Growth-inhibiting conditions slow growth plate senescence

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Abstract

The mammalian growth plate undergoes programed senescence during juvenile life, causing skeletal growth to slow with age. We previously found that hypothyroidism in rats slowed both growth plate chondrocyte proliferation and growth plate senescence, suggesting that senescence is not dependent on age *per se* but rather on chondrocyte proliferation. However, one alternative explanation is that the observed slowing of growth plate senescence is a specific consequence of hypothyroidism. We reasoned that, if delayed senescence is a general consequence of growth inhibition, rather than a specific result of hypothyroidism, then senescence would also be slowed by other growth-inhibiting conditions. In this study, we therefore used tryptophan deficiency to temporarily inhibit growth in newborn rats for 4 weeks. We then allowed the animals to recover and studied the effects on growth plate senescence. We found that structural, functional, and molecular markers of growth plate senescence were delayed by prior tryptophan deficiency, indicating that the developmental program of senescence had occurred more slowly during the period of growth inhibition. Taken together with previous studies in hypothyroid rats, our findings support the hypothesis that delayed senescence is a general consequence of growth inhibition and hence that growth plate senescence is not simply a function of time *per se* but rather depends on growth.

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Introduction

Mammalian body length is primarily determined by longitudinal bone growth that occurs at the growth plate, which is a thin layer of cartilage found near the ends of long bones. The process of longitudinal bone growth occurs by endochondral ossification, in which cartilage is formed and then remodeled into bone tissue (Kronenberg 2003). The growth plate is spatially organized into three layers: the resting zone, the proliferative zone, and the hypertrophic zone (Schenk & Hunziker 1991). The resting zone chondrocytes are stem-like cells capable of generating new clones of proliferative chondrocytes (Abad *et al.* 2002). The proliferative zone and hypertrophic zone contain clones of chondrocytes, arranged in columns parallel to the long axis of the bone. The proliferative chondrocytes have a high rate of replication, and when they get farther from the epiphysis they stop dividing and become hypertrophic chondrocytes (Walker & Kember 1972). Hypertrophic chondrocytes later undergo apoptosis, and the hypertrophic cartilage is invaded by blood vessels, osteoclasts, and differentiating osteoblasts, which remodel the cartilage into bone tissue (Kronenberg 2003).

Longitudinal bone growth is rapid in early life but gradually slows with age, until growth velocity eventually approaches zero in adulthood. The decline in longitudinal bone growth with age is associated with functional, structural, and molecular changes in the growth plate known as growth plate senescence (Nilsson & Baron 2004). These changes include a decline in the chondrocyte proliferation rate (Walker & Kember 1972), overall growth plate height, proliferative and hypertrophic zone heights, column density, and extensive changes in gene expression (Gafni *et al.* 2001, Schrier *et al.* 2006, Marino *et al.* 2008, Lui *et al.* 2010*a*).

The physiological process that drives growth plate senescence is not known. One possibility is that senescence is a function of time *per se*, implying the existence of a biological clock in the growth plate. Another possibility is that senescence is actually a function of growth. For example, growth plate chondrocytes might have a finite proliferative capacity, which is gradually exhausted, leading to the decline in growth rate and other senescent changes in the growth plate. The latter hypothesis that senescence is a function of growth can be tested by investigating whether inhibition of longitudinal bone growth affects growth plate senescence. If senescence were a function of growth, then growth

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inhibition would be expected to slow senescence; if senescence were simply a function of time, then growth inhibition would not slow senescence. We previously showed that inhibiting bone growth in newborn rats by inducing hypothyroidism slowed both growth plate chondrocyte proliferation as well as growth plate senescence, suggesting that senescence is driven by growth (Marino *et al.* 2008). However, one alternative explanation for these findings is that the observed slowing of growth plate senescence is a specific consequence of hypothyroidism, which exerts complex and widespread effects on growth plate cartilage, rather than a general consequence of growth inhibition.

The goal of this study is to determine whether delayed growth plate senescence is a specific consequence of hypothyroidism or a more general consequence of growth inhibition. We therefore used another growth-inhibiting condition, tryptophan deficiency, to temporarily suppress growth in newborn rats for 4 weeks. Afterwards, growth in these rats was allowed to recover by switching to a replete diet. These rats were then studied to determine whether the prior period of growth inhibition had slowed growth plate senescence by examining structural, functional, and molecular markers of growth plate senescence.

Materials and Methods

Animal care

All animal procedures were approved by the National Institute of Child Health and Human Development Animal Care and Use Committee (National Research Council 2003).

Animal procedures for normal development assessment

Sprague–Dawley rats (Harlan, Indianapolis, IN, USA) received standard rodent chow (Zeigler Bros, Gardners, PA, USA) and water, made available *ad libitum*. Male rats (six per time point) were killed by carbon dioxide inhalation at ages 1, 3, 6, 9, and 12 weeks. To study the growth plate without the complicating effects of sex steroids, 3-, 6-, 9-, and 12-week-old rats were castrated at 18 days of age. Proximal tibial growth plates were excised, embedded in optimum cutting temperature (OCT) compound (Electron Microscopy Sciences, Hatfield, PA, USA), and stored at −80 °C until subsequent microdissection.

Animal procedures for growth inhibition by tryptophan deficiency

Sprague–Dawley rats were obtained from Charles River Laboratories (Wilmington, MA, USA) and provided with food and water *ad libitum*. Growth was inhibited by providing a tryptophan deficient (Trp^-) diet to the lactating mother from birth of the male pups until they reached 4 weeks of age as previously described (Lui *et al.* 2010*b*). Male pups of lactating mothers receiving a diet with adequate tryptophan for normal nutritional needs were used as controls. At 4 weeks of age, all animals were switched to regular chow. Control and treated animals were killed at 1, 3, 4, 5, 6, 8, 12, 16, and 20 weeks of age and tibial length was measured using a digital caliper. From each animal, one proximal tibial growth plate was excised, embedded in OCT compound, and stored at −80 °C until subsequent microdissection and the other was fixed in formalin for quantitative histology.

Animal procedures for growth inhibition by hypothyroidism

Sprague–Dawley rats were obtained from Harlan Laboratories and provided with food and water *ad libitum*. Growth was inhibited in newborn male pups by introducing propylthiouracil (PTU, supplied by Sigma–Aldrich) into the drinking water of the mother at a concentration 1 g/l beginning when the pups were 1 day of age as previously described (Marino *et al.* 2008). PTU treatment was discontinued at 5 weeks of age. The PTU-treated animals were not weaned during the experiment because preliminary studies showed that they were not mature enough to support their own nutritional needs. Pups from lactating mothers not receiving PTU were used as controls and weaned at the age of 3 weeks. All rats were given depot leuprolide acetate (6 mg/kg, s.c.; Tap Pharmaceuticals, Deerfield, IL, USA) every 3 weeks, starting at 3 weeks of age. Leuprolide acetate is a long-acting GnRH agonist that downregulates gonadotropin secretion and thus gonadal steroid production (Ogawa *et al.* 1989). Control and PTU-treated animals were killed and studied after the end of the treatment period, at 7 and 9 weeks of age ($n=6$, each age group). The proximal tibiae growth plate was excised and frozen in dry ice, stored at −80 °C until subsequent microdissection.

Growth plate microdissection, RNA extraction

Growth plate microdissection was performed as previously described (Nilsson *et al.* 2007). Briefly, 50 μm frozen sections were thawed, fixed in ethanol, stained with eosin, dehydrated, and placed in xylene. Using an inverted microscope, the proliferative plus early hypertrophic zone was collected by manual microdissection. RNA (100–150 ng) was extracted from growth plate samples as previously described (Nilsson *et al.* 2007). RNA quality was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

Quantitative real-time PCR

RNA was reverse transcribed using Superscript III reverse transcriptase (Invitrogen) following the manufacturer's instructions. Quantitative real-time PCR was performed using commercially available 6-carboxyfluorescein (FAM) or VIC-labeled Taqman assays, or custom-designed primers coupled with SYBR green real-time PCR reagent (Applied Biosystems, Foster City, CA, USA). The assay ID or sequence

information for different assays and primers are as follow: *18S*, 4319413E; insulin-like growth factor-binding protein 7 (*Igfbp7*), Rn01413246_m1; HtrA serine peptidase 1 (*Prss11*), Rn00581870_m1; PYD and CARD domain containing (*Pycard*), Rn00597229_g1; retinoid X receptor, gamma (*RxRg*), Rn01483466_m1; *Igf2*, Rn01454518_m1; calcitonin (*Calca*), Trp^- experiment: Rn01511353_g1, hypothyroidism experiment: Rn00569199_m1; cyclin-dependent kinase inhibitor 2A (*Cdkn2a*), Trp^- experiment: Rn00580664_m1, hypothyroidism experiment: forward primer 5′-GGGTCACCGACAGGCATAAC, reverse primer 5′-GCCTAACTTA GCGCTGCTTTG; ankyrin-repeat and suppressor of cytokine signaling box-containing protein 4 (*Asb4*), Trp^- experiment: Rn01501048_m1, hypothyroidism experiment: forward primer 5′-TC-GTCTGTGCCAAGCAGTTG, reverse primer 5′-CC-TGGTTGTTGGTTTTCATGTTC. Reactions were performed in triplicate on cDNA derived from individual animals using the ABI prism 7900 Sequence Detection System instrument (Applied Biosystems). The relative quantity of each mRNA was calculated using the formula: relative expression $= 2^{-\Delta C_t} \times 10^9$, where C_t represents the threshold cycle and $\Delta C_t = (C_t \text{ of gene of interest}) - (C_t \text{ of } 18S \text{ rRNA})$. Values were multiplied by 10^9 for convenience of comparison. For the comparison between Trp^- animals and controls, three to five animals were studied per treatment and time point. For the comparison between hypothyroid animals and controls, six animals were studied per treatment and time point.

Quantitative histology

Growth plate samples fixed in formalin were decalcified in 0·5 M pH 7·4 EDTA (KD Medical, Columbia, MD, USA), embedded in paraffin, cut into 10 μm sections, and stained with Masson's trichrome stain. ScanScope CS digital scanner (Aperio Technologies, Inc., Vista, CA, USA) was used to visualize and analyze the stained sections. Measurements were taken as previously described (Marino *et al.* 2008) with some modifications. The following measurements were made by an observer blinded to the age and treatment group: overall growth plate height, measured from the margin of the metaphyseal bone to the margin of the epiphyseal bone; resting zone height, measured from the edge of the margin of the epiphyseal bone to the first cell of a proliferative column; hypertrophic zone height, measured from the margin of the methaphyseal bone to the last cell bigger or equal to 10 μm; proliferative zone height, measured from the first cell minor to 10 μm to the first resting chondrocytes; the number of resting zone chondrocytes, assessed as the number of cells per 200 μm growth plate width (measured parallel to the epiphyseal margin); column density, assessed as the number of proliferative columns per 500 μm growth plate (measured parallel to the metaphyseal margin); and the height of the terminal hypertrophic chondrocyte lacuna (the intact lacuna closest to the metaphyseal bone). All histological measurements were performed in the central two-thirds of the growth plate sections for avoiding regions close to the perichondrium. Heights were measured parallel to the chondrocyte columns in three areas of each growth plate section and averaged. Column density was calculated in two areas per growth plate section. The number of proliferative and hypertrophic cells was counted in three to four intact columns per growth plate. Hypertrophic chondrocytes were operationally defined by a height ≥ 10 μm. The terminal hypertrophic cell height was measured in eight to ten different columns per growth plate section. For each animal, three different growth plate sections were analyzed and then all measurements from that animal were averaged. Four to five animals were studied per treatment and time point.

Statistical analysis

The SigmaStat 3.1 statistical program was used to perform all statistical measurements. One-way ANOVA was used to assess the effect of age during normal development and in untreated controls. Two-way ANOVA was used to assess the effect of prior treatment in the hypothyroidism and tryptophan-deficient experiments. For these analyses, we analyzed time points after the end of the treatment period using time and prior treatment as independent variables. All real-time PCR data were log transformed prior to statistical testing.

Results

Tryptophan deficiency delayed structural and functional senescent changes in the growth plate

Early postnatal growth was delayed in rats from birth until 4 weeks of age by providing a Trp^- diet to the lactating mother. At the end of the 4-week treatment period, the tibial length was 16·5 ± 0·4 mm in Trp^- rats versus 26·6 ± 0·2 mm in control rats fed with a replete diet ($P < 0·001$). The rats were then placed on normal chow, allowed to recover, and then assessed to determine whether the prior period of tryptophan deficiency and growth inhibition had delayed growth plate senescence. In control animals, many structural features of the growth plate changed significantly ($P < 0·05$) with age (Fig. 1, solid lines), including the growth plate height, the proliferative zone height, the number of proliferative chondrocytes per column, and the number of resting zone chondrocytes per 200 μm. Other structural features (such as resting zone height), however, did not show significant changes, which might be due to the limited age range (4–8 weeks) of this study. In animals that had previously been tryptophan deficient, most of these changes were significantly delayed compared to controls (Fig. 1A) except for the number of hypertrophic zone chondrocytes per column ($P = 0·053$) and column

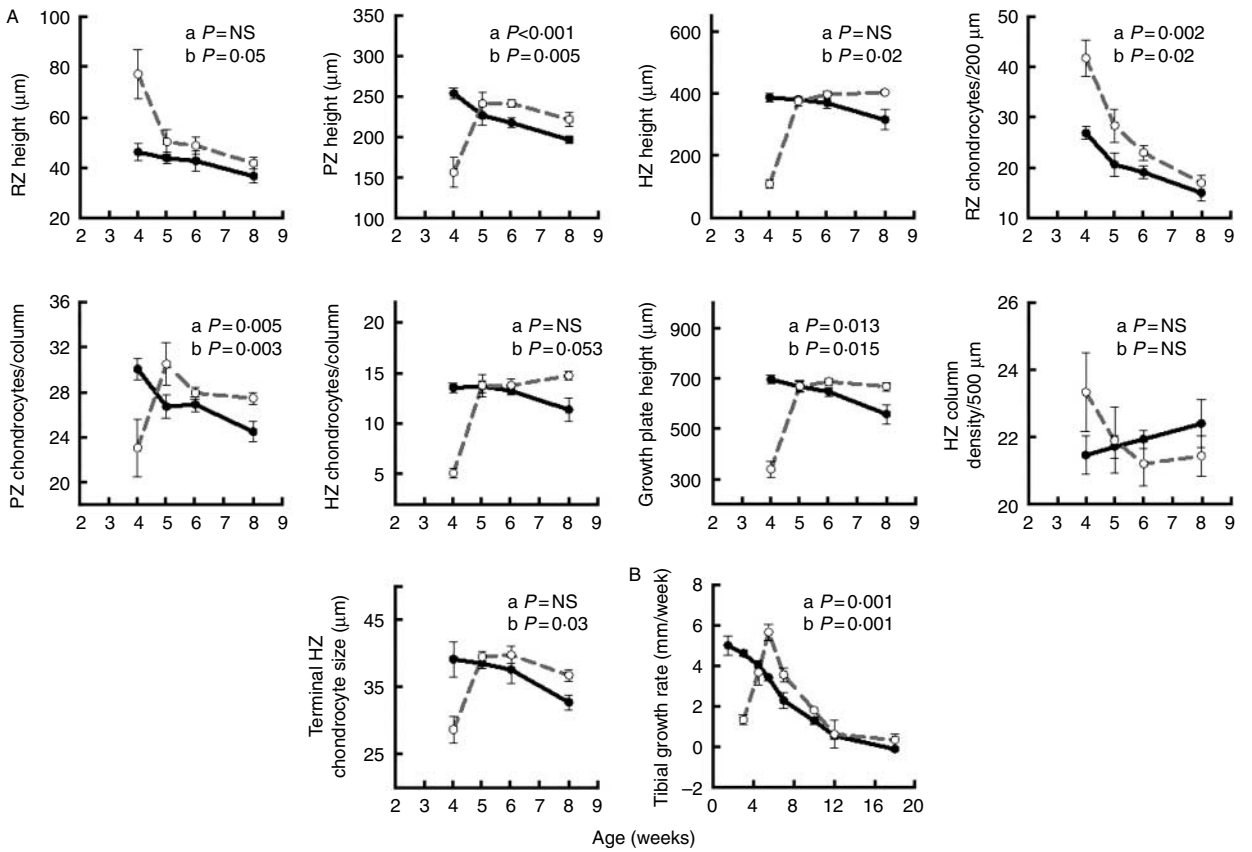

Figure 1 Structural and functional senescent changes in the growth plate were delayed by prior tryptophan deficiency. Markers of growth plate senescence were compared in rat proximal tibia's growth plate Masson's trichrome-stained sections for control or untreated rats (solid lines) and rats that received a Trp^- diet from birth to 4 weeks of age (dotted line). Between 4 and 5 weeks of age, the Trp^- rats were going through a transition between being growth inhibited and resuming normal growth. (A) Structural markers were measured at different ages, including resting, proliferative, and hypertrophic zones height; number of resting zone chondrocytes per 200 μm of growth plate width; number of proliferative and hypertrophic chondrocytes per column; growth plate height; number of hypertrophic columns or column density per 500 μm of growth plate width; and terminal hypertrophic chondrocytes height. Each experimental group contained four to five animals. (B) Rate of proximal tibia growth per week, defined as the increase in proximal tibial height from the previous time point divided by the number of weeks between the two time points. a, effect of age, one-way ANOVA; b, effect of treatment during the recovery period, two-way ANOVA. RZ, resting zone; PZ, proliferative zone; HZ, hypertrophic zone; NS, not significant.

density. The delay in structural changes also was evident by visual inspection of the growth plate sections (Fig. 2). For example, the overall growth plate height during tryptophan deficiency (at 4 weeks of age) was much shorter than in controls, but after the tryptophan deficiency resolved (at 6–8 weeks of age), the growth plate height exceeded the height in control animals. Similarly, the tibial growth rate, which is a functional marker of growth plate senescence, declined in control animals ($P<0·001$; Fig. 1B) and was delayed by prior tryptophan deficiency ($P<0·001$; Fig. 1B).

Tryptophan deficiency delayed senescence-associated changes in gene expression

We next asked whether tryptophan deficiency also delayed senescence-associated changes in gene expression. We recently reported that hundreds of genes show significant changes in gene expression with postnatal age in the rat proliferative zone plus early hypertrophic zone, as assessed by expression microarray analysis (Lui *et al*. 2010*a*). Based on that expression data (GEO accession number GSE16981), we focused on five genes that showed increasing expression with age (Table 1): *Igfbp7*; *Pycard*, which is involved in apoptotic signaling pathways; *Cdkn2a*, a tumor suppressor gene; *Prss11*, which regulates availability of IGFs; and *Calca*, which regulates extracellular calcium. We also studied three molecular markers that decrease their expression during senescence (Table 1), including *Igf2*; *Asb4*; and *RxRg*, which modulates the actions of multiple nuclear receptors. Firstly, we used real-time PCR to verify the microarray findings. In the control animals, real-time PCR confirmed that all these genes showed significant ($P<0·05$) changes in expression with age (Fig. 3, left column of graphs). We then asked whether the animals that were previously Trp^- but had then

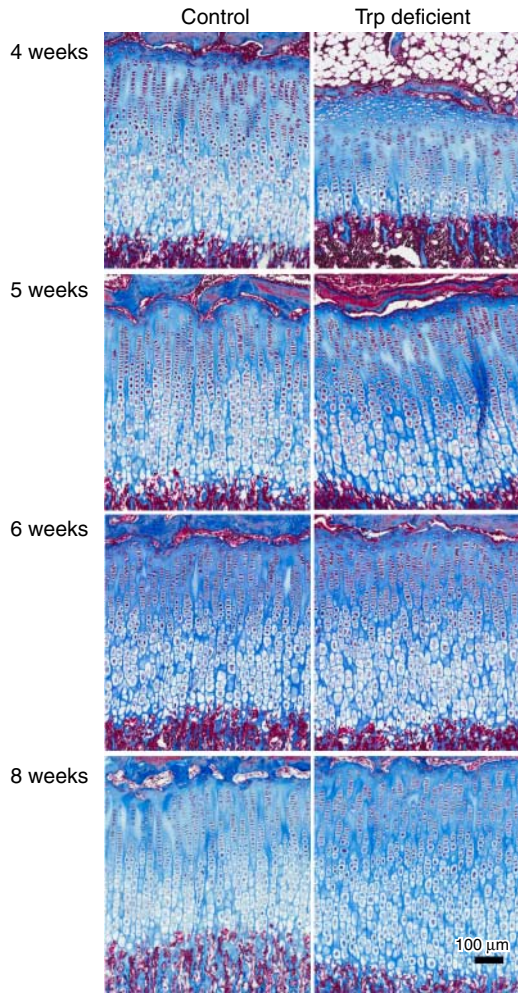

Figure 2 Growth plate structure in 4-, 5-, 6-, and 8-week-old control and Trp^- animals. Photomicrographs of Masson's trichrome-stained longitudinal sections of proximal tibial growth plate from control and Trp^- animals at different ages. During the period of growth inhibition (4 weeks), the overall growth plate height in the Trp^- animals was smaller than the control animals, despite having significantly more resting chondrocytes. At ~5 weeks of age, the experimental group was in the process of recovering from the growth inhibition (see Fig. 1B), which may explain why the growth plate height media was similar to that of control animals. Afterwards, at 6 and 8 weeks of age, the growth plate height was greater in animals that had previously received a Trp^- diet than in control animals. Each experimental group contained four to five animals. Bar represents 100 µm. Full colour version of this figure available via http://dx.doi.org/10.1677/JOE-10-0302.

resumed normal growth showed a delay in the age-dependent changes in gene expression compared to controls. At 5–8 weeks of age, the expression of *Igfbp7*, *Calca*, *Prss11*, and *Pycard* was significantly ($P<0{\cdot}05$) lower in the previously Trp^- animals than in controls. As the expression of these genes normally rises during senescence, the findings indicate that prior tryptophan deficiency delayed the normal increase (Fig. 3A, middle column of graphs). Conversely, for *Igf2*, prior tryptophan deficiency delayed the normal decrease in expression (Fig. 3B, middle column of graphs). For *Cdkn2a*, *Asb4*, and *RxRg*, gene expression did not differ significantly between control and treated animals during the recovery period. However, for these three genes, the changes in expression in the control group were modest during the time period studied. Therefore, a delayed change in expression in the experimental group might be more difficult to detect.

Growth inhibition due to hypothyroidism also delayed senescence-associated changes in gene expression

We then used a second model of growth inhibition and assessed the effects on the same set of genes. Newborn rats were rendered hypothyroid by addition of PTU to the drinking water. We previously demonstrated that this intervention does cause hypothyroidism, growth inhibition, and delay of structural and functional markers of growth plate senescence (Marino *et al.* 2008). In this study, we shortened the duration of the PTU treatment from 0 to 8 weeks to 0 to 5 weeks, and studied the effects of gene expression. mRNA levels were measured by real-time PCR at 7 and 9 weeks of age, that is after the removal of PTU from drinking water at 5 weeks. The normal senescence-associated changes in expression were delayed significantly ($P<0{\cdot}05$) by prior hypothyroidism for *Igfbp7*, *Prss11*, *Calca*, *Pycard*, *Igf2*, and *Asb4*. Thus, hypothyroidism, similar to tryptophan deficiency, caused a delay in most of the senescence-associated genes studied. Expression of *Cdkn2a* and *RxRg* was not significantly different in control versus treated animals. However, for these two genes, there was mininal change in expression in the control group during the time period studied, which might make a delayed change in expression more difficult to detect.

Discussion

We previously showed that hypothyroidism slows both growth plate chondrocyte proliferation and growth plate senescence in newborn rats (Marino *et al.* 2008), suggesting that growth plate senescence is a function of growth. However, one alternative explanation is that the observed effect on growth plate senescence is a specific consequence of hypothyroidism, which exerts complex and widespread effects on growth plate cartilage (Nilsson *et al.* 2005*a*), including direct effects on proliferation and hypertrophy (Stevens *et al.* 2000), and indirect effects mediated by decreased GH (Martin *et al.* 1985, Katakami *et al.* 1986) and IGF1 (Kindblom *et al.* 2001). If delayed growth plate senescence is a general consequence of growth inhibition, rather than a specific consequence of hypothyroidism, then senescence would also be delayed by other growth-inhibiting conditions. We therefore used a second model of growth inhibition in this study by inducing tryptophan deficiency in newborn rats. We then allowed the animals to recover and studied the effects on growth

Table 1 Changes in gene expression during senescence by Affymetrix Rat Genome Array 230 2.0

	Fold change* (3 to 6 weeks)	***P* value** (3 vs 6 weeks)	**Fold change*** (3 to 9 weeks)	***P* value** (3 vs 9 weeks)	**Fold change*** (3 to 12 weeks)	***P* value** (3 vs 12 weeks)
Gene symbol						
Igfbp7	2·490	$2{\cdot}54\times10^{-3}$	4·296	$2{\cdot}11\times10^{-5}$	5·010	$5{\cdot}39\times10^{-6}$
Htra1	2·236	$3{\cdot}50\times10^{-4}$	3·568	$1{\cdot}56\times10^{-6}$	4·486	$1{\cdot}60\times10^{-7}$
Pycard	4·018	$8{\cdot}61\times10^{-6}$	8·18	$2{\cdot}98\times10^{-8}$	8·286	$1{\cdot}96\times10^{-8}$
Cdkn2a	1·677	$2{\cdot}10\times10^{-3}$	2·564	$3{\cdot}83\times10^{-6}$	2·887	$7{\cdot}22\times10^{-7}$
Calca	2·009	$8{\cdot}18\times10^{-4}$	3·617	$8{\cdot}30\times10^{-7}$	4·182	$1{\cdot}69\times10^{-7}$
Igf2	−43·204	$3{\cdot}46\times10^{-11}$	−78·823	$1{\cdot}90\times10^{-11}$	−77·605	$1{\cdot}41\times10^{-12}$
Rxrg	−1·748	$7{\cdot}58\times10^{-5}$	−1·933	$6{\cdot}71\times10^{-6}$	−1·961	$3{\cdot}97\times10^{-6}$
Asb4	−5·871	$1{\cdot}66\times10^{-8}$	−11·083	$1{\cdot}27\times10^{-10}$	−9·948	$1{\cdot}67\times10^{-10}$

*Negative fold changes indicate declining expression with age.

plate senescence. We found that structural, functional, and molecular markers of growth plate senescence were delayed by prior tryptophan deficiency, indicating that the developmental program of senescence had occurred more slowly during the period of growth inhibition. Taken together, our findings in the hypothyroidism model and in the tryptophan-deficient model support the hypothesis that delayed senescence is a general consequence of growth inhibition.

The tryptophan-deficient model that we chose in this study may inhibit bone growth through multiple mechanisms. Firstly, it has been shown that single amino acid restriction may inhibit the nutrient-sensing mechanistic target of rapamycin pathway in the growth plate (Kim *et al.* 2009), which inhibits chondrocyte proliferation and differentiation (Phornphutkul *et al.* 2008, 2009, Kim *et al.* 2009). Secondly, tryptophan deficiency causes depletion of brain serotonin (Biggio *et al.* 1974) and thereby diminishes appetite and food intake, causing malnutrition (D'Souza *et al.* 2004). This generalized malnutrition could in turn have systemic effects on the growth plate, in part through diminished GH and IGF1 levels (Heinrichs *et al.* 1997, Gat-Yablonski *et al.* 2009). Hypothyroidism has a direct effect on growth plate but can also decrease GH and IGF1 levels (Nilsson *et al.* 2005*a*). Therefore, there may be some overlap in the mechanisms by which tryptophan deficiency and hypothyroidism affect the growth plate and thus the two models may not be completely independent. However, the fact that both tryptophan deficiency and hypothyroidism, as well as a third model using dexamethasone excess in rabbit, though with more limited data (Baron *et al.* 1994, Gafni *et al.* 2001), all showed delayed growth plate senescence, strongly support the hypothesis that delayed growth plate senescence is a general consequence of growth inhibition, which in turn suggests that growth plate senescence is not simply a function of time *per se* but rather depends on growth.

The underlying cellular mechanisms by which growth may drive senescence remain to be elucidated. Several lines of argument suggest that growth plate senescence may be dependent on the proliferative history of the resting zone chondrocytes. Firstly, the resting chondrocyte appears to be the only long-term cellular resident in the growth plate because proliferative chondrocytes differentiate into hypertrophic chondrocytes, which then undergo apoptosis (Kronenberg 2003). Therefore, the resting chondrocyte is a good candidate to store long-term information regarding the growth history of the growth plate. Secondly, the resting zone chondrocytes appear to act as a stem-like cell in the postnatal growth plate, capable of generating new clones of proliferative and hypertrophic chondrocytes (Abad *et al.* 2002). Therefore, senescence-associated changes in the resting chondrocyte could affect its proliferative and hypertrophic cellular progeny, thus accounting for the widespread changes characteristic of growth plate senencences. Thirdly, with age, there is a gradual decline in the number of resting chondrocytes as well as in resting chondrocyte proliferation, which may contribute to growth plate senescence (Schrier *et al.* 2006). Thus, resting zone chondrocytes may have a finite proliferative capacity, which is gradually exhausted. Consequently, growth-inhibiting conditions, by slowing resting zone chondrocyte proliferation, may preserve the future proliferative potential of these progenitor cells and thereby slow senescence.

Similarly, the molecular mechanism by which growth may advance the senescence program is unknown. One possibility is that proliferation leads to alterations in epigenetic marks, which are known to participate in cellular memory (Ringrose & Paro 2004, Bantignies & Cavalli 2006). Limited evidence suggests that the level of DNA methylation in the resting zone chondrocytes decreases with age (Nilsson *et al.* 2005*b*) but it is unclear whether this observation is causally related to their replicative potential. In other juvenile tissues, as somatic growth slows, there is a decline in histone H3K4 methylation in the promoter region of growth-promoting genes, which may lead to their downregulation (Lui *et al.* 2010*b*).

A postnatal decline in growth rate is observed not only in the long bones, but also in many other organs. It is yet unclear how growth in different parts of the body slows concordantly to maintain body proportion. In transplantation experiments, growth of the transplanted organs generally depends on the age of the donor, suggesting that the growth deceleration in other organs may involve a local mechanism similar to that of the growth plate (Cooke *et al.* 1986, Pape *et al.* 2006). Interestingly, recent studies suggest that growth deceleration

in the liver, kidney, lung, and heart depends on a genetic program involving the downregulation of multiple growth-promoting genes and suggest that this program can be delayed by prior growth inhibition (Lui *et al*. 2008, 2010*b*, Finkielstain *et al*. 2009). Thus, the mechanism slowing growth of nonskeletal tissues also appears to be driven by growth, rather than by time alone. It will be intriguing to determine whether the two processes – growth plate senescence and growth deceleration in the soft tissue – is governed by similar mechanisms, and if certain types of cells in the soft tissue may function as 'stem-like' progenitor cells analogous to the resting zone chondrocytes in the growth plate.

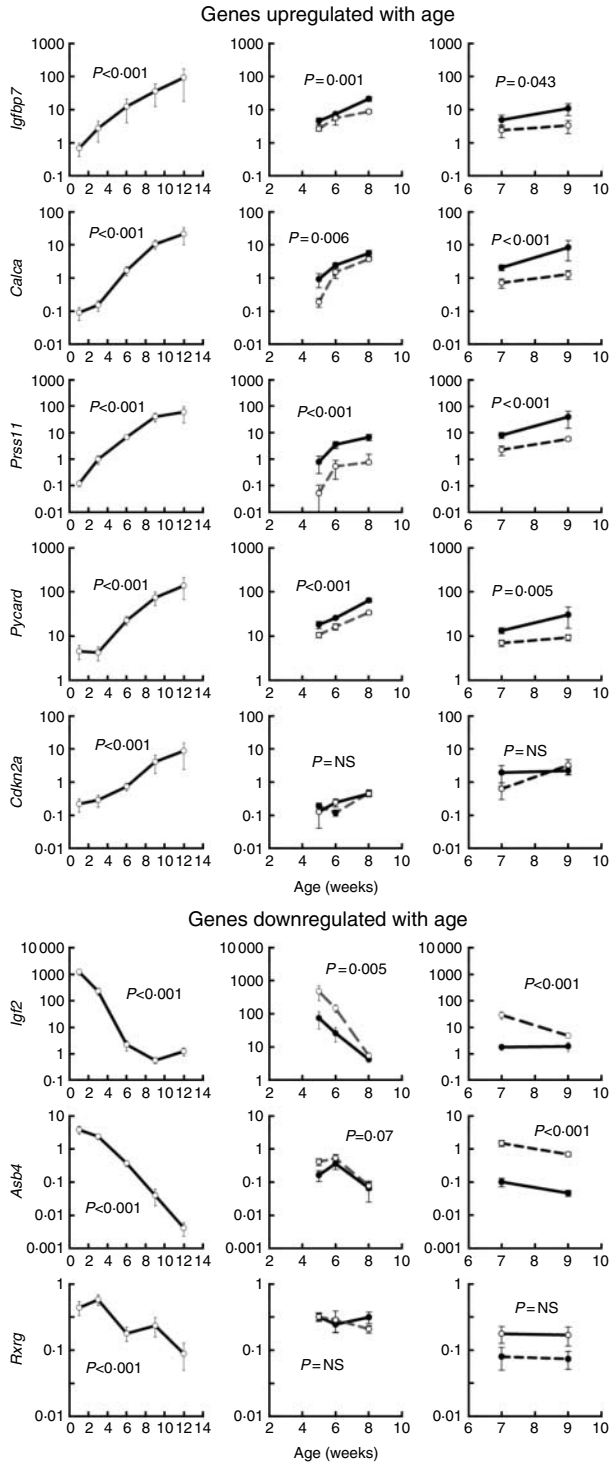

Figure 3 Prior tryptophan deficiency or prior hypothyroidism delayed senescent changes of gene expression in the growth plate. mRNA levels were measured by real-time PCR at various ages in castrated male rats (left column) and in rats that had previously received a tryptophan-deficient diet (dotted line, middle column) or received PTU to induce hypothyroidism (dotted line, right column) along with corresponding controls (solid lines). Each experimental group contained three to six animals. NS, not significant.

Finally, our findings also provide important insights into the mechanism of catch-up growth. As previously suggested (Nilsson & Baron 2004), delayed growth plate senescence may partly explain catch-up growth, which refers to accelerated growth above the normal rate for age after remission from growth inhibitory conditions. It is yet unclear whether catch-up growth after different types of growth inhibition is explained by delayed growth plate senescence. This hypothesis has previously been studied in animal models involving hypothyroidism and glucocorticoid excess to inhibit growth (Gafni *et al*. 2001, Marino *et al*. 2008). One clinical study has shown that the pattern of catch-up growth in children recovering from celiac disease is consistent with the hypothesis of delayed growth plate senescence (Emons *et al*. 2005). In this study, we found that animals previously receiving a Trp^- diet had tibial growth rates higher than the control animals (Fig. 1B). The Trp^- animals appeared to have a delayed decline in tibial growth rate of ~2 weeks (Fig. 1B). Thus, for example, at 9 weeks of age, the rats exhibited a rate of longitudinal growth similar to that of a 7-week-old control animal. Notably, this delay of ~2 weeks was similar in magnitude to the delay in structural and molecular markers of the growth plates. Therefore, our findings here provide strong support for the hypothesis that catch-up growth after malnutrition can be explained at least in part by a delay in the program of growth plate senescence. Taken together with previous findings that delayed growth plate senescence contributes to catch-up growth after hypothyroidism and glucocorticoid excess, our data suggest that delayed growth plate senescence is a general mechanism that contributes to catch-up growth after a variety of growth-inhibiting conditions.

In summary, we used tryptophan deficiency to temporarily inhibit growth in newborn rats from birth until 4 weeks of age. We then studied structural, functional, and molecular markers of growth plate senescence and found that they were delayed by prior tryptophan deficiency. This finding indicates that the developmental program of senescence had occurred more slowly during the period of growth inhibition.

Taken together with previous studies in hypothyroid rats, our findings support the hypothesis that delayed senescence is a general consequence of growth inhibition, suggesting that growth plate senescence is not simply a function of time *per se* but rather depends on growth.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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References

Abad V, Meyers JL, Weise M, Gafni RI, Barnes KM, Nilsson O, Bacher JD & Baron J 2002 The role of the resting zone in growth plate chondrogenesis. *Endocrinology* **143** 1851–1857. (doi:10.1210/en.143.5.1851)

Bantignies F & Cavalli G 2006 Cellular memory and dynamic regulation of polycomb group proteins. *Current Opinion in Cell Biology* **18** 275–283. (doi:10.1016/j.ceb.2006.04.003)

Baron J, Klein KO, Colli MJ, Yanovski JA, Novosad JA, Bacher JD & Cutler GB Jr 1994 Catch-up growth after glucocorticoid excess: a mechanism intrinsic to the growth plate. *Endocrinology* **135** 1367–1371. (doi:10.1210/en.135.4.1367)

Biggio G, Fadda F, Fanni P, Tagliamonte A & Gessa GL 1974 Rapid depletion of serum tryptophan, brain tryptophan, serotonin and 5-hydroxyindoleacetic acid by a tryptophan-free diet. *Life Sciences* **14** 1321–1329. (doi:10.1016/0024-3205(74)90440-8)

Cooke PS, Yonemura CU, Russell SM & Nicoll CS 1986 Growth and differentiation of fetal rat intestine transplants: dependence on insulin and growth hormone. *Biology of the Neonate* **49** 211–218. (doi:10.1159/000242533)

D'Souza DN, Zhang Y, Garcia F, Battaglia G & Van de Kar LD 2004 Fluoxetine-induced changes in body weight and 5-HT1A receptor-mediated hormone secretion in rats on a tryptophan-deficient diet. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology* **286** R390–R397. (doi:10.1152/ajpregu.00335.2003)

Emons JA, Boersma B, Baron J & Wit JM 2005 Catch-up growth: testing the hypothesis of delayed growth plate senescence in humans. *Journal of Pediatrics* **147** 843–846. (doi:10.1016/j.jpeds.2005.07.033)

Finkielstain GP, Forcinito P, Lui JC, Barnes KM, Marino R, Makaroun S, Nguyen V, Lazarus JE, Nilsson O & Baron J 2009 An extensive genetic program occurring during postnatal growth in multiple tissues. *Endocrinology* **150** 1791–1800. (doi:10.1210/en.2008-0868)

Gafni RI, Weise M, Robrecht DT, Meyers JL, Barnes KM, De-Levi S & Baron J 2001 Catch-up growth is associated with delayed senescence of the growth plate in rabbits. *Pediatric Research* **50** 618–623. (doi:10.1203/00006450-200111000-00014)

Gat-Yablonski G, Yackobovitch-Gavan M & Phillip M 2009 Nutrition and bone growth in pediatrics. *Endocrinology and Metabolism Clinics of North America* **38** 565–586. (doi:10.1016/j.ecl.2009.07.001)

Heinrichs C, Colli M, Yanovski JA, Laue L, Gerstl NA, Kramer AD, Uyeda JA & Baron J 1997 Effects of fasting on the growth plate: systemic and local mechanisms. *Endocrinology* **138** 5359–5365. (doi:10.1210/en.138.12.5359)

Katakami H, Downs TR & Frohman LA 1986 Decreased hypothalamic growth hormone-releasing hormone content and pituitary responsiveness in hypothyroidism. *Journal of Clinical Investigation* **77** 1704–1711. (doi:10.1172/JCI112490)

Kim MS, Wu KY, Auyeung V, Chen Q, Gruppuso PA & Phornphutkul C 2009 Leucine restriction inhibits chondrocyte proliferation and differentiation through mechanisms both dependent and independent of mTOR signaling. *American Journal of Physiology. Endocrinology and Metabolism* **296** E1374–E1382. (doi:10.1152/ajpendo.91018.2008)

Kindblom JM, Gothe S, Forrest D, Tornell J, Tornell J, Vennstrom B & Ohlsson C 2001 GH substitution reverses the growth phenotype but not the defective ossification in thyroid hormone receptor alpha 1−/−beta−/− mice. *Journal of Endocrinology* **171** 15–22. (doi:10.1677/joe.0.1710015)

Kronenberg HM 2003 Developmental regulation of the growth plate. *Nature* **423** 332–336. (doi:10.1038/nature01657)

Lui JC, Finkielstain GP, Barnes KM & Baron J 2008 An imprinted gene network that controls mammalian somatic growth is down-regulated during postnatal growth deceleration in multiple organs. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology* **295** R189–R196. (doi:10.1152/ajpregu.00182.2008)

Lui JC, Andrade AC, Forcinito P, Hegde A, Chen W, Baron J & Nilsson O 2010*a* Spatial and temporal regulation of gene expression in the mammalian growth plate. *Bone* **46** 1380–1390. (doi:10.1016/j.bone.2010.01.373)

Lui JC, Forcinito P, Chang M, Chen W, Barnes KM & Baron J 2010*b* Coordinated postnatal down-regulation of multiple growth-promoting genes: evidence for a genetic program limiting organ growth. *FASEB Journal* **24** 3083–3092. (doi:10.1096/fj.09-152835)

Marino R, Hegde A, Barnes KM, Schrier L, Emons JA, Nilsson O & Baron J 2008 Catch-up growth after hypothyroidism is caused by delayed growth plate senescence. *Endocrinology* **149** 1820–1828. (doi:10.1210/en.2007-0993)

Martin D, Epelbaum J, Bluet-Pajot MT, Prelot M, Kordon C & Durand D 1985 Thyroidectomy abolishes pulsatile growth hormone secretion without affecting hypothalamic somatostatin. *Neuroendocrinology* **41** 476–481. (doi:10.1159/000124222)

Nilsson O & Baron J 2004 Fundamental limits on longitudinal bone growth: growth plate senescence and epiphyseal fusion. *Trends in Endocrinology and Metabolism* **15** 370–374. (doi:10.1016/j.tem.2004.08.004)

Nilsson O, Marino R, De Luca F, Phillip M & Baron J 2005*a* Endocrine regulation of the growth plate. *Hormone Research* **64** 157–165. (doi:10.1159/000088791)

Nilsson O, Mitchum RD Jr, Schrier L, Ferns SP, Barnes KM, Troendle JF & Baron J 2005*b* Growth plate senescence is associated with loss of DNA methylation. *Journal of Endocrinology* **186** 241–249. (doi:10.1677/joe.1.06016)

Nilsson O, Parker EA, Hegde A, Chau M, Barnes KM & Baron J 2007 Gradients in bone morphogenetic protein-related gene expression across the growth plate. *Journal of Endocrinology* **193** 75–84. (doi:10.1677/joe.1.07099)

Ogawa Y, Okada H, Heya T & Shimamoto T 1989 Controlled release of LHRH agonist, leuprolide acetate, from microcapsules: serum drug level profiles and pharmacological effects in animals. *Journal of Pharmacy and Pharmacology* **41** 439–444.

Pape L, Hoppe J, Becker T, Ehrich JH, Neipp M, Ahlenstiel T & Offner G 2006 Superior long-term graft function and better growth of grafts in children receiving kidneys from paediatric compared with adult donors. *Nephrology, Dialysis, Transplantation* **21** 2596–2600. (doi:10.1093/ndt/gfl119)

Phornphutkul C, Wu KY, Auyeung V, Chen Q & Gruppuso PA 2008 mTOR signaling contributes to chondrocyte differentiation. *Developmental Dynamics* **237** 702–712. (doi:10.1002/dvdy.21464)

Phornphutkul C, Lee M, Voigt C, Wu KY, Ehrlich MG, Gruppuso PA & Chen Q 2009 The effect of rapamycin on bone growth in rabbits. *Journal of Orthopaedic Research* **27** 1157–1161. (doi:10.1002/jor.20894)

Ringrose L & Paro R 2004 Epigenetic regulation of cellular memory by the Polycomb and Trithorax group proteins. *Annual Review of Genetics* **38** 413–443. (doi:10.1146/annurev.genet.38.072902.091907)

Schenk RK & Hunziker EB 1991 Growth plate: histophysiology, cell and matrix turnover. In *Rickets*, pp 63–76. Ed. FH Glorieux. New York, NY: Vevey/Raven Press.

Schrier L, Ferns SP, Barnes KM, Emons JA, Newman EI, Nilsson O & Baron J 2006 Depletion of resting zone chondrocytes during growth plate senescence. *Journal of Endocrinology* **189** 27–36. (doi:10.1677/joe.1.06489)

Stevens DA, Hasserjian RP, Robson H, Siebler T, Shalet SM & Williams GR 2000 Thyroid hormones regulate hypertrophic chondrocyte differentiation and expression of parathyroid hormone-related peptide and its receptor during endochondral bone formation. *Journal of Bone and Mineral Research* **15** 2431–2442. (doi:10.1359/jbmr.2000.15.12.2431)

Walker KV & Kember NF 1972 Cell kinetics of growth cartilage in the rat tibia. I. Measurements in young male rats. *Cell and Tissue Kinetics* **5** 401–408. (doi:10.1111/j.1365-2184.1972.tb00378.x)

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