective agonist GC-1 also rescued the chondrocyte differentiation and bone mineralization defects but was unable to restore normal growth plate architecture.

In vivo analysis of mechanisms that account for T₃ action in the growth plate is difficult because of the complex interactions between chondrocytes and other cell types and because of the interplay between T₃ and other endocrine and paracrine systems, which may be affected by TR genetic modifications. In particular, local concentrations of thyroid hormone can be affected by these mutations: TR β ^{-/-} mice have been shown not only to have elevated TH levels, but also to have altered expression of de-iodinases in some tissues, including liver (Ammar *et al.* 2001) and cochlea (Campos-Barros *et al.* 2000), thus modifying the local metabolism of TH. The aim of this study was to determine the roles of TR α and TR β in T₃-stimulation of chondrocyte growth, differentiation and maturation, in an *in vitro* system, which allows a precise control of TH concentration. For this purpose, we isolated chondrocytes from the ribs of newborn mice. Murine rib chondrocytes cultured in a monolayer maintain a differentiated phenotype of round or polygonal cells that express type II collagen (Lefebvre *et al.* 1994). Cells

differentiate in response to bone morphogenetic protein-2 (BMP-2) (Valcourt *et al.* 2003, Yagi *et al.* 2003), but may de-differentiate after several passages if cultured at low density (Lefebvre *et al.* 1994) or when maintained in the presence of TGF β 1 (Valcourt *et al.* 2002). To analyse the roles of TR α and TR β , we isolated rib chondrocytes from mice, which lack either TR α (TR α ^{0/0} mice) (Gauthier *et al.* 2001) or TR β (TR β ^{-/-} mice) (Gauthier *et al.* 1999). We measured the expression of TR isotypes at different times of the culture. We monitored the growth of the cultures by measuring the amount of DNA and evaluated the differentiation by measuring the activity of alkaline phosphatase (ALP) and the expression of collagen X.

Materials and Methods

Animals and cell culture

Mice were maintained with a ratio of 12 h light:12 h darkness cycle at 22–26 °C and experiments performed according to requirements of the regional committee of Ethics and the National Committee of Genetics. Homozygous wild type, TR α ^{0/0} and TR β ^{-/-} mice were crossed to obtain homozygous offspring. Neonatal ribs were dissected as described (Lefebvre *et al.* 1994). Cells were dissociated in 2 mg/ml protease (Sigma) for 20 min, washed in PBS, incubated in 2 mg/ml collagenase (Sigma) for 10 min, washed thrice in PBS, and incubated in 2 mg/ml collagenase for 5 h. The cell pellet was resuspended in DMEM/F12 medium (Gibco, Life Technology) supplemented with 10% heat-inactivated foetal bovine serum and 2 mM L-glutamine (Gibco), penicillin, streptomycin and gentamycin. Cells were seeded into 75 cm² flasks at a density of 50 000/cm². Confluent cells were trypsinized and seeded into 96- or 6-well plates at the same density and after 48 h the medium was replaced by a serum-free medium containing 1:500 ITS (Bio-Whittaker, Walkersville, Cambrex), 1 mg/ml BSA and combinations of 10⁻⁷ M T₃ (Sigma) and 100 ng/ml BMP-2 (Peprotech, Tebu).

Cell growth and ALP activity

Cells were harvested at several time points after seeding by washing twice in PBS and freeze-thawing in 50 μ l Hoechst 33258 solution (5 μ g/ml in 10 mM Tris-HCl, pH 8.0, 150 mM NaCl and 0.1 mM EDTA). DNA concentration was determined in a Cytofluorometer2 multi-well fluorometer (excitation 360 nm/emission 460 nm) compared with a standard curve prepared from calf thymus DNA. ALP activity was determined by freeze-thawing cells in 50 μ l 0.5% Triton X-100 and incubating them for 1–30 min at 37 °C with 50 μ l ALP substrate (20 mM p-nitrophenyl phosphate) in alkaline buffer solution (Sigma). Absorbance was determined at 405 nm in a fluorescence microplate reader (MRX,

Dynex Technologies) and ALP activity was estimated compared with a standard curve prepared from a p-nitrophenol solution (Sigma), normalized to DNA concentration and expressed as nanomoles/minute per microgram DNA.

Analysis of gene expression by real-time quantitative RT-PCR (qRT-PCR)

RNA was extracted using an RNeasy Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Reverse transcription was performed with Superscript II reverse transcriptase (Invitrogen) using 1·25 µg RNA template. One microlitre of tenfold diluted cDNA was used for qRT-PCR, using specific primers (Table 1) to amplify hypoxanthine phosphoribosyl transferase (HPRT), TRβ1 (Boelen *et al.* 2005), TRα1 and collagen X (Cormier *et al.* 2003). Reactions were performed in duplicate for each cDNA using the iQ SYBR Green supermix in an i-cycler IQ thermocycler (Bio-Rad). Quantitation was performed using the Optical System Software (Bio-Rad). Ct values were determined in all amplifications and the mean Ct value for each duplicate was calculated. For each RNA extraction, the mean ΔCt between the gene of interest and the HPRT gene was calculated. For each series of analyses, the reference sample was derived from untreated wild-type cells at the earliest time point of the culture, or from tissues of untreated wild-type mice. This reference value (ΔCt₀) was subtracted from the ΔCt values of all other samples to give the ΔΔCt value; thus, the ΔΔCt value of the reference sample was zero. The relative amount of cDNA in each sample was calculated using the formula $RQ = 2^{-\Delta\Delta C_t}$; thus the quantity of cDNA in the reference sample was 1.

Statistical analysis

Statistical analysis was performed using unpaired two-tailed Student's *t*-tests and a *P* value <0·05 was considered significant. These analyses were performed by comparing the data obtained from multiple wells for each experimental condition, within one primary culture. All the results presented in this manuscript have been repeated at least in three independent primary cultures for each genotype.

Results

Expression of TRα and TRβ isoforms in rib chondrocytes

Expression of TR isoforms in rib chondrocytes was determined over 21 days in culture by qRT-PCR. In the absence of T₃, TRβ1 expression (Fig. 1A) was similar in wild type and TRα^{0/0} cells and did not vary significantly with time, other than a 50% decrease at day 12 compared with day 6 in wild-type cells. In the presence of T₃, TRβ1 expression increased significantly at days 12 and 21 in the wild-type cells and at days 6 and 21 in TRα^{0/0} cells, as compared with the control untreated cells. TRβ1 expression in the presence of T₃ did not differ between wild type and TRα^{0/0} cells at any time of the culture. In wild-type chondrocytes in the absence of T₃, TRα1 expression (Fig. 1B) remained constant until day 21. In T₃-treated cells, TRα1 expression tended to be lower at day 6 and significantly decreased at days 12 and 21 compared with the control untreated cells. In untreated TRβ^{-/-} chondrocytes, TRα1 expression was lower than in untreated wild-type cells at all times. T₃ decreased TRα1 expression at day 6 but did not have any effect at days 12 and 21.

T₃ inhibits growth of wild-type and TRα-deficient, but not TRβ-deficient rib chondrocytes in the presence of BMP-2

When wild-type rib chondrocytes were grown in 10% foetal bovine serum, cell numbers increased during the first 7 days of culture but remained stable until day 21 (Fig. 2A). In serum-free medium, cell numbers declined between day 7 and day 14, but remained stable until day 21. T₃ treatment had no effect on cell growth in serum-free medium. In contrast, treatment with BMP-2 increased cell number by 40–100% at days 7, 14 and 21 (four separate cultures from four different litters, eight wells per treatment for each culture). T₃ significantly decreased the number of cells measured in the presence of BMP-2 at days 7, 14 and 21 by 20–40% in four separate experiments. Thus, T₃ inhibits BMP-2-induced growth of wild-type mouse rib chondrocytes. Chondrocytes from TRα^{0/0} mice cultured in 10% serum or in the absence of T₃ displayed a similar pattern of growth as wild-type cells (Fig. 2B). Treatment with T₃ alone had no significant effect on cell growth. BMP-2 stimulated cell growth by 40–120% (three separate experiments, eight wells per treatment for each culture), and T₃ reduced the cell number obtained in the presence of BMP-2 by 20–30% (three separate experiments).

Table 1 DNA sequences of primers used in real-time PCR

	Upstream primer	Downstream primer
Gene products		
HPRT	5' GCAGTACAGCCCCAAAATGG 3'	5' AACAAAGTCTGGCCTGTATCCAA 3'
TRα1	5' CATCTTTGAAGTGGGCAAGT 3'	5' CTGAGGCTTTAGACTTCTCTGATC 3'
TRβ1	5' CACCTGGATCCTGACGATGT 3'	5' ACAGGTGATGCAGCGATAGT 3'
CollagenX	5' CAAACGGCCTCTACTCCTCTGA 3'	5' CGATGGAATTGGGTGGAAAG 3'

Figure 1 Expression of TR isoforms in rib chondrocytes. (A) TR β 1 mRNA concentrations normalized to HPRT, in primary wild-type and TR α ^{0/0} mouse rib chondrocytes cultured for 6, 12 and 21 days in the absence or presence of 10^{-7} M T₃. Results are shown as mean $\pm$ S.E.M. $n \geq 3$ for all conditions except for T₃-treated TR α ^{0/0} cells at day 21 ($n = 2$). (B) Expression of TR α 1-mRNA normalized to HPRT in TR β ^{-/-} chondrocytes cultured for 6, 12 and 21 days in the absence or presence of 10^{-7} M T₃. Results are shown as mean $\pm$ S.E.M. $n \geq 2$ for all conditions. Data were analysed by two-tailed Student's *t*-test. NS, not significant; * $P < 0.001$; † $P < 0.05$; ‡ $P < 0.01$.

In 10% serum and in the absence of T₃, chondrocytes from TR β ^{-/-} mice had a similar growth pattern to that observed in wild-type cells (Fig. 2C). T₃ did not modify the growth of TR β ^{-/-} chondrocytes. BMP-2 stimulated chondrocyte growth by 25–100%, but T₃ did not significantly affect this stimulation in three separate experiments (eight wells per treatment for each culture).

T₃ stimulates differentiation of wild type and TR α -deficient, but not TR β -deficient rib chondrocytes

Chondrocyte differentiation was monitored by measuring ALP activity at days 14 and 21 (Fig. 3), and collagen X expression at days 6, 12 and 21 (Fig. 4). When wild-type chondrocytes were cultured in the absence of T₃, ALP activity increased by fourfold between day 14 and day 21 (Fig. 3A). ALP activity was increased five- and threefold at days 14 and 21 respectively, following T₃ treatment. A two- to fivefold stimulation of ALP activity by T₃ was observed at day 21 in five separate experiments. Treatment with BMP-2 elicited a

Figure 2 Effect of T₃ and BMP-2 on growth of primary rib chondrocytes. Results are expressed as micrograms DNA per well. Cells were seeded on day 0 (50 000 cells/cm²) and maintained in the absence or presence of T₃ (100 nM) $\pm$ BMP-2 (100 ng/ml) for 21 days. All experiments were performed four times (four independent cultures). For each culture, eight wells per condition were seeded. The graphs show a single typical experiment for each genotype. The bars indicate mean $\pm$ S.E.M. Results were analysed by two-tailed Student's *t*-test. NS, not significant; * $P < 0.001$; † $P < 0.01$. (A) Wild-type chondrocytes. (B) TR α ^{0/0} chondrocytes. (C) TR β ^{-/-} chondrocytes. Hatched bars, 10% FCS; white bars, control, serum-free medium; black bars, serum-free medium with T₃ (10^{-7} M); vertically hatched bars, serum-free medium with BMP-2 (100 ng/ml); horizontally hatched bars, serum-free medium with T₃ (10^{-7} M) and BMP-2 (100 ng/ml).

nine- and a fivefold increase in ALP activity at days 14 and 21 respectively, compared with untreated cells. A further 2- to 2.5-fold activation was observed when cells were treated with both BMP-2 and T₃. In TR α ^{0/0} chondrocytes, basal ALP activity was stable between days 14 and 21. T₃ increased ALP

Figure 3 Effect of T₃ on alkaline phosphatase activity in primary rib chondrocytes. Chondrocytes were cultured for 21 days in the absence or presence of 10^{-7} M T₃ $\pm$ 100 ng/ml BMP-2. Alkaline phosphatase activity is expressed as nanomoles of substrate hydrolysed per minute per micrograms of DNA $\pm$ S.E.M. ($n=4$ experiments, eight wells per condition). Results were analysed by two-tailed Student's t -test. NS, not significant. * $P<0.001$; † $P<0.01$. (A) Wild-type chondrocytes. (B) TR $\alpha^{0/0}$ chondrocytes. (C) TR $\beta^{-/-}$ chondrocytes. Hatched bars, 10% FCS; white bars, control, serum-free medium; black bars, serum-free medium with T₃ (10^{-7} M); vertically hatched bars, serum-free medium with BMP-2 (100 ng/ml), horizontally hatched bars, serum-free medium with T₃ (10^{-7} M) and BMP-2 (100 ng/ml).

activity by 30% at day 14 and by 2.5-fold at day 21 (Fig. 3B). In four separate experiments, ALP activity was stimulated by T₃ by two- to fivefold in TR $\alpha^{0/0}$ chondrocytes. BMP-2 stimulated ALP activity at days 14 and 21 by two- and fourfold respectively. T₃ further increased this activity by 40% at day

14 and twofold at day 21. Notably, ALP activity was lower in TR $\alpha^{0/0}$ chondrocytes than wild-type cells in all cultures. In untreated TR $\beta^{-/-}$ chondrocytes, ALP activity displayed a similar pattern as in wild-type cells, with an increase between days 14 and 21. However, T₃ failed to stimulate ALP activity in TR $\beta^{-/-}$ chondrocytes (Fig. 3C). In contrast, BMP-2 stimulated ALP activity 22-fold at day 14 and eightfold at day 21, indicating that TR $\beta^{-/-}$ chondrocytes retain their differentiation capacity. T₃ had no additional effect on ALP activity in the presence of BMP-2.

Expression of collagen X, another specific marker of chondrocyte differentiation, was also determined (Fig. 4). Basal expression of collagen X in the absence of T₃ was similar in wild type, TR $\alpha^{0/0}$ and TR $\beta^{-/-}$ chondrocytes throughout the 21-day period of culture. Collagen X expression was increased by T₃ at all times in wild type and TR $\alpha^{0/0}$ chondrocytes, but this stimulation was less in TR $\alpha^{0/0}$ cells at day 6 compared with wild type. In contrast, T₃ failed to stimulate collagen X expression in TR $\beta^{-/-}$ chondrocytes.

Discussion

Chondrocytes are sensitive to thyroid hormones *in vivo* and *in vitro* (Nilsson *et al.* 2005). *In vitro*, T₃ inhibits cell proliferation but accelerates hypertrophic chondrocyte differentiation; however, the mechanisms responsible for these effects are poorly understood. In order to understand the mechanisms of T₃ action in chondrocytes, it is important to define which TR mediates its biological effects, and thus we investigated chondrocyte proliferation and differentiation in primary cells obtained from TR α and TR β knockout mice. Inhibition of chondrocyte proliferation by T₃ has been demonstrated in several cell culture systems, including epiphyseal chondrocyte monolayer (Ishikawa *et al.* 1998) and three-dimensional cultures (Ballock *et al.* 2000, Robson *et al.* 2000). When mouse rib chondrocytes were cultured in serum-free medium, cell proliferation declined within the first 48 h (data not shown) before reaching a plateau. Addition of T₃ had no effect on cell growth in serum-free medium, which is not surprising because T₃ has not been reported to exert effects on quiescent chondrocyte cultures, although it is has been shown to inhibit expansion of proliferating chondrocyte cultures (Ballock *et al.* 2000, Robson *et al.* 2000). In contrast and consistent with studies showing that BMP-2 stimulates mouse growth plate chondrocyte proliferation *in vivo* (Minina *et al.* 2001), BMP-2 not only prevented cell loss but also stimulated growth of primary rib chondrocytes. Importantly, T₃ prevented the BMP-2-induced increase in cell number, indicating that T₃ inhibits BMP-2-stimulated growth of mouse rib chondrocytes. We also demonstrated that growth of TR α - and TR β -deficient chondrocytes in serum-free medium and in the presence of BMP-2 was similar to the growth of wild-type cells, suggesting that TRs do not play a role in T₃-independent chondrocyte growth. As observed in wild-type cells, T₃ had no effect on the growth of TR $\alpha^{0/0}$ or TR $\beta^{-/-}$ cells in basal medium. Addition of T₃ to BMP-2-treated cultures impaired

Figure 4 Effect of T₃ on collagen X mRNA expression in rib chondrocytes. Wild type, TR $\alpha^{0/0}$ and TR $\beta^{-/-}$ chondrocytes were grown for 6, 12 and 21 days in the absence (white bars) or presence (black bars) of 10^{-7} M T₃. ColX mRNA was normalized to the concentration of HPRT mRNA. Data are expressed as mean $\pm$ S.E.M. ($n=2$ each condition). Results were analysed by two-tailed Student's *t*-test. NS, not significant. * $P<0.05$; † $P<0.01$.

growth of TR $\alpha^{0/0}$ chondrocytes, as observed with wild-type cells, but had no effect on BMP-2-treated TR $\beta^{-/-}$ chondrocytes. These data suggest that TR β 1 mediates the inhibitory action of T₃ on the BMP-2-stimulated proliferation of mouse rib chondrocytes.

T₃ has been shown to stimulate terminal differentiation of chondrocytes in a variety of systems (Alini *et al.* 1996, Leboy *et al.* 1997, Ishikawa *et al.* 1998, Rosenthal & Henry 1999, Robson *et al.* 2000, Stevens *et al.* 2000, Okubo & Reddi 2003). In particular, T₃ stimulates ALP activity and increases the expression of collagen X in these models. When mouse rib chondrocytes were cultured for 21 days in serum-free medium in the absence of T₃, ALP activity and collagen X expression were similar to the levels at the onset of culture, indicating a lack of hypertrophic differentiation. In contrast, in the presence of T₃, ALP activity increased rapidly and collagen X expression was stimulated at all time points indicating that T₃ stimulates differentiation of primary mouse rib chondrocytes in monolayer cultures.

ALP activity in TR $\alpha^{0/0}$ chondrocytes was consistently lower than in wild-type cells in all culture conditions; however, the amplitude of responses to T₃, BMP-2 and both agents was similar in wild type and TR α -deficient cells. Collagen X expression was also stimulated by T₃ to a similar degree in both TR $\alpha^{0/0}$ and wild-type chondrocytes. In contrast, T₃-stimulation of ALP and collagen X was abolished in TR $\beta^{-/-}$ chondrocytes. Explanations for this discrepancy include an absence or low proportion of chondrocyte precursors within the ribs of TR $\beta^{-/-}$ mice, or an impaired intrinsic inability of TR $\beta^{-/-}$ cells to differentiate into mature chondrocytes. However, BMP-2 stimulated the differentiation of TR $\beta^{-/-}$ cells to the same extent as in wild type and TR α -deficient cells, indicating that TR β -deficient cells have the intrinsic capacity to differentiate into mature chondrocytes. Furthermore, whereas T₃ could enhance the ALP response of wild type and TR $\alpha^{0/0}$

chondrocytes to BMP-2, it had no effect in BMP-2-treated TR $\beta^{-/-}$ cells. All these data suggest that the T₃-dependent differentiation pathway is selectively impaired in TR β -deficient cells.

The predominant role of TR β 1 in the mediation of T₃ action in mouse rib chondrocytes is consistent with the pattern of expression of TR isoforms during differentiation of wild type and TR-deficient cells. In wild-type cells, T₃ increased expression of TR β 1 and decreased expression of TR α 1. This differential regulation of TR-isotype expression by T₃ suggests TR β 1 as the main mediator of T₃ action in these cells. The observations that, following treatment with T₃, increased TR β 1 expression is preserved in TR α -deficient cells, whereas the decrease in TR α 1 expression is attenuated in TR β -deficient cells also support a key role for TR β 1. Our observations do not exclude the possibility that in wild-type chondrocytes, both TRs can mediate T₃ action and that TR β 1 can substitute for TR α 1 in TR $\alpha^{0/0}$ cells, whereas TR α 1 cannot substitute for TR β 1 in TR $\beta^{-/-}$ cells. Indeed, in the absence of T₃, the level of expression of TR α 1 was lower in TR $\beta^{-/-}$ than in wild-type cells. One explanation for this low amount of TR α 1 in TR β -deficient cells is that TR β 1 as an aporeceptor positively regulates the expression of TR α 1. The absence of TR β would therefore decrease the expression of TR α 1 and diminish the response to T₃. Consequently, it is possible that TR α 1 is active in wild-type cells but, because less abundant, unable to mediate T₃ action in TR $\beta^{-/-}$ cells. In TR α -deficient cells, the response to T₃ may be mediated by TR β 1, however, no compensatory overexpression of this isotype was observed in TR $\alpha^{0/0}$ cells. Although it is not possible to exclude that TR α 1 is involved in T₃ action in wild-type chondrocytes, upregulation of TR β 1 and the downregulation of TR α 1 by T₃ in these cells are more consistent with a major role of TR β 1. Such a key role of TR β 1 in the modulation of chondrocyte differentiation by T₃ is supported by *in vivo* studies using the TR β -selective agonist

GC-1 (Freitas *et al.* 2005). In these studies, young hypothyroid rats displayed altered organization of chondrocyte columns within the growth plate with impaired hypertrophic differentiation, as assessed by the decreased size of the hypertrophic zone and reduced expression of collagen X. GC-1, like T₃, restored normal morphometric parameters of the growth plate and normal expression of collagen X, strongly suggesting that TR β 1 mediates the effect of T₃ on chondrocyte differentiation. These findings are consistent with an important role for TR β 1 in the control of chondrocyte differentiation and, thus, the role of TR α 1 in chondrocyte biology remains to be elucidated. In hypothyroid rats, T₃, but not the TR β -selective agonist GC-1, restored normal organization of the growth plate, suggesting that TR α 1 mediates the effects of T₃ on the spatial organization of chondrocytes (Freitas *et al.* 2005). However, our findings using rib chondrocytes in monolayer cultures did not demonstrate a major role for TR α in the regulation of cell proliferation and differentiation. It is possible that, *in vivo* or in a three-dimensional environment, TR α 1 is the key mediator of T₃ action, whereas in monolayer cultures, TR β 1 becomes predominant. Nonetheless, in TR α ^{0/0}-chondrocytes, ALP activity was blunted compared with wild-type cells, raising the possibility that TR α may be a permissive factor that facilitates chondrocyte differentiation. The importance of TR α is further suggested by the growth plate phenotype of mice lacking the TR α gene (Fraichard *et al.* 1997, Gauthier *et al.* 2001) or expressing a dominant negative TR α 1 isoform (O'Shea *et al.* 2005). These mice have disorganized chondrocyte columns and reduced width of the hypertrophic zone. However, they also have mild hypothyroxinemia (Macchia *et al.* 2001, Weiss *et al.* 2002), raising the possibility that the observed phenotype results from reduced local TH availability.

Altogether, our data are consistent with the observations from TR-knockout and knock-in mice, and suggest that TR β 1 mediates important T₃ effects on hypertrophic chondrocyte terminal differentiation, whereas TR α 1 is likely to be involved in the control of cellular and molecular interactions that are important for the three-dimensional organization of growth plate cartilage and the initiation of chondrocyte differentiation. These findings may have important applications for the potential use of T₃ analogues as therapeutic agents for the treatment of bone and articular cartilage lesions.

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