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# 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 chondocyte 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*$^{fx}$) 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 ± 0.09 gm, transgenic) (Fig.1B). Shortening of endochondral bones in *Col2-Twist1* transgenic

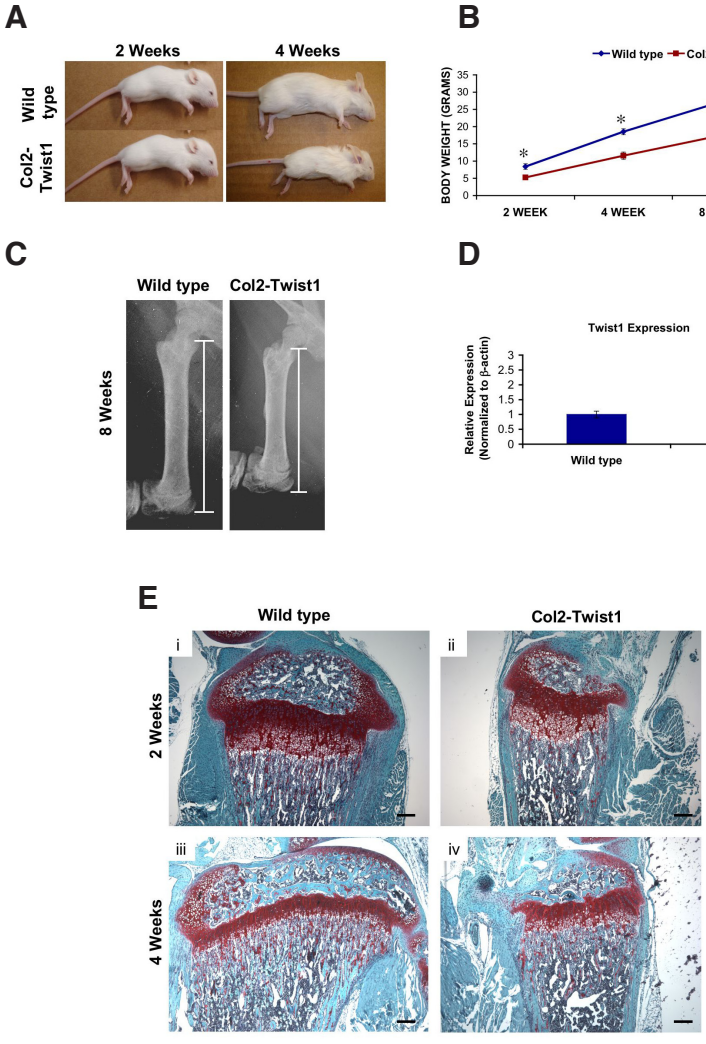


**Fig. 1. Persistent, chondrocyte-specific Twist1 expression causes growth retardation. (A)** *Appearance of wild type and runted* Col2-Twist1 *littermates at two and four weeks.* **(B)** *Growth curves of wild type (blue) and* Col2-Twist1 *mice (red). Mean ± standard error (SE) from 6-7 mice. *P < 0.05* **(C)** *Radiographs of femurs from wild type and* Col2-Twist1 *mice at eight weeks.* **(D)** *Q-PCR analysis of* Twist1 *mRNA expression in growth plates from two-week wild type (blue) and* Col2-Twist1 *(red) mice showed increased* Twist1 *transcript expression in transgenic mice. *P<3.6 x 10$^{-5}$.* **(E)**. *Safranin O-fast green staining of tibia sections from two and four week old mice showed reduced secondary ossification site formation in* Col2-Twist1 *mice. Scale bar, 200 μm.*

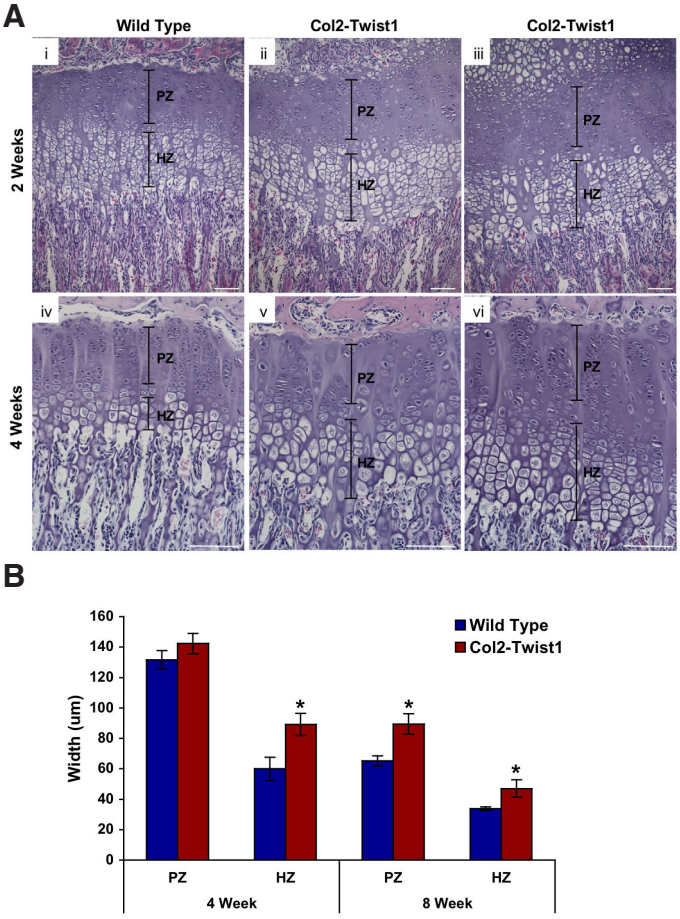


**Fig. 2. Aberrant growth plate organization in *Col2-Twist1* transgenic mice. (A)** *H&E staining revealed hypertrophic zone expansion in the proximal tibia growth plate of* Col2-Twist1 *mice (ii, iii, v, vi) compared to wild type mice (i, iv), at two (i-iii) and four (iv-vi) weeks. Scale bar, 100 μm.* **(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.*

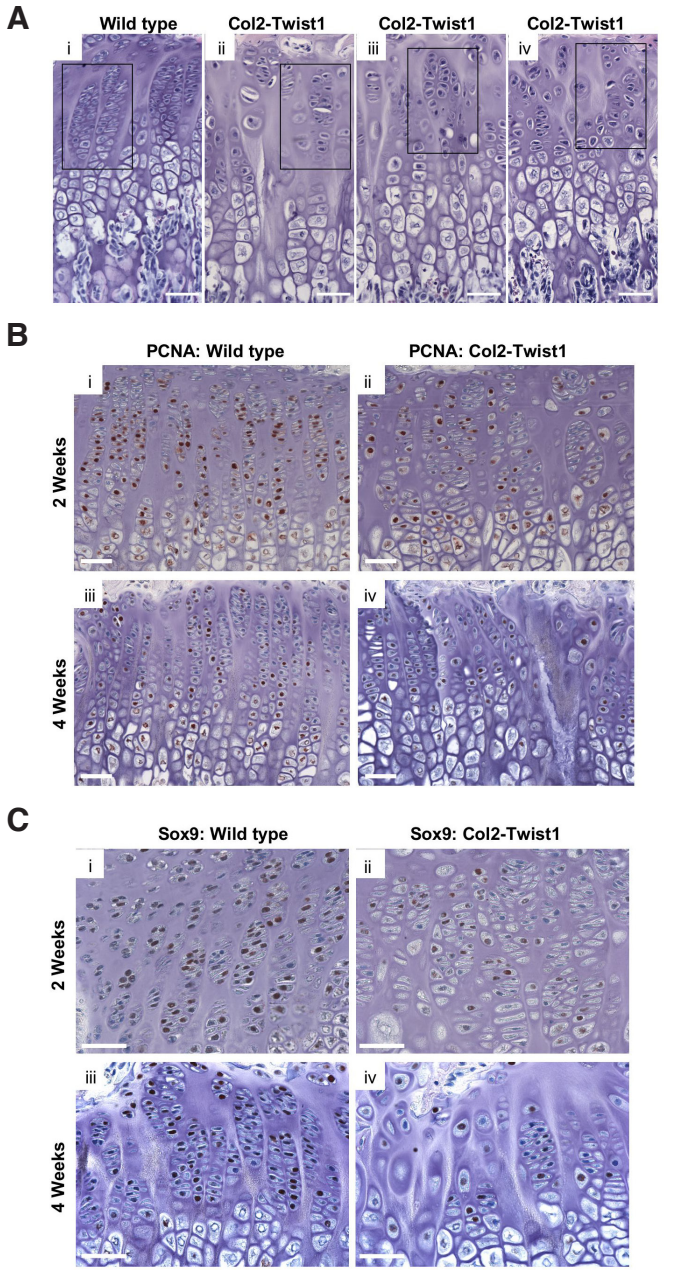


**Fig. 3. Growth plate abnormalities are associated with persistent Twist1 expression in chondrocytes. (A)** *H&E staining of proximal tibia growth plates from four week old wild type and* Col2-Twist1 *mice revealed disorderly columnar arrays of proliferating chondrocytes and acellular regions in* Col2-Twist1 *growth plates. Scale bar, 50 μm.* **(B)** *Reduced numbers of PCNA positive nuclei in tibia sections of two and four week old* Col2-Twist1 *mice (ii, iv) as compared to control mice (i, iii). Scale bar, 50 μm.* **(C)** *Immunohistochemical staining showed reduced numbers of Sox9-positive cells in tibia sections from* Col2-Twist1 *(ii, iv) versus wild type mice (i, iii) at two and four weeks. Scale bar, 50 μm.*

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 ($p<3.6 \times 10^{-5}$) (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 mice 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 as 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 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).

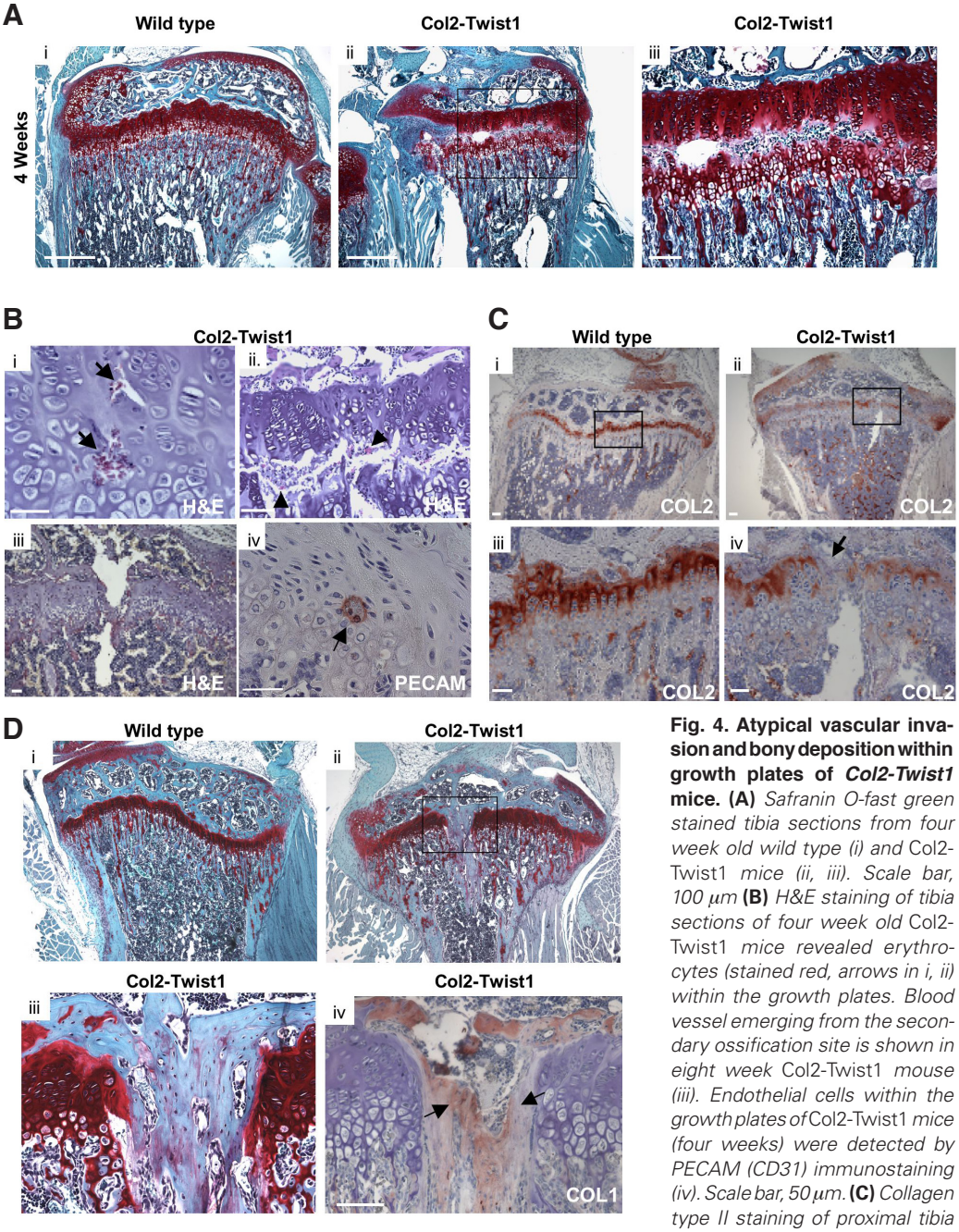


**Fig. 4. Atypical vascular invasion and bony deposition within growth plates of *Col2-Twist1* mice. (A)** *Safranin O-fast green stained tibia sections from four week old wild type (i) and* Col2-Twist1 *mice (ii, iii). Scale bar, 100 μm* **(B)** *H&E staining of tibia sections of four week old* Col2-Twist1 *mice revealed erythrocytes (stained red, arrows in i, ii) within the growth plates. Blood vessel emerging from the secondary ossification site is shown in eight week* Col2-Twist1 *mouse (iii). Endothelial cells within the growth plates of* Col2-Twist1 *mice (four weeks) were detected by PECAM (CD31) immunostaining (iv). Scale bar, 50 μm.* **(C)** *Collagen type II staining of proximal tibia sections from wild type (i,iii) and* Col2-Twist1 *mice (ii,iv) at eight weeks showed impaired structural integrity of the collagen type II matrix in* Col2-Twist1 *mice. Arrow in (iv) demarcates regions of reduced collagen type II matrix formation in* Col2-Twist1 *mice. Scale bar, 100 μm.* **(D)** *Focal bony deposition within growth plates of* Col2-Twist1 *mice. Safranin O-fast green staining of tibia sections from wild type (i) and* Col2-Twist1 *mice (ii, iii) at four weeks illustrated regions of bony deposition (shown in green) within the cartilaginous growth plates (red) of* Col2-Twist1 *mice (ii, iii). Boxed areas in (ii) magnified in (iii). Collagen type I immunostaining (iv) showed bone tissue deposition within growth plates of four week* Col2-Twist1 *mouse. Scale bar, 100 μm.*

***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 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 bony 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 ± 1.2%) 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 ± 0.25 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 $\alpha$*1(II) Collagen-Cre; Twist1*$^{fx}$ embryos (E13.5 and E16.5), showed no obvious phenotype attributable to *Twist1* gene inactivation in chondrocytes (Hinoi *et al.*, 2006). However, conditional *Twist1*

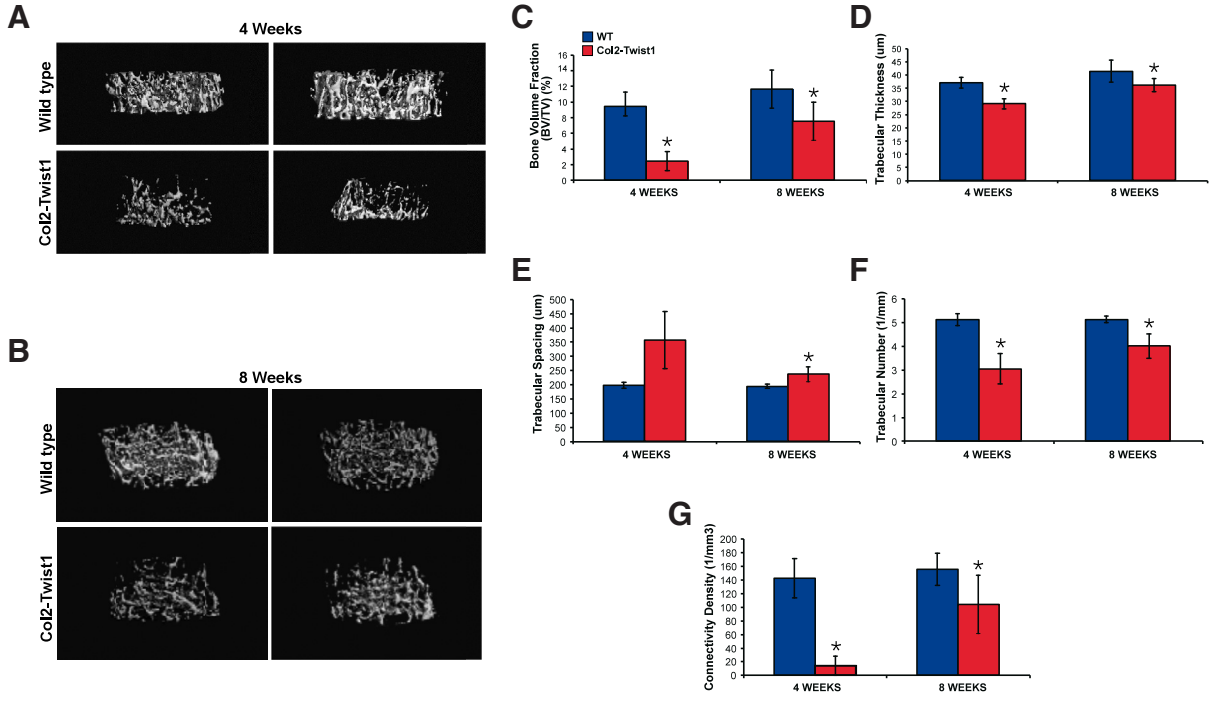


**Fig. 5. Persistent chondrocyte-specific Twist1 expression causes postnatal decrease in trabecular bone formation.** *μCT scans of wild type and transgenic femurs at four* **(A)** *and eight weeks* **(B)** *illustrate reduced trabecular bone volume and density in* Col2-Twist1 *mice.* **(C-G)** *Quantitative 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*$^{fx}$ 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 chondroprogenitor 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, μCT 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 intermembranous 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 clips 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'-GCAGTGGTGGAATGCCTTTA-3'; reverse primer, 5'-TGTGGTATGGCTGATTATGATCTC-3'), and 9-actin (forward primer, 5'-AGATGTGGATCAGCAAGCAG-3'; reverse primer, 5'-GCGCAAGTTAGGTTTTGTCA-3'). Data was 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*

Deparaffinized sections were first incubated in antigen retrieval solution (4N HCL or pepsin) 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). Image acquisition used a Q-Imaging Retig 2000R camera connected to Nikon Eclipse 50i microscope and image analyses performed using NIS Elements B$ 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 Gausian filtering, and rendered within a 12.3 mm field of view at 578,704 voxels/mm$^3$ (isometric 12 mm voxels). Standard algorithms describing trabecular morphometry were applied for measures of bone architecture and mineral density.

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