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Prx1 and 3.2 kb *Col1a1* promoters target distinct bone cell populations in transgenic mice



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ABSTRACT

Bones consist of a number of cell types including osteoblasts and their precursor cells at various stages of differentiation. To analyze cellular organization within the bone, we generated *Col1a1CreER-DsRed* transgenic mice that express, in osteoblasts, CreER and DsRed under the control of a mouse 3.2 kb *Col1a1* promoter. We further crossed *Col1a1CreER-DsRed* mice with *Prx1CreER-GFP* mice that express CreER and GFP in osteochondro progenitor cells under the control of a 2.4 kb *Prx1* promoter. Since the 3.2 kb *Col1a1* promoter becomes active in osteoblasts at early stages of differentiation, and *Prx1CreER-GFP*-expressing periosteal cells show endogenous *Col1a1* expression, we expected to find a cell population in which both the 2.4 kb *Prx1* promoter and the 3.2 kb *Col1a1* promoter are active. However, our histological and flow cytometric analyses demonstrated that these transgenes are expressed in distinct cell populations. In the periosteum of long bones, *Col1a1CreER-DsRed* is expressed in the innermost layer directly lining the bone surface, while *Prx1CreER-GFP*-expressing cells are localized immediately outside of the *Col1a1CreER-DsRed*-expressing osteoblasts. In the calvaria, *Prx1CreER-GFP*-expressing cells are also localized in the cranial suture mesenchyme. Our experiments further showed that *Col1a1CreER-DsRed*-expressing cells lack chondrogenic potential, while the *Prx1CreER-GFP*-expressing cells show both chondrogenic and osteogenic potential. Our results indicate that *Col1a1CreER-DsRed*-expressing cells are committed osteoblasts, while *Prx1CreER-GFP*-expressing cells are osteochondro progenitor cells. The *Prx1CreER-GFP* and *Col1a1CreER-DsRed* transgenes will offer novel approaches for analyzing lineage commitment and early stages of osteoblast differentiation under physiologic and pathologic conditions.

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Introduction

Bone formation takes place by two major ossification processes, endochondral ossification and intramembranous ossification [1,2]. In endochondral ossification, the skeletal element first develops as a cartilaginous template that is subsequently replaced by bone. In intramembranous ossification, mesenchymal cells directly differentiate into bone-forming osteoblasts, and the ossification process does not require pre-existing cartilage. Bones in the calvaria, such as the frontal and parietal bones, ossify through intramembranous ossification, while bones such as the long bones in the limb ossify through endochondral ossification. Periosteum that covers most of the external bone surface contains osteochondro progenitor cells that participate in bone and cartilage formation under physiologic states and pathologic conditions

such as fracture healing [3,4]. Osteochondro progenitor cells can give rise to cells in both the chondrocyte and osteoblast lineages.

Histologically, periosteum consists of at least two layers: an outer fibrous layer and an inner cambium layer [4,5]. It is believed that the outer fibrous layer consists of fibroblastic cells, while the inner cambium layer contains a number of cell types including fibroblasts, multipotential stem cells, osteochondro progenitor cells, and cells in the osteoblast lineage at various stages of differentiation. Indeed, molecular analyses in chicks have identified a number of distinct cell populations in the periosteum, indicating heterogeneity in cellular organization [6]. To analyze the cellular composition of the periosteum, we previously generated *Prx1CreER-GFP* transgenic mice that express CreER and GFP under the control of a 2.4 kb *Prx1* promoter [7]. *Prx1* is a *paired*-related homeobox gene that is expressed in undifferentiated mesenchyme in the developing limbs [8,9]. Our previous study showed that the *Prx1CreER-GFP* transgene is expressed in a subset of periosteal cells in the cambium layer surrounding the long bones and that these cells can differentiate into chondrocytes and osteoblasts *in vitro* and *in vivo*, indicating that the *Prx1CreER-GFP* transgene marks osteochondro

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progenitor cells in the periosteum of long bones. In addition to its expression in the long bones, the *Prx1CreER-GFP* transgene is also expressed in the calvaria. However, the precise localization of the transgene-expressing cells and their differentiation potential have remained uncharacterized. Because other *Prx1*-driven transgenes have been reported to inhibit or enhance bone formation at the cranial suture [10–13], the *Prx1* promoter may be active in osteoprogenitor cells in the cranial suture mesenchyme.

Our previous study also showed that *Prx1CreER-GFP*-expressing periosteal cells express the genes for type I collagen, *Col1a1* and *Col1a2* [7]. Regulatory elements of *Col1a1*, the gene for the alpha-1 chain of type I collagen, have been shown to direct transgene expression in osteoblasts [14–16]. While the 2.3 kb mouse and rat promoter elements are active only in differentiated osteoblasts, the 3.2 kb mouse and 3.6 kb rat promoter sequences become active at the early stages of osteoblast differentiation [15,16]. A number of studies have indicated that *Col1a1*-driven transgenes are expressed in the periosteum [17–20]. Because 3.2 kb mouse and 3.6 kb rat promoters become active in early osteoblasts, and *Prx1CreER-GFP*-expressing cells also express endogenous *Col1a1*, we hypothesized that the *Prx1*-driven transgenes and *Col1a1*-driven transgenes are expressed in distinct but overlapping domains in the periosteum. These transgenes may allow the identification of distinct cell populations and cell layers in the periosteum.

In this study, we generated *Col1a1CreER-DsRed* transgenic mice that express CreER and DsRed under the control of a mouse 3.2 kb *Col1a1* promoter to further delineate the cellular organization of the periosteum and specifically to determine the relationship between *Prx1CreER-GFP*-expressing osteochondro progenitor cells and *Col1a1*-expressing osteoblasts. We also examined the localization of *Prx1CreER-GFP*- and *Col1a1CreER-DsRed*-expressing cell populations in the calvaria. We show here that *Prx1CreER-GFP* and *Col1a1CreER-DsRed* transgenes are expressed in distinct cell populations both in the long bones and calvaria and that *Prx1CreER-GFP*-expressing cells are precursor cells for the *Col1a1CreER-DsRed*-expressing osteoblasts. We also show that the *Prx1CreER-GFP* transgene marks osteochondro progenitor cells in the cranial suture mesenchyme, while the *Col1a1CreER-DsRed* transgene is expressed in committed osteoblasts. These transgenes will provide novel approaches for analyzing the biology of the periosteum and cranial suture mesenchyme under physiologic and pathologic conditions.

Materials and methods

The institutional animal care and use committee of Case Western Reserve University approved all animal procedures.

DNA construction, screening, and transgenic mice

The *Prx1CreER-GFP* transgenic mice were described previously in our laboratory [7]. To express CreER and DsRed in osteoblasts in the periosteum, we cloned cDNAs for CreER and DsRed Express2 downstream of a 3.2 kb *Col1a1* promoter (Fig. 1A). CreER is a fusion molecule of Cre recombinase and the ligand binding domain of a mutated estrogen receptor [21]. The internal ribosome entry site (IRES), *DsRed Express2* cDNA, and SV40 polyadenylation signal were excised from pIRES2 DsRed-Express2 (Clontech) and subcloned downstream of CreER cDNA. The construct was injected into the fertilized C57BL6 or C57BL6 × SJL F2 hybrid eggs at the Case Transgenic and Targeting Facility. Transgenic founders were identified by Polymerase Chain Reaction (PCR) using the primer set (forward) 5'-TGCAACGAGTGATGAGGTTCC-3' and (reverse) 5'-CATGTTAGCTGCCCAATGT-3' that amplifies the 241 bp sequence of Cre recombinase. Transgene expression was assessed in offspring mice by examining the calvaria under the fluorescence microscope (Leica, DM-IRB).

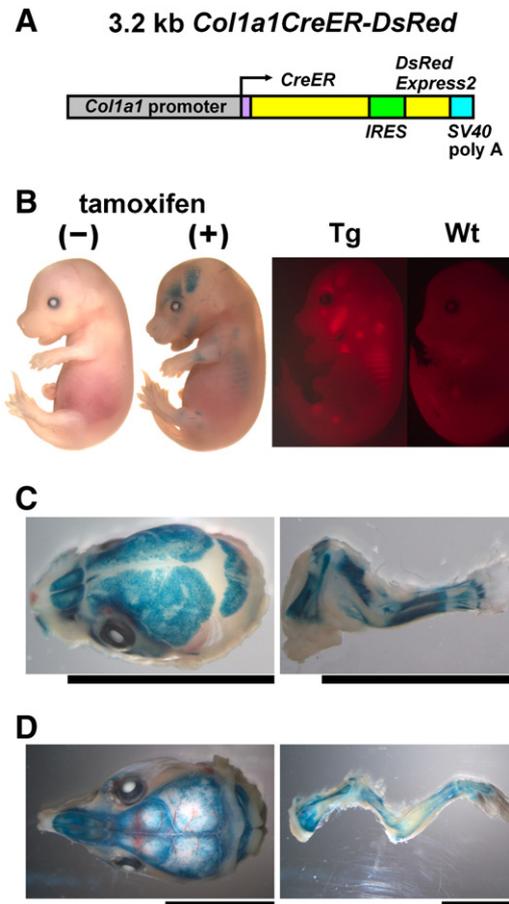


Fig. 1. Generation of *Col1a1CreER-DsRed* transgenic mice. (A) Schematic representation of the *Col1a1CreER-DsRed* transgene. cDNAs for CreER and DsRed were cloned downstream of a 3.2 kb *Col1a1* promoter. (B) X-gal staining and DsRed fluorescence of E15.5 embryos. Tamoxifen injection at E13.5 caused widespread X-gal staining in the bones of *Rosa26 LacZ*; *Col1a1CreER-DsRed* embryos at E15.5. These embryos did not stain positive for X-gal without tamoxifen injection. (Tg) transgenic; (Wt) wild-type. (C) X-gal staining of the calvaria and forelimb of E18.5 *Rosa26 LacZ*; *Col1a1CreER-DsRed* embryos showing positive staining in the bones and tendons. Tamoxifen was injected into the mother at E15.5 and E16.5. Scale bars indicate 10 mm. (D) X-gal staining of the calvaria and forelimb of P15 *Rosa26 LacZ*; *Col1a1CreER-DsRed* mice showing positive staining in the bones. Tamoxifen was injected at P11 and P13. Scale bars indicate 10 mm.

Tamoxifen injection, X-gal staining, and histological analysis

Tamoxifen-inducible Cre activity was evaluated using the *Rosa26 LacZ* reporter mice (Jackson Laboratories). 1 mg/100 μ l/day tamoxifen (Sigma) was injected into the pregnant mother or offspring mice via intraperitoneal or subcutaneous injection at indicated time points. Mice were sacrificed 2–3 days after the last tamoxifen injection. For X-gal staining, tissues were fixed with 0.2% glutaraldehyde, 5% formalin, 2 mM MgCl₂, 5 mM EDTA, 0.02% NP40 in phosphate buffered saline (PBS), washed in rinse buffer (0.1% sodium deoxycholate, 0.2% NP40, 2 mM MgCl₂, 0.1 M phosphate buffer, pH 7.3), and stained in X-gal solution (1 mg/ml X-gal, 5 mM ferricyanide, 5 mM ferrocyanide in the rinse buffer). X-gal-stained tissues were postfixed in 10% formalin in PBS, demineralized in 0.5 M EDTA, and embedded in paraffin. Sections were made at 7 μ m and counterstained with hematoxylin and eosin. For histological analysis of GFP and DsRed fluorescence, tissues were harvested from *Prx1CreER-GFP*; *Col1a1CreER-DsRed* double transgenic mice, fixed in 10% formalin in PBS for 20 h at 4 °C, and demineralized in 0.5 M EDTA for 18 days. The tissues were then embedded in OCT compound (Sakura Finetek) and sectioned at 7 μ m using a cryostat (Leica CM1850 UV). Sections were mounted with Fluoroshield with DAPI (Sigma). All images were acquired with a Leica DM 6000B

microscope. Photographs were taken with a digital camera (Leica DC500) using Leica Application Suite 1.3 software. For simultaneous visualization of GFP and DsRed, a G/R filter (Leica) was used. The use of the dual bandpass filter allowed discrimination of GFP and autofluorescence of the bone [22]. The intensity of GFP and DsRed signal was adjusted using Photoshop software (Adobe) by applying adjustments to the whole image, with the strict intent of not obscuring, eliminating, or misrepresenting any information present in the original, including background.

Isolation of transgene-expressing cells

Transgene-expressing bone cells were isolated either from the long bones or from the calvariae.

For isolation from the long bones, the diaphyses of tibiae, femora, humeri, radii, and ulnae were dissected out from 6- to 13-day-old *Prx1CreER-GFP*; *Col1a1CreER-DsRed* double transgenic mice, and cells were enzymatically released as described previously [7]. For isolation from the calvariae, the frontal, parietal, and interparietal bones were dissected out from *Prx1CreER-GFP*; *Col1a1CreER-DsRed* double transgenic mice at E18.5–P3. Calvariae were sequentially digested with collagenase B (Roche) at 3 mg/ml for 1 h or at 0.5 mg/ml overnight followed by 0.25% Trypsin/EDTA (Invitrogen) for an additional 30 min – 1 h at 37 °C. The released cells were collected by centrifugation, resuspended in PBS, 5% FCS, and filtered through a 70 µm cell strainer. Then the cells were sorted by fluorescence-activated cell sorting at the Case Western Reserve University Comprehensive Cancer Center Facility.

Osteogenic and chondrogenic differentiation

For assessing the osteogenic potential of calvaria-derived *Prx1CreER-GFP*-expressing cells, GFP (+); DsRed (–) cells were isolated from the calvaria by flow cytometry, plated in a 6-well plate at 30,000 cells/cm², and cultured in αMEM, 10% FCS with 5% CO₂ at 37 °C. After reaching confluence, osteogenic differentiation was induced by supplementing αMEM, 10% FCS with 5 mM β-glycerophosphate and 100 µg/ml ascorbic acid. The medium was changed three times per week. After 3 weeks of induction, osteogenic differentiation was examined by DsRed fluorescence, calcein blue staining, and alizarin red staining. For calcein blue staining, the medium was supplemented with 30 µM calcein blue [23]. For alizarin red staining, cells were fixed with 70% ethanol and stained in 1% alizarin red solution.

For chondrogenic differentiation, 8 × 10⁴ of sorted GFP (+); DsRed (–) and GFP (–); DsRed (+) cells were each resuspended in a 10 µl droplet and plated in micromass at the center of a 48-well plate. Cells were incubated for 1.5 h with 5% CO₂ at 37 °C. Then, 200 µl of DMEM and 10% FCS were added onto the micromass droplet. Chondrogenic differentiation was induced by 500 ng/ml BMP2 (R&D). The medium was replaced every 3 days. After 7 days of chondrogenic induction, RNA was extracted and subjected to real-time PCR.

RNA extraction and real-time PCR

Total RNA was extracted using an RNeasy micro kit (Qiagen). RNA was reverse-transcribed into cDNA with a High Capacity cDNA Archive Kit (Applied Biosystems) or Ovation Pico WTA System V2 (NuGEN). Real-time PCR was performed on the Applied Biosystems 7500 real-time PCR detection system. TaqMan probe sets were designed and synthesized by Applied Biosystems (*Alcam*; Mm00711623_m1, *Asporin*; Mm00445945_m1, *Col1a1*; Mm00801666_g1, *Cxcl12*; Mm00445553_m1, *Gapdh*; 4352932E, *Hapln1*; Mm00488952_m1, *Ibsp*; Mm00492555_m1, *Ly6a*; Mm00726565_s1, *Prx1*; Mm00599934_m1, *Thy1*; Mm00493681_m1, *Osteopontin*; Mm00436767_m1, *Osterix*; Mm00504574_m1, *Periostin*; Mm00450111_m1, *Runx2*; Mm00501584_m1, *Satb2*; Mm00507337_m1, *Sulf1*; Mm00552283_m1, *Thbs2*; Mm01279240_m1, *4Cyt11*; Mm01217843_m1). To compare gene expression levels, the comparative

cycle threshold (Ct) method was used. *Gapdh* was used as an endogenous control to correct for potential variation in RNA loading or in efficiency of amplification. A *t* test was used to compare expression levels between GFP (+); DsRed (–) and GFP (–); DsRed (+) cells. *p* values < 0.05 were considered statistically significant.

Microarray analysis

Microarray analysis was performed at the Gene Expression and Genotyping Facility of the Case Comprehensive Cancer Center at Case Western Reserve University. Total RNA was converted into double-stranded cDNA and amplified cDNA was labeled with biotin and fragmented using Encore Biotin Module (NuGEN). Approximately 12 ng of total RNA was amplified using standard protocols found in Ovation Pico WTA System V2 (NuGEN) in preparation for use on the latest vintage of Affymetrix Whole Transcript Mouse Gene array ST 1.0 microarrays. Briefly, the RNA was reverse transcribed using random primers and Superscript II enzyme. Second strand synthesis was carried out using RNase H and DNA Polymerase I enzymes. Biotin labeled cDNA was added with carrier molecules, buffers and control spikes to make a hybridization cocktail. Samples were hybridized to Affymetrix mouse Gene Array ST 1.0, containing more than 35,000 probe sets.

Expression signals were generated using proprietary software algorithms contained within Affymetrix' Expression Console (EC) software (Affymetrix, Inc.). Criteria for changed expression were three fold, as follows: Data were subjected to quantile normalization using EC feature named Robust Multichip Analysis (RMA). Data were exported from EC as tab delimited text files containing log₂ transformed signals. These files were opened using MS Excel. Signal log ratios were generated between binary paired files and these were converted in to fold changes. The data were imported into MS access and queried for fold changes whose absolute values were greater than or equal to 1.5.

Results

Screening of transgenic mice

Among 94 founder mice, we identified 17 transgenic founders by PCR. These transgenic lines were further crossed with the *Rosa26 LacZ* reporter mice, and tamoxifen-inducible Cre activity was evaluated in offspring embryos/mice by X-gal staining. Four of the 17 lines showed intense X-gal staining upon induction with tamoxifen. Out of 4 lines that showed similar Cre activity, 1 line was chosen for further analysis. The selected line was further mated to homozygosity. Both heterozygous and homozygous mice developed and bred normally, indicating that the transgene did not disrupt genes that are essential for normal development.

Col1a1CreER-DsRed transgene is expressed in osteoblasts, odontoblasts, and some of the tendon cells

Expression domains of the transgene were further analyzed by the fluorescence of DsRed and tamoxifen-inducible Cre recombinase activity. At E15.5, DsRed fluorescence was observed in the ribs, the mandible, the frontal and parietal bones in the calvaria, and the diaphyses of the long bones, corresponding to the location of bone formation (Fig. 1B). We further crossed the *Col1a1CreER-DsRed* transgenic mice with the *Rosa26 LacZ* reporter mice and examined Cre-*loxP* mediated recombination in the offspring mice by X-gal staining. Tamoxifen was injected at various time points between E11.5 and 3 months of age. When tamoxifen was injected into the pregnant mother at E11.5 and the embryos were stained with X-gal at E15.5, not all of the *Col1a1CreER-DsRed*; *Rosa26 LacZ* embryos stained positive for X-gal, most likely reflecting the variation in development among embryos (data not shown). When tamoxifen was injected into the pregnant mother at E13.5 and embryos were stained with X-gal at

E15.5, all of the *Col1a1CreER-DsRed*; *Rosa26 LacZ* embryos showed X-gal staining in a pattern that is very similar to that of DsRed fluorescence (Fig. 1B). Littermate *Rosa26 LacZ* embryos without the *Col1a1CreER-DsRed* transgene did not stain positive for X-gal (data not shown). In addition, none of the E15.5 *Col1a1CreER-DsRed*; *Rosa26 LacZ* embryos stained positive for X-gal without tamoxifen injection (Fig. 1B). Similarly, tamoxifen injection at E15.5 and E16.5 (Fig. 1C) and P11 and P13 (Fig. 1D) resulted in intense X-gal staining in the bones of *Col1a1CreER-DsRed*; *Rosa26 LacZ* mice at E18.5 and P15, respectively. Tendons also stained positive for X-gal at E18.5 (Fig. 1C). These observations indicate that E11.5 is the critical time point after which *Cre-loxP* mediated recombination is widely induced in skeletal elements. *Cre-loxP* mediated recombination was induced by tamoxifen at least up to 3 months of age (data not shown).

Histological analysis further revealed DsRed fluorescence in osteoblasts lining the cortical and trabecular bones as well as some of the osteocytes within the bone matrix (Fig. 2A). Consistent with this observation, X-gal staining was observed in osteoblasts and some of the osteocytes of tamoxifen-injected *Col1a1CreER-DsRed*; *Rosa26 LacZ* mice both in the cranial bones and long bones (Figs. 2B,C,D). X-gal staining was also observed in odontoblasts in the developing teeth and in some cells in the ligaments, in the meniscus of the knee joint, and in muscle fascia (Fig. 2D). No chondrocytes in the articular and epiphyseal cartilage stained positive for X-gal (Figs. 2C,D).

Col1a1CreER-DsRed transgene shows no germline recombination

Since germline recombination has been reported in transgenic mice expressing Cre recombinase under the control of the 2.3 kb and 3.6 kb

rat *Col1a1* promoter [24], we examined whether the *Col1a1CreER-DsRed* transgene shows Cre activity in the germline or early embryogenesis in the absence of tamoxifen. E15.5 embryos were harvested from the wild-type female that was crossed with the *Col1a1CreER-DsRed*; *Rosa26 LacZ* male and from the *Col1a1CreER-DsRed*; *Rosa26 LacZ* female that was crossed with the wild-type male. In neither case was X-gal staining observed in *Col1a1CreER-DsRed*; *Rosa26 LacZ* embryos, indicating that *Cre-loxP*-mediated recombination does not take place in the germline or in early embryogenesis in the absence of tamoxifen (data not shown).

Prx1CreER-GFP and *Col1a1CreER-DsRed* transgenes show complementary expression

We previously generated *Prx1CreER-GFP* transgenic mice that express CreER and GFP under the control of a 2.4 kb *Prx1* promoter [7]. The transgene is expressed in osteochondro progenitor cells that can give rise to osteoblasts, and flow-sorted *Prx1CreER-GFP*-expressing periosteal cells show endogenous *Col1a1* expression, raising the possibility that the *Prx1CreER-GFP* and *Col1a1CreER-DsRed* transgenes are expressed in an overlapping cell population. Therefore, we examined and compared expression domains of the respective transgene in *Prx1CreER-GFP*; *Col1a1CreER-DsRed* double transgenic mice. At E15.5, the *Col1a1CreER-DsRed* transgene is expressed in the developing frontal and parietal bones in the calvaria, while *Prx1CreER-GFP* transgene is expressed in the interposing cranial sutures (Fig. 3A). The same pattern of complementary expression was also observed in the calvaria at P12. We further examined the localization of DsRed and GFP fluorescence by histological analysis. While the *Col1a1CreER-DsRed* transgene is expressed

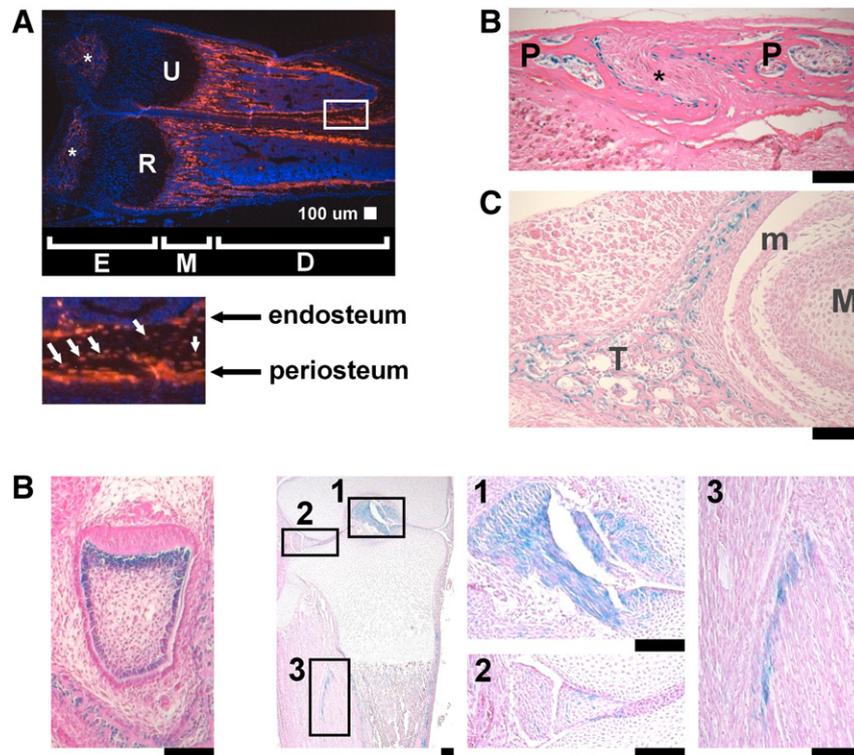


Fig. 2. Tissue distribution of DsRed fluorescence and Cre recombinase activity of the *Col1a1CreER-DsRed* transgenic mice. (A) DsRed fluorescence in the distal ulna (U) and radius (R) of *Col1a1CreER-DsRed* transgenic mice at P12. The boxed area in the upper panel is magnified in the lower panel. Osteoblasts in the endosteum and periosteum as well as some of the osteocytes (white arrows) show DsRed fluorescence. E: epiphysis, M: metaphysis, D: diaphyses. Asterisks (*) indicate secondary ossification centers. (B) Coronal section of the sagittal suture of E18.5 *Rosa26 LacZ*; *Col1a1CreER-DsRed* embryo showing X-gal staining in the osteoblasts of the parietal bones (P). Cells in the cranial suture mesenchyme (*) are negative for X-gal. Tamoxifen was injected into the pregnant mother at E15.5 and E16.5. (C) Coronal section of the temporomandibular joint of E18.5 *Rosa26 LacZ*; *Col1a1CreER-DsRed* embryo showing X-gal staining in osteoblasts. Cells in the developing articular cartilage and meniscus (m) are negative for X-gal. Temporal bone (T), mandible (M). (D) Odontoblasts in the incisor (left), cells in the cruciate ligaments (box 1), meniscus of the knee joint (box 2), and muscle fascia (box 3) stain positive for X-gal. Boxes 1–3 are magnified in the right panels. The incisor and knee samples were harvested at E17.5 and E18.5, respectively. Tamoxifen was injected into the pregnant mother 2 days and 3 days prior to X-gal staining. Scale bars at the right bottom of each panel indicate 100 μ m.

in osteoblasts lining the cranial bone, the *Prx1CreER-GFP* transgene-expressing cells were found in the cranial suture mesenchyme (Fig. 3B). In the long bones, the *Col1a1CreER-DsRed* transgene is expressed in the osteoblasts lining the cortical and trabecular bones and some of the osteocytes, while the *Prx1CreER-GFP* transgene is expressed in the periosteum (Fig. 3C). Interestingly, while both transgenes are expressed in the inner cambium layer of the periosteum, *Col1a1CreER-DsRed* and *Prx1CreER-GFP* transgenes are expressed in distinct cell populations. The *Col1a1CreER-DsRed* transgene is expressed in the innermost layer lining the cortical bone. In contrast, *Prx1CreER-GFP*-expressing cells are localized immediately outside of the *Col1a1CreER-DsRed*-expressing cells. In addition to the bone and periosteum, *Col1a1CreER-DsRed* and *Prx1CreER-GFP* transgenes are also expressed in tendon cells (Fig. 3C). Similar to their expression in the periosteum, these transgenes are expressed in distinct cell populations in the tendons, and only a few cells expressed both transgenes. The distinct localization of *Col1a1CreER-DsRed* and *Prx1CreER-GFP*-expressing cells in the periosteum was further confirmed in the respective mouse line by using the *Rosa26 LacZ* reporter (Fig. 4). Histological measurement indicated that *Prx1CreER-GFP*-expressing cells are localized at a greater distance from the cortical bone surface compared with the *Col1a1CreER-DsRed*-expressing cells (Fig. 4B).

Prx1CreER-GFP and *Col1a1CreER-DsRed*-expressing cells are distinct cell populations

To further characterize *Col1a1CreER-DsRed* and *Prx1CreER-GFP*-expressing cells, cells were isolated from the calvariae of wild type and *Col1a1CreER-DsRed*; *Prx1CreER-GFP* double transgenic mice by sequential digestion with collagenase and trypsin/EDTA. The released cells were plated in the culture dish, and GFP and DsRed fluorescence was examined under the microscope (Fig. 5A, Supplemental Fig. 1A). Consistent with the histological observation, only a few cells showed both GFP and DsRed fluorescence in the culture of *Col1a1CreER-DsRed*;

Prx1CreER-GFP double transgenic cells. Wild type cells showed minimal background for GFP and DsRed fluorescence. The specificity of GFP and DsRed fluorescence was also confirmed in C3H10T1/2 cells expressing either GFP or DsRed alone (Supplemental Fig. 1B). The non-overlapping expression of the *Col1a1CreER-DsRed* and *Prx1CreER-GFP* transgenes was further confirmed by flow cytometry (Fig. 5B). Very similar results were obtained when the cells were isolated from the diaphyses of long bones (data not shown).

Prx1CreER-GFP and *Col1a1CreER-DsRed*-expressing cells express endogenous *Prx1* and *Col1a1*

We next examined whether the expression of *Col1a1CreER-DsRed* and *Prx1CreER-GFP* transgenes reflects endogenous *Col1a1* and *Prx1* mRNA expression. GFP (–); DsRed (+), GFP (+); DsRed (–), and GFP (–); DsRed (–) cells were sorted from the diaphyses of long bones by flow cytometry, and *Prx1* and *Col1a1* expression was examined by real-time PCR.

Interestingly, endogenous *Prx1* and *Col1a1* expression did not strictly correlate with the transgene expression (Fig. 5C). Although GFP (–); DsRed (+) cells do not express the *Prx1CreER-GFP* transgene, these cells express endogenous *Prx1* at relatively high levels. Similarly, GFP (+); DsRed (–) cells that do not express the *Col1a1CreER-DsRed* transgene express endogenous *Col1a1*, albeit at a reduced level.

Prx1CreER-GFP-expressing calvaria cells show both chondrogenic potential and osteogenic potential, while *Col1a1CreER-DsRed*-expressing calvaria cells lack chondrogenic potential

Our previous study indicated that *Prx1CreER-GFP*-expressing periosteal cells in the long bones are osteochondro progenitor cells that can give rise to chondrocytes and osteoblasts [7]. Interestingly, the *Prx1CreER-GFP* transgene is also expressed in the developing calvaria. We therefore examined the osteogenic and chondrogenic

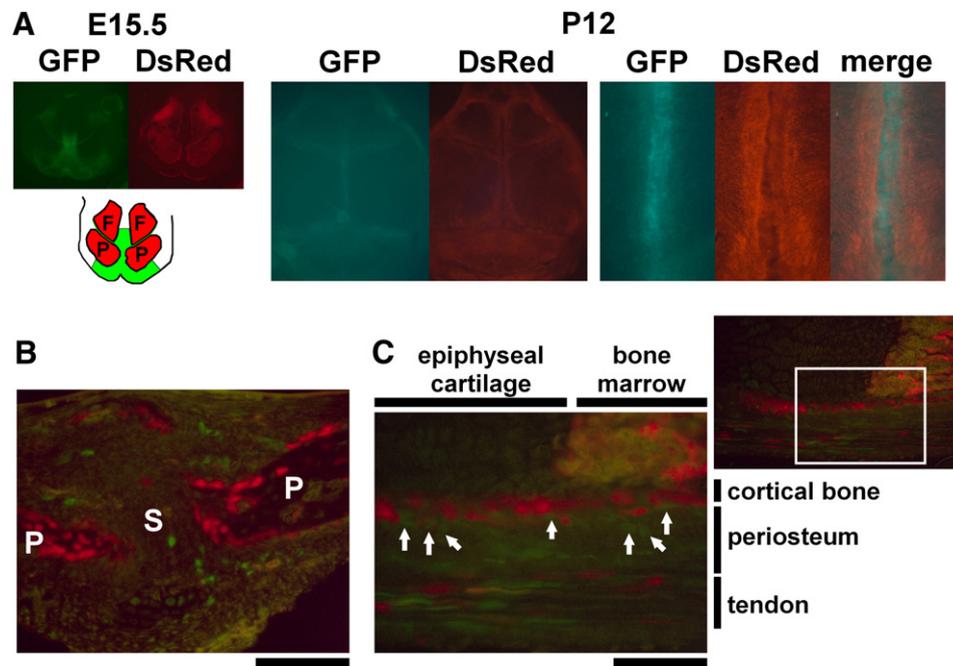


Fig. 3. Complementary expression of the *Prx1CreER-GFP* and *Col1a1CreER-DsRed* transgenes. (A) GFP and DsRed fluorescence of the calvaria of *Prx1CreER-GFP*; *Col1a1CreER-DsRed* at E15.5 and P12 showing complementary expression of the transgenes. The right panel shows magnified view of the sagittal suture. While the *Col1a1CreER-DsRed* transgene is expressed in the bone, the *Prx1CreER-GFP* transgene is expressed in the cranial suture. F: frontal bone, P: parietal bone. (B) GFP and DsRed fluorescence of the sagittal suture of *Prx1CreER-GFP*; *Col1a1CreER-DsRed* mice at P12. GFP-expressing cells are localized in the cranial suture mesenchyme. P: parietal bone, S: sagittal suture. (C) GFP and DsRed fluorescence of the distal ulna at the level of chondro-osseous junction at P12. The boxed area in the upper right panel is magnified in the lower left panel. Osteoblasts lining the developing cortical bone show DsRed fluorescence, while GFP-expressing cells are found in the periosteum immediately adjacent to the DsRed-expressing osteoblasts (white arrows). Some of the periosteal cells further outside also show GFP fluorescence. GFP and DsRed-expressing cells are also found in the tendon.

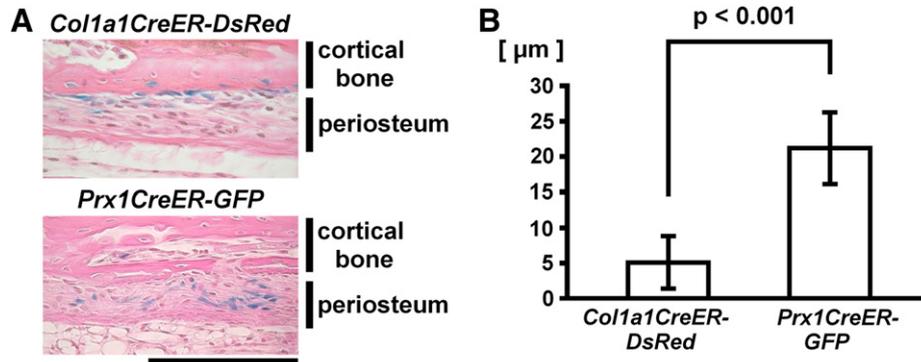


Fig. 4. Distinct distribution of Cre recombinase activity in the periosteum of *Prx1CreER-GFP* and *Col1a1CreER-DsRed* transgenic mice. (A) X-gal staining of the cortical bone and periosteum of distal radius of *Rosa26 LacZ*; *Col1a1CreER-DsRed* (upper panel) and *Rosa26 LacZ*; *Prx1CreER-GFP* mice (lower panel) at P27. Tamoxifen was injected twice at P23 and P25. While osteoblasts lining the bone cortex stain positive in *Rosa26 LacZ*; *Col1a1CreER-DsRed* mice, periosteal cells overlying X-gal-negative osteoblasts stain positive in *Rosa26 LacZ*; *Prx1CreER-GFP* mice. Scale bar at the right bottom of panel indicates 100 μm . (B) Localization of X-gal-positive cells in the periosteum was expressed as the distance from the outer surface of the cortical bone. Histological measurement in the radius of tamoxifen-injected *Rosa26 LacZ*; *Col1a1CreER-DsRed* and *Rosa26 LacZ*; *Prx1CreER-GFP* mice indicated that *Prx1CreER-GFP*-expressing cells ($n = 52$) are localized at a greater distance from the cortical bone compared with *Col1a1CreER-DsRed*-expressing cells ($n = 53$) that are localized adjacent to the cortical bone.

potential of *Prx1CreER-GFP*-expressing cells isolated from the calvaria. For osteogenic differentiation, we first isolated GFP (+); DsRed (-) cells from the calvaria of *Prx1CreER-GFP*; *Col1a1CreER-DsRed* double transgenic mice by flow cytometry. The cells were plated in culture and treated with β -glycerophosphate and ascorbic acid after reaching confluence. After 3 weeks of osteogenic induction, these cells formed mineralized bone nodules as indicated by calcein blue fluorescence and alizarin red staining (Fig. 6A and data not shown). The cells in

the mineralized nodules showed DsRed fluorescence, indicating the osteogenic differentiation of *Prx1CreER-GFP*-expressing cells.

For chondrogenic differentiation, we flow-sorted GFP (+); DsRed (-) and GFP (-); DsRed (+) cells from the calvariae of *Prx1CreER-GFP*; *Col1a1CreER-DsRed* double transgenic mice, and each cell population was plated in micromass and maintained in parallel. The cells were treated with BMP2 at 500 ng/ml for 7 days to induce chondrogenic differentiation. In GFP (+); DsRed (-) cells, BMP2 caused

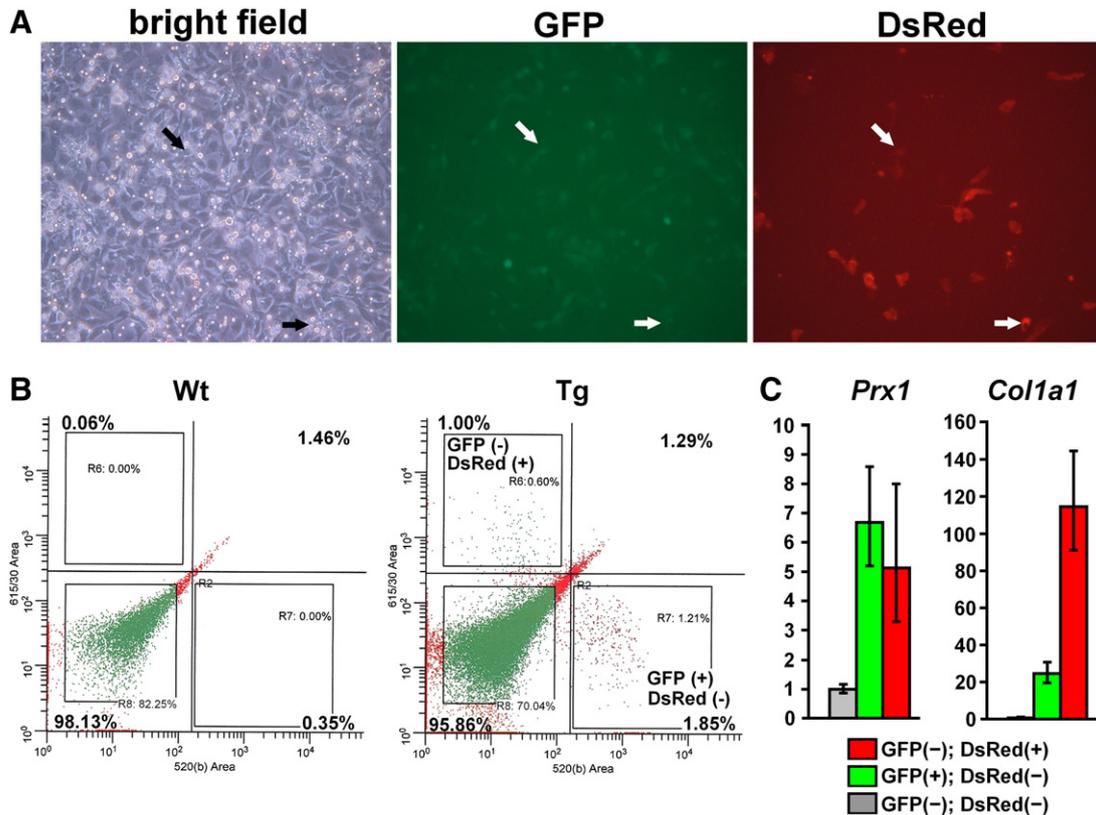


Fig. 5. *Prx1CreER-GFP* and *Col1a1CreER-DsRed* transgenes are expressed in mostly distinct cell populations. (A) GFP and DsRed fluorescence and corresponding bright field view of calvaria cells isolated from E18.5 *Prx1CreER-GFP*; *Col1a1CreER-DsRed* embryos one day after plating. GFP and DsRed are expressed in distinct cell populations, and only a few cells express both GFP and DsRed (arrow). (B) Flow cytometry analysis of calvaria cells isolated from wild type (Wt) and *Prx1CreER-GFP*; *Col1a1CreER-DsRed* (Tg) mice at P12. The proportion of cells in each quadrant is indicated. The proportion of the upper right quadrant corresponding to GFP and DsRed double positive cells is similar between wild type and *Col1a1CreER-DsRed*; *Prx1CreER-GFP* double transgenic cells, indicating that the fluorescence is mostly background. (C) Real-time PCR analysis of GFP (-); DsRed (+), GFP (+); DsRed (-), and GFP (-); DsRed (-) cells isolated from the diaphyses of long bones. Both GFP (+); DsRed (-) and GFP (-); DsRed (+) cells show relatively high *Prx1* expression compared with GFP (-); DsRed (-) cells, while GFP (-); DsRed (+) cells show highest expression of *Col1a1*. Data represent mean \pm standard deviations. The figure presents data from one of three experiments with similar results.

a 20-fold and 4-fold increase in *Aggrecan* and *Col2a1* expression, while the same treatment did not induce *Aggrecan* and *Col2a1* expression in GFP (-); DsRed (+) cells (Fig. 6B). Expression of osteoblast markers *Ibsp* and *Satb2* remained relatively unaffected in BMP2-treated GFP (+); DsRed (-) cells. These results indicate the chondrogenic potential of GFP (+); DsRed (-) cells and the lack of chondrogenic potential in GFP (-); DsRed (+) cells.

Prx1CreER-GFP-expressing calvaria cells give rise to chondrocytes and osteoblasts in vivo

Since *Prx1CreER-GFP-expressing calvaria cells* showed osteogenic and chondrogenic potential in culture, we further examined whether *Prx1CreER-GFP-expressing cells* differentiate into osteoblasts and

chondrocytes in vivo. We crossed *Prx1CreER-GFP* transgenic mice with *Rosa26 LacZ* reporter mice, and tamoxifen was injected into the pregnant mother at E13.5. X-gal staining of calvariae at P15 showed intense staining in the frontal, parietal, and interparietal bones (Fig. 7A). Histological analysis confirmed X-gal staining in the osteoblasts and some of the osteocytes, indicating that *Prx1CreER-GFP-expressing cells* differentiate into the osteoblast lineage (Fig. 7B). Furthermore, in the interparietal bone that at least partially forms through endochondral ossification [25], some of the chondrocytes stained positive for X-gal, indicating that *Prx1CreER-GFP-expressing cells* in the calvaria also differentiate into the chondrocyte lineage (Fig. 7C). Similar to the calvaria, X-gal staining of the long bones in the limb also showed positive staining both in osteoblasts and chondrocytes (Fig. 7D).

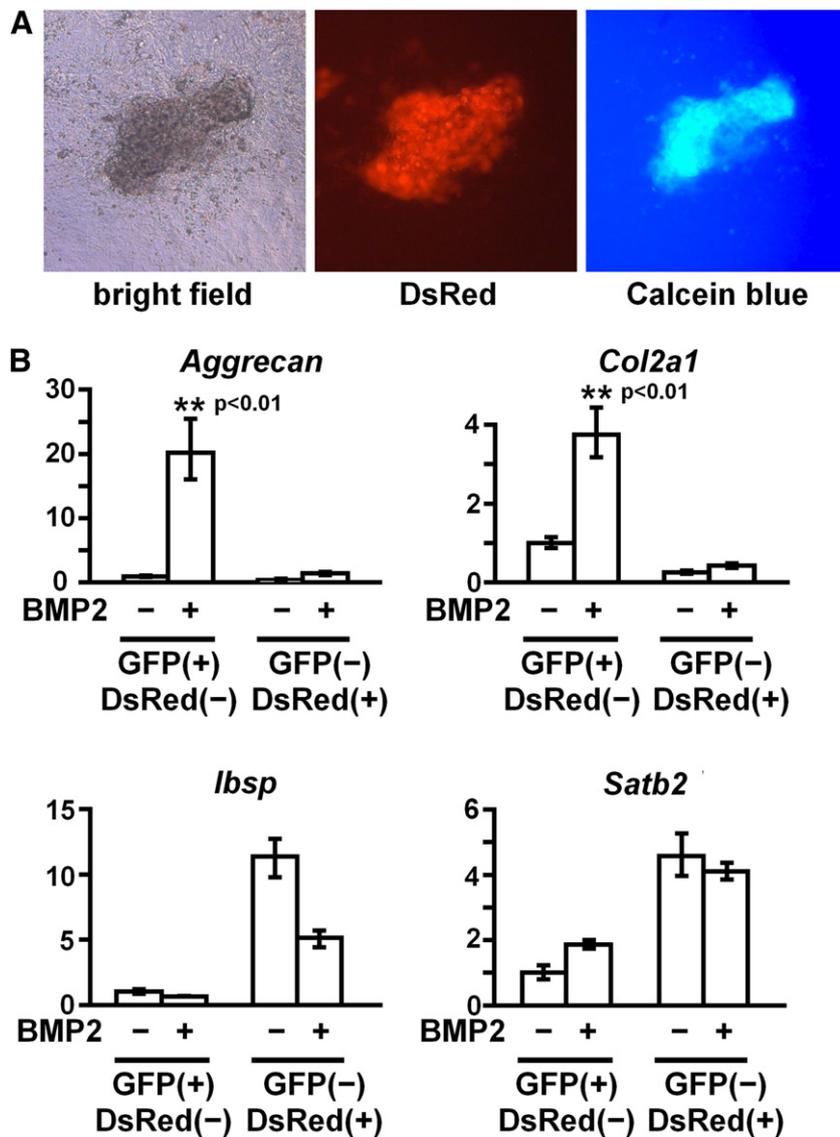


Fig. 6. GFP (+); DsRed (-) cells show osteogenic and chondrogenic potential, while GFP (-); DsRed (+) cells lack chondrogenic potential. (A) GFP (+); DsRed (-) calvaria cells form mineralizing bone nodules consisting of DsRed-expressing osteoblasts in culture. GFP (+); DsRed (-) cells were isolated from the calvariae of *Prx1CreER-GFP; Col1a1CreER-DsRed* mice at P0-2 by enzymatic digestion followed by flow cytometry, and osteogenic differentiation was induced with ascorbic acid and β -glycerophosphate. After 25 days of osteogenic induction, GFP (+); DsRed (-) cells formed bone nodules containing DsRed-expressing osteoblasts (middle panel), and their matrix showed mineralization as indicated by calcein blue fluorescence (right panel). (B) GFP (+); DsRed (-) calvaria cells, but not GFP (-); DsRed (+) cells, show chondrogenic differentiation in micromass culture. GFP (+); DsRed (-) and GFP (-); DsRed (+) cell populations were isolated from the calvariae of *Prx1CreER-GFP; Col1a1CreER-DsRed* mice at P0-1 by enzymatic digestion followed by flow cytometry. The cells were cultured in micromass, and chondrogenic differentiation was induced with 500 ng/ml BMP2 for 7 days. Real-time PCR showed remarkable upregulation of *Aggrecan* and *Col2a1* in BMP2-treated GFP (+); DsRed (-) cells, but not in GFP (-); DsRed (+) cells. Osteoblast markers *Ibsp* and *Satb2* remained relatively unchanged in BMP2-treated GFP (+); DsRed (-) cells. Data represent mean \pm standard deviations. The figure presents data from one of two experiments with similar results.

Prx1CreER-GFP-expressing cells express genes associated with chondrocytes, periosteal cells, and mesenchymal stem cells, while *Col1a1CreER-DsRed*-expressing cells express osteoblast markers

To identify differentially expressed genes between *Col1a1CreER-DsRed* and *Prx1CreER-GFP*-expressing cells, we performed microarray analysis. GFP (+); DsRed (-) and GFP (-); DsRed (+) cells were isolated from the calvaria of *Prx1CreER-GFP*; *Col1a1CreER-DsRed* double transgenic mice by flow cytometry, and RNA was subjected to microarray analysis using Affymetrix Mouse Gene array ST1.0 Chips. We identified 497 genes that reproducibly showed a difference of 1.5 fold or greater in two independent experiments (Supplemental Table 1). Contrary to our expectations, *Col1a1* and *Prx1* were not among the 497 genes; *Col1a1* was not identified in one of the two experiments, and *Prx1* was not identified in either experiment. Nevertheless, we found a number of osteoblast-related genes that are expressed at higher levels in GFP (-); DsRed (+) cells. We also found that GFP (+); DsRed (-) cells express genes associated with chondrocytes, periosteal cells, and mesenchymal stem cells at higher levels compared with GFP (-); DsRed (+) cells. The differential expression was further confirmed by real-time PCR for some of the representative genes (Fig. 8). While the GFP (-); DsRed (+) cells showed higher expression of osteoblast markers *Runx2*, *Osterix*, *Satb2*, *Col1a1*, *Spp1*, and *Ibsp*, the GFP (+); DsRed (-) cells showed higher expression of chondrocyte markers *Sox9*, *Sulf-1*, *Aggrecan*, *Hapln1*, *4Cyt1-1*, and *Col2a1*, of periosteal markers *Asporin*, *Periostin*, and *Thrombospondin 2*, and of markers associated with mesenchymal stem cells, *Cxcl12* (*SDF-1*), *Ly6a* (*Sca-1*), *Thy1* (*CD90*), and *Alcam* (*CD166*).

Discussion

Several lines of evidence had indicated that the *Prx1* promoter is active in progenitor cells that give rise to osteoblasts. When genes essential for osteoblast differentiation were disrupted by using the *Prx1Cre* transgene, the gene inactivation resulted in severe impairment of bone formation, as observed in the case of β -catenin and ERK1/ERK2 [10,11]. In addition, our previous experiments using the *Prx1CreER-GFP* transgene showed that the transgene-expressing cells in the periosteum give rise to osteoblasts in the fracture callus [7]. In the present study, we further showed that *Prx1CreER-GFP*-expressing cells give rise to osteoblasts during normal development both in the long bones and the calvariae, and that flow-sorted *Prx1CreER-GFP*-expressing cells differentiate into *Col1a1CreER-DsRed*-expressing osteoblasts

in culture. These observations indicate that *Prx1CreER-GFP*-expressing cells are precursor cells for *Col1a1CreER-DsRed*-expressing osteoblasts.

Since the 3.2 kb *Col1a1* promoter is active in immature osteoblasts, and *Prx1CreER-GFP*-expressing periosteal cells express endogenous *Col1a1*, we expected to find a cell population in which both the 2.4 kb *Prx1* promoter and the 3.2 kb *Col1a1* promoter are active. However, our histological and flow cytometric analyses demonstrated that these transgenes are virtually expressed in distinct cell populations. The non-overlapping expression of the transgenes indicates that either the *Prx1CreER-GFP* transgene is turned off before the *Col1a1CreER-DsRed* transgene is activated or the cells express both *Prx1CreER-GFP* and *Col1a1CreER-DsRed* transgenes only for a short period of time during the course of osteoblast differentiation.

Calvaria-derived *Prx1CreER-GFP* and *Col1a1CreER-DsRed*-expressing cells showed a distinct difference in chondrogenic potential in vitro. While *Prx1CreER-GFP*-expressing cells underwent chondrogenic differentiation in micromass culture as evidenced by the remarkable induction of *Col2a1* and *Aggrecan* with BMP2 treatment, the same culture conditions did not induce chondrogenic differentiation in *Col1a1CreER-DsRed*-expressing cells isolated in parallel, implying that *Col1a1CreER-DsRed*-expressing cells are already committed to the osteoblast lineage. This is also consistent with our experiments using the *Rosa26 LacZ* reporter, in which no chondrocytes showed positive staining for X-gal in tamoxifen-injected *Col1a1CreER-DsRed*; *Rosa26 LacZ* embryos and mice (Fig. 2). In the periosteum of long bones, the *Col1a1CreER-DsRed*-expressing cells are localized in the innermost layer directly lining the bone matrix, while *Prx1CreER-GFP*-expressing cells are localized immediately on the outer side of the *Col1a1CreER-DsRed*-expressing cells (Figs. 3C, 4). Apparently, osteochondro progenitor cells maintain close association with committed osteoblasts within the periosteum.

Our microarray experiments and real-time PCR analyses demonstrated distinct gene expression profiles of the two cell populations. Consistent with their differentiation potential and lineage commitment, *Col1a1CreER-DsRed*-expressing cells show high levels of osteoblast marker expression and very low levels of chondrocyte marker expression, while *Prx1CreER-GFP*-expressing cells express both osteoblast and chondrocyte markers at modest levels. In addition, *Prx1CreER-GFP*-expressing cells also express periosteal markers and some of the mesenchymal stem cell markers. This is also consistent with their periosteal localization and undifferentiated progenitor status. Based on the gene expression profile, *Prx1CreER-GFP*-expressing cells may best be described as osteochondro progenitor cells

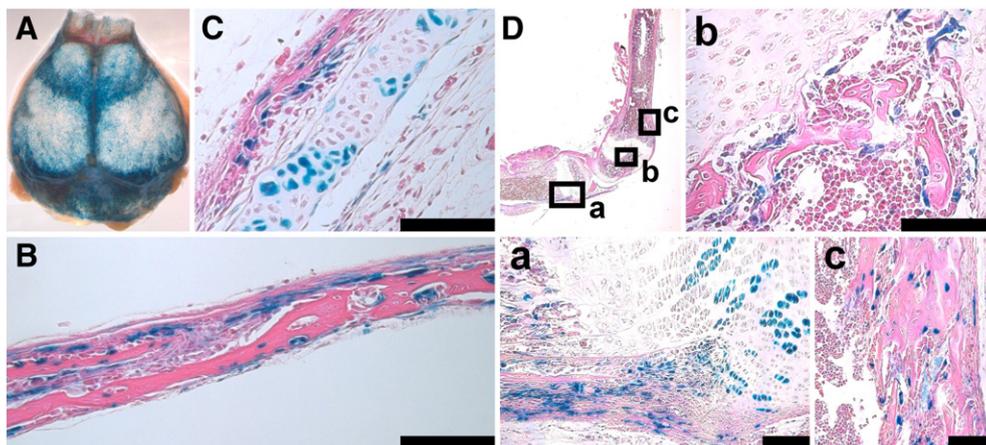


Fig. 7. *Prx1CreER-GFP*-expressing cells give rise to both chondrocytes and osteoblasts in the calvaria and long bone. (A) X-gal staining of the calvaria of *Rosa26 LacZ*; *Prx1CreER-GFP* mice at P15. Tamoxifen was injected into the mother at E13.5. (B) Coronal section of the parietal bone shown in (A). Some of the osteoblasts, osteocytes and periosteal cells stained positive for X-gal. (C) Section of the interparietal bone shown in (A). In addition to osteoblasts, some of the chondrocytes stained positive for X-gal, indicating osteogenic and chondrogenic potential of *Prx1CreER-GFP*-expressing cells. (D) X-gal staining of the tibia and femur of the *Rosa26 LacZ*; *Prx1CreER-GFP* mouse shown in (A). Boxed areas (a–c) in the upper left panel are magnified in the corresponding panel. (a) Some of the chondrocytes, osteoblasts, and periosteal cells stained positive in the proximal tibia. (b) Osteoblasts lining the bone trabeculae stained positive in the secondary ossification center of the distal femur. (c) Osteoblasts stained positive in the anterior cortex of the distal femur. Scale bars at the right bottom of each panel indicate 100 μ m.

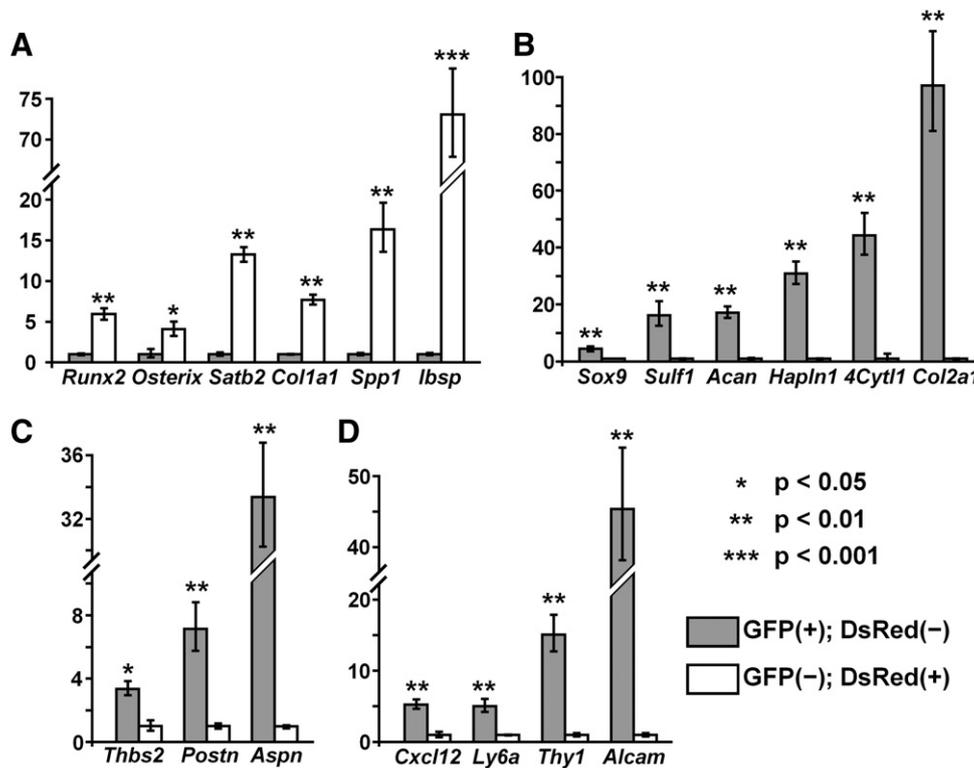


Fig. 8. Real-time PCR analyses of GFP (+); DsRed (-) and GFP (-); DsRed (+) cells isolated from the calvariae of E18.5 *Prx1CreER-GFP; Col1a1CreER-DsRed* embryos. While GFP (-); DsRed (+) cells show higher expression for osteoblast markers (A), GFP (+); DsRed (-) cells express chondrocyte markers (B), periosteal markers (C), and markers associated with mesenchymal stem cells (D) at higher levels. Data represent mean \pm standard deviations. The figure presents data from one of three experiments with similar results. Acan: Aggrecan, Thbs2: Thrombospondin 2, Postn: Periostin, Aspn: Asporin.

with some molecular characteristics of periosteal cells and mesenchymal stem cells.

Bones in the calvaria are derived from both cranial neural crest and paraxial mesoderm [26,27]. Bones anterior to the coronal suture, such as frontal bones, are derived from the neural crest, while bones posterior to the coronal suture, such as parietal and interparietal bones, are mostly derived from the paraxial mesoderm [28,29]. Cells that stain positive for X-gal were uniformly distributed among cranial bones, implicating that the *Prx1CreER-GFP* transgene is expressed in the mesenchyme derived from both origins, which is consistent with the known expression patterns of endogenous *Prx1* [30]. Our analysis also indicated that *Prx1CreER-GFP*-expressing cells are localized in the cranial suture mesenchyme. Cranial bones grow by the apposition of bone tissue at the cranial suture [27]. Cranial suture mesenchyme provides osteoblasts that participate in this appositional growth, while at the same time the mesenchyme remains undifferentiated and separates the growing cranial bones. The precise regulation of the differentiation of *Prx1CreER-GFP*-expressing cells would be essential for the proper growth of the calvaria, since premature ossification of the cranial suture leads to the growth disturbance known as craniosynostosis. The aberrant differentiation of osteochondro progenitor cells also accounts for the ectopic chondrocyte marker expression and cartilage formation in the calvariae of genetically engineered mouse models [10,31,32].

In summary, we have generated transgenic mice that express CreER and DsRed in osteoblasts under the control of a 3.2 kb *Col1a1* promoter. Histological and flow cytometric analyses of *Prx1CreER-GFP; Col1a1CreER-DsRed* double transgenic mice indicated that *Prx1CreER-GFP* and *Col1a1CreER-DsRed* transgenes are expressed in distinct cell populations in the long bones and calvariae. While the *Prx1CreER-GFP* transgene marks osteochondro progenitor cells, the *Col1a1CreER-DsRed* transgene is expressed in committed osteoblasts. The *Prx1CreER-GFP* and *Col1a1CreER-DsRed* transgenes will be useful in inducing Cre-*loxP*-

mediated recombination in respective cell populations. These transgenes will allow the visualization and isolation of transgene-expressing cells and thus facilitate the analysis of the lineage commitment of progenitor cells in a variety of mouse models for bone disorders.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.bone.2013.10.016>.

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