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Tamoxifen-inducible gene deletion reveals a distinct cell type associated with trabecular bone, and direct regulation of *PTHrP* expression and chondrocyte morphology by Ihh in growth region cartilage

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Abstract

Indian hedgehog (Ihh) controls multiple aspects of endochondral skeletal development by signaling to both chondrocytes and perichondrial cells. Previous efforts to delineate direct effects of Ihh on chondrocytes by *Col2-Cre*-mediated ablation of *Smoothened* (*Smo*, encoding a transmembrane protein indispensable for Ihh signaling) has been only partially successful, due to the inability to discriminate between chondrocytes and perichondrial cells. Here we report a transgenic line (*Col2-Cre*™) expressing under the control of the *Colα1(II)* promoter an inert form of Cre that is activatable by exogenous tamoxifen (TM); TM administration at proper times during embryogenesis induced Cre activity in chondrocytes but not in the perichondrium. By using this mouse line, we deleted *Smo* within subsets of chondrocytes without affecting the perichondrium, and found that *Smo* removal led to localized disruption of the expression of *parathyroid hormone related protein* (*PTHrP*) and the morphology of chondrocytes. Unexpectedly, TM invariably induced Cre activity in a subset of cells associated with the trabecular bone surface of long bones. These cells, when genetically marked and cultured in vitro, were capable of producing bone nodules. Expression of the *Col2-Cre*™ transgene in these cells likely reflected the endogenous *Colα1(II)* promoter activity, as similar cells were found to express the *IIA* isoform of *Colα1(II)* mRNA endogenously. In summary, the present study has not only provided evidence that Ihh signaling directly controls *PTHrP* expression and chondrocyte morphology in the growth region cartilage, but has also uncovered a distinct cell type associated with the trabecular bone that appears to possess osteogenic potential.

Keywords

Ihh; Smo; PTHrP; Type IIA procollagen; Cre; tamoxifen; cartilage; mouse

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INTRODUCTION

Much of the vertebrate skeleton arises from cartilage templates that originate from mesenchymal condensations and eventually undergo endochondral ossification. Prior to overt chondrocyte differentiation, the condensing mesenchyme expresses the IIA splice form of type II collagen (Ng et al., 1993; Sandell et al., 1991; Sandell et al., 1994); subsequently cells in the core of the condensation switch to express the IIB splice form characteristic of chondrocytes, whereas the peripheral cells upregulate expression of type I collagen forming the perichondrium. Following formation of the cartilage anlage, chondrocytes initially proliferate, but progressively exit the cell cycle and undergo hypertrophy starting at the center of the element. The hypertrophic cartilage is eventually removed upon the invasion of blood vessels and replaced by bone and the marrow cavity. The continued orderly progression of chondrocytes from proliferation to hypertrophy results in the characteristic growth plates located at both ends of the cartilage template, where the round chondrocytes are near the articular surface, followed by the flat chondrocytes arranged in columns (columnar chondrocytes), and finally the hypertrophic chondrocytes adjacent to the marrow cavity. The repeated process of hypertrophy and removal of chondrocytes eventually gives rise to the final form of an endochondral bone in which much of the cartilage is replaced by bone. Thus, proper formation of an endochondral bone requires coordinated regulation of chondrocyte proliferation and maturation, vascular invasion as well as osteoblast differentiation.

Indian hedgehog (Ihh) critically controls multiple aspects of the development of endochondral bones. In the developing cartilage, Ihh is produced by prehypertrophic (immediately before hypertrophic) as well as early hypertrophic chondrocytes, and it signals to both the immature chondrocytes and the overlying perichondrial cells (St-Jacques et al., 1999; Vortkamp et al., 1996). *Ihh* homozygous null mutant mice exhibited a marked reduction in chondrocyte proliferation, profound dysregulation of chondrocyte maturation, a severe defect in cartilage vascularization as well as a complete lack of osteoblasts (Long et al., 2001; St-Jacques et al., 1999). More recently, genetic experiments have implicated Ihh in positively regulating the length of the columnar region, as ectopic *Ihh* expression induced by mosaic ablation of *parathyroid hormone-related-protein receptor* (*PTHrP-R*) in the growth plate lengthened the columnar region (Kobayashi et al., 2005). On the other hand, *Col2-Cre*-mediated removal of *Smoothened* (*Smo*), which encodes a transmembrane protein indispensable for all Hh signaling (Alcedo et al., 1996; van den Heuvel and Ingham, 1996; Zhang et al., 2001), abolished the same region within the growth plate (Long et al., 2006).

Genetic manipulation of *Smo* in the mouse has revealed that Ihh functions via either direct or indirect signaling on target cells. For instance, direct Ihh input was required for proper proliferation of chondrocytes as well as development of the osteoblast lineage (Long et al., 2004; Long et al., 2001), whereas regulation of chondrocyte maturation appeared to be mediated primarily via a secondary signal, parathyroid hormone related protein (PTHrP) (Karp et al., 2000; Long et al., 2001). Although *PTHrP* expression in the periarticular cartilage is known to depend upon Ihh signaling (St-Jacques et al., 1999), which most likely functions by antagonizing Gli3 repressor activity (Hilton et al., 2005; Koziel et al., 2005), it is not clear whether direct Ihh input in the periarticular chondrocytes is required for this regulation. Indeed, although earlier work suggested that Ihh may indirectly regulate *PTHrP* by signaling through the adjacent perichondrium (Vortkamp et al., 1996) via a secondary signal such as TGFβ2 (Alvarez et al., 2002), a recent report challenged this view as exogenous TGFβ1 failed to activate *PTHrP* expression in the absence of Ihh signaling in an organ culture model (Koziel et al., 2004). Moreover, although removal of *Smo* using a *Col2-Cre* line abolished *PTHrP* expression in the periarticular chondrocytes but not in the periarticular perichondrium (Long et al., 2001), it could not be conclusive whether the loss of *PTHrP* was specifically due to the removal of Ihh responsiveness in chondrocytes, as *Col2-Cre* was also active in at least a subset

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of perichondrial cells (Long et al., 2004). For the same reason, the loss of columnar region in the growth plate of the *Col2-Cre; Smo*$^{n/c}$ mutant embryo could not be attributed specifically to the removal of *Smo* in chondrocytes (Long et al., 2006). Thus, a clear delineation of direct effects of Ihh signaling in chondrocytes awaits genetic tools better distinguishing them from perichondrial cells.

Compared to growth plate development, relatively little is understood about endochondral (within the cartilage) bone formation *per se*. The process begins with vascular invasion of the hypertrophic cartilage and produces the trabecular bone, and is distinct from ossification within the perichondrium that initiates slightly earlier and contributes to the cortical bone. A conventional view holds that the osteoblastic cells (osteoblasts and their progenitors) at both locations differentiate and behave similarly. On the other hand, as the trabecular versus the periosteal bone cells reside in distinct microenvironment, it is conceivable that they are regulated and maintained differently. Recent evidence supports this view as constitutive activation of PTHrP signaling in all osteoblasts in the mouse increased bone formation in the trabecular but not the cortical region (Calvi et al., 2001). However, no molecular features have been reported to distinguish the cortical versus trabecular osteoblastic cells.

Here we report the generation of a transgenic mouse line (*Col2-Cre*™) that can be used to restrict Cre activity to chondrocytes versus perichondrial cells upon proper administration of tamoxifen (TM). By using the *Col2-Cre*™ line to locally remove *Smo*, we have provided evidence that Ihh likely regulates *PTHrP* expression and chondrocyte morphology in a direct manner. In addition, we demonstrate that the *Col2-Cre*™ transgene is expressed by a subset of cells that are associated with the trabecular bone surface and possess osteogenic potential.

MATERIALS AND METHODS

Mouse strains

To generate the *Col2-Cre*™ transgene, a 2 kb cDNA encoding *Cre*™ (Danielian et al., 1998) was ligated into the *Eco*RV site of a modified form of the *Col2a1* expression vector (Horton et al., 1987). The transgene was released by *Not*I and *Eco*RI digestion, purified and injected into the pronuclei of fertilized eggs from CBA/BL6-F1/J females (Jackson Laboratory). Pronuclear injection was performed by the Mouse Genetics Core (Washington University Medical School). Founder mice and their progenies were genotyped by PCR using primers specific for *Cre*.

Mice carrying *Smo*^{n} (Zhang et al., 2001) or *Smo*^{c} (Long et al., 2001) alleles were as previously described.

Tamoxifen (TM) administration

TM (Sigma, St. Louis) dissolved in corn oil (Sigma) was injected intraperitoneally into either pregnant females or postnatal pups. For the initial screening of founders, 100 μg TM per gram of body weight was injected into pregnant females. Dosages for other injections were as indicated in each experiment.

Analysis of mouse embryos

Lac Z stainings were performed on either whole embryos or frozen sections. Whole embryos were stained as previously described (Whiting et al., 1991). Frozen sections were prepared and stained using a protocol based on a published procedure (Lobe et al., 1999). The sections were counterstained with nuclear fast red.

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For histology and *in situ* hybridization, limbs were prepared and sectioned as described previously (Hilton et al., 2005; Long et al., 2001). The *in situ* probe for *Colα1(II)A* was generously provided by Dr. Linda Sandell (Washington University Medical School) (Zhu et al., 1999). All other *in situ* hybridization probes were as previously described (Hilton et al., 2005; Long et al., 2001).

For co-localization studies, frozen sections were first stained for Lac Z activity and then subjected to in situ hybridization using ^{35}S-labeled riboprobes. After in situ signals were developed, the sections were counterstained with nuclear fast red.

Bone marrow stromal cell cultures

Bone marrow stromal cells were isolated from the femur and the tibia of 2-month-old mice at 12 hrs after TM administration via oral gavage at 125 μg per gram of body weight. Briefly, upon surgical removal of the epiphyseal ends, the bone shafts were flushed with α-MEM with a 25-gauge needle. Cells from a single mouse were centrifuged, treated with the red blood cell lysis buffer (Roche), rinsed and resuspended in α-MEM containing 20% fetal bovine serum, before being filtered through a 70 μm cell strainer (Falcon) and plated at 2×10^6/well in 12-well plates. Half of the medium was changed at day 3 and all medium changed at day 6 of culture to remove floating cells. Multiple cultures were performed in parallel for each animal to allow Lac Z staining at different time points. For bone nodule assays, cells cultured to confluence were switched to mineralization medium containing ascorbic acid and β-glycerophosphate, and further cultured for 10 days. For Lac Z staining, the cultures were fixed for 5 minutes before proceeding with the protocol (Lobe et al., 1999). For von Kossa staining, cultures previously subjected to Lac Z staining were incubated with 1% silver nitrate under bright light and finally washed with PBS.

RESULTS

Generation of a *Col2-Cre*™ transgenic mouse line

To generate genetic tools expressing Cre specifically in chondrocytes but not in perichondrial cells, we seeked to bypass the transient *Colα1(II)* promoter activity present in the precursors of perichondrial cells. To this end, we took advantage of a modified form of Cre (Cre™) in which the recombinase was fused to a mutated ligand binding domain of the estrogen receptor and rendered inactive but activatable by exogenous TM (Danielian et al., 1998). Specifically, we established transgenic mice (*Col2-Cre*™) by injecting DNA expressing *Cre*™ under the *Colα1(II)* promoter/enhancer (Fig.1A) into the pronuclei of fertilized eggs. Fifteen male founder mice carrying the transgene were obtained and screened by crossing with the *Rosa26 reporter* (*R26R*) mouse, which expresses β-galactosidase specifically in cells that presently or previously express Cre activity (Soriano, 1999). Embryos were harvested at E12.5 without exposure to TM and those carrying the genotype of *Col2-Cre*™; *R26R* were assayed for "leaky" Cre activity. Through this procedure, 6 founders were found to express at least some level of Cre activity without TM induction, and were therefore eliminated from further analyses. The remaining 9 founders were again crossed with *R26R* females, which this time received intraperitoneal injections of TM at E10.5; embryos were harvested at E12.5 and assayed for β-galactosidase activity. Two founders that exhibited the most robust TM-dependent β-galactosidase activity were selected for further analyses. Since the two lines behaved identically in all assays, here we only report results obtained from one such line.

Temporal control of Cre activity

To assess the onset of the transgene expression during embryogenesis, we generated *Col2-Cre*™*; R26R* embryos that were induced with TM at progressively earlier time points and harvested at E12.5 to assay for β-galactosidase activity. With injection at E10.5, we detected

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Cre activity in all cartilage throughout the embryo (Fig. 1, C). In particular, both forelimbs and hindlimbs expressed robust activities in all skeletal elements (Fig. 1, C1 and C2, respectively). However, when TM was administered at E9.5, in the limb, only the elements including and proximal to the zeugopod exhibited Cre activity, whereas the autopod had little or no activity (Fig. 1, B1 and B2). Earlier injections up to E6.5 produced a similar Lac Z staining pattern to the E9.5 injection (data not shown). In all cases, *Col2-Cre*™*; R26R* embryos not induced with TM showed no β-galactosidase activity (data not shown). Thus, expression of the *Col2-Cre*™ transgene in principle tracks the proximal-to-distal progression of chondrogenesis in the limb.

We next examined whether proper timing of TM administration during embryogenesis could restrict Cre activity to chondrocytes without inducing a significant level in the perichondrium. For consistency we have focused the analyses on the humerus. As preliminary analyses of the embryos induced at E10.5 had revealed Cre activity in both chondrocytes and perichondrial cells (data not shown), we next examined *Col2-Cre*™*; R26R* embryos exposed to TM at E11.5, E12.5, E13.5 or E14.5, and harvested at E17.5. With E11.5 injection, the proximal half of the humerus showed robust Cre activity in the chondrocytes (red arrow) in contrast to a minor level in the perichondrium (green arrowhead) (Fig 2, A1), but the distal half still exhibited strong activities in both chondrocytes (red arrow) and the perichondrium (green arrowhead) (Fig. 2, A2). In addition, Cre activity was also evident in the primary spongiosa (purple arrow, Fig. 2, A1) and within the elbow joint capsule (asterisk, Fig. 2, A2). On the other hand, when TM was administered at E12.5, the proximal half showed no detectable Cre activity in the perichondrium (green arrowhead) despite robust levels in chondrocytes (red arrow) (Fig. 2, B1). Within the distal half, Cre activity in both the perichondrium (green arrowhead) and the elbow joint region (asterisk) was markedly reduced (Fig. 2, B2). However, clear Cre activity remained within the primary spongiosa (purple arrow, Fig. 2, B1) (discussed below). Similar results were obtained with TM injection at E13.5 (Fig. 2, C, C1, C2) or E14.5 (Fig. 2, D, D1, D2), but Cre activity in the distal perichondrium was further reduced. Interestingly, a small number of the distal perichondrial cells remained positive even with the later injections; reasons for the discrepancy between distal and proximal perichondrium are not known but may be unique to the humerus, as a similar difference was not evident in the radius or the ulna (supplementary data, Fig. S1). On the other hand, the progressive restriction of Cre activity to chondrocytes and the primary spongiosa was confirmed in other long bones including the radius and the ulna (Fig. S1). Thus, TM administration at or after E12.5 restricted Cre largely to chondrocytes and the primary spongiosa in long bones.

We next assessed whether the *Col2-Cre*™ line could be useful for postnatal gene removal. To this end, *Col2-Cre*™*; R26R* animals were injected TM at postnatal day 12 (P12) and 14 (P14), and then harvested at P16 for Lac Z staining. Strong signals were observed in a large number of chondrocytes at the proximal end of the humerus (Fig. 3, A). In particular, nearly 90% of the columnar chondrocytes expressed robust Cre acvitiy (Fig. 3, A2). Similarly, a high percentage of the articular chondrocytes exhibited strong activities (Fig. 3, A3). In addition, Cre activity was induced within both primary (Fig. 3, A1) and secondary ("2°", Fig. 3, A) ossification centers. In contrast, TM injection in animals carrying *Col2-Cre*™ but not the *R26R* allele did not induce any β-galactosidase activity in any chondrocytes (Fig. 3, B–B3). Similarly, in the absence of TM animals with the genotype *Col2-Cre*™*; R26R* did not show any activity in chondrocytes (data not shown). However, the control animals showed a weak β-galactosidase-like activity within both primary and secondary ossification centers (Fig. 3, B and B1). The activity, judged by the intensity of Lac Z staining, was noticeably weaker than that induced by TM, and appeared to be transient because it was not detected either in embryonic bones, or in 2-month-old animals (see below). Overall, successful gene deletion can be achieved in the postnatal cartilage using the *Col2-Cre*™ transgenic line.

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Expression of *Col2-Cre*™ in trabecular bone surface cells

The persistent TM-inducible β-galactosidase activity within the ossification centers of *Col2-Cre*™*; R26R* animals prompted us to examine whether bone-associated cells expressed the *Col2-Cre*™ transgene. To confirm that the Lac Z-positive cells in the primary spongiosa were not derived from Lac Z-postive perichondrial cells, we administered TM to *Col2-Cre*™*; R26R* embryos at E13.5 to minimize Cre activity in the perichondrium. As expected, when the embryos were assayed at E17.5, virtually no perichondrial cell within the proximal half of the humerus showed any Lac Z activity (green arrowhead, Fig. 4, A, B). However, robust Lac Z staining was detected in the primary spongiosa (purple arrow, Fig. 4, A, B). At a higher magnification, most Lac Z-positive cells appeared to adhere to the surface of the trabecular bone (green contour), assuming either flat (red arrow) or cuboidal (green arrow) morphology (Fig. 4, A1), whereas some others appeared to be osteocytes embedded within the bone matrix (blue arrows, Fig. 4, B1). Thus, in *Col2-Cre*™*; R26R* embryos, TM induced Cre activity in trabecular bone-associated cells, independent of the activity in the perichondrium.

To exclude the possibility that the Lac Z-positive cells in the primary spongiosa were progenies of chondrocytes that previously expressed Cre, we minimized the time between TM injection and embryo harvest. TM injection 6 hours prior to the harvest at E17.5 induced Cre in a small number of immature chondrocytes (red arrow) and very few hypertrophic cells ("H") in *Col2-Cre*™*; R26R* embryos (Fig. 4, C). However, a significant number of Lac Z-positive cells were detected within the primary spongiosa (purple arrow, Fig. 4, C). Consistent with the earlier observation, most positive cells appeared to associate with the trabecular bone surface (green contour), exhibiting either flat (red arrow) or cuboidal (green arrow) morphology (Fig. 4, C1). However, no Lac Z-positive osteocytes were noticed in these samples (Fig.4, C1, and data not shown), indicating that the positive osteocytes observed earlier were probably progenies of the positive cells on bone surfaces. As a control, a littermate embryo carrying *R26R* but no *Col2-Cre*™ did not show any activity in any cells (Fig. 4, D, D1). Furthermore, TM injection 3 hours prior to the harvest also induced Cre activity in trabecular bone surface cells of *Col2-Cre*™*; R26R* embryos (data not shown). Finally, Cre activity in these cells was confirmed in a second *Col2-Cre*™ transgenic line (supplementary data, Fig. S2). Thus, in *Col2-Cre*™*; R26R* embryos, a pool of trabecular bone surface cells appears to actively express the transgene.

To examine whether expression of the *Col2-Cre*™ transgene associated with the trabecular bone reflects endogenous activity of the *Colα1(II)* promoter, we performed *in situ* hybridization for *Colα1(II)* mRNA on sections of developing long bones. In E18.5 embryos, a conventional probe that recognized both the *IIA* and the *IIB* form revealed a consistent but relatively weak signal in the trabecular osteoblastic cells, compared to the overwhelming levels in the growth plate chondrocytes (data not shown). On the other hand, a probe exclusively against exon 2 and therefore specific for the *IIA* splice form detected a markedly stronger signal in the primary spongiosa (green arrow) than in chondrocytes (asterisk), both in the tibia (Fig. 5, A) and in the humerus (data not shown). In addition, *Colα1(II)A* was also detected on the endosteal surface (black arrow) of the cortical bone, with little, if any, expression on the periosteal surface (purple arrow) (Fig. 5, A). Similar to the Lac Z-positive cells in *Col2-Cre*™*; R26R* embryos, the *Colα1(II)A*-positive cells (red arrow, Fig. 5, A1) represented a subset of cells associated with the trabecular bone surface whereas the others (blue arrow, Fig. 5, A1) did not show any expression. Thus, expression of the *Col2-Cre*™ transgene in the trabecular bone surface cells likely reflects the endogenous *Colα1(II)* promoter activity in those cells.

To explore the molecular identity of the *Col2-Cre*™-expressing bone surface cells, we examined the expression of *Colα1(I)* and *bone sialoprotein* (*Bsp*), two known osteoblast markers. Specifically, sections from E17.5 *Col2-Cre*™*; R26R* embryos induced with TM for 6 hrs were first stained for Lac Z activity and then subjected to *in situ* hybridization. As expected, both *Colα1(I)* and *Bsp* were expressed at high levels in the primary spongiosa (Fig

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5, B, C). However, at a higher magnification, it appeared that no Lac Z-positive cells expressed high levels of either osteoblast marker (Fig. 5, B1, C1). Thus, the *Col2-Cre*™-positive cells associated with the trabecular bone surface appear to be distinct from the cells expressing high levels of *Colα1(I)* or *Bsp*.

Osteogenic potential of *Col2-Cre*™-positive cells

The finding that the *Col2-Cre*™-expressing cells associate with the bone surface but lack high-level *Colα1(I)* or *Bsp* expression prompted us to investigate whether they could be precursors for osteoblasts. To this end, we administered TM at 2 months of age and harvested the animals after 12 hrs. With one hindlimb of each animal, we performed Lac Z staining on sections and confirmed that acute TM treatment induced Lac Z-positive cells on trabecular bone surfaces in the *Col2-Cre*™*; R26R* animals (arrows, Fig. 6A′) but not in the *Col2-Cre*™ controls (Fig. 6B). With the other hindlimb, we isolated and cultured bone marrow stromal cells from the femur and the tibia. Lac Z staining of sub-confluent cultures consistently identified positive cells in the *Col2-Cre*™*; R26R* samples (Fig. 6C) but not in the controls (Fig. 6D). Importantly, when confluent stromal cells were further cultured in mineralization media to induce osteoblast differentiation, followed by Lac Z and von Kossa staining, certain von Kossa-positive bone nodules in the *Col2-Cre*™*; R26R* cultures were found to consist predominantly of Lac Z-positive cells (Fig. 6E-E′), whereas the control cultures, as expected, did not have any positive cells (Fig. 6F-F′). Thus, *Col2-Cre*™-positive cells isolated from the bone marrow can differentiate in vitro to produce mineralized matrix, a hallmark of osteoblasts.

TM dose-dependence of Cre activity

To explore the potential utility of the *Colα1(II)-Cre*™ line in gene removal within subsets of chondrocytes, we examined the dose-response of Cre activity to TM. Here, embryos were administered varying amounts of TM at E16.5 and assayed for Cre activity at E17.5. A direct correlation was observed between the TM dosage and the percentage of chondrocytes expressing β-galactosidase in *Col2-Cre*™*; R26R* embryos. In particular, whereas a higher dosage induced activity in nearly 80% of the growth region chondrocytes (Fig. 7, A–A2), a lower amount of TM activated Cre in less than 10% of the chondrocytes within the proximal half of the humerus (Fig. 7, B, B1), although a slightly higher percentage was found within the distal growth plate (Fig. 7, B, B2). At the lower dosage, the positive cells were often found in small clusters (red arrow) although single positive cells were also seen at a lower rate (green arrow) (Fig. 7, B1). Thus, the amount of TM positively correlates with the percentage of chondrocytes undergoing Cre recombination in the *Col2-Cre*™ mouse.

Direct regulation of *PTHrP* expression and chondrocyte morphology by Ihh

To examine the effects of a direct Ihh input in chondrocytes, we genetically removed *Smo* from subsets of cells within the growth region. To this end, we induced Cre recombination in embryos of *Col2-Cre*™*; Smo*$^{n/c}$ that carried the *Col2-Cre*™ transgene, one *Smo* null allele (*Smo*^{n}) and one *Smo* conditional allele (*Smo*^{c}). In particular, we administered an intermediate dose of TM at E11.5 and analyzed at E14.5 the proximal growth plate of the humerus, where Cre induction was found to be largely restricted to chondrocytes (see Fig. 2, A1). Since up-regulation of *Patched 1* (*Ptch1*), a direct transcriptional target of the Hh pathway (Agren et al., 2004), requires Smo function, we have monitored *Ptch1* expression to assess the removal of *Smo*. In the wild type embryo, *Ptch1* was detected at high levels in the perichondrium (green arrowheads, Fig. 8, A6) flanking the *Ihh*-expressing domain (Fig. 8, A5). In addition, *Ptch1* was expressed by immature chondrocytes in a graded pattern, with the highest level present in cells adjacent to the Ihh source (Fig. 8, A6). In the TM-induced *Col2-Cre*™*; Smo*$^{n/c}$ embryos, the high-level expression was maintained in the perichondrium (green arrowheads, Fig. 8, B6, C6), confirming a normal response to Ihh in this tissue. However, groups of immature

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chondrocytes were found to be devoid of *Ptch1* (asterisks, Fig. 8, B6, C6), indicating effective removal of *Smo* in those cells. Notably, the size and location of the affected region varied between the two *Col2-Cre*™*; Smo*$^{n/c}$ embryos of the same litter (Fig. 8, B6 vs. C6), revealing a stochastic aspect of TM-induced gene deletion. Overall, proper administration of TM induced deletion of *Smo* within subpopulations of the growth region chondrocytes without disrupting Ihh signaling in the perichondrium.

We next examined the effect of the local removal of *Smo* on *PTHrP* expression. For this, *in situ* hybridization was performed on adjacent sections for *Ptch1* and *PTHrP*. At the proximal end of the humerus of E14.5 embryos, *PTHrP* was normally detected in a group of periarticular chondrocytes that also expressed a low level of *Ptch1* (yellow contour, Fig. 8, A6, A7). Remarkably, in the TM-induced *Col2-Cre*™*; Smo*$^{n/c}$ embryos, areas devoid of *Ptch1* within the periarticular domain showed little to no expression of *PTHrP* (red contour, Fig. 8, B6, B7), whereas the adjacent domains maintaining low levels of *Ptch1* also retained *PTHrP* expression (purple contour, Fig. 8, B6, B7). Importantly, in embryos where *Ptch1* expression was not disturbed in the periarticular area but severely reduced in other domains of the growth region, *PTHrP* expression was not disrupted (green contour, Fig. 8, C6, C7). In fact, the *PTHrP*-expressing domain appeared to be expanded in these embryos (Fig. 8, compare A7 and C7), perhaps reflecting further Ihh movement into the periarticular territory (competent to express *PTHrP*) due to the decrease in sequestration by Ptch1 (Chen and Struhl, 1996). Thus, *PTHrP* expression by periarticular chondrocytes correlated with their own responsiveness to Ihh, but not with that in the perichondrium or other parts of the growth region.

We next examined whether localized removal of *Smo* affected the morphology of growth plate chondrocytes in the TM-induced *Col2-Cre*™*; Smo*$^{n/c}$ embryos. Histology of the humerus revealed obvious anomalies within subdomains of the growth region in the mutant embryo. In particular, certain flat chondrocytes (Fig. 8, A2) were replaced with cells with heterogeneous morphology (red arrows) and irregular spacing (asterisks) in the mutant sample (Fig. 8, B2, C2). The abnormal morphology could not be accounted for by overt acceleration of chondrocyte maturation, as no ectopic expression of *Ihh* (Fig. 7, B5, C5) or *PTHrP-R* (data not shown) was detected, and *Colα1(X)* was only weakly detectable in a scattered subset of the affected cells with no correlation with the size of the cell (orange arrows, Fig. 8, B4, C4, data not shown). Importantly, the anomaly was found exclusively within areas devoid of *Ptch1* expression (asterisks, Fig. 8, B6, C6), whereas adjacent regions that maintained *Ptch1* expression were not affected (Fig. 8, B3, C3). Thus, removal of Ihh responsiveness from subsets of cells within the growth region locally disrupted the morphology and organization of immature chondrocytes. In summary, these results support the notion that Ihh directly controls both *PTHrP* expression and chondrocyte morphology in the growth region cartilage.

DISCUSSION

We have described a TM-inducible *Col2-Cre*™ line that can be used to selectively activate Cre in chondrocytes, overcoming a limitation of current *Col2-Cre* transgenic lines that express Cre in both chondrocytes and the perichondrium (Long et al., 2004; Sakai et al., 2001). Serendipitously, we found that the new line also targets a subset of cells associated with the trabecular bone surface, likely reflecting the endogenous activity of the *Colα1(II)* promoter that directs expression of the prechondrogenic *Colα1(II)A* isoform in these cells. Finally, utilization of the *Col2-Cre*™ line in localized removal of *Smo* has provided evidence that direct Ihh input is likely required for both *PTHrP* expression in the periarticular chondrocytes, and the proper morphology of columnar chondrocytes.

The versatility of the *Col2-Cre*™ line lies in the fact that both cell type-selectivity (chondrocytes vs. perichondrial cells) and localized activity (subsets of chondrocytes) can be

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achieved by adjusting the timing or dosage of TM administration. It should be noted however, that although a higher TM dosage generally correlated with more cells expressing Cre activity, certain variability has been observed among embryos from pregnant females receiving the same amount of TM. In addition, the location of Cre-active cells within the growth plate also varied among embryos. These observations reflect a stochastic aspect of TM-mediated Cre activation, likely resulted from the variable availability of TM to each cell especially when a lower dosage was used.

In addition to its utility in embryonic studies, the *Col2-Cre*™ line also promises to be a useful tool for postnatal genetic studies. In particular, our results with young pups showed that nearly all of columnar chondrocytes activated Cre after receiving two doses of TM. Similarly, a high percentage of the articular chondrocytes exhibited TM-induced Cre activity. Thus the *Col2-Cre*™ line may be useful for postnatal studies involving either the growth plate or the joints.

Several lines of evidence from the present study support that direct Ihh input is likely required for periarticular chondrocytes to express *PTHrP*. First, loss of Ihh response in subsets of periarticular chondrocytes correlated with the loss of *PTHrP* in those cells, whereas cells outside the affected domains maintained their normal *PTHrP* expression profile. Second, loss of Ihh response in chondrocytes outside the normal *PTHrP*-expressing periarticular domain did not diminish *PTHrP* expression. Third, Ihh response in the perichondrium did not correlate with periarticular *PTHrP* expression. However, the current data does not exclude the possibility that other signals may emanate from within the *PTHrP*-expressing group of cells and function in either an autocrine or a paracrine manner.

The immediate regulator of *PTHrP* downstream of Ihh is presently unknown. In an effort to examine potential direct regulation of the *PTHrP* promoter by Ihh, we identified several putative Gli binding sites approximately 4 kb upstream of the first exon of the murine *PTHrP* gene. However, studies of promoter fragments containing these potential sites by transient transfections in C3H10T1/2 cells have not revealed a consistent response to exogenous Hh. The most trivial explanation for the negative results is that the Hh responsive elements were not included in the tested fragments. However, since *PTHrP* promoter activity in the embryonic growth plate generally correlates with low- but not high-level Ihh signaling, as indicated by the low level of *Ptch1* in the *PTHrP*-expressing cells, it is possible that a "proper" level of Hh signaling required for activating *PTHrP* expression was not achieved in the cell culture model. Finally, Hh may not directly control the promoter activity of *PTHrP*. Future studies are required to distinguish these possibilities.

The present study indicates that direct Ihh input may be required for the proper morphology of columnar chondrocytes. Local removal of Ihh responsiveness resulted in abnormal cell morphology and disorganization of chondrocytes within the affected regions. Specifically, the number of flat cells was greatly reduced and the normal "stacks" of cells were no longer evident. The morphological defect was reminiscent to that observed in *Col2-Cre; Smo*$^{n/c}$ embryos where the entire columnar region was absent (Long et al., 2006), but the underlying mechanism remains unknown. Ihh could directly control the cell morphology, or, as previously suggested, regulate a "differentiation" process that encompasses the morphological changes (Kobayashi et al., 2005). Alternatively, the loss of flat cells could be secondary to the marked decrease in cell number within the region in the absence of Ihh signaling (Long et al., 2001). In any case, the regulation appears to be mediated by antagonizing Gli3 repressor activity, as removal of *Gli3* restored the columnar chondrocytes in the absence of *Ihh* (Hilton et al., 2005).

Finally, the current study has identified a *Col2-Cre*™-positive cell population primarily associated with the trabecular, but not the cortical, bone surface. These cells appear to represent only a subset of the bone surface cells, and are morphologically heterogeneous as appearing

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on tissue sections. The role of these cells in vivo remains unknown at present, but they are capable of producing mineralized bone nodules in vitro. Molecularly, they do not express high levels of *Colα1(I)* or *Bsp*. However, it should be noted that in the "co-localization" experiment, tissue sections were first subjected to Lac Z staining prior to in situ hybridization for *Colα1 (I)* or *Bsp* mRNA. Because we have noticed that the staining procedure notably reduces the in situ hybridization signals, it is possible that the *Col2-Cre*™-positive cells express a lower level of *Colα1(I)* or *Bsp* that is below the current sensitivity threshold. Finally, since the endogenous *Colα1(II)* promoter is known to be active in skeletal precursors during embryogenesis, the *Col2-Cre*™-expressing cells may represent a progenitor population uniquely associated with the trabecular bone both in the embryo and in postnatal life. Future studies are necessary to determine whether this is indeed the case.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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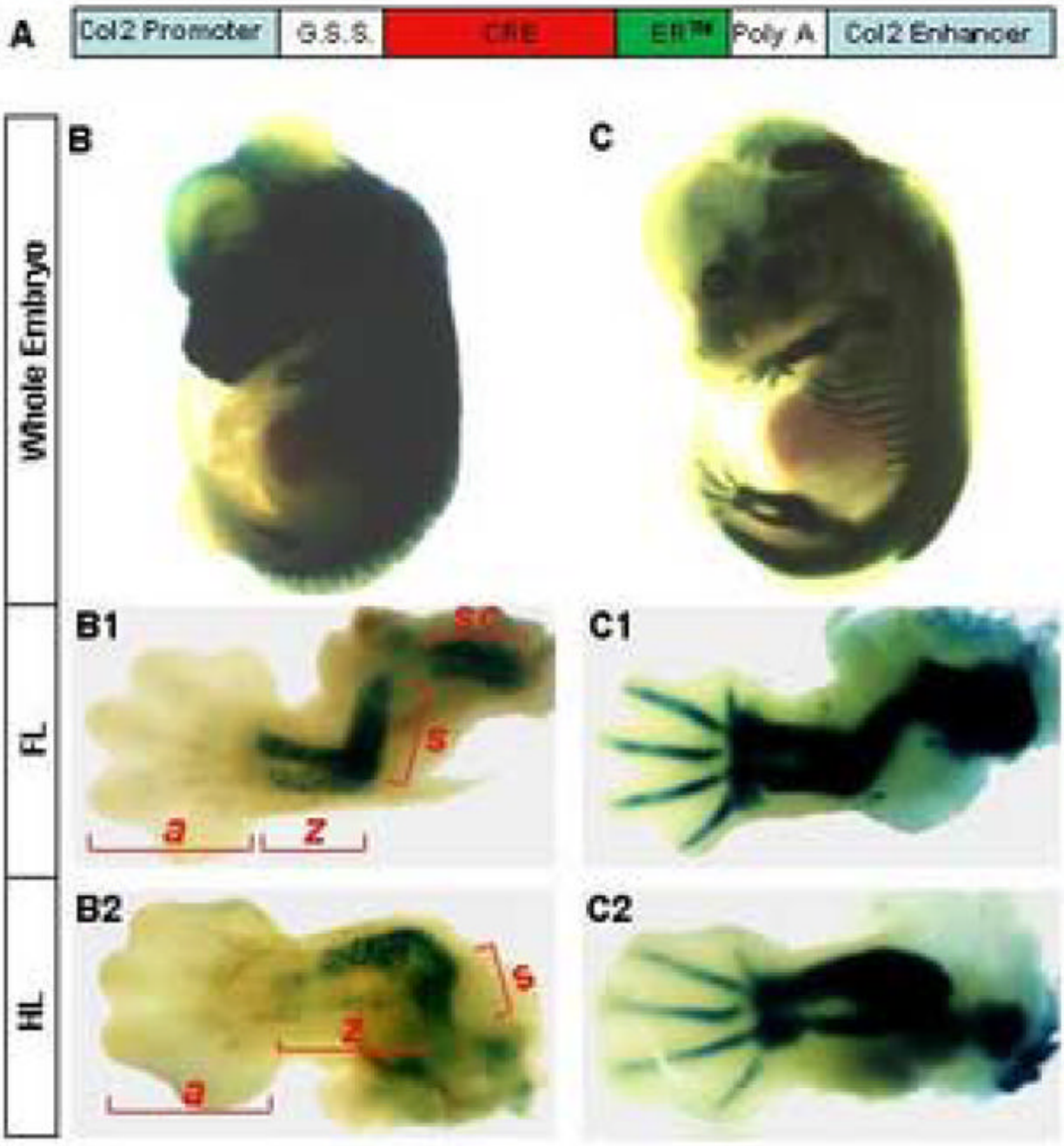

Figure 1.

Tamoxifen (TM) inducible *Col2-Cre*™ transgenic mouse. (A) Schematic of the *Col2-Cre*™ transgene. G.S.S., β-globin splicing sequence. ER™, Modified form of the estrogen receptor responsive only to exogenous tamoxifen. (B–C) β-galactosidase activity assays (Lac Z staining) indicating induction of Cre by TM in *Col2-Cre*™*; R26R* embryos. Whole embryos were stained at E12.5 after pregnant females were injected with 75 μg/g TM (75 μg TM per gram of body weight) at either E9.5 (B) or E10.5 (C). Forelimbs (FL) (B1–C1) and hindlimbs (HL) (B2–C2) were shown at a higher magnification beneath the corresponding whole embryos. a: autopod, z: zeugopod, s: stylopod, sc: scapula.

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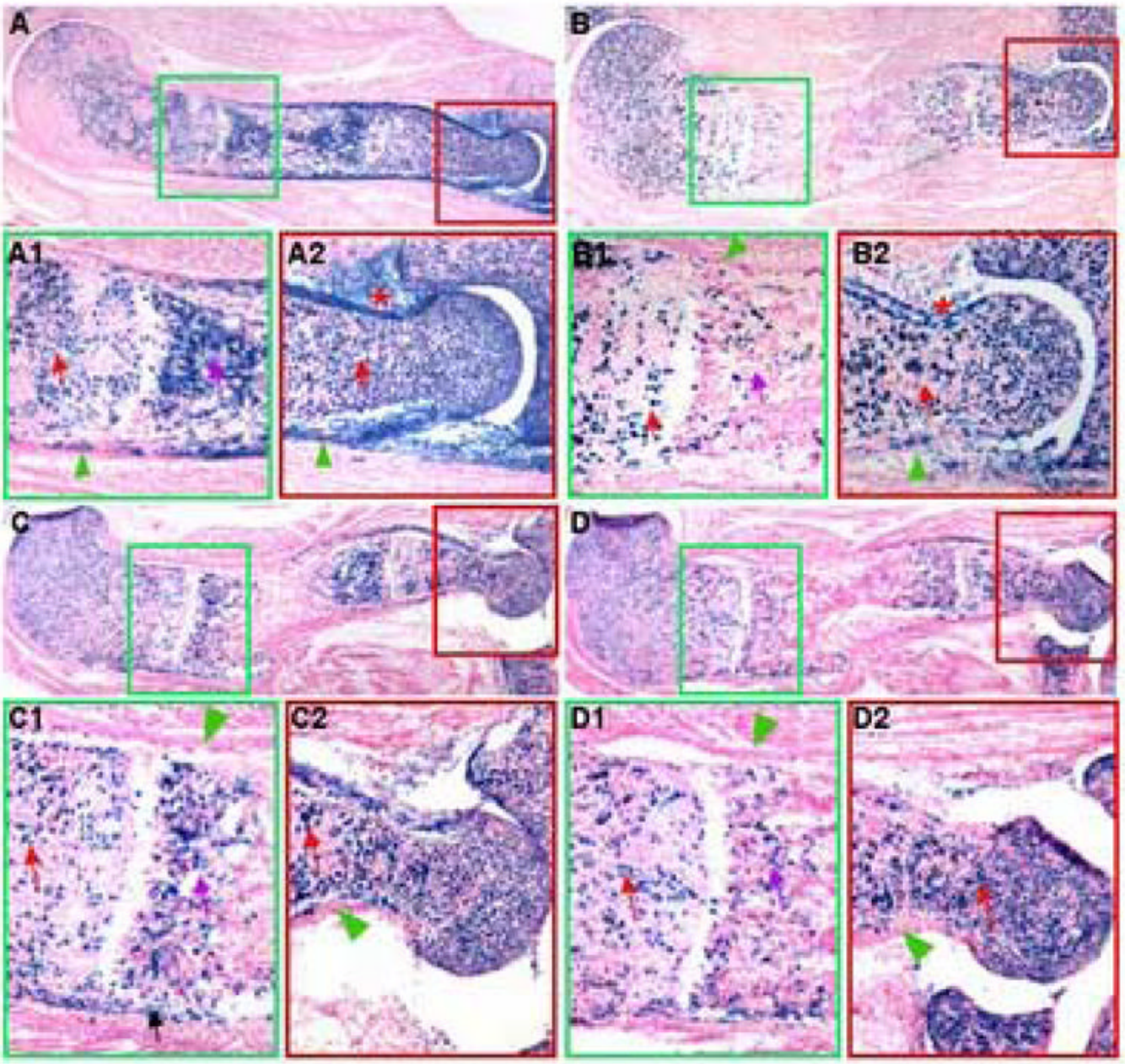

Figure 2.

Control of cell-type specific Cre activity in the embryo. Lac Z staining was performed on cryostat longitudinal sections of the humerus from E17.5 *Col2-Cre*™*; R26R* embryos. The embryos were harvested after pregnant females were injected with 25 μg/g TM (25 μg TM per gram of body weight) at E11.5 (A), E12.5 (B), E13.5 (C) or E14.5 (D). Green and red boxes in A–D identify areas that are shown below at a higher magnification. The proximal end is to the left in all panels. Red arrows: activity in chondrocytes; purple arrows: activity in primary spongiosa; black arrow: chondrocytes of deltoid tuberocity; green arrowheads: perichondrium; asterisks: activity around joint capsule.

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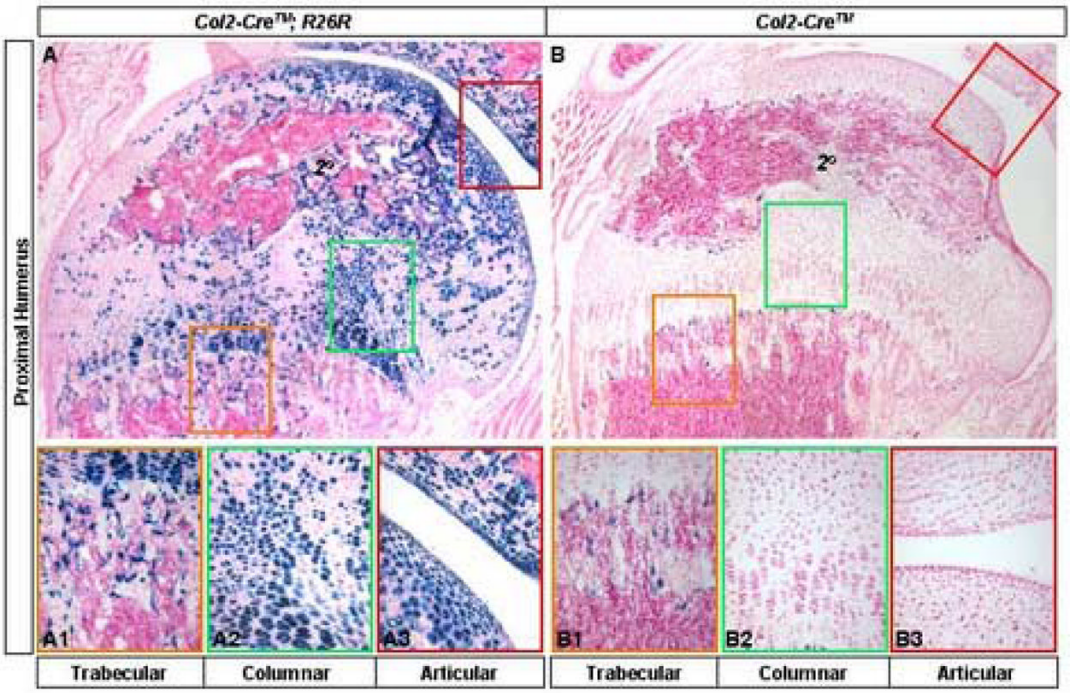

Figure 3.

Induction of Cre activity in postnatal mice. (A, B) Lac Z staining on cryostat longitudinal sections through the proximal end of the humerus from P16 animals. Mice with the genotype of either *Col2-Cre*™; *R26R* (A) or *Col2-Cre*™ (B) were injected 400 μg/g TM (400 μg TM per gram of body weight) at P12 and P14. Color-coded rectangular boxes in A and B identify areas shown at a higher magnification below (A1–A3 and B1–B3 respectively). The proximal end is to the top in all panels. 2°: secondary ossification center.

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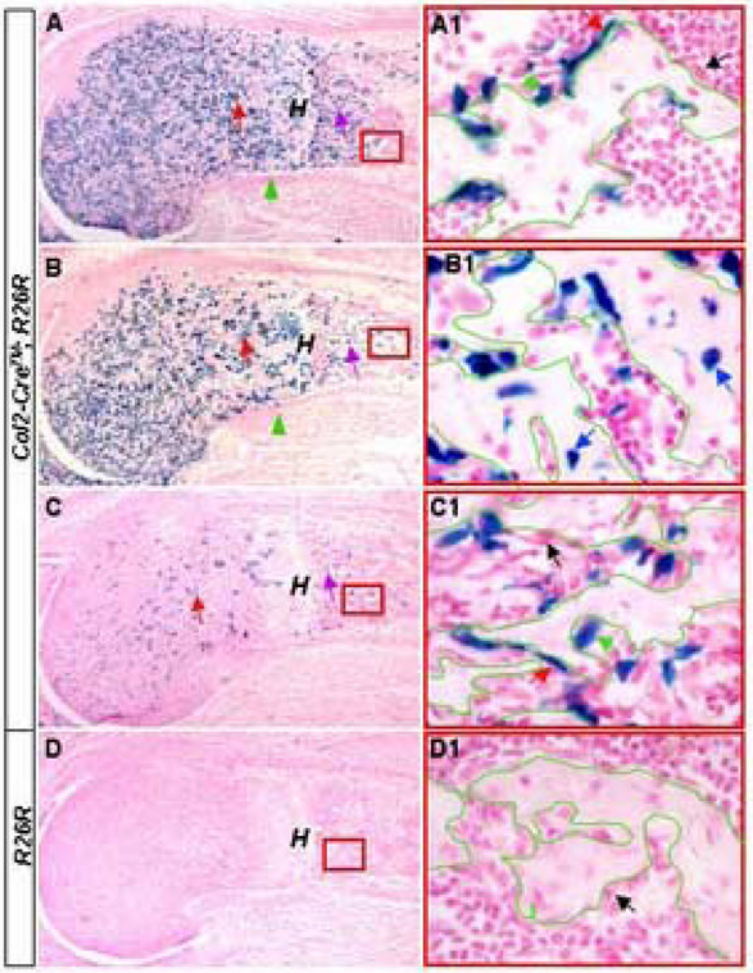

Figure 4.

Cre activity in trabecular osteoblast-lineage cells. Lac Z staining on cryostat longitudinal sections through the humerus from E17.5 embryos with the indicated genotypes. The pregnant females were injected with 25 μg/g TM (25 μg TM per gram of body weight) at either E13.5 (A, B) or 6 hours prior to harvest (C, D). The proximal half of the humerus is shown in A–D. Red boxes in A–D indicate areas shown at a higher magnification (A1–D1, respectively). Red arrows in A–C: activity in chondrocytes; purple arrows in A–C: activity in primary spongiosa; green arrowheads in A and B: perichondrium; red arrows in A1 and C1: Lac Z-positive flat cells; green arrows in A1 and C1: Lac Z-positive cuboidal cells; black arrows in A1, C1 and D1: Lac Z-negative cells; blue arrows in B1: Lac Z-positive osteocytes; green contours in A1–D1: outlines of bone surface; H: hypertrophic zone. The proximal end is to the left in all panels.

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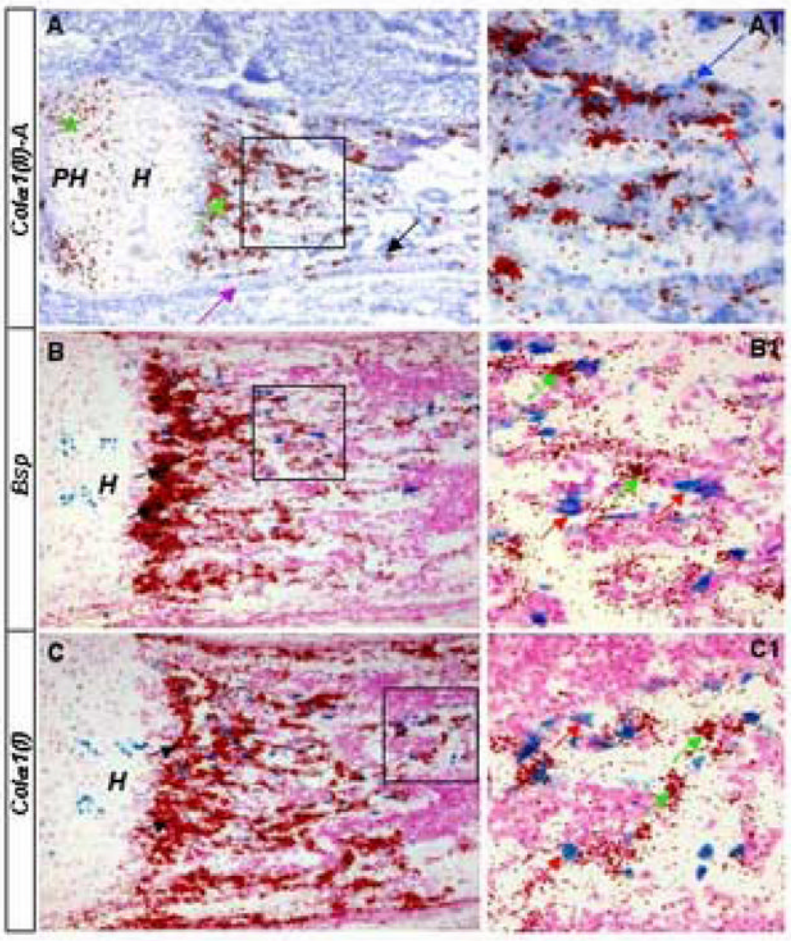

Figure 5.

Expression of *Colα1(II)A* by trabecular osteoblast-lineage cells. (A) *In situ* hybridization using ^{35}S-labeled riboprobe specific to *(II)A* on longitudinal sections through the tibia of E18.5 wild type embryos. Signal is in red and counterstain in blue. Black arrow in A: endosteal surface of cortical bone; purple arrow in A: periosteal surface of cortical bone; asterisk in A: signal in chondrocytes; green arrows in A: primary spongiosa; blue arrow in A1: cell without signal; red arrow in A1: cell with signal. (B–C) Lac Z staining followed by in situ hybridization on frozen sections through the humerus of E17.5 *Col2-Cre*™; *R26R* embryos induced with 25 μg/g TM for 6 hrs. Lac Z signal in blue, in situ signal in red, counterstain in magenta. Note that bone trabeculae were not stained. Also note that black arrows in B and C point to dark specks representing oversaturated in situ signal not Lac Z staining. Red arrows in B1 and C1: Lac Z-positive cells; green arrows in B1 and C1: high-expressers of *Bsp* or *Colα1(I)*. The proximal end is to the left in all panels. Boxed regions are shown at a higher magnification to the right (A1–C1). “PH”: prehypertrophic chondrocytes; “H”: hypertrophic chondrocytes.

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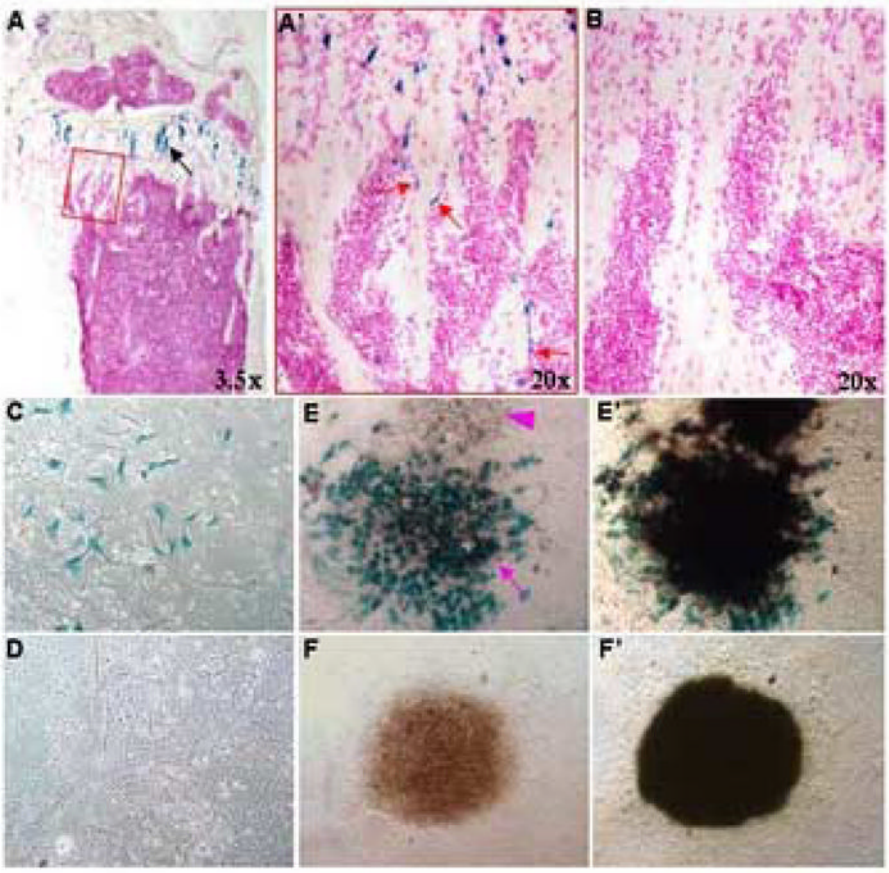

Figure 6.

Osteogenic potential of *Col2-Cre*™-positive cells isolated from the bone marrow. 2-month-old mice were analyzed at 12 hrs after TM delivery via oral gavage at 125 μg per gram of body weight. (A) Lac Z staining on a longitudinal section through the tibia of a *Col2-Cre*™*; R26R* mouse. Black arrow denotes Lac Z-positive chondrocytes. (A′) The boxed area in (A) viewed at a higher magnification. Red arrows denote Lac Z-positive cells on trabecular bone surfaces. (B) An equivalent region from a *Col2-Cre*™ mouse containing no Lac Z-positive cells. (C–D) Lac Z staining of sub-confluent cultures of bone marrow stromal cells isolated from *Col2-Cre*™*; R26R* (C) versus *Col2-Cre*™ (D) animals. (E–F) Lac Z staining of bone nodules from *Col2-Cre*™*; R26R* (E) or *Col2-Cre*™ (F) cultures. Purple arrow denotes a bone nodule consists predominantly of Lac Z-positive cells; purple arrowhead denotes an adjacent nodule with no Lac Z-positive cells. (E′–F′) von Kossa staining of the same bone nodules following Lac Z staining in (E–F).

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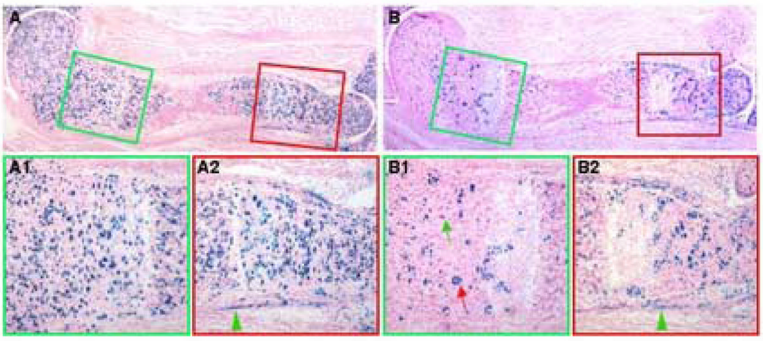

Figure 7.

TM dose-dependence of Cre activity in the embryo. Lac Z staining was performed on cryostat longitudinal sections of the humerus from E17.5 *Col2-Cre*™*; R26R* embryos. The embryos were harvested after pregnant females were injected at E16.5 with either 100 (A) or 12.5 (B) μg/g TM (100 or 12.5 μg TM per gram of body weight). Green and red boxes in A and B identify areas shown below at a higher magnification (A1–A2, B1–B2, respectively). The proximal end to the left in all panels. Red arrow: a cluster of positive cells; green arrow: a single positive cell; green arrowheads: residual activity in perichondrium.

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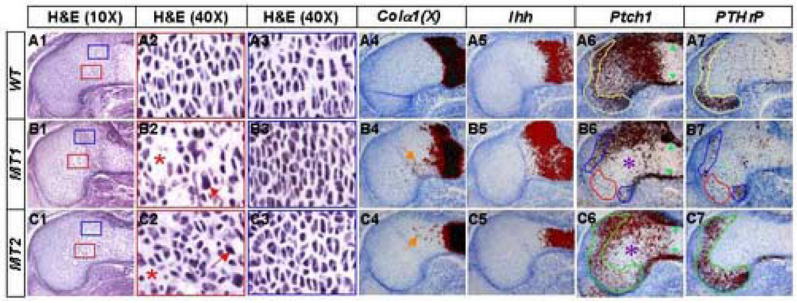

Figure 8.

Direct regulation of *PTHrP* expression and chondrocyte morphology by Ihh. Histology (A1–A3, B1–B3, C1–C3) and *in situ* hybridization (A4–A7, B4–B7, C4–C7) performed on adjacent longitudinal sections of humerus from E14.5 wild type (WT) (A1–A7) and *Col2-Cre*™*; Smo*$^{n/c}$ (MT1 and MT2) (B1–B7, C1–C7 respectively) littermate embryos. The embryos were harvested after pregnant females were injected 50 μg/g TM (50 μg TM per gram of body weight) at E11.5. The proximal half of each section is shown with the articular surface to the left. The color-coded boxes in A1–C1 denote areas shown at a higher magnification in A2–A3, B2–B3 and C2–C3, respectively. Red arrows in B2 and C2 denote abnormal chondrocyte morphology; asterisks in B2 and C2 indicate irregular spacing between cells. ^{35}S-labeled probes used for *in situ* hybridization are as indicated directly above the panels. Signal is in red and counterstain in blue. Orange arrows in B4 and C4 denote ectopic *Colα1(X)* expression; color-coded contours in A6–A7, B6–B7 and C6–C7 demarcate equivalent areas in adjacent sections; asterisks in B6 and C6 indicate areas devoid of *Ptch1* expression; green arrowheads in A6–C6 denote signal in perichondrium.