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REGULATORY ELEMENTS DRIVING THE EXPRESSION OF SKELETAL LINEAGE REPORTERS DIFFER DURING BONE DEVELOPMENT AND ADULTHOOD

Pieter-Jan Stiers^{a,b}, Nick van Gestel^{a,b}, Karen Moermans^a, Ingrid Stockmans^a, Geert Carmeliet^{a,b,*}

^a Laboratory of Clinical and Experimental Endocrinology, Department of Clinical and Experimental Medicine, KU Leuven, Leuven, Belgium

^b Prometheus, Division of Skeletal Tissue Engineering, KU Leuven, Leuven, Belgium

* Author of correspondence: Geert Carmeliet

Laboratory of Clinical and Experimental Endocrinology,

Herestraat 49 bus 902, 3000 Leuven, Belgium

E-mail address: geert.carmeliet@kuleuven.be

Tel +32 16 330731

Highlights:

- Fate mapping provides insight in cell behavior during bone development and regeneration.
- Promoter fragments of collagen type I and II drive reporter expression in respectively osteoblasts and chondrocytes.
- Different regulatory elements in collagen type I and II promoter are required to target cells in adult versus embryonic mice.
- Labeling and targeting of specific skeletal cell types during bone repair in adult mice remains challenging.

Keywords:

Bone repair, endochondral ossification, lineage tracing, promoter constructs, fluorescent reporter

ABSTRACT

To improve bone healing or regeneration more insight in the fate and role of the different skeletal cell types is required. Mouse models for fate mapping and lineage tracing of skeletal cells, using stage-specific promoters, have advanced our understanding of bone development, a process that is largely recapitulated during bone repair. However, validation of these models is often only performed during development, whereas proof of the activity and specificity of the used promoters during the bone regenerative process is limited. Here, we show that the regulatory elements of the 6kb collagen type II promoter are not adequate to drive gene expression during bone repair. Similarly, the 2.3kb promoter of collagen type I lacks activity in adult mice, but the 3.2kb promoter is suitable. Furthermore, Cre-mediated fate mapping allows the visualization of progeny, but this label retention may hinder to distinguish these cells from ones with active expression of the marker at later time points. Together, our results show that the lineage-specific regulatory elements driving gene expression during bone development differ from those required later in life and during bone repair, and justify validation of lineage-specific cell tracing and gene silencing strategies during fracture healing and bone regenerative applications.

1. INTRODUCTION

Bone has a remarkable capacity to regenerate by recapitulating the molecular and cellular pathways that regulate its development [1]. Nonetheless, up to one in ten fractures will result in a delayed or non-union, and this figure is only expected to increase as the general population ages and comorbidities are more often present [2, 3]. These patients with non-healing fractures suffer from severe disability and require extensive hospitalization and revalidation as the current treatments, such as bone auto- or allografts, are often unsatisfactory [4-7]. Bone tissue engineering is a promising therapeutic alternative for non-healing bone defects, but its clinical applicability remains low. A major cause is the limited insight into the fate of the implanted cells and how they contribute to successful bone regeneration. Tracing of individual cells into different skeletal lineages is a very valuable tool for studying not only the process of fracture healing, but also the *in vivo* fate of cells implanted in bone tissue-engineered constructs that are designed to recapitulate the healing process.

The transgenic Cre-*loxP* recombination system driven by regulatory elements that are active during specific stages of differentiation of mesenchymal stem cells (MSC) has provided important information on the contribution of this lineage to *in vivo* endochondral ossification (reviewed in [8]). The earliest MSC and progenitors in developing limbs can be tagged by their expression of paired related homeobox 1 (*Prx1*) and in a later stage by sex-determining region Y box 9 (*Sox9*). These progenitors then condense and differentiate into chondrocytes, marked by high expression of the extracellular matrix protein collagen type II (*Col2*). At the same time, the MSC surrounding these condensations differentiate into the osteogenic lineage and produce large amounts of collagen type I (*Col1*), which makes up most of the organic bone matrix. Long bone trauma leads to the activation of cell populations that largely recapitulate these processes, resulting in the formation of a cartilaginous fracture callus that is subsequently replaced by bone matrix and remodeled into mature mineralized bone.

Although the molecular pathways governing the bone regenerative process appear similar to those active during development, the discussed genetic fate mapping strategies have in many cases only been validated during embryogenesis and around birth. Proof that they also target the skeletal cell populations contributing to fracture healing or other bone regenerative processes, such as in tissue-engineered implants, is limited. Skeletal cells that are critical for the healing of long bone trauma are the periosteal cells [9], making them an attractive MSC source for bone regenerative and tissue engineering applications [10, 11]. However, evidence on the expression of transgenic lineage markers in these cells especially during bone repair remains limited.

In this study, we have performed an in-depth investigation of chondrogenic and osteogenic lineage markers commonly used for cell tracing during development, in order to validate them for application in fracture healing and the study of bone tissue engineering *in vivo*. Our results show that several regulatory elements driving gene expression in specific lineages during bone development are not adequate to induce gene expression in young-adult periosteal cells or during bone regeneration, underscoring the need for careful validation of genetic tools when experimental conditions differ from the verified ones.

2. MATERIALS & METHODS

2.1 Mouse strains

The following mouse strains were analyzed: Gt(ROSA)26Sor^{tm4(ACTB-tdTomato,-EGFP)Luo} mice (hereafter referred to as mTmG) [12] crossed with either the Tg(Col1a1-cre)1Kry mice (referred to as (2.3kb)Col1Cre) [13] or Tg(Col2a1-cre)1Bhr mice (referred to as (6kb)Col2Cre) [14]. Transgenic reporter lines directly expressing a lineage-specific fluorophore were Tg(Col1a1-cre/ERT2,-DsRed)1Smkm mice (referred to as (3.2kb)Col1-dsRed) [15] and the Col2-GFP mouse (referred to as the (6kb)Col2GFP mouse) [16]. All strains were 100% C57Bl/6J background, except (6kb)Col2Cre mice which were 100% CD1. At least three animals per genotype were analyzed at each time point. Animal experiments were conducted according to the regulations and with approval of the Animal Ethics Committee of the KU Leuven.

2.2 Histochemistry.

The tibiae of postnatal day (P) 5 pups or 8-week-old male mice were dissected and fixed in 2% paraformaldehyde overnight at 4°C, decalcified in EDTA for 14 days at 4°C, embedded in NEG-50 frozen section medium (Richard-Allen Scientific, Thermo Fisher Scientific, Kalamazoo, MI) and sectioned with a cryostat at 7 µm. Sections were counterstained with Hoechst or stained with haematoxylin & eosin (H&E). Immunohistochemistry for anti-green fluorescent protein (GFP) and anti-collagen type II was performed as published previously [17, 18]. Images were taken on a Zeiss Axioplan 2 light microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) using a Plan-Neofluar 20x/0.5 objective or on a Zeiss LSM510 confocal microscope using a Plan-Apochromat 63x/1.4 oil objective. eGFP and mGFP were excited using a 488 nm Argon laser and imaged through a 500 – 550 nm band pass filter. mTomato and dsRed were excited with a 546 nm HeNe laser and imaged through a 565 – 615 nm band pass filter. Hoechst 33342 nuclear counterstain was excited using a Mai Tai Ti:sapphire multiphoton laser (Spectra-Physics, Santa Clara, CA, USA) tuned to 760 nm and

imaged through a 435 – 485 nm band pass filter. Confocal images were processed using ImageJ v1.49 software.

2.3 Cell cultures

Murine periosteum-derived cells (mPDC) were isolated as described previously [19]. Briefly, tibiae and femora were dissected and cleaned, their epiphyses were coated in 5% low melting point agarose (SeaPlaque, Lonza, Verviers, Belgium) and mesenchymal cells were released from the periosteal surface by collagenase-dispase digest. Cells were cultured in α MEM medium containing 2 mM GlutaMAX-I and supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin (all from Gibco, Life Technologies, Ghent, Belgium). For *in vitro* osteogenic differentiation experiments, cells were seeded at a density of 3×10^4 per cm^2 in Lab-Tek II glass-bottom chamber slides (Nunc A/S, Roskilde, Denmark) and cultured in medium supplemented with 10 mM β -glycerophosphate and 50 $\mu\text{g/ml}$ ascorbic acid (Sigma-Aldrich, Diegem, Belgium) for 14 days. For *in vivo* cartilage formation assays, mPDC were cultured in medium supplemented with 5 U/ml heparin (LEO Pharma, Ballerup, Denmark) and 5 ng/ml human recombinant basic fibroblast growth factor (R&D Systems, Minneapolis, MN). Following expansion, cells were embedded in collagen type I and implanted as described [17]. To investigate that recombination of the mTmG reporter was efficient and specific, *in vitro* recombination of mTmG reporter was tested by incubating mPDC with 500 plaque-forming units of adenovirus expressing empty control or Cre (Gene Transfer Vector Core, University of Iowa, IA) as previously described [20]. Cells were then fixed in 4% paraformaldehyde for 30 minutes at 4°C and nuclei were counterstained with Hoechst 33342.

3. RESULTS

3.1 The (6kb)Col2 promoter labels osteochondrogenic cells during endochondral ossification

To confirm earlier studies showing that the (6kb)Col2Cre transgene targets mainly chondrogenic cells during endochondral bone development, (6kb)Col2Cre mice were mated to mTmG reporter mice. Histological analysis of P5 offspring showed that the reporter was activated in all types of cartilage of the long bones, including articular cartilage as well as proliferating and hypertrophic chondrocytes of the growth plate (Figure 1A-C). Furthermore, numerous GFP⁺ cells were also found in the perichondrium, the primary spongiosa and the endosteum (Figure 1D, E, G), whereas GFP⁺ cells were sparse in the bone marrow and the periosteum (Figure 1F-H), confirming that the reporter is active in a population of osteochondral progenitors during bone development [21]. Recombination was not observed in the tibiae of (6kb)Col2Cre-negative mTmG littermates (Figure 1I-L). These observations were in line with *in vitro* data showing that recombination occurred in mPDC derived from mTmG mice only after transduction with adenovirus expressing Cre (Figure S1).

3.2 (6kb)Col2Cre-labeled cells are present in adult bone but insufficiently mark cartilage during bone repair

The (6kb)Col2Cre x mTmG reporter expression was then assayed in the long bones of 8-week-old mice, before being analyzed during fracture repair. In line with previous reports, chondrocytes in the articular and growth plate cartilage were GFP⁺ (Figure 2A-C), as well as some cells in the primary spongiosa, the trabecular bone and bone marrow, the endosteum and the periosteum (Figure 2D-H). However, this Cre-mediated genetic strategy does not allow distinction between cells actively expressing Cre, driven by the lineage-specific promoter, and progeny derived from osteochondral progenitors where recombination occurred earlier and thus remained GFP-positive. No labeled cells were found in (6kb)Col2Cre-negative mTmG mice (Figure 2I-L). To determine whether this approach labels cartilage formed during endochondral bone repair, a mid-tibial fracture was stabilized with a

medullary pin resulting in the formation of a cartilaginous callus surrounded by woven bone one week after surgery (Figure 3A, Figure S2). Unexpectedly, hardly any chondrocyte in the callus was labeled by the (6kb)Col2Cre x mTmG reporter (Figure 3B-C). Some GFP⁺ cells were observed in the woven bone that was formed at the callus edges (Figure 3D-E). These observations suggest that the labeled cells likely derived from cells in which the (6kb)Col2Cre-transgene had been active before, but that they did not express the transgene any longer. The absence of labeling of newly formed chondrocytes in adult mice was confirmed in a model for ectopic cartilage formation. The implanted mPDC, derived from (6kb)Col2Cre x mTmG reporter mice, differentiated into collagen type II-positive chondrocytes but did not become GFP-positive (Figure S3).

3.3 The (6kb)Col2GFP transgene is not actively transcribed during bone repair

To further confirm that the (6kb)Col2Cre transgene is not actively transcribed during endochondral bone repair, tibiae of (6kb)Col2GFP mice were analyzed. In these mice, the same transgenic promoter construct drives expression of a fluorophore that will not be genetically propagated into the lineage and thus labels cells only when they actively express the reporter. In tibiae of P5 mice, the (6kb)Col2GFP transgene was expressed in articular cartilage, proliferating chondrocytes, and a few cells lining the endosteal and periosteal bone surfaces (Figure 4A-D), which resembled the labeling pattern of (6kb)Col2Cre x mTmG mice. However, expression of the (6kb)Col2GFP reporter was not observed in adult cartilage or bone (Figure 4E-H) or in any part of the fracture callus at day 7 post injury, even when staining was enhanced with anti-GFP immunocytochemistry (Figure 4I-J). Together these data indicate that the regulatory elements in the (6kb)Col2 promoter are not sufficient to mark newly differentiated chondrocytes during fracture healing.

3.4 The (2.3kb)Col1 promoter labels osteogenic cells during endochondral ossification

We next investigated possible reporters to trace osteogenic cells during fracture healing. To confirm previous data that the (2.3kb)Col1Cre transgene is expressed in osteoblasts during

long bone development, these mice were mated to mTmG reporter mice. Histological analysis of tibiae at P5 revealed that GFP⁺ cells were present at sites of active bone formation, including the perichondrium, the primary spongiosa, trabecular bone, and the endosteal and periosteal bone surfaces (Figure 5D-H). As expected, recombination had not occurred in any of the cartilaginous tissues, including articular cartilage and growth plate chondrocytes (Figure 5A-C). In addition, GFP⁺ cells were not detected in developing tibiae of (2.3kb)Col1Cre-negative mTmG littermates (Figure 5I-L).

3.5 Expression driven by the (2.3kb)Col1 promoter becomes increasingly restricted with age

The reporter expression in long bones of (2.3kb)Col1Cre x mTmG mice was further analyzed at 8 weeks of age. As expected, no recombination was observed in cartilage (Figure 6A) or in (2.3kb)Col1Cre-negative mTmG littermates (Figure 6I-L). In bone tissue, most cells in the cortex as well as some osteoblasts lining the endosteal surface were GFP-positive (Figure 6E-G). On the other hand, no reporter activation was apparent in the secondary ossification center, the primary spongiosa, the trabecular bone or the periosteal bone surface (Figure 6B-D, Figure 6H), suggesting that the regulatory elements in the 2.3kb promoter are insufficient to drive collagen type I expression in many osteogenic cells, particularly those in the periosteum. This finding was confirmed *in vitro*, showing that mPDC isolated from 8-week-old (2.3kb)Col1Cre x mTmG mice expressed collagen type I when cultured in osteogenic conditions but did not become GFP-positive (Figure S4).

3.6 The (3.2kb)Col1-dsRed reporter retains its expression after development

As a possible solution to label osteogenic cells in adult long bones, the reporter expression pattern, driven by a longer collagen type I promoter, was assayed in the (3.2kb)Col1-dsRed mouse. In line with previous reports, many cells were labeled in newborn pups at sites of active bone formation, such as the primary spongiosa, trabecular bone, and the endosteal and periosteal bone surfaces (Figure 7D-H), whereas reporter expression was not observed

in chondrocytes (Figure 7A-C). In 8-week-old mice, abundant reporter expression was still found in all areas of active bone formation, including the secondary ossification center, the primary spongiosa, trabecular bone, the endosteum and the periosteum, as well as in osteocytes that had become embedded into the cortex (Figure 7J-P). No dsRed-positive cells were found in cartilage (Figure 7I). Furthermore, when mPDC were isolated from these mice and cultured *in vitro* only few cells expressed Col1-dsRed under normal growth conditions, whereas in osteogenic conditions the expression of the (3.2kb)Col1-dsRed reporter became active in almost all cells (Figure 7Q-R), confirming the active expression of this transgene in periosteal cells differentiating into the osteogenic lineage.

4. DISCUSSION

In this study, we report that the lineage-specific regulatory elements in gene promoters, which are commonly used for cell tracing during bone development, are often not adequate to label skeletal progeny generated during adulthood. Our results confirm the validity of the (2.3kb)Col1 and (6kb)Col2 promoter fragments to target respectively osteoblasts and chondrocytes during development, but also show that other DNA fragments are required to label these cells during fracture healing and bone repair. The longer (3.2kb)Col1 promoter is sufficient to label osteoblasts in adult mice, whereas a shorter Col2 promoter fragment can target newly-formed adult chondrocytes [22].

Knowledge of the fate and behavior of skeletal cells during bone repair may help to improve the development of bone regenerative strategies. Cell lineage tracing is a powerful tool to address this issue. We noticed however that the Col1 promoter fragments that label collagen type I-expressing cells, like osteoblasts, in fetal and young mice are not sufficient to mark these cells in adult mice. On the other hand, the longer (3.2kb)Col1-dsRed reporter remained actively expressed in osteoblasts in adult mice, indicating that the spatiotemporal expression pattern of Col1 during bone development and homeostasis requires temporary contribution of distinct regulatory fragments. Furthermore, reporters driven by the 3.6kb and 2.3kb rat collagen type I promoters are expressed in murine long bone osteoblasts until at least 12 weeks of age [23-25], and the rat 2.3kb Col1 promoter was found to drive expression of GFP in fracture callus, indicating that it contains regulatory elements active during bone repair and can thus be used for fate mapping [26]. Given the disparate effect between the mouse and rat 2.3kb Col1 promoters, we performed a cursory sequence comparison between them, which revealed almost 90% homology, but also suggested that the rat promoter may contain additional putative binding sites for FoxD3, NFκB, and the SOX family of transcription factors. More in-depth molecular analysis of these promoters could lead to the identification of the regulatory elements responsible for the differential transcriptional activity during adult life and bone repair.

Comparable findings are obtained with respect to a cartilage-specific promoter active during adult endochondral bone repair or tissue engineering applications. Indeed, our data show that the Cre-mediated recombination induced by the (6kb)Col2 promoter is propagated into the osteochondrogenic lineage, as previously reported [21], but they also indicate that recombination activity is no longer observed in fracture callus cartilage. Interestingly, the few labeled cells were found primarily in woven bone and not in cartilage. These observations suggest that at least two types of periosteal progenitor populations are activated during bone repair: an unipotent osteogenic population derived from (6kb)Col2Cre-expressing progenitors, and bipotent osteochondrogenic cells wherein (6kb)Col2Cre was never actively expressed. Other Col2Cre(ERT2) mice have been generated using the same 6kb Col2 promoter-intron sequence [27, 28], and a recent study demonstrated that the expression of this transgene is almost undetectable when induced during fracture healing [29]. On the other hand, a 1.6kb Col2CreERT2 transgene was shown to be expressed by chondrocytes in callus cartilage, although labeling efficiency remained suboptimal [22]. Interestingly, the (6kb)Col2Cre mouse analyzed in the present study has been generated using longer fragments from the same promoter-enhancer region, but is not expressed in fracture callus. A possible explanation is that repressor elements are present in regions of the longer fragments that become active after birth, such as those responsive to NF κ B signaling or ETV factors [30, 31]. An alternative approach is the Sox9-CreERT2 transgene, which can be induced during fracture repair, but it targets both chondrocytes and osteoblasts making it less appropriate for detailed lineage tracing [32]. On the other hand, the Aggrecan-CreERT2 knock-in generated by Henry *et al.* [33] was demonstrated to be efficiently inducible in fracture callus [29, 34]. In addition, hypertrophic chondrocytes in the callus cartilage can be labeled by using a transgenic mouse with knock-in of the mCherry fluorophore into the Col10 gene locus [35, 36]. Several other promoters have been used to target chondrocytes, including parts of the rat [37, 38] or human [39] Col2 promoter, collagen type XI [40] and Aggrecan [41], but their validation is restricted to the developmental stage.

The approach of conditional Cre-mediated reporter expression is a useful tool for fate mapping and lineage tracing, but is less appropriate to detect active gene expression at later time points, indicating that findings in adult mice have to be interpreted with caution. Indeed, using this strategy it is very difficult to distinguish between a cell that is actively expressing the lineage-specific transgene and one that is derived from a precursor that expressed Cre at an earlier stage. More precisely, the (6kb)Col2Cre transgene is expressed in chondrocytes and early osteochondral progenitors before birth, which will result in propagation of the genetically induced label expression in all derived lineages. This outcome may clarify the positive staining of not only articular and growth plate cartilage, but also of many other skeletal cell types. On the other hand, when the (6kb)Col2GFP reporter was used, which labels only cells with active collagen type II transcription, almost no reporter expression was observed in bone, cartilage or fracture callus of mice at 8 weeks of age. These findings indicate that depending on the research question, versus detecting active expression, the most appropriate reporter system has to be chosen to allow correct interpretation of experimental results.

The data obtained in this study have also implications on the use of these Cre drivers for gene silencing during adult life. The described Cre drivers have been thoroughly validated and successfully used for this goal during development, but any transgene driven by these promoters will likely become inactive later in life resulting in progressive loss of the designed effect. Furthermore, inducible genetic models are commonly used to avoid interference of a developmental phenotype, when study of the function of the gene of interest during bone homeostasis, pathology or repair is intended. Since these promoter fragments cannot activate transcription in adult tissue, they would also not be suited to induce gene expression at later ages. Further identification of osteogenic lineage-specific transgenic promoters that drive efficient gene expression in adult mice will enhance insight in post-developmental bone repair and bone tissue engineered constructs.

In conclusion, we have demonstrated that the lineage-specific regulatory promoter elements that drive expression during bone development are often not sufficient to induce cell-specific expression later in life. Other promoter fragments have to be used to generate genetic models for bone repair and tissue engineering. Most importantly, our results affirm that genetic lineage tracing and targeting requires verification if used in applications that differ significantly from those validated before.

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FIGURE LEGENDS

Figure 1: Histological analysis of long bones in P5 (6kb)Col2Cre x mTmG mice. (A-H) Cre recombinase activity was visualized via conversion of mTomato to mGFP expression in mTmG reporter mice, analyzed in articular cartilage (A), proliferating chondrocytes (B), hypertrophic chondrocytes (C), perichondrium (D), primary spongiosa (E), bone marrow and trabecular bone (F), and the endosteal (G) and periosteal (H) bone surfaces. (J-L) Leaky mGFP expression was not detected in articular cartilage (I), proliferating chondrocytes (J), bone marrow (K) and periosteum (L) of Cre-negative mTmG littermates. The joint space (j) and muscle (m) adjacent to the bone are indicated; scale bars are 20 μ m.

Figure 2: Histological analysis of long bones in 8-week-old (6kb)Col2Cre x mTmG mice. (A-H) Previous or current Cre activity was assayed as mGFP-positive cells in articular cartilage (A), growth plate cartilage (B-C), primary spongiosa (D), trabecular bone (E), bone marrow (F), and at the endosteal (G) and periosteal (H) bone surfaces. (I-L) No recombination of the reporter was found in articular cartilage (I), growth plate cartilage (J), bone marrow (K) or periosteum (L) of Cre-negative mTmG littermates. The joint space (j) and muscle (m) adjacent to the bone are indicated; scale bars are 20 μ m.

Figure 3: Histological analysis of fracture calluses in 8-week-old (6kb)Col2Cre x mTmG mice. (A) H&E-stained overview of fracture callus 7 days post-injury, showing tissue composition and cell morphology (fracture line is indicated). (B-E) Cre-mediated mTmG recombination was analyzed in the cartilaginous fracture callus (B-C) and in the woven bone formed at the callus edges (D-E). Left panels in A-E are H&E stainings on the same sections. Scale bars are 20 μ m.

Figure 4: Histological analysis of long bones in P5 and of long bones and fracture calluses in 8-week-old (6kb)Col2GFP mice. (A-D) Active expression of GFP driven by the (6kb)Col2 promoter was analyzed in the articular cartilage (A), proliferating chondrocytes (B), and at the

endosteal (C) and periosteal (D) bone surfaces of P5 pups. (E-H) At 8 weeks of age, (6kb)Col2-driven GFP expression was assayed in articular cartilage (E), growth plate cartilage (F), and at the endosteal (G) and periosteal (H) bone surfaces. (I-J) Seven days after fracture in 8-week-old (6kb)Col2GFP mice, expression of GFP was analyzed by anti-GFP immunocytochemistry in the callus cartilage (I) and callus woven bone (J). Left panels in I, J are H&E stainings on the same sections and lower panel is H&E overview of fracture site. The joint space (j) and muscle (m) adjacent to the bone are indicated; scale bars are 20 μ m.

Figure 5: Histological analysis of long bones in P5 (2.3kb)Col1Cre x mTmG mice. (A-H) Cre recombinase activity was assayed in articular cartilage (A), proliferating chondrocytes (B) hypertrophic chondrocytes (C), perichondrium (D), primary spongiosa (E), trabecular bone (F), endosteum (G) and the periosteum (H). (I-L) mGFP expression was not detected in articular cartilage (I), proliferating chondrocytes (J), the endosteum (K) and the periosteum (L) of Cre-negative mTmG littermates. The joint space (j) and muscle (m) adjacent to the bone are labeled; scale bars are 20 μ m.

Figure 6: Histological analysis of long bones in 8-week-old (2.3kb)Col1Cre x mTmG mice. (A-H) Cre recombinase activity was analyzed in cells of the articular cartilage (A), the secondary ossification center (SOC; B), the primary spongiosa (C), trabecular bone (D), the endosteal bone surface (E-F), the bone cortex (G), and the periosteum (H). (I-L) No leaky Cre expression was found in articular cartilage (I), growth plate (J), endosteum (K) or periosteum (L) of Cre-negative mTmG littermates. The joint space (j) and muscle (m) adjacent to the bone are labeled; scale bars are 20 μ m.

Figure 7: Histological analysis of long bones in P5 or 8-week-old (3.2kb)Col1-dsRed mice. (A-H) Expression of dsRed was assayed in articular cartilage (A), proliferating chondrocytes (B), hypertrophic chondrocytes (C), primary spongiosa (D), trabecular bone (E), endosteum

(F), the bone cortex (G), and the periosteal bone surface (H) of P5 pups. (I-P) At 8 weeks of age (3.2kb)Col1-dsRed reporter activity was analyzed in articular cartilage (I), the secondary ossification center (SOC; J), the primary spongiosa (K), trabecular bone (L), endosteum (M), the bone cortex (N) and the periosteum (O-P). (Q-R) Expression of (3.2kb)Col1-dsRed was assayed in periosteal cells cultured in normal growth medium (Q) and following 14 days of osteogenic differentiation (R). Immunocytochemistry for endogenous collagen type I production was used as control. The joint space (j) and muscle (m) adjacent to the bone are labeled; scale bars are 20 μ m.

Figure S1: In vitro validation of the mTmG Cre reporter. (A-B) Basal expression of mTomato and Cre-induced recombination leading to expression of mGFP were assayed in periosteal cells after transduction with adenovirus expressing empty control (A) or Cre recombinase (B). Scale bars are 20 μ m.

Figure S2: Histological overview of mid-tibial fracture stabilized by medullary pin. (A) H&E staining at day 7 post-fracture in (6kb)Col2Cre x mTmG mice shows that the callus consists mainly of cartilaginous tissue and woven bone. (B) The presence of cartilage matrix, shown on H&E staining, was confirmed by collagen type II immunohistochemistry on the adjacent section, but contains hardly any mGFP-positive chondrocyte (right panel). (C) Enlargement of boxed area in B, with white dotted lines indicating the fracture ends and yellow dashed line the cartilaginous fracture callus (ca: cartilage, wb: woven bone). Scale bars are 100 μ m.

Figure S3: *In vivo* chondrogenesis assay using the (6kb)Col2Cre x mTmG reporter. Periosteal cells were isolated from (6kb)Col2Cre x mTmG mice, and expression of collagen type II and Cre-mediated reporter recombination were assayed after implantation in a collagen gel for 10 days. Differentiated chondrocytes were observed at the implant border (top panels) and in the center (bottom panels), but very few cells expressed mGFP. Scale bars are 100 μ m.

Figure S4: *In vitro* osteogenesis assay using the (2.3kb)Col1Cre x mTmG reporter. The expression of mTomato, Cre-mediated induction of mGFP, and of collagen type I by immunocytochemistry was analyzed in (2.3kb)Col1Cre x mTmG periosteal cells following 14 days of culture in osteogenic medium.

Figure 1

Figure 2

Figure 3

Figure 4

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Figure 7