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Dual Reporter Transgene Driven by 2.3Col1a1 Promoter is Active in Differentiated Osteoblasts

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Aim. As quantitative and spatial analyses of promoter reporter constructs are not easily performed in intact bone, we designed a reporter gene specific to bone, which could be analyzed both visually and quantitatively by using chloramphenicol acetyltransferase (CAT) and a cyan version of green fluorescent protein (GFPcyan), driven by a 2.3-kb fragment of the rat collagen promoter (Col2.3).

Methods. The construct Col2.3CATiresGFPcyan was used for generating transgenic mice. Quantitative measurement of promoter activity was performed by CAT analysis of different tissues derived from transgenic animals; localization was performed by visualized GFP in frozen bone sections. To assess transgene expression during in vitro differentiation, marrow stromal cell and neonatal calvarial osteoblast cultures were analyzed for CAT and GFP activity.

Results. In mice, CAT activity was detected in the calvaria, long bone, teeth, and tendon, whereas histology showed that GFP expression was limited to osteoblasts and osteocytes. In cell culture, increased activity of CAT correlated with increased differentiation, and GFP activity was restricted to mineralized nodules.

Conclusion. The concept of a dual reporter allows a simultaneous visual and quantitative analysis of transgene activity in bone.

Key words: *cell differentiation; cells, cultured; chloramphenicol O-acetyltransferase; mice, transgenic; osteoblasts; promoter regions (genetics); ribosome; transgenes*

Osteoprogenitor cells arise from multipotential mesenchymal stem cells that progress to a number of committed and restricted cell lineages, including those for osteoblasts, chondroblasts, adipocytes, and myoblasts (1). By morphological and histochemical criteria, four maturational stages in osteoblast development have been identified in the bone: preosteoblast, osteoblast, osteocyte, and bone-lining cell (2). Osteoblasts are the skeletal cells responsible for the synthesis, deposition, and mineralization of the extracellular matrix of bone. They are post-proliferative, cuboidal, strongly alkaline phosphatase-positive cells lining the bone matrix at sites of active matrix production. Acquisition and maintenance of normal bone mass is dependent on an adequate osteoblast number and activity (3). If osteoporosis is viewed as an inability of the lineage to generate sufficient number of functional osteoblasts, then an approach for understanding the pathophysiological basis of the disease would be to identify each cellular stage of osteoblast differentiation.

We have been developing tools to assess the progression of cells within the osteoprogenitor lineage to full osteoblast differentiation. We have previously identified stage-specific fragments of the rat Col1a1 promoter, which were used to distinguish different stages of osteoblast development (4-8). Transgenic mice harboring a 3.6-kb fragment of collagen promoter driving chloramphenicol O-acetyltransferase (Col3.6 CAT) show transgene activity in osseous (bone and tooth) and nonosseous tissues (tendon, skin, and lung). The 2.3-kb fragment of the same promoter in the Col2.3 CAT transgenic mouse has a more restricted pattern of expression, with strong activity in bones, low activity in tendons, and very low or undetectable activity in other tissues (4). When calvarial osteoblast cultures and marrow stromal cultures derived from these mice are grown in differentiating conditions (2,9,10), they show early expression of Col3.6, which remains permanently active in the culture, whereas Col2.3 becomes active later and is associated with the mature osteoblast (5-7).

Subsequently, we have produced transgenic mice containing pOBCol3.6 and pOBCol2.3 that included a 1.6-kb fragment of the first intron of collagen driving a green fluorescent protein (GFP) (8). pOB

Col3.6 directed strong expression of GFP messenger RNA (mRNA) to bone and isolated tail tendon and lower expression in nonosseous tissues, whereas pOBCol2.3 expressed GFP mRNA in bone and tail tendon only. Cell cultures and histology confirmed our previous hypothesis that the 3.6-kb fragment of the collagen promoter was active in fibroblastic cells as well as osteoblastic cells, whereas the activity of 2.3-kb fragment was restricted specifically to osteoblasts and osteocytes (8).

Each reporter has its advantages and problems. CAT provides a quantitative readout for the entire tissue but is difficult to image with immunostaining. GFP provides exquisitely detailed transgene expression at cellular level, but makes the quantitation of the total activity of the tissue difficult to perform. To overcome this problem and develop a version of GFP that can be distinguished from the green-shifted GFPs, we designed a construct for a dual reporter transgene (CATiresGFPcyan) under the control of the rat Col2.3 promoter to follow collagen-expressing cells in transgenic animals. Transgene expression was assessed by measuring CAT activity in protein extracts of collagen-producing tissues and by fluorescent microscopy of frozen bone sections from transgenic mice. Strong transgene expression was found in mouse calvarial osteoblast and marrow stromal cell cultures when grown under conditions inducing osteoblastic differentiation. Our results confirmed that this dual reporter was both active and regulated in transgenic animals and therefore a useful tool in the analysis of the Col2.3 activity in bone cell cultures and intact bone.

Material and Methods

Cloning Strategy and Generation of Transgenic Mice

pIRESeGFP vector (Clontech, Paolo Alto, CA, USA) was modified by inserting the CAT gene as a BamHI/XhoI fragment into BglII/SalI compatible cohesive ends of the multicloning site upstream of internal ribosome entry site (IRES). The cytomegalovirus (CMV) promoter was cut out with AseI and NheI, and an AseI-NotI-EcoRV-XbaI-NheI linker was inserted. The enhanced GFP and simian virus 40 (SV40) polyadenylation signals were cut out with BstXI and AflII, and a BstXI-SnaBI-NotI-blunt end linker was inserted. GFPcyan (ECFP, Clontech) linked with bovine growth hormone (bGHPA) as a BstXI/EcoRV fragment was placed into BstXI/SnaBI sites. The 2.3Col1A1 promoter was ligated as a HindIII/blunt end fragment into an EcoRV site and clones were selected for the correct orientation with PvuII. This construct showed GFP expression in vitro by transient transfection (Lipofectamine 2000, Life Technologies, Rockville, MD, USA) in 293GPG cells (not shown). The transgene Col2.3CATires GFP cyan was excised from the plasmid backbone as a NotI fragment and transgenic mice were produced by microinjection (11) of the isolated DNA into pronuclei of fertilized CD1 mouse embryos at the institutional transgenic animal facility.

Primary Marrow Stromal Cell Culture

Bone marrow was harvested from 2-month-old heterozygous mice. Femurs and tibiae were dissected from surrounding tissue and the epiphyseal growth plate was removed. The marrow was collected by flushing with α-modified essential culture medium (α-MEM) containing 100 U/mL penicillin, 100 µg/mL streptomycin (Life Technologies), and 10% fetal bovine serum (FBS; Summit Biotechnologies, Ft. Collins, CO, USA) with a 25-gauge needle. The cells were dispersed by several cycles of aspiration and expulsion through an 18-gauge needle, filtered through a 70-µm cell strainer, counted, and plated at a density of 2×10^7 cells per 35-mm culture dish. On day 4, half of the culture medium was removed and replaced with fresh medium. On day 7, the medium was replaced with α-MEM supplemented with 25 µg/mL ascorbic acid, 10 nmol/L dexamethasone, and 8 mmol/L β-glycerophosphate (Sigma-Aldrich, St. Louis, MO, USA). The medium was changed every 2 days for the duration of the experiment. Cells were examined by using an Olympus IX50 microscope (Olympus America Inc, Melville, NY, USA) equipped with a filter set for fluorescence detection of GFPcyan (excitation at 433 nm) and an x10 objective.

Primary Calvarial Osteoblast Culture

Calvariae were isolated from 6-8-day-old heterozygous mice. After the removal of the sutures, calvariae were subjected to four sequential 15-minute digestions in a mixture containing 0.05% trypsin and 0.1% collagenase-P at 37 °C, rotating at a speed of 80 revolutions per minute (rpm). Cell fractions 2-4 were collected and resuspended in Dulbecco's modified Eagle's medium (DMEM, Life Technologies) containing 10% FBS (Atlas, Ft. Collins, CO, USA), 100 U/mL penicillin, and 100 mg/mL streptomycin. Cells were filtered through a 70-µm cell strainer, centrifuged, resuspended in the medium, and plated at a density of 1.5 $\times 10^5$ cells per 35 mm culture dish. The medium was changed at 24 h and at day 4 after plating. At day 7, DMEM was changed to differentiation medium (α-MEM containing 10% FBS, 25 µg/mL ascorbic acid, and 4 mmol/L β-glycerophosphate); this medium was changed every 2 days for the duration of the experiment. Cells were again examined by using an Olympus IX50 microscope, equipped with a filter set for fluorescence detection of GFPcyan.

End Point Markers of Differentiation

Alkaline Phosphatase Staining. Cells were rinsed with phospohate buffered saline (PBS), fixed with citrate-acetone formaldehyde fixative, rinsed with water, and stained for enzyme activity with 0.25% AS-BI phosphate alkaline solution and fast red violet LB base (Sigma-Aldrich, procedure No. 86). Plates were scanned with an Umax Astra 4000U scanner (Umax Technologies, Dallas, TX, USA).

Von Kossa Staining. After alkaline phosphatase staining, the same cells were exposed for 45 minutes to bright light in a 5% solution of $AgNO^3$ (Sigma-Aldrich). The reaction was stopped with sodium thiosulphate; cells were washed and photographed. Plates were scanned with an Umax Astra 4000U scanner.

CAT Assays

Tissues were dissected from 2-month-old transgenic mice and dispersed in 0.5 mL of extraction buffer (0.25 mol/L Tris-HCl, pH 7.8, containing 0.5% Triton X-100). The samples were subjected to three cycles of freezing and thawing followed by heating at 65 °C for 15 min to inactivate endogenous deacetylases. Samples were centrifuged and supernatants were used to determine CAT activity and protein concentration as described previously (6,12). CAT activity was normalized to the protein content of the extract as measured by the BCA assay (Pierce, Rockford, IL, USA). The CAT assay for the cultured cells was carried out in the same manner, except the cells were harvested by scraping in 0.15 mol/L NaCl, 40 mmol/L Tris-Cl, pH 7.4, and 1 mmol/L ethylenediaminetetraacetic acid (EDTA).

Histology and Fluorescent Microscopy

Femurs from 2-month-old transgenic mice were fixed in 4% paraformaldehyde at 4 °C, with constant agitation for 3 days. Fixed bones were decalcified in a 14% EDTA solution at 4 °C with constant agitation for 3-5 days, with change of decalcifying solution every 24 h. Bones were washed for 2 h in PBS. After overnight soaking in 30% sucrose in PBS at 4 °C with constant agitation, bones were embedded in Cryomatrix (Thermo-Shandon, Pittsburgh, PA, USA) and cut in 5 µm thick frozen sections by using a cryostat (CryoJane System, Instrumedics Inc, Hackensack, NJ, USA). Sections were rinsed in PBS two times for 5 min, soaked in 0.1 mol/L $MgCl_2$ in PBS for 30 min, and rinsed again in PBS for 5 min. Covered slides were mounted with 50% glycerol in PBS. Slides were examined by using a Zeiss Axiovert 200 inverted microscope (Zeiss, Göttingen, Germany), at x20 magnification. A specific excitation wavelength was obtained by using filters for GFPcyan (exciter, D436/20; dichroic, LP 455; emitter, D480/40).

Results

Transgenic mice were generated by using a 2.3ColCATiresGFPcyan construct (Fig. 1A), which contains 2,296 bp of the rat Col1A1 promoter, 116 bp of transcribed Col1A1 DNA, a CAT gene, viral IRES sequence, GFPcyan, and a polyadenylation site. No differences in health or development between transgenic and wild type mice were observed. Transgenic mice were genotyped by fluorescence microscopy of freshly isolated tail clips. Mice positive for the transgene showed fluorescent signal in the vertebral bone of the tail (Figs. 1B and 1C). Three founders were generated but only one of them passed the transgene to its offspring.

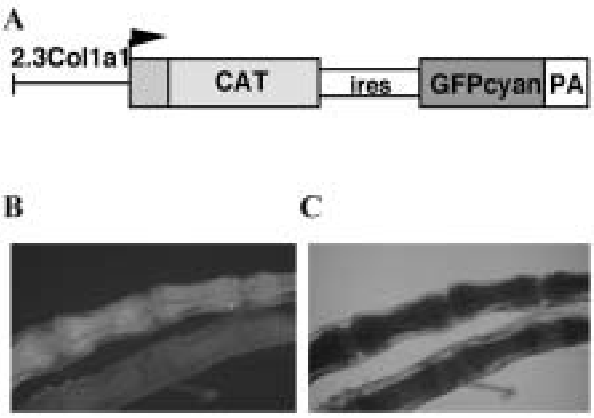

Figure 1. Generation of transgenic mice. **A.** The Col2.3CA TiresGFP construct contains 2296 bp of rat Col1A1 5′ flanking DNA. 116 bp of transcribed Col1A1 DNA, chloramphenicol acetyltransferase (CAT) gene, internal ribosome entry site (IRES) sequence derived from encephalomyocarditis virus (EMCV), GFPcyan and polyadenylation site (PA) derived from bovine growth hormone. **B.** Fluorescent and **C.** transmitted image of freshly stripped tails from 4 week-old Col2.3CATiresGFP transgenic mouse showing a strong expression of transgene in bone compared to a negative signal in the wild type mouse.

In vivo analysis of transgene expression performed by CAT assay of protein extracts from different tissues of 8-week-old heterozygous mice revealed that the activity of the Col2.3 promoter was limited to collagen-producing tissues, such as tendon, calvaria, long bones, and teeth. The expression was stronger in osseous tissues than in the tendon. Transgene expression was low or not detected in the skin, brain, heart, lung, liver, kidney, bladder, and muscle (Fig. 2).

Histological analysis of the femurs obtained from 2-month-old transgenic mice required frozen sectioning; strong GFPcyan signal was detected in most osteoblasts lining the endosteal surface and some osteocytes in the cortical bone (Fig. 3). The intensity of the signal was clearly above the blue autofluoresent background.

To assess transgene expression during in vitro differentiation, marrow stromal cell cultures were established from 2-month-old transgenic heterozygous

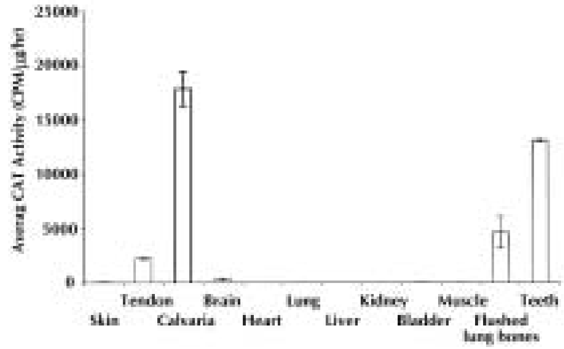

Figure 2. Analysis of transgene expression in different tissues. Chloramphenicol acetyltransferase (CAT) assay from tissues of Col2.3CATiresGFP transgenic mice is showing increased CAT activity in the tendon, calvaria, flushed long bones and teeth, marking tissues with high collagen production. Measurements are the mean±SEM of triplicate determinations.

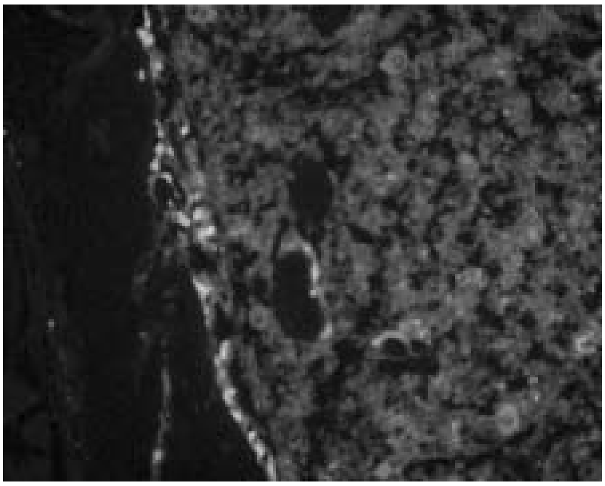

Figure 3. Histology of femoral bone of Col2.3CATires GFP cyan mouse. Frozen section of diaphyseal region of the femur from a 2-month-old transgenic mouse (×20 magnification). GFPcyan-expressing osteocytes and osteoblasts can be observed in the regions close to the endosteal surface.

animals (Fig. 4). Alkaline phosphatase and Von Kossa staining of sister plates were used to follow osteoblast differentiation of the cultures (Fig. 4A). Examination of the same culture dishes over time for GFP fluorescence revealed the presence of the first GFP positive cells at day 14 coincidently with the first mineralized nodules detected by Von Kossa staining at day 14. The strength of the signal increased as differentiation proceeded at day 18. The signal was localized in multilayered areas of cuboidal cells, which are associated with mineralization (Fig. 4B). Measurement of CAT activity is a more sensitive assay than visual assessment of GFP. Increased CAT levels were detected at day 11 of the culture and continued to increase as the culture differentiated (Fig. 4C).

Comparable findings were observed in neonatal calvarial cultures derived from 5-8-day-old transgenic heterozygous animals (Fig. 5). At day 14 and at later

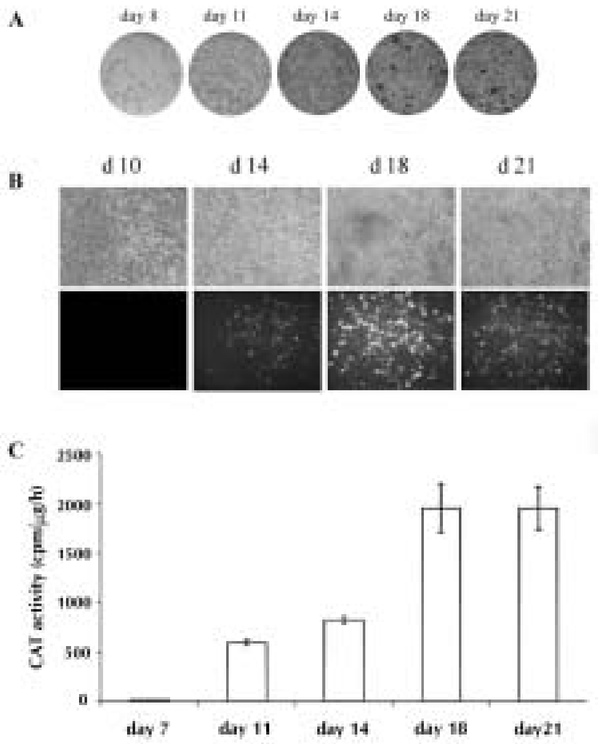

Figure 4. Expression of the dual transgene in primary marrow stromal cell culture. **A.** Differentiation of marrow stromal cell culture shows increasing intensity of alkaline phosphatase staining and Von Kossa staining over time. **B.** Cells from marrow stromal culture are starting to express GFP cyan as starting to differentiate into osteoblasts; activity of the Col2.3 promoter is localized within mineralized nodules. **C.** Chloramphenicol acetyltransferase (CAT) activity is increasing as marrow stromal culture differentiates and it is detectable earlier than GFPcyan due to higher sensitivity of the assay. Values are the mean±SEM of triplicate determinations.

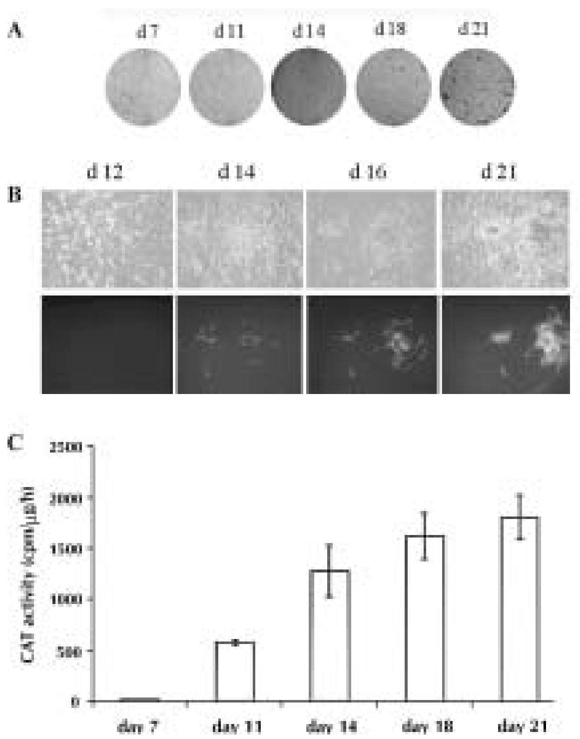

Figure 5. Expression of the dual transgene in primary calvarial osteoblast cell culture. **A.** Differentiation of calvarial osteoblast cell culture shows increasing intensity of alkaline phosphatase staining and Von Kossa staining over time. **B.** Cells from calvarial osteoblast cell culture are starting to express GFPcyan as starting to differentiate into osteoblasts; activity of the Col2.3 promoter is localized within mineralized nodules. **C.** Chloramphenicol acetyltransferase (CAT) activity is increasing as calvarial culture differentiates and it is detectable earlier than GFPcyan due to higher sensitivity of the assay. Values are the mean±SEM of triplicate determinations.

time points, the expression of the GFP was detected in the nodule areas of cuboidal-shaped cells undergoing mineralization (Fig. 5B). The progression of culture differentiation and the appearance of the first mineralized nodules at day 14 was shown by alkaline phosphatase and Von Kossa staining (Fig. 5A). CAT activity was detectible earlier than GFP signal, as expected, and it continued to increase until the last time point at day 21 (Fig. 5C).

Discussion

The new marker transgene CAT-IRES-GFP was implemented to measure and localize the activity of promoter constructs in primary bone cell cultures derived from mouse calvarial and marrow stromal fibroblasts. This approach will be very useful because both reporters demonstrate increasing activity concomitant with culture differentiation; the GFP signal first appears in nodules undergoing mineralization. The Col2.3CATiresGFP is expressed in a tissue-specific manner as the other previously described collagen promoter-reporter constructs (6,8,13-15). CAT activity is high in COL1A1-producing tissues, such as bone and tendon. Unfortunately, in vivo use is limited due to GFPcyan inability to survive paraffin embedding, as is the case with eGFP (Clontech) (16), but frozen sections of bones show GFP positive osteoblasts and osteocytes. The 2.3-kb fragment of the collagen promoter marks cells in later stages of the osteoblast lineage, which extend into mature osteocytes. We also evaluated a double reporter construct with the 3.6-kb fragment of the collagen promoter. It did show activity in preosteoblastic cells and osteoblasts cells, as expected from our previous studies (8). However, our recent experience underlies the importance of including the first intron in the transgene design. The constructs discussed in this report are inherently less robust than those that contain the collagen first intron (8). Studies by Liska et al (17) provide support for the concept that different cell types require different regions of the COL1A1 promoter for activity. They found that the first intron of the human COL1A1 is required for high-level expression in the dermis of transgenic mice, but not for the expression in the calvariae and tendon.

We have previously shown in transgenic mice that the pOBCol2.3GFPemerald construct was active in cells within the mineralized portion of the nodule (8). In vivo, only bone-lining cells and cells within the matrix were GFP-positive. The pOBCol3.6GFPtopaz transgene became active earlier, coincident with Col1a1 and alkaline phosphatase expression and in vitro and in vivo lower level of expression was detected in preosteoblastic cells, which have the potential to become fully differentiated bone cells (8). As differentiation occurred, the promoter was up-regulated and higher levels of expression were detected in osteoblastic cells. The identification of subpopulations of cells of the osteoblastic lineage within the same animal requires two different fluorescent reporter proteins, which can be clearly distinguished with filters. GFPemerald and GFPtopaz could not be distinguished with their respective filters, and GFP cyan was therefore introduced as a reporter.

The IRES approach for expression of bicistronic RNA was previously studied and used in transgenic mice (18-22). IRES is a viral sequence that enables translation of both reporter proteins from one bicistronic mRNA. The first protein, CAT, is translated via CAP-dependent translation using 5′ pppG methylated position 7 as a signal for ribosome entry, and the second protein, GFP, is translated by using IRES as a recognition sequence for ribosome entry (23-25). There are some advantages of a double reporter over the breeding of single transgenic lines: it requires less time and space, while increasing the percentage of offspring carrying both transgenes. This new marker transgene CAT-IRES-GFP quantitatively evaluates promoter activity using the CAT assay and in parallel visually distinguishes by GFP in which cells of osteoblastic lineage that activity is present.

The utility of GFP colors, such as GFPcyan or GFPsaphire, will lead to multiplexed colors for assigning different stages of differentiation in the same culture dish. Col2.3CATiresGFPcyan construct showed consistency in the intensity and expression pattern with previously developed (pOB)Col2.3 reporter constructs (6,8,14,15,26) and will complement them in identifying and assessing activity of a cell populations within the osteoblast lineage. It can be a useful tool in understanding the process of bone cell maturation.

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