REVIEW

The skeletal phenotypes of TR α and TR β mutant mice

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Abstract

Analysis of mice harbouring deletions or mutations of T₃ receptor α (TR α) and β (TR β) have clarified the complex relationship between central and peripheral thyroid status and emphasised the essential but contrasting roles of T₃ in skeletal development and adult bone. These studies indicate that TR α 1 is the predominant TR expressed in bone and that T₃ exerts anabolic actions during growth but catabolic actions in the adult skeleton. Examination of key skeletal regulatory pathways in TR mutant mice has identified GH, IGF-1 and fibroblast growth factor signalling and the Indian hedgehog/parathyroid hormone-related peptide feedback loop as major targets of T₃ action in chondrocytes and osteoblasts. Nevertheless, although increased osteoclastic resorption is a major feature of thyrotoxic bone loss and altered osteoclast activity is central to the skeletal phenotype of TR mutant mice, it remains unclear whether T₃ has direct actions in osteoclasts. Detailed future analysis of the molecular mechanisms of T₃ action in bone will enhance our understanding of this emerging field and has the potential to identify novel strategies for the prevention and treatment of osteoporosis.

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Introduction

Thyroid hormone is essential for normal skeletal development and the maintenance of adult bone mass. Recent studies of T₃ receptor (TR) mutant mice have advanced our understanding and demonstrated the relevance of this field to osteoporosis, a major public health burden that affects half of all women and one-fifth of men over the age of 50, costing the European community €31 billion per annum (Kanis & Johnell 2005).

Systemic thyroid hormone levels are maintained by the hypothalamus–pituitary–thyroid (HPT) feedback axis. The cellular actions of 3,5,3'-L-triiodothyronine (T₃) are mediated by TRs, which act as hormone-inducible transcription factors. Unliganded TRs bind T₃ response elements (TREs) in T₃-target genes and mediate transcriptional repression. T₃ binding results in derepression and activation of gene transcription (Yen 2001). The *THRA* and *THRB* genes encode three functional receptors TR α 1, TR β 1 and TR β 2 as well as a non-T₃-binding isoform of unknown function TR α 2 (Fig. 1). TR α 1 and TR β 1 are expressed widely and the ratio of TR α 1 to TR β 1 is spatio-temporally regulated. Thus, T₃-target tissues may predominantly display either TR α 1 or TR β 1 responsiveness or show no

TR-isoform specificity. TR β 2 has a more restricted pattern of expression and regulates sensory organ development (Jones *et al.* 2007) as well as the HPT axis. In the skeleton, chondrocyte and osteoblast lineages express TR α and TR β mRNAs, but in osteoclasts the position is less clear as studies have been restricted to precursor cells or *in situ* hybridisation analysis of osteoclastomas (Williams *et al.* 1994, Abu *et al.* 2000, Stevens *et al.* 2000, Kanatani *et al.* 2004). Several studies have demonstrated apparent expression of TR proteins in all bone cell lineages, but it is well recognised within the field that available TR antibodies are of low affinity, thus compromising the detection of endogenous TRs (Abu *et al.* 1997, Robson *et al.* 2000). For these reasons, a comprehensive understanding of TR expression in bone is lacking and a detailed analysis of cell-specific and temporal expression of TR isoforms is required.

Long bones are formed by endochondral ossification and the skull by intramembranous ossification. During endochondral ossification, mesenchyme-derived chondrocytes form a cartilage model, undergo hypertrophic differentiation and then apoptose. The surrounding collagen X-rich cartilage matrix calcifies and forms a scaffold for bone formation by osteoblasts. Organised columns of proliferating and differentiating

Figure 1 Thyroid hormone receptor isoforms. (A) Schematic of the four functional domains of the thyroid hormone receptor. (B) Products of the *Thra* and *Thrβ* genes in the mouse. *Thra* has two promoters, TRα1 and TRα2 are derived from the 5' promoter whereas TRΔα1 and TRΔα2 are transcribed from an internal promoter in intron 7. TRα2 and TRΔα2 result from alternative splicing. TRα1 is a functional receptor whereas TRΔα1, TRα2 and TRΔα2 do not bind T₃ and act as antagonists. *Thrβ* also has two promoters and both N-terminal variants TRβ1 and TRβ2 bind T₃ and act as functional receptors.

chondrocytes persist in the growth plate until adolescence and mediate linear growth and the acquisition of peak bone mass. By contrast, in intramembranous ossification, osteoblasts differentiate from mesenchyme to form bone directly. Adult bone structure and mechanical strength are preserved by a continuous process of skeletal remodelling during which precise coupling of osteoclastic bone resorption and subsequent osteoblastic bone formation is maintained.

The established view that skeletal responses to abnormal thyroid status result exclusively from altered T₃ action in bone has been challenged recently by studies proposing a direct role for TSH in bone. We recently discussed this issue elsewhere (Bassett & Williams 2008) and the present review will therefore focus on the analysis of T₃ and TR action in bone.

Thyroid hormone receptor mutant mice

Several TRα knockout mice have been generated and this led to the identification of additional TRα isoforms expressed from a promoter within intron 7 of the *Thra* gene (Chassande *et al.* 1997). As a result only TRα^{0/0} mice lack all TRα isoforms whereas other TRα mutants retain truncated isoforms with dominant-negative activity (Fig. 1 and Table 1). This review will focus on mice lacking all TRα (TRα^{0/0}) or all TRβ (TRβ^{-/-}) isoforms and those harbouring dominant-negative

mutations of either TRα (TRα1^{PV/+}, TRα1^{R384C/+}) or TRβ (TRβ^{PV/PV}).

Thyroid hormone actions during skeletal development are anabolic

The developing skeleton is exquisitely sensitive to thyroid status and childhood hypothyroidism is characterised by growth retardation, delayed bone age and short stature, whereas juvenile thyrotoxicosis accelerates growth and advances bone age but results in short stature due to premature fusion of the epiphyses (Rivkees *et al.* 1988, Boersma *et al.* 1996, Segni & Gorman 2001).

Although TRα^{0/0} mice are systemically euthyroid, juveniles display post-natal growth retardation with delayed endochondral ossification characterised by impaired chondrocyte differentiation and decreased mineral deposition (Bassett *et al.* 2007b). TRα1^{R384C/+} mice, which harbour a dominant-negative mutation of TRα, are also euthyroid. They display a similar period of growth retardation and delayed endochondral ossification, but additionally have reduced cortical bone thickness, abnormal cortical bone remodelling and impairment of intramembranous ossification (Fig. 2; Bassett *et al.* 2007a). TRα1^{PV/+} mice, which express a potent dominant-negative TRα mutant, display a more severe skeletal phenotype. TRα1^{PV/+} mice have persistent post-natal growth retardation, markedly

Table 1 Skeletal phenotypes of TH receptor mutant mice

Model	Genotype	Endocrine status	Juvenile skeleton	Adult skeleton	Skeletal thyroid status	References
TRα mutants TR α ^{-/-}	No TR α 1/ α 2 TR $\Delta\alpha$ 1 and $\Delta\alpha$ 2 preserved	Hypothyroid (T ₄ 0.1 × T ₃ 0.4 ×, TSH 2 ×) GH normal	Severe growth delay; delayed endochondral ossification; impaired chondrocyte differentiation; reduced mineralisation	Die by weaning unless T ₃ treated	NR	Chassande <i>et al.</i> (1997), Fraichard <i>et al.</i> (1997) and Gauthier <i>et al.</i> (1999)
	TR α 1 ^{-/-}	Mild hypothyroidism (T ₄ 0.7 × T ₃ 1 ×, TSH 0.8 ×)	No growth retardation of skeletal phenotype	NR	NR	Wikstrom <i>et al.</i> (1998)
	TR α 2 ^{-/-}	Mild hypothyroidism (T ₄ 0.8 × T ₃ 0.7 ×, TSH 1 ×)	No growth retardation; normal endochondral ossification	Reduced bone mineral density; reduced cortical bone	NR	Salto <i>et al.</i> (2001)
	TR α 1/ $\Delta\alpha$ 1 preserved; TR α 1 over-expression No TR α transcripts TR β unaffected	0.8 × T ₃ 0.7 ×, TSH 1 × GH normal IGF1 low Euthyroid; normal GH and IGF1	Transient growth delay; delayed endochondral ossification; impaired chondrocyte differentiation; reduced mineralisation	Osteosclerosis; increased trabecular bone volume; reduced osteoclastic bone resorption	Hypothyroid	Gauthier <i>et al.</i> (2001) and Bassett <i>et al.</i> (2007b)
TR α 1 ^{PV/+}	Heterozygous dominant-negative TR α receptor	Mild thyroid failure (T ₄ 1 × T ₃ 1.2 ×, TSH 1.7 ×) GH normal	Severe persistent growth retardation; delayed intramembranous and endochondral ossification; impaired chondrocyte differentiation; reduced mineralisation	NR	Hypothyroid	Kaneshige <i>et al.</i> (2001) and O'Shea <i>et al.</i> (2005)
TR α 1 ^{R384C/+}	Heterozygous dominant-negative TR α receptor (10-fold reduced affinity for T ₃)	Euthyroid adults; mild hypothyroidism P10-35 (T ₄ 0.8 × T ₃ 0.7 ×, TSH 0.7 ×) (GH reduced in juveniles)	Transient growth delay; delayed intramembranous and endochondral ossification; impaired chondrocyte differentiation	Osteosclerosis; increased trabecular bone volume; increased mineralisation; reduced osteoclastic bone resorption	Hypothyroid	Tinnikov <i>et al.</i> (2002) and Bassett <i>et al.</i> (2007a)
TR α 1 ^{R398H/+}	Heterozygous dominant-negative TR α receptor	Euthyroid (T ₄ 1.1 × T ₃ 1.1 ×, TSH 3.4 ×)	NR	NR	NR	Liu <i>et al.</i> (2003)
TR α 1 ^{AM1/+} SYCP1-Cre	Cre-inducible dominant-negative receptor TR α 1 ^{L400R} (early global expression)	Euthyroid (T ₄ 1 × T ₃ 0.9 ×, TSH 0.3 ×) Reduced GH (0.4 ×)	Severe persistent growth retardation; delayed endochondral ossification	NR	NR	Quignodon <i>et al.</i> (2007)

(continued)

Table 1 Continued

Genotype	Endocrine status	Juvenile skeleton	Adult skeleton	Skeletal thyroid status	References
TR β mutants TR $\beta^{-/-}$	RTH and goitre (T_4 4 $\times$, T_3 4 $\times$, TSH 8 $\times$) ¹ (T_4 3 $\times$, T_3 3 $\times$, TSH 2.6 $\times$) ²	Persistent short stature; advanced endochondral and intramembranous ossification; increased mineralisation No reported growth abnormality	Osteoporosis; reduced mineralisation; increased osteoclastic bone resorption	Thyrotoxic	Forrest <i>et al.</i> (1996), ¹ Gauthier <i>et al.</i> (2001), ² Ng <i>et al.</i> (2001) and Bassett <i>et al.</i> (2007a,b)
TR $\beta 2^{-/-}$	Mild RTH (T_4 3 $\times$, T_3 1.3 $\times$, TSH 2.5 $\times$) ¹ Small decrease in GH (T_4 1 $\times$, T_3 1.5 $\times$, TSH 1.2 $\times$) ²		NR	NR	¹ Abel <i>et al.</i> (1999) and ² Ng <i>et al.</i> (2001)
TR $\beta^{PV/PV}$	Severe RTH and goitre (T_4 15 $\times$, T_3 9 $\times$, TSH > 400 $\times$); reduced GH	Accelerated prenatal growth; persistent postnatal growth retardation; advanced intramembranous and endochondral ossification; increased mineralisation No growth phenotype	NR	Thyrotoxic	Kaneshige <i>et al.</i> (2000), O'Shea <i>et al.</i> (2003, 2005) and Bassett <i>et al.</i> (2007a)
TR $\beta^{\Delta 377T/\Delta 377T}$	Severe RTH and goitre (T_4 15 $\times$, T_3 10 $\times$, TSH > 50 $\times$)		NR	NR	Hashimoto <i>et al.</i> (2001)
TR α and TR β compound mutants TR $\alpha^{-/-}\beta^{-/-}$	RTH and small goitre (T_4 10 $\times$, T_3 10 $\times$, TSH > 100 $\times$)	Growth delay similar to TR $\alpha^{-/-}$; delayed endochondral ossification; impaired chondrocyte differentiation; reduced mineralisation Persistent growth retardation; delayed endochondral ossification; reduced mineralisation	Die at or near weaning	NR	Gauthier <i>et al.</i> (1999) and Bassett <i>et al.</i> (2007b)
TR $\alpha 1^{-/-}\beta^{-/-}$	RTH and large goitre (T_4 60 $\times$, T_3 60 $\times$, TSH > 160 $\times$); reduced GH/IGF1		Reduced trabecular and cortical bone mineral density. GH treatment corrects growth but not ossification defect	NR	Gothe <i>et al.</i> (1999), Kindblom <i>et al.</i> (2001), O'Shea <i>et al.</i> (2003, 2005) and Kindblom <i>et al.</i> (2005)
TR $\alpha 2^{-/-}\beta^{-/-}$	Mild hypothyroidism (T_4 0.7 $\times$, T_3 0.8 $\times$, TSH 1 $\times$)	Transient growth delay	NR	NR	Ng <i>et al.</i> (2001)
TR $\alpha^{0/0}\beta^{-/-}$	RTH and goitre (T_4 13 $\times$, T_3 14 $\times$, TSH > 200 $\times$); reduced GH/IGF1	More severe phenotype than TR $\alpha^{0/0}$; growth delay; delayed endochondral ossification; impaired chondrocyte differentiation; reduced mineralisation	NR	Hypothyroid	Gauthier <i>et al.</i> (2001) and Flamant <i>et al.</i> (2002)

(continued)

Table 1 Continued

Genotype	Endocrine status	Juvenile skeleton	Adult skeleton	Skeletal thyroid status	References
TR α , TR β and Pax8 compound mutants Pax8 ^{-/-} Apo TR α and TR β receptors	No thyroid (No T ₄ or T ₃ , TSH 1900 ×)	More severe growth defect than TR α ^{0/0} β ^{-/-} ; severe persistent growth retardation; severely delayed endochondral ossification; impaired chondrocyte differentiation	Majority die by weaning; coarse plate-like trabeculae with impaired trabecular remodelling; reduced cortical thickness; reduced mineralisation	Hypothyroid	Mansouri <i>et al.</i> (1998), Flamant <i>et al.</i> (2002) and Bassett <i>et al.</i> (2008)
Pax8 ^{-/-} TR α 1 ^{-/-}	No T ₃ or TR α 1/ $\Delta\alpha$ 1 TR α 2/ $\Delta\alpha$ 2 preserved Apo TR β receptors	Severe growth retardation similar to Pax8 ^{-/-} ; skeletal phenotype not reported	Die by weaning	NR	Mittag <i>et al.</i> (2005)
Pax8 ^{-/-} TR α ^{0/0}	No thyroid (No T ₄ or T ₃ , TSH >400 ×)	Growth retardation less than Pax8 ^{-/-} and similar to TR α ^{0/0} β ^{-/-} ; delayed endochondral ossification; mice survive to adulthood	NR	NR	Flamant <i>et al.</i> (2002)
Pax8 ^{-/-} TR β ^{-/-}	No thyroid (No T ₄ or T ₃ , TSH >400 ×)	Severe growth retardation similar to Pax8 ^{-/-} ; severely delayed endochondral ossification	Die by weaning	NR	Flamant <i>et al.</i> (2002)

TR, T₃ receptor; T₄, thyroxine; T₃, triiodothyronine; NR, not reported; IGF1, insulin-like growth factor 1; RTH, resistance to thyroid hormone; P10-35, postnatal day 10 to 35.

Figure 2 Skeletal development and growth in TR mutant mice. (A) Skull vaults from P1 mutant mice and littermate controls stained with alizarin red (bone) and alcian blue (cartilage) showing sutures and anterior and posterior fontanelles. Arrows indicate delayed intramembranous ossification in $TR\alpha^{PV/+}$ and $TR\alpha^{R384C/+}$ mice and advanced ossification in $TR\beta^{-/-}$ and $TR\beta^{PV/PV}$ mice. Note that two $TR\beta^{-/-}$ strains in different genetic backgrounds were analysed $TR\beta^{-/-}$ (a) (Gauthier *et al.* 2001, Bassett *et al.* 2007b) and $TR\beta^{-/-}$ (b) (Forrest *et al.* 1996, Ng *et al.* 2001, Bassett *et al.* 2007a). Scale bar, 1mm. (B) Graphs of longitudinal growth in mutant mice and littermate controls; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ mean tibia/ulna length in mutant versus control; two-tailed Student's *t*-test.

delayed endochondral ossification, decreased mineralisation, reduced cortical bone thickness and impaired intramembranous ossification (O'Shea *et al.* 2005). Similar findings have been reported in mice expressing the potent dominant-negative receptor $TR\alpha^{L400R}$ (Quignodon *et al.* 2007). Consistent with the delayed bone development in juvenile $TR\alpha^{0/0}$, $TR\alpha^{R384C/+}$ and $TR\alpha^{PV/+}$ mice, skeletal expression of the T_3 -target genes, fibroblast growth factor receptors 1 and 3 (FGFR1/3; Stevens *et al.* 2003, Barnard *et al.* 2005, O'Shea *et al.* 2007) were reduced (Barnard *et al.* 2005, O'Shea *et al.* 2005, Bassett *et al.* 2007a,b). Deletion or mutation of $TR\alpha$ does not affect systemic thyroid status but causes local skeletal hypothyroidism while the presence of a dominant-negative $TR\alpha$ leads to a more severe skeletal phenotype than receptor deficiency alone.

Two $TR\beta^{-/-}$ strains have been generated in different genetic backgrounds and both show similar skeletal phenotypes (Table 1; Forrest *et al.* 1996, Gauthier *et al.* 1999). Deletion of $TR\beta$ results in elevated circulating TSH, T_4 and T_3 levels consistent with resistance to thyroid hormone (RTH). In contrast to $TR\alpha$ mutants, juvenile $TR\beta^{-/-}$ mice display advanced

endochondral ossification, accelerated chondrocyte differentiation, increased mineral deposition and persistent short stature due to premature growth plate quiescence. Furthermore, cortical thickness is increased and intramembranous ossification advanced in $TR\beta^{-/-}$ mice (Fig. 2; Bassett *et al.* 2007b). $TR\beta^{PV/PV}$ mice express a potent dominant-negative $TR\beta$ and display severe RTH with a 400-fold elevation of TSH and 15-fold elevation of T_4 . $TR\beta^{PV/PV}$ animals exhibit a more severe phenotype than $TR\beta^{-/-}$ mice with accelerated intrauterine growth characterised by advanced endochondral and intramembranous ossification. Premature ossification results in persistent post-natal growth retardation, premature growth plate quiescence, increased mineral deposition and craniosynostosis (O'Shea *et al.* 2003). Consistent with advanced skeletal development in $TR\beta^{-/-}$ and $TR\beta^{PV/PV}$ mice, expression of the T_3 -target genes *Fgfr1* and *Fgfr3* was increased (O'Shea *et al.* 2003, Barnard *et al.* 2005, Bassett *et al.* 2007a,b). Thus, deletion or mutation of $TR\beta$ disrupts the HPT axis resulting in increased circulating thyroid hormone levels and skeletal thyrotoxicosis. The presence of a dominant-negative $TR\beta$ leads to a greater elevation of systemic

thyroid hormone concentration and a more severe skeletal phenotype than receptor deficiency alone.

In the developing skeleton, reduced T_3 action in $TR\alpha$ mutant mice results in delayed ossification and reduced mineralisation whereas increased T_3 action in $TR\beta$ mutant mice leads to advanced ossification and increased mineralisation. Thus, during growth, T_3 actions in bone are anabolic.

Thyroid hormone actions in adult bone are catabolic

Adult thyrotoxicosis results in both increased bone resorption and formation, but uncoupling of these processes favours osteoclastic resorption and leads to a 10% net bone loss per remodelling cycle (Mosekilde *et al.* 1990). Consequently, thyrotoxicosis is an important cause of secondary osteoporotic fracture (Mosekilde *et al.* 1990, Cummings *et al.* 1995, Franklyn *et al.* 1998, Vestergaard *et al.* 2000, Vestergaard & Mosekilde 2002, Murphy & Williams 2004) and even subclinical hyperthyroidism has been associated with decreased bone mineral density and increased fracture risk in postmenopausal women (Bauer *et al.* 2001, Quan *et al.* 2002, Murphy & Williams 2004, Heemstra *et al.* 2006, Kim *et al.* 2006, Morris 2007).

Remarkably, delayed ossification and reduced mineralisation in juvenile $TR\alpha^{0/0}$ mice were accompanied by greatly increased trabecular bone mass in adults (Fig. 3; Bassett *et al.* 2007b). Moreover, the robust and plate-like trabeculae contained highly mineralised calcified cartilage indicating a trabecular bone remodelling defect. Consistent with such a defect, $TR\alpha^{0/0}$ mice displayed reduced osteoclast numbers and activity (Bassett *et al.* 2007b). Trabecular bone mass increased progressively with age in $TR\alpha^{R384C/+}$ mice with adults showing osteosclerosis (Bassett *et al.* 2007a). Consistent with a remodelling defect, trabeculae were of increased thickness and connectivity, showed increased mineralisation with extensive retention of calcified cartilage and reduced osteoclast numbers and activity (Fig. 4). Remarkably, brief T_3 supplementation during growth, sufficient to overcome transcriptional repression by $TR\alpha^{R384C}$, ameliorated the adult skeletal phenotype (Bassett *et al.* 2007a; Table 1). These data indicate that during development even transient relief from the transcriptional repression mediated by unliganded $TR\alpha$ (apo- $TR\alpha$) has long-term consequences for adult bone structure and mineralisation. Thus, in the adult skeleton, deletion or mutation of $TR\alpha$ results in persistently impaired bone remodelling. Similarly, the presence of a dominant-negative $TR\alpha$ leads to a more severe skeletal phenotype than receptor deficiency alone.

Despite a juvenile phenotype of accelerated growth and increased ossification, adult $TR\beta^{-/-}$ mice became progressively osteoporotic (Fig. 3; Bassett *et al.* 2007a).

Figure 3 Trabecular bone micro-architecture in adult TR mutant mice. (A) Backscattered electron scanning electron microscopy (BSE-SEM) views showing trabecular bone in thyrotoxic wild-type (WT) mice treated with daily sc injections of T_3 (30 ng T_3 /gram body weight) and congenitally hypothyroid $Pax8^{-/-}$ mice. (B) Animals lacking either $TR\alpha$ or $TR\beta$ and (C) mice harbouring a dominant-negative mutation of $TR\alpha$ (R384C). Scale bar, 200 μ m.

Two $TR\beta^{-/-}$ strains in different genetic backgrounds were analysed (Gauthier *et al.* 2001, Bassett *et al.* 2007b). In one strain, cortical and trabecular bone mineralisation were both reduced and osteoclast numbers and activity increased (Bassett *et al.* 2007a,b). However, in the other strain, only trabecular bone mineralisation was affected but this was also accompanied by an increase in osteoclast numbers and activity (Bassett *et al.* 2007a; Fig. 4). Thus, in the adult skeleton, deletion of $TR\beta$ results in accelerated remodelling and bone loss although the severity may depend on genetic background.

In the adult skeleton reduced T_3 action in $TR\alpha$ mutant mice results in osteosclerosis whereas increased T_3 action in $TR\beta$ mutant mice leads to osteoporosis. Thus, the actions of T_3 are catabolic in adult bone.

Mechanism of T_3 action

The opposing skeletal phenotypes in $TR\alpha$ and $TR\beta$ mutant mice provide compelling evidence for the complex interaction between central and peripheral

Figure 4 Trabecular bone micro-mineralisation in adult TR mutant mice. Quantitative backscattered electron scanning electron microscopy (qBSE-SEM) images showing mineralisation densities of trabecular bone from mice lacking TRβ and mice harbouring a dominant-negative mutation of TRα (R384C) (A). Mineralisation densities were derived from halogenated standards and images pseudo-coloured according to the palette shown in which high mineralisation density is grey. (B) Relative and cumulative frequency histograms of bone micro-mineralisation densities are shown. Black arrow indicates the increased relative frequency of high mineralisation densities that correspond to retained calcified cartilage in TRα1^{R384C/+} mice. (C) Higher power views of trabecular bone are shown. Dashed white arrows indicate normal bone cement lines. Solid white arrows indicate retained calcified cartilage within which the outlines of chondrocyte lacunae remain evident. Scale bar 200 μm in all panels. ****P* < 0.001 micro-mineralisation density in mutant versus WT; Kolmogorov–Smirnov test.

thyroid status (Fig. 5). Thus, delayed ossification and impaired bone remodelling in TR α mutant mice are secondary to the disruption of T₃ action in bone, whereas advanced skeletal development and osteoporosis in TR β mutant mice are due to disruption of the HPT axis, elevated systemic thyroid hormone levels and local supraphysiological stimulation of TR α in bone (O'Shea *et al.* 2006). This model is supported by T₃-target gene expression in skeletal cells of TR mutant mice and by the demonstration of higher levels of TR α mRNA expression in bone compared with TR β (O'Shea *et al.* 2003, 2005, Barnard *et al.* 2005, Bookout *et al.* 2006, Bassett *et al.* 2007a,b). Nevertheless, it is apparent that TR α ^{0/0} TR β ^{-/-} mice have a more severe skeletal phenotype than TR α ^{0/0} mice, while TR α ^{0/0} mice also remain sensitive to T₄ treatment, thus suggesting a residual role for TR β in skeletal cells. In this context, quantitative RT-PCR analysis has shown that TR α expression is 10- to 100-fold greater than TR β expression in adult whole bone (O'Shea *et al.* 2003, Bookout *et al.* 2006). However, since the temporospatial patterns of TR α and TR β expression in skeletal cells are unknown, a role for TR β is possible. Furthermore, it is unclear whether individual skeletal cell co-express both TR isoforms or whether their patterns of expression are cell type specific.

Present understanding is that TR α 1 is functionally predominant in bone.

T₃ action in the developing skeleton

In vitro T₃ inhibits chondrocyte proliferation and promotes differentiation (Robson *et al.* 2000, Shao *et al.* 2006; Fig. 6). Since growth plate architecture and linear growth are frequently disrupted in TR mutant mice, key regulators of endochondral ossification have been investigated as targets of thyroid hormone action.

GH and insulin-like growth factor 1 signalling pathways are thought to act as a point of convergence for many growth promoting factors since more than 80% of somatic growth can be attributable to the actions of the GH/IGF1 axis (Lupu *et al.* 2001). IGF1 is the major determinant of post-natal growth, it mediates both GH-dependent and -independent effects and is implicated in chondrocyte recruitment, proliferation and hypertrophic differentiation (van der Eerden *et al.* 2003). Examination of the GH/IGF1 pathway revealed that GH receptor (GHR) and *Igf1* receptor (IGF1R) mRNA expression was reduced in growth plate chondrocytes in TR α ^{0/0} and TR α 1^{PV/+} mice. Furthermore, phosphorylation of their secondary messengers signal transducer and activator of transcription 5 (STAT5) and protein kinase B (AKT) was also impaired (O'Shea *et al.* 2005, Bassett *et al.* 2007b). By contrast, GHR and IGF1R expression was increased in TR β ^{-/-}

and TR β ^{PV/PV} mice (O'Shea *et al.* 2005, Bassett *et al.* 2007b). Thus, GH/IGF1 signalling is also a local downstream mediator of T₃ action in the growth plate (Fig. 6).

FGFs and their receptors have key roles in skeletal development with activating mutations of FGFR3 causing achondroplasia, the commonest form of genetic dwarfism. In the developing growth plate, FGFR3 is expressed in reserve and proliferating chondrocytes and negatively regulates their proliferation and differentiation (Murakami *et al.* 2004). By contrast, FGFR1 is expressed in prehypertrophic and hypertrophic chondrocytes and its location suggests a role in differentiation, matrix synthesis and apoptosis (Ornitz 2005). FGFR1 is also expressed in the osteoblast lineage with activating mutations result in Pfeiffer craniosynostosis. Investigation of the FGF/FGFR signalling pathway in TR mutant mice demonstrated that *Fgfr3* and *Fgfr1* expression was reduced in growth plates of TR α ^{0/0}, TR α 1^{R384C/+} and TR α 1^{PV/+} mice and *Fgfr1* expression was reduced in osteoblasts from TR α ^{0/0} and Pax8^{-/-} mice (Stevens *et al.* 2003, Barnard *et al.* 2005, O'Shea *et al.* 2005, Bassett *et al.* 2007a,b, 2008). By contrast, *Fgfr3* and *Fgfr1* expression was increased in growth plates of TR β ^{-/-} and TR β ^{PV/PV} mice and *Fgfr1* expression was increased in osteoblasts from TR β ^{PV/PV} mice (O'Shea *et al.* 2003, Barnard *et al.* 2005, Bassett *et al.* 2007a,b). Thus, FGF/FGFR signalling is a downstream mediator of T₃ action in chondrocytes and osteoblasts (Fig. 6).

The pace of chondrocyte differentiation is precisely regulated by the Indian hedgehog/parathyroid hormone-related peptide paracrine (Ihh/PTHrP) negative feedback loop. Prehypertrophic chondrocytes secrete Ihh which diffuses to periarticular cells to induce synthesis of PTHrP. PTHrP, acting via its receptor PTHrP1, then completes the loop by stimulating chondrocyte proliferation and inhibiting further hypertrophic differentiation (Vortkamp *et al.* 1996, Dentice *et al.* 2005). Although this pathway has not been studied in TR mutant mice, previous experiments in thyroid-manipulated rats demonstrated increased *PTHrP* mRNA expression in hypothyroid animals and decreased *PTHrP* receptor mRNA expression in growth plates of thyrotoxic animals (Stevens *et al.* 2000). Furthermore, recent studies in chicken tibia explants have shown that Ihh stimulates degradation of the type 2 deiodinase enzyme resulting in an induction of PTHrP expression (Dentice *et al.* 2005). Together, these findings suggesting that thyroid hormone can inhibit chondrocyte proliferation and promote differentiation by local regulation of the Ihh/PTHrP negative feedback loop (Fig. 6).

T₃ is essential for normal cartilage matrix synthesis. Heparan sulphate proteoglycans (HSPGs) are a key matrix component and are essential for functional

FGF/FGFR signalling and extracellular diffusion of *Ihh*. Studies in thyroid manipulated rats and $TR\alpha^{0/0}\beta^{-/-}$ and $Pax8^{-/-}$ mice revealed reduced HSPG expression in thyrotoxic animals, increased expression in $TR\alpha^{0/0}\beta^{-/-}$ mice and more markedly increased

expression in hypothyroid rats and congenitally hypothyroid $Pax8^{-/-}$ mice (Bassett *et al.* 2008).

These studies suggest T_3 coordinately regulates FGF/FGFR and *Ihh*/PTHrP signalling within the growth plate.

T₃ actions in the bone remodelling cycle

In vitro studies suggest that thyroid hormone stimulates osteoblast proliferation, differentiation and apoptosis by direct and indirect actions. Thus, T₃ increases synthesis of osteocalcin, type 1 collagen, alkaline phosphatase and matrix metalloproteinases 9 and 13 (Pereira *et al.* 1999, Huang *et al.* 2000, Gouveia *et al.* 2001, Bassett & Williams 2003) and also regulates IGF1, parathyroid hormone (PTH) and FGF signalling (Huang *et al.* 2000, Gu *et al.* 2001, Pepene *et al.* 2001, Bassett & Williams 2003, Stevens *et al.* 2003, O'Shea *et al.* 2007; Fig. 6). *In vivo*, activation of FGFR1 stimulates osteoblast proliferation and differentiation (Zhou *et al.* 2000). Consistent with this, *Fgfr1* expression in osteoblasts is reduced in the hypothyroid skeleton and increased in thyrotoxic bone (O'Shea *et al.* 2003, Stevens *et al.* 2003, Bassett *et al.* 2007a). Although osteoclasts have been reported to express TRs (Allain *et al.* 1992, Abu *et al.* 1997, 2000, Kanatani *et al.* 2004), it remains uncertain whether T₃ regulates osteoclast differentiation directly or indirectly (Siddiqi *et al.* 1998, Miura *et al.* 2002, Varga *et al.* 2004). Previous studies are contradictory; some demonstrating that T₃ acts directly in osteoclasts while others report indirect effects mediated via osteoblasts.

Future directions

Analyses of TR mutant and congenitally hypothyroid Pax8^{-/-} mice have identified a key role for thyroid hormone and TRα1 in both the developing skeleton and adult bone. However, it remains unclear how the skeletal effects of apo-TRα1 differ from those of TRα1 deficiency and to what extent the skeletal effects of thyroid hormones result from central, systemic and local actions of T₃. The more severe skeletal phenotype observed in congenitally hypothyroid Pax8^{-/-} mice as compared with TRα^{0/0}β^{-/-} mice suggests that unliganded TRs may be more detrimental to skeletal development than TR deficiency (Mansouri *et al.* 1998, Flamant *et al.* 2002, Bassett *et al.* 2008; Table 1). In support of this, amelioration of the Pax8^{-/-} skeletal phenotype in Pax8^{-/-}TRα^{0/0} mice but not in Pax8^{-/-}TRβ^{-/-} mice suggests that some of the

detrimental effects of hypothyroidism are mediated by unliganded TRα1 in bone (Flamant *et al.* 2002). Despite this, it is important to note from an additional study (Mittag *et al.* 2005) that deletion of TRα1 alone in Pax8^{-/-}TRα1^{-/-} mice did not prevent weight loss, early mortality and pituitary abnormalities although the skeletal consequences were not investigated. Thus, it remains possible that TRα2 has an additional and essential role in the manifestation of the Pax8^{-/-} phenotype. This importance of apo-TRα1 is further supported by the more severe skeletal phenotype present in mice harbouring dominant-negative mutations of TRα1 (TRα1^{R384C/+} and TRα1^{PV/+}) as compared with mice lacking all TRα isoforms (TRα^{0/0}; O'Shea *et al.* 2005, Bassett *et al.* 2007a,b) and by the amelioration of the adult skeletal phenotype in TRα1^{R384C/+} mice following a transient reversal of TRα1R384C apo-receptor activity during development (Bassett *et al.* 2007a). Nevertheless, a complete understanding of the molecular mechanism of thyroid hormone action in bone will require at least two experimental approaches. First, it is clear that phenotyping of the existing mouse models is incomplete and more detailed studies including quantitative histomorphometry, mechanical testing and analysis of primary bone cell cultures will help to clarify the picture. However, such an approach cannot identify the cellular targets of T₃ action in the skeleton *in vivo* and this will require the use of cell-specific gene-targeting strategies.

Analysis of TRα and TRβ mutant mice has demonstrated the complex relationship between central and peripheral thyroid status and established the predominant role of TRα1 in bone. These studies also highlight contrasting responses of the skeleton to T₃ during developing and in adulthood. Although understanding of the molecular mechanism of T₃ action in bone is still limited, coordinate regulation of key signalling pathways has now been identified in chondrocytes and osteoblasts. By contrast, the molecular mechanism of T₃ action in the osteoclast lineage remains unclear. A more detailed understanding of the molecular basis of T₃ action in bone will provide the rational for the development of novel strategies for the prevention and treatment of osteoporosis.

Figure 5 Proposed molecular mechanism for TRα and TRβ mutant mice. The pituitary expresses predominantly TRβ. T₃ and TRβ act via a negative TRE to repress TSH transcription. The skeleton expresses predominantly TRα1 and T₃ acts via TRα1 to activate target gene expression. Since the levels of TRα in the pituitary are low, its absence in TRα^{0/0} mice does not disrupt pituitary negative feedback and systemic TSH, T₄ and T₃ concentrations are normal. By contrast, because TRα predominates in bone, its absence in TRα^{0/0} mice results in impaired T₃ responsiveness, skeletal hypothyroidism and reduced expression of T₃-target genes. Similarly, in TRα1^{PV/+} and TRα1^{R384C/+} mice, the levels of the dominant-negative TRα receptor in the pituitary are insufficient to interfere with TRβ and disrupt TSH repression. However, in bone, the higher concentrations of mutant receptor interfere with the actions of wild-type TRα1. Thus, T₃ responses are severely impaired and the skeleton is hypothyroid. In TRβ^{-/-} mice, by contrast, the absence of TRβ disrupts pituitary T₃ responses and impairs TSH repression leading to high circulating TSH, T₄ and T₃ concentrations. Since TRβ levels are low in bone, T₃ responses are unaffected in TRβ^{-/-} mice and high circulating levels of TH act via wild-type TRα1 to induce skeletal thyrotoxicosis. Similarly in TRβ^{PV/PV} mice, the dominant-negative TRβ disrupts TSH repression in the pituitary and results in grossly elevated TSH, T₄ and T₃ concentrations. The low levels of the mutant receptor in bone are insufficient to interfere with TRα1 and impair T₃ responses and thus high circulating levels of TH increase expression of T₃-target genes resulting in severe skeletal thyrotoxicosis.

Figure 6 Role of TH in endochondral ossification and bone remodelling. (A) The role of T₃ in the growth plate is illustrated and the pattern of TR expression is shown. Reserve cells undergo clonal expansion to form columns of proliferating chondrocytes that secrete cartilage matrix stained blue in this section. Committed prehypertrophic chondrocytes undergo hypertrophic differentiation, enlarge and subsequently apoptose, the residual mineralised cartilage matrix forming the scaffold for trabecular bone formation. In chondrocyte cultures, T₃ inhibits proliferation and increases the expression of cyclin-dependent kinase inhibitors P21^{clp-1} P27^{kip-1}. T₃ stimulates matrix synthesis and expression of the differentiation markers collagen X, alkaline phosphatase (ALP), matrix metalloproteinase 13 (MMP13) and aggrecanase 2. GH/insulin-like growth factor 1 (GH/IGF1), Indian hedgehog/parathyroid hormone-related peptide (Ihh/PTHrP) and fibroblast growth factor/fibroblast growth factor receptor (FGF/FGFR) pathways are T₃ targets. Expression of heparan sulphate proteoglycans (HSPGs), which are essential for FGF and Ihh signalling, is negatively regulated by T₃. (B) The role of T₃ in the bone remodelling cycle, which is characterised by osteoclastic bone resorption followed by osteoblast migration, osteoid matrix deposition and mineralisation, is shown. Osteoclast differentiation requires direct osteoblast–osteoclast interaction mediated by receptor activator of nuclear factor-κB (RANK) and its ligand RANKL. Osteoprotegerin (OPG) is a decoy receptor secreted by osteoblasts that antagonises this interaction. Osteoclast differentiation also requires co-stimulation by osteoblast-derived cytokines. Thyroid hormone stimulates osteoblast proliferation, differentiation and apoptosis directly and also indirectly by regulating IGF1 and FGF signalling. It remains uncertain whether osteoclast differentiation is regulated directly by T₃ or indirectly via T₃ actions in osteoblasts.

Declaration of interest

There is no conflict of interest.

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