Persistent expression of Twist1 in chondrocytes causes growth plate abnormalities and dwarfism in mice

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ABSTRACT Evidence from various in vitro gain and loss of function studies indicate that the bHLH transcription factor Twist1 negatively regulates chondrocyte differentiation; however limited information regarding Twist1 function in postnatal cartilage development and maintenance is available. Twist1 expression within the postnatal growth plate is restricted to immature, proliferating chondrocytes, and is significantly decreased or absent in hypertrophic chondrocytes. In order to examine the effect of maintaining the expression of Twist1 at later stages of chondrocyte differentiation, we used type II collagen Cre (Col2-Cre) mice to activate a Cre-inducible Twist1 transgene specifically in chondrocytes (Col2-Twist1). At two weeks, postnatal growth was inhibited in Col2-Twist1 mice, as evidenced by limb shortening. Histological examination revealed abnormal growth plate structure, characterized by poor columnar organization of proliferating cartilaginous cells, decreased cellularity, and expansion of the hypertrophic zone. Moreover, structural defects within the growth plates of Col2-Twist1 transgenic mice included abnormal vascular invasion and focal regions of bony formation. Quantitative analysis of endochondral bone formation via micro-computed topography revealed impaired trabecular bone formation in the hindlimbs of Col2-Twist1 transgenic mice at various timepoints of postnatal development. Taken together, these findings indicate that regulated Twist1 expression contributes to growth plate organization and endochondral ossification to modulate postnatal longitudinal bone growth.

KEY WORDS: twist1, chondrocyte, growth plate

Transition of chondrocytes from proliferation to terminal maturation within the growth plate is vital for longitudinal bone growth. Proliferating, immature chondrocytes synthesize an extracellular matrix (ECM) abundant in type II collagen and aggrecan. As cells differentiate, they mature into hypertrophic, postmitotic chondrocytes that enrich the ECM in type X collagen. Following hypertrophy, chondrocytes terminally mature and the calcified cartilage matrix is degraded by proteases and is infiltrated by blood vessels. Terminally mature chondrocytes then undergo apoptosis, thus facilitating the remodeling of the vascularized calcified matrix and its invasion by osteoblast precursors. Various families of transcription factors and signaling molecules, including transforming growth factor beta (TGF-β) and Wnts mediate this fine balance of chondrocyte proliferation, hypertrophy and terminal maturation necessary for longitudinal bone growth (Wuelling and Vortkamp, 2010). Further elucidation of the downstream effectors of these pathways may provide insights into the mechanisms controlling the transition of proliferating chondrocytes to hypertrophy.

There is substantial evidence demonstrating that crosstalk between the TGF-β and Wnt signals modulate cartilage formation and endochondral ossification (Dong et al., 2005). The repressive activities of TGF-β and Wnt signaling on chondrogenesis are mediated by the Twist1 transcription factor. In vitro gain and loss of function studies by Reinhold et al., demonstrated that Twist1 negatively regulates chondrogenesis and chondrocyte gene expression downstream of activated canonical Wnt signaling (Reinhold et al., 2006). Our previously published studies using in vitro models of chondrocyte maturation, indicated that Twist1

Abbreviations used in this paper: bHLH, basis helix-loop-helix; Col2, type II collagen; TG, transgenic; BV/TV, bone volume/total volume; PZ, proliferating zone; HZ, hypertrophic zone; μCT, micro-computed tomography.

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transcripts are down-regulated as chondrocytes mature and that differential regulation of Twist1 and Runx2 mediate stage-specific cooperative or antagonistic effects of TGF-β and canonical Wnt signaling during chondrocyte maturation (Dong et al., 2007). Moreover, Twist1 exerted repressive effects on in vitro chondrocyte maturation (Dong et al., 2007).

**TWIST1** haploinsufficiency results in Saethre-Chotzen, a syndrome characterized by craniosynostosis and limb abnormalities (Paznekas et al., 1998). Trisomy of the **TWIST1** locus also results in craniofacial defects (Papadopoulou et al., 2006), and interestingly both of these conditions are associated with short stature, indicating that small changes in Twist1 expression have significant effects on skeletal formation and growth. Twist1 impedes osteoblast differentiation, partially through inhibition of Runx2 activity (Bialek et al., 2004). Moreover, Twist1 plays a role in immature chondrocytes of the perichondrium during skeletal development by regulating Runx2 activation of FGF18 expression (Hinoi et al., 2006). Two studies using several Twist1 mutant alleles demonstrated that incremental reduction of Twist1 activity during early limb bud patterning alters Shh expression domains and markedly affects limb and girdle development (Krawchuk et al., 2010, Zhang et al., 2010). Removal of Twist1 activity from the limb mesenchyme using a floxed conditional null allele (Twist1<sup>-/-</sup>) crossed with the Prx-Cre transgene resulted in forelimb patterning defects, hypoplastic scapula and clavicle, tibial aplasia and preaxial polydactyly exhibiting disorganized cartilage elements (Krawchuk et al., 2010). These observations raise the possibility of spatial and temporal regulation of cartilage formation by Twist1 in a dose-dependent manner.

In this study, we assessed the effects of persistent Twist1 expression in chondrocytes in vivo via tissue-specific activation of a Twist1 transgene in type II collagen-expressing cells. We found that maintenance of Twist1 expression in mature chondrocytes resulted in postnatal growth retardation and growth plate defects. Moreover, micro-computed tomography (μCT) of hindlimbs from Twist1 transgenic mice further substantiated a role for Twist1 during endochondral ossification.

**Results**

**Impaired skeletal growth in mice with persistent chondrocyte-specific Twist1 expression**

Restricted postnatal expression of Twist1 proteins in immature, proliferating growth plate chondrocytes and its exclusion from hypertrophic chondrocytes (Supplemental Fig. 1), suggested a role for Twist1 in the regulation of skeletal growth and maintenance of cartilage tissue. Moreover, these observations indicated that decreased expression of Twist1 may be required for proper chondrocyte maturation. To test this, we used a Twist1 transgene that is conditionally activated by Cre recombinase (Chakraborty et al., 2010, Connerney et al., 2008, Connerney et al., 2006). Twist1 mice were crossed with Col2a1-Cre mice, which express Cre in proliferating chondrocytes, to activate constitutive Twist1 expression in these cells and their progeny. Col2-Twist1 mice displayed a runted phenotype as compared to their wild type littermates as early as two weeks after birth (Fig. 1A). Growth curve analysis revealed a significant reduction (36-41% reduction versus wild type) in the body mass of Col2-Twist1 transgenics at two weeks (8.42 ± 0.80 gm, wild type versus 5.25 ± 0.62 gm, transgenic), four weeks (18.5 ± 0.87 gm, wild type versus 11.6 ± 1.06 gm, transgenic) eight weeks (27.2 ± 1.62 gm, wild type versus 17.5 ± 2.51 gm, transgenic) and twelve weeks (28.7 ± 1.11 gm, wild type versus 17.1 ± 0.93 gm, transgenic) (Fig. 1B). Shortening of endochondral bones in Col2-Twist1 transgenic mice.
Overexpression of Twist1 in chondrocytes

643

Fig. 1. Aberrant growth plate organization in Col2-Twist1 transgenic mice. (A) H&E staining revealed hypertrophic zone expansion in the proximal tibia growth plate of Col2-Twist1 mice (ii, iii, v, vi) compared to wild type mice (i, iv), at two (i-iii) and four (iv-vi) weeks. Scale bar, 100 μm. (B) Widths of proliferating zone (PZ) and hypertrophic zone (HZ) within growth plates of wild type and Col2-Twist1 mice at four and eight weeks determined by histomorphometry. Significantly wider HZ was observed in transgenic (red) versus wild type mice (blue). * P < 0.05.

mice was further confirmed by X-ray analysis (Fig. 1C). At eight weeks, the length of both the femur and tibia were 14.2 ± 2.9% (p < 0.01) and 13.5 ± 2.6% (p < 0.01), respectively shorter in Col2-Twist1 transgenics relative to wild type mice (data not shown). Safranin-O/Fast Green staining of proximal tibia sections from two and four week old mice consistently showed that the proportion of cartilage to bone appeared higher in the secondary ossification site of Col2-Twist1 transgenics compared to wild type littermates at two weeks. These differences were not obvious by histology at four weeks, suggesting that secondary ossification site formation was delayed in Col2-Twist1 mice (Fig. 1E).

To evaluate the comparative level of Twist1 expression following transgene activation, we isolated RNA from tissue encompassing the growth plates within the distal femur and proximal tibia of two-week old Col2-Twist1 transgenic mice and wild type littermates. Q-PCR analysis revealed a 2.63 (± 0.132) fold increase in Twist1 transcript expression in transgenic mice relative to wild type mice (p<3.6 x 10⁻⁵) (Fig. 1D). Analysis of the proximal tibia of 4-day old Col2-Twist1 mice that also contained the R26R reporter, which expresses β-galactosidase following Cre recombination, indicated transgene activation in all chondrocytes within the growth plate and perichondrium (data not shown).
Impaired growth plate organization in the Col2-Twist1 transgenic mice

We next evaluated the effects of chondrocyte-specific persistent Twist1 expression on growth plate organization and structure. Histological analyses showed that the width of the hypertrophic zone within the proximal tibia growth plate was enlarged in Col2-Twist1 transgenic mice compared to wild type littermates at two and four weeks (Fig. 2A). These observations were validated by quantitative histomorphometric analyses, revealing significant increases in the width of the tibia growth plate hypertrophic zone in transgenic mice at four weeks (60.0 ± 7.59 μm, wild type mouse versus 89.2 ± 7.25 μm, Col2-Twist1, P = 0.04) and eight weeks (33.7 ± 1.20 μm, wild type versus 47.0 ± 5.81 μm, Col2-Twist1, P = 0.05) (Fig. 2B). Elevated chondrocyte-specific Twist1 expression within cartilage also appeared to have a significant effect on the width of the proliferating zone in older (eight weeks) animals (65.3 ± 3.27 μm, wild type versus 89.5 ± 6.71 μm, Col2-Twist1 mice, P = 0.02).

Hematoxylin-eosin (H&E) staining of the proximal tibia from four-week old wild type mice revealed a growth plate with orderly columns of proliferating chondrocytes (Fig. 3A). In contrast, transgenic tibial growth plates exhibited disorderly columnar arrays of chondrocytes as well as acellular regions in the proliferating zone (Fig. 3A). To address whether the acellularity observed within the growth plates of Col2-Twist1 transgenic mice was attributed to reduced chondrocyte proliferation, immunohistochemical staining for proliferating cell nuclear antigen (PCNA) was performed. These studies showed fewer PCNA-positive populations of growth plate chondrocytes (Fig. 3B) in Col2-Twist1 transgenic mice compared to control mice at two and four weeks.

Sox9, which is expressed in all chondroprogenitor cells and differentiated chondrocytes, has also been shown to be critical for the alignment of columnar arrays of proliferating chondrocytes parallel to the longitudinal axis of the bone (Akiyama et al., 2002, Bi et al., 1999). Growth plates from the proximal tibias of Col2-Twist1 transgenic mice showed fewer Sox9 immune positive cells versus age-matched wild-type mice (Fig. 3C).

Structural cartilage anomalies in Col2-Twist1 transgenic mice

Disruption of the typical contiguous columns of growth plate chondrocytes by areas of vascular invasion (Fig. 4A,B) was
observed within the proliferating zone of the Col2-Twist1 transgenic growth plate, as evidenced by the presence of erythrocytes and PECAM (CD31) immune positive cells (Fig. 4B). Immunohistochemical staining for type II collagen expression revealed further evidence of structural anomalies indicative of a vascular response within the transgenic cartilaginous growth plate (Fig. 4C). The growth plates within the proximal tibias of Col2-Twist1 transgenic mice exhibited focal regions of bony deposition emanating from the secondary ossification site (Fig. 4D). Deposition of bone matrix within the proximal tibia growth plate of transgenic mice was confirmed by type I collagen immunostaining (Fig. 4D).

**Chondrocyte-specific Twist1 overexpression leads to decreased trabecular bone formation**

In view of the hindlimb shortening and abnormal growth plate organization in Col2-Twist1 transgenic mice, we speculated that elevated chondrocyte-specific Twist1 expression impedes postnatal trabecular bone formation. μCT scans of femurs isolated from wild type and Col2-Twist1 transgenic mice revealed a diminution in trabecular bone formation in Col2-Twist1 transgenic mice at four (Fig. 5A) and eight weeks (Fig. 5B). At four weeks, quantitative μCT analysis of femurs showed a significant decrease in bone volume fraction (bone volume/total volume, BV/TV; %) in Col2-Twist1 transgenics (2.4 ± 1.8%) as compared to control mice (9.4 ± 1.8%, *P=0.007). Similarly, a significant decrease in BV/TV was also observed in transgenic mice (7.5 ± 2.4%) as compared to wild type mice (11.6 ± 2.4%) at eight weeks (*P = 0.007) (Fig. 5C). μCT analysis also showed significant reductions in trabecular thickness in Col2-Twist1 transgenics at four weeks (37 ± 2 μm, wild type versus 29 ± 2 μm, transgenic *P = 0.02) and eight weeks (41.3 ± 4.2 μm, wild type mice versus 36 ± 2.5 μm transgenic mice, *P = 0.009) (Fig. 5D). Trabecular number (1/mm) was also reduced in Col2-Twist1 transgenic mice at four weeks (5.11 ± 2.4 μm, wild type versus 3.04 ± 0.64 μm, transgenic, *P = 0.02) and eight weeks (5.12 ± 0.14 μm, wild type versus 4 ± 0.51 μm, transgenic, *P = 0.005) (Fig. 5F). Moreover trabecular spacing (μm) was significantly increased in Col2-Twist1 transgenic mice relative to wild type mice at eight weeks (194 ± 6.69 μm, wild type versus 236 ± 26.58 μm, transgenic, *P = 0.005) (Fig. 5E), further validating the reduction in trabecular bone formation in transgenic mice. These morphometric measurements demonstrated that mice with persistent Twist1 expression in chondrocytes develop a runted phenotype as a result of impaired endochondral ossification.

**Discussion**

Gene expression data and studies using normal and diseased cartilage suggest that Twist1 is a key regulator of chondrocyte function (Dong et al., 2007, Karlsson et al., 2010). Genetic studies have sought to define the possible function of Twist1 during cartilage and limb development. Hinoi et al. previously reported high levels of Twist1 expression within immature perichondrial cells in vivo, but failed to detect Twist1 transcripts in mature chondrocytes by in situ hybridization (Hinoi et al., 2006). Moreover, their histological analyses of the developing ribs and limbs in α1(II) Collagen-Cre: Twist1"""" embryos (E13.5 and E16.5), showed no obvious phenotype attributable to Twist1 gene inactivation in chondrocytes (Hinoi et al., 2006). However, conditional Twist1

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**Fig. 5. Persistent chondrocyte-specific Twist1 expression causes postnatal decrease in trabecular bone formation.** μCT scans of wild type and transgenic femurs at four (A) and eight weeks (B) illustrate reduced trabecular bone volume and density in Col2-Twist1 mice. (C-G) Quantitative analysis reveals significant reduction in bone volume fraction (C); trabecular thickness (D); trabecular number (F); and connectivity density (G) in Col2-Twist1 mice (red) versus wild type mice (blue) at four and eight weeks. Col2-Twist1 mice displayed increased trabecular spacing (E) versus wild type mice at four and eight weeks *P < 0.05.
abrogation from the limb mesenchyme by crossing Twist1\textsuperscript{fx} mice with mice containing the Prx1-Cre transgene, which activates Cre expression in prechondrocytes, resulted in forelimb patterning defects, hypoplastic scapula and clavicle, tibial aplasia, and preaxial polydactyly characterized by severe disorganization of cartilage elements (Krawchuk et al., 2010, Zhang et al., 2010). We speculated that the temporal requirement for Twist1 during postnatal chondrocyte formation may provide novel insight into stage- and dose-dependent roles of Twist1 during cartilage maturation and maintenance. Thus, we hypothesized that sustained expression of Twist1 in chondrocytes during embryonic and postnatal development will delay chondrocyte differentiation and impair endochondral ossification.

The rationale for examining the effects of persistent Twist1 expression on cartilage development in vivo stems from our in vitro functional studies demonstrating that Twist1 acts as a potent inhibitor of chondrocyte hypertrophy (Dong et al., 2007). Others have shown that ectopic expression of Twist1 in the murine prechondrogenic ATDC5 cell line repressed chondrocyte gene expression and activity; whereas Twist1 knockdown by RNA interference increased the expression of early chondrogenic gene markers (Reinhold et al., 2006). Moreover, using human primary mesenchymal stem cells (MSC), Isenmann et al., demonstrated that enforced expression of Twist1 sustained an immature stromal phenotype and inhibited the chondrogenic potential of human MSCs, as shown by a reduced capacity to synthesize glycosaminoglycans and down-regulated expression of Sox9 and collagen type X (Isenmann et al., 2009). Increased expression of different forms of Twist1 in mesenchymal progenitors of the limb resulted in runted limb growth (Firulli et al., 2007), however the phenotype of the limb cartilage of these mice was not analyzed. Thus, we initiated our studies to explore the dose-dependant and temporal function of Twist1 during cartilage formation and maturation in vivo. We used a conditional Cre-induced transgene approach to constitutively express Twist1 in collagen II-expressing cells and their progeny. Activation of the transgene resulted in a modest (2.6 fold) increase in Twist1 transcripts over endogenous Twist1 expression, which was the same level as found using this transgene in other tissues (Chakraborty et al., 2010), yet the expression of the transgene was maintained as chondrocytes matured. Sustained expression of Twist1 in cartilage led to a growth phenotype, characterized by shortening of the limbs and reduced body mass. Various Twist1 loss of function studies have also reported growth abnormalities and limb dismorphogenesis (Firulli et al., 2005, Firulli et al., 2007, Krawchuk et al., 2010, Zhang et al., 2010), thus demonstrating that in addition to its established function during intramembranous bone formation, Twist1 also plays a role in endochondral ossification.

Histological assessments of the proximal tibias from Col2-Twist1 transgenic mice showed a disruption of the regular columnar arrangement of the proliferating and hypertrophic chondrocytes. Immunohistochemical analyses further revealed decreased PCNA and Sox9 stained populations of growth plate chondrocytes in Col2-Twist1 transgenic mice. Sox9, expressed by all chondrogenic progenitor cells and differentiating chondrocytes, is critical for the expression of components of the cartilaginous ECM (Bi et al., 1999) and promotes chondrocyte proliferation and alignment of the proliferative clones into columnar arrays parallel to longitudinal axis of bone (Akiyama et al., 2002). Disruption of the growth plate chondrocyte columnar arrays in Col2-Twist1 transgenic mice may be attributed to reduced Sox9 expression and/or insufficient number of proliferating growth plate chondrocytes.

While an effect of increased Twist1 expression was predicted to inhibit Sox9 expression, and thus indirectly affect chondrocyte proliferation (Akiyama et al., 2002), Col2-Twist1 transgenic mice also displayed an unexpected expansion of the hypertrophic zone in postnatal growth plates. It is plausible that accumulation of hypertrophic chondrocytes in vivo may be attributed to delayed chondrocyte terminal maturation and apoptosis induced by ossification fronts. However, we did not observe decreased apoptosis in Col2-Twist1 mice (data not shown). Chondrocyte hypertrophy and subsequent ossification is coordinately regulated by Runx2 and Mef2c transcription factors (Wuelling and Vortkamp, 2010). Interestingly, both of these factors are direct targets of Twist1 inhibition (Bialek et al., 2004, Spicer et al., 1996), which could explain the phenotype of the Col2-Twist1 mice. Importantly, all of the in vitro studies identifying an important role for Twist1 in chondrocyte specification and early differentiation events were done by expressing Twist1 in MSCs or prechondrocytes, while Col2-Twist1 mice initiate expression of Twist1 at a later stage of chondrocyte differentiation, which may have allowed us to identify a later role for Twist1 in chondrocyte differentiation. These studies indicate that deregulation of Twist1 expression within the growth plate negatively impacts endochondral ossification.

Histologic and immunohistochemical analyses revealed additional growth plate abnormalities in Col2-Twist1 transgenics, including blood vessel and focal bony formation within the cartilaginous matrix. Postnatal chondrocyte-specific ablation of Ihh (Maeda et al., 2007) led to similar features as Col2-Twist1 mice, including growth retardation, decreased PCNA-stained chondrocytes, loss of chondrocyte columnar organization and aberrant vascular invasion within the growth plate, attributed to the premature differentiation of mutant chondrocytes. However, microCT analyses of femurs from wild type and Col2-Twist1 transgenic mice showed that chondrocyte-specific Twist1 expression led to significant decreases in bone volume fraction, trabecular thickness, trabecular number and connectivity density. These findings argue that aberrant vascular invasion and bony bridge trabeculae within Col2-Twist1 transgenic growth plates are not due to advanced replacement of mineralized cartilage by bone. Rather, we postulate that these defects arise due to growth plate fracture or trauma. In support of this, animal models of growth plate fracture have displayed structural disorganization, formation of vertical septa, bone bridge formation and longitudinal shortening (Wattenbarger et al., 2002). Moreover, marrow-derived osteogenic precursors are recruited to undergo intramembranous bone formation at the growth plate defect site (Xian et al., 2004). We speculate that Col2-Twist1 transgenic growth plate may be prone to trauma, due to impaired cartilage ECM integrity or production, resulting in decreased trabecular bone formation.

As previous studies have not addressed Twist1 function in postnatal chondrocyte development, the effects of chondrocyte-specific loss of Twist1 function in postnatal development warrant further investigation. While future experiments will explore the cellular and molecular mechanisms underlying Twist1 function during chondrocyte differentiation, our findings argue that regulated expression of Twist1 within growth plate chondrocytes is important for proper endochondral ossification.
Materials and Methods

Mice and genotyping
Female CAGCAT-Twist1 mice were bred with Col2a1-Cre or with Col2a1-Cre;R26R males to generate offspring heterozygous for each transgene. Col2a1-Cre and R26R mice were obtained from The Jackson Laboratory, Bar Harbor, ME. Genotyping for the Cre and Twist1 transgenes was performed by PCR of genomic DNA from 3-week postnatal tail tips using Cre, CAT and LacZ specific primers (Connerney et al., 2008).

RNA isolation and real-time PCR
Growth plates from Col2-Twist1 and wild type mice were homogenized in TRIzol reagent (Invitrogen) using the TissueLyserII (Qiagen). Total RNA was extracted, reverse transcribed and Q-PCR performed using mouse specific primers for Twist1 (forward primer, 5’-GCAGTGTTGGAAATGCCTTA-3’; reverse primer, 5’-TGTGGATATGGATATGATCCT-3’), and 9-actin (forward primer, 5’-AGATGGATACACGCAAGCAG-3’; reverse primer, 5’-GGCGCAAAGTATTGTGTGCTA-3’). Data analysis was performed using the StepOne Software v2.1.

Histological staining
Hindlimbs from 2, 4 and 8 week old mice were dissected and fixed in 4% paraformaldehyde for 2-5 days at 4°C. Specimens were decalcified in 15% EDTA (pH 7.1) for 5 days. Samples were embedded in paraffin and 5um sections were collected. Sections were deparaffinized in xylene and rehydrated through graded alcohols to water, then stained with hemotoxylin-eosin, Safranin O/Fast green or 1% Alcian blue (pH 2.5) (PolyScientific).

Immunohistochemical studies
Densafrapparation sections were first incubated in antigen retrieval solution (4N HCL or pepsim) for 15 minutes at 37°C. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide for 30 minutes. Sections were permeabilized in 0.1% Triton-X-100 and non-specific binding sites were blocked with 1% bovine serum albumin and 10% goat serum in PBS. Sections were incubated with or without primary antibodies, including mouse anti-collagen type II (1:200, Millipore), rabbit anti-PCNA (1:200, Abcam), rabbit anti-Sox9 (1:75, Abcam), mouse anti-Twist1 3E11 (1:50, Novus Biologicals) and goat anti-PECAM (1:75, Santa Cruz). Labeling was visualized with the appropriate peroxidase-conjugated secondary antibody and Zymed Aminoethyl Carbazole Substrate Kit (Invitrogen). Digital images were acquired using a Q-Imaging Retig 2000R camera connected to Nikon Eclipse 50i microscope and image analyses performed using NIS Elements B5.3.0 software.

X-ray and micro-computed tomography
Femurs were disarticulated at the hip and knee, dissected of soft tissue, and fixed in 10% formalin. Bone morphometry was measured using micro-focus conebeam X-ray computed tomography (μCT40, Scanco Medical AG, Switzerland). Scanning was performed at 55 kV and 145 μA, collecting 1000 projections/rotation at 300 msec integration time. Three-dimensional images were reconstructed using standard convolution and back-projection algorithms with Gaussian filtering, and rendered within a 12.3 mm field of view at 578,704 voxels/mm² (isometric 12 mm voxels). Standard algorithms describing trabecular morphometry were applied for measures of bone architecture and mineral density.

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