

β -Galactosidase staining on bone marrow. The osteoclast pitfall

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Summary. The enzyme β -galactosidase, encoded by the bacterial gene *lacZ*, is commonly used as a histochemical reporter to track transplanted cells *in vivo* or to analyze temporospatial gene expression patterns by coupling expression of specific target genes to β -galactosidase activity. Previously, endogenous β -galactosidase activity has been recognized as a confounding factor in the study of different soft tissues, but there is no description of the typical background on bone marrow sections when using the chromogenic substrate 5-Bromo-4-chloro-3-indolyl β -D-Galactoside (X-Gal). In this report, we show that osteoclasts in bone marrow sections specifically and robustly stain blue with X-Gal. This leads to a typical background when bone marrow is examined that is present from the first day post partum throughout the adult life of experimental mice and can be confused with transgenic, bacterial β -galactosidase expressing hematopoietic or stromal cells. Experimental variations in the X-Gal staining procedure, such as pH and time of exposure to substrate, were not sufficient to avoid this background. Therefore, these data demonstrate the need for strenuous controls when evaluating β -galactosidase positive bone marrow cells. Verifiable bacterial β -galactosidase positive bone marrow cells should be further identified using immunohistological or other approaches. Specifically, β -galactosidase positive hematopoietic or stromal cells should be proven specifically not to be osteoclasts by co-staining or staining adjacent sections for specific markers of hematopoietic and stromal cells.

Key words: β -galactosidase, X-Gal staining, Bone marrow, Osteoclasts

Introduction

The use of *E. coli* β -galactosidase (β -gal, encoded by the bacterial gene *lacZ*) has become a routine histological method for the identification of transplanted cells *in vivo* or for studying the expression of transgenic genes in mice where the expression of β -gal is driven by the promoter of a target gene. A common problem in identifying β -gal-positive cells by development with the chromogenic substrate 5-Bromo-4-chloro-3-indolyl β -D-Galactoside (X-Gal) is the presence of cells expressing endogenous mammalian β -gal activity (Coates et al., 2001). This problem has been addressed in the literature and optimized staining protocols have been suggested for soft tissues (Weiss et al., 1999; Ma et al., 2002). The occurrence of endogenous cells containing a level of β -gal activity comparable to that seen in transgenic mice has been identified as "the biggest risk" in studies where transplanted cells are tracked (Brazelton and Blau, 2005).

X-Gal stains of bone marrow typically appear in the scientific literature either as small cut-outs or high magnification images of single cells, while low magnification images are rarely seen. In our previous work with transgenic mice where the Tie2 promoter drives β -gal expression, we noticed the presence of flat cells, which were adherent to the endosteal bone surface, and initially thought of them as Tie2-positive, bacterial β -gal expressing cells (Kopp et al., 2005). However, re-examination and close comparison of slides from transgenic as well as wild type control mice revealed that these cells are also present in wild type mice and that they fulfill all morphologic and histochemical criteria of osteoclasts. Osteoclasts are bone-resorbing cells, which occur more rarely than osteoblasts and are derived from hematopoietic cells. Typically, endosteum-adherent mononuclear osteoclasts fuse to create big, multinucleated osteoclasts (Miyamoto and Suda, 2003). Osteoclasts can be detected by histochemically staining

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for tartrate-resistant acid phosphatase (TRAP) activity. In long bones, they are most abundant in the resorption zone of the epiphyseal growth plates, where they phagocytose apoptosing chondrocytes (Bronckers et al., 2000) as well as along the endosteal surfaces of cortical and trabecular bone.

In order to establish common knowledge of this potentially misleading artifact, we systematically examined the bones of different wild type mouse strains at various stages of development. The same pattern of background staining was observed in C57bl/6, FvB/NJ, 129P3/J, and CD1 strains of mice. In addition, we examined the effect of pH and time of exposure to substrate and observed no changes in endogenous mammalian β -gal activity in the bone marrow. Finally, neonate wild type mouse bones were examined for β -gal activity, and the same pattern of osteoclast positivity was observed. We conclude from these data that there is a field-wide need for strenuous controls and further classification when evaluating β -galactosidase positive bone marrow cells.

Materials and methods

Animals

Animal experiments were performed with the authorization of the institutional review board and Institutional Animal Care and Use Committee of Weill Medical College of Cornell University. The mice were purchased from Jackson Laboratories, Bar Harbor, ME and maintained in air-filtered Thoren units.

Reagents

X-gal (5-Bromo-4-chloro-3-indolyl β -D-galactoside) was purchased from Biosynth AG (Staad, Switzerland).

Tissue processing for cryosections

Adult murine bones were dissected and fixed in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) for 4 hours at 4°C. Whole adult femur samples were decalcified in 10% EDTA/PBS for 3-4 days and after extensive washing were cryoprotected in 30% sucrose/PBS overnight. Thereafter, the decalcified whole femur bones were embedded in Tissue Tek OCT (Sakura Finetek USA, Torrance, CA). Newborn murine bones were embedded in OCT without decalcification. Five micron cryosections were prepared on a Leica CM3050S (Leica, Inc., Nussloch, Germany), placed onto Superfrost Plus slides (VWR, West Chester, PA), and dried for 2 hours at room temperature. Slides were washed in LacZ Wash Buffer (PBS containing 0.01% sodium deoxycholic acid, 0.02% Nonidet-P40, 2 mmol/l MgCl_2) three times for 5 min. and subsequently incubated in X-gal staining solution (1 mg/ml 5-Bromo-4-chloro-3-indolyl β -D-Galactoside, 2 mmol/l MgCl_2 , 5 mmol/l potassium ferrocyanide, 5 mmol/l potassium

ferricyanide, 0.01% sodium deoxycholate, 0.02% Nonidet-P40) at 30°C for 6-12 hours. X-gal staining solution was titrated to pH 7.0, 7.5, and 8.0, respectively, with sodium hydroxide. Alternatively, a commercially available β -gal staining set was used according to the instructions of the manufacturer (Roche Diagnostics Corp., Indianapolis, IN). After 3 washes in PBS, the samples were postfixed in 0.2% Glutaraldehyde (Electron Microscopy Sciences) washed in distilled water, counterstained with Nuclear Fast Red (NFR, Biomed Foster City, CA), dehydrated in graded alcohols, cleared in xylene, and coverslipped.

Tissue processing for paraffin sections

Freshly dissected femurs were carefully cut open lengthwise with a scalpel blade and incubated in 0.2% glutaraldehyde/PBS or alternatively in 4% paraformaldehyde/PBS for 4 hours at 4°C. Femur pieces were washed in PBS for 5 min. and subsequently incubated in X-gal staining solution as above. After a 10 min postfixation in 0.2% glutaraldehyde/PBS or alternatively in 4% paraformaldehyde/PBS, samples were decalcified in EDTA for 3-4 days and subsequently dehydrated in gradual ethyl alcohols from 50% to 100%, incubated in xylene, and infiltrated with three changes of paraffin at 58°C followed by embedding in paraffin. Femurs were sectioned at 5 μm , deparaffinized and rehydrated for immunostaining with rat anti-mouse anti-pan endothelial cell monoclonal antibody, clone MECA32 (BD Pharmingen, San Diego, CA) as reported previously (Kopp et al., 2005). Additional sections were prepared for TRAP staining (Sigma, St. Louis, MO) per kit instructions. Counterstaining was done with Lillie's Modification of Mayer's hematoxylin (DAKO, Carpinteria, CA).

Brightfield images of H&E, β -gal, TRAP and immunostained tissues were visualized using an Olympus BX51 microscope (Olympus America, Melville, NY) and digitalized with a Qimaging camera (Retiga EX; Qimaging, Burnaby, BC). Images were recorded using Mac Qcapture Acquisition software (Version 2.68.6; Qimaging).

Results

Wild type murine bones were stained with X-gal resulting in a typical epiphyseal β -gal positive stain line seen on whole mount preparations (Fig. 1A,B). With low magnification light microscopy of H&E stained sections, the endogenous β -gal staining is observed in the epiphyseal resorption zone as well as along the endosteal surfaces of trabecular bones (Fig. 1C). It has been reported previously that the growth plate closure observed in humans is not found in experimental rodents. In rats, a process of bony bridging as an equivalent of growth plate closure occurs between 8-10 months of age in the proximal tibia (Martin et al., 2003). Whether and to what extent growth plate fusion takes

place in other rodent long bones has not been established. However, epiphyseal growth plates including resorption zones with an abundance of osteoclasts will always be present in experimental mice at the typical age of 6-12 weeks. Higher magnification revealed that the β -gal positive cells are located along the endosteum adjacent to trabecular bone and in particularly high concentrations in the epiphyseal resorption zone (Fig. 2). In addition, the cells are multinucleated and display the typical foamy cytoplasm of osteoclasts (Fig. 2 and inset).

It is possible to do concomitant immunohisto-

chemistry, if whole mount bone is first stained with X-gal, then sectioned and finally subjected to immunohistochemical staining. When an antibody against a well-known and previously published marker of the vasculature, pan-endothelial cell antigen (clone MECA32), was used in wild type bone, the X-gal positive osteoclasts could be found in their typical location in epiphyseal resorption pits and closely located to the bone marrow vasculature (Fig. 3A and inset). In fact, when β -gal positivity was compared to the gold standard osteoclast stain of tartrate resistant acid phosphatase (TRAP, Fig. 3B), we found X-gal staining

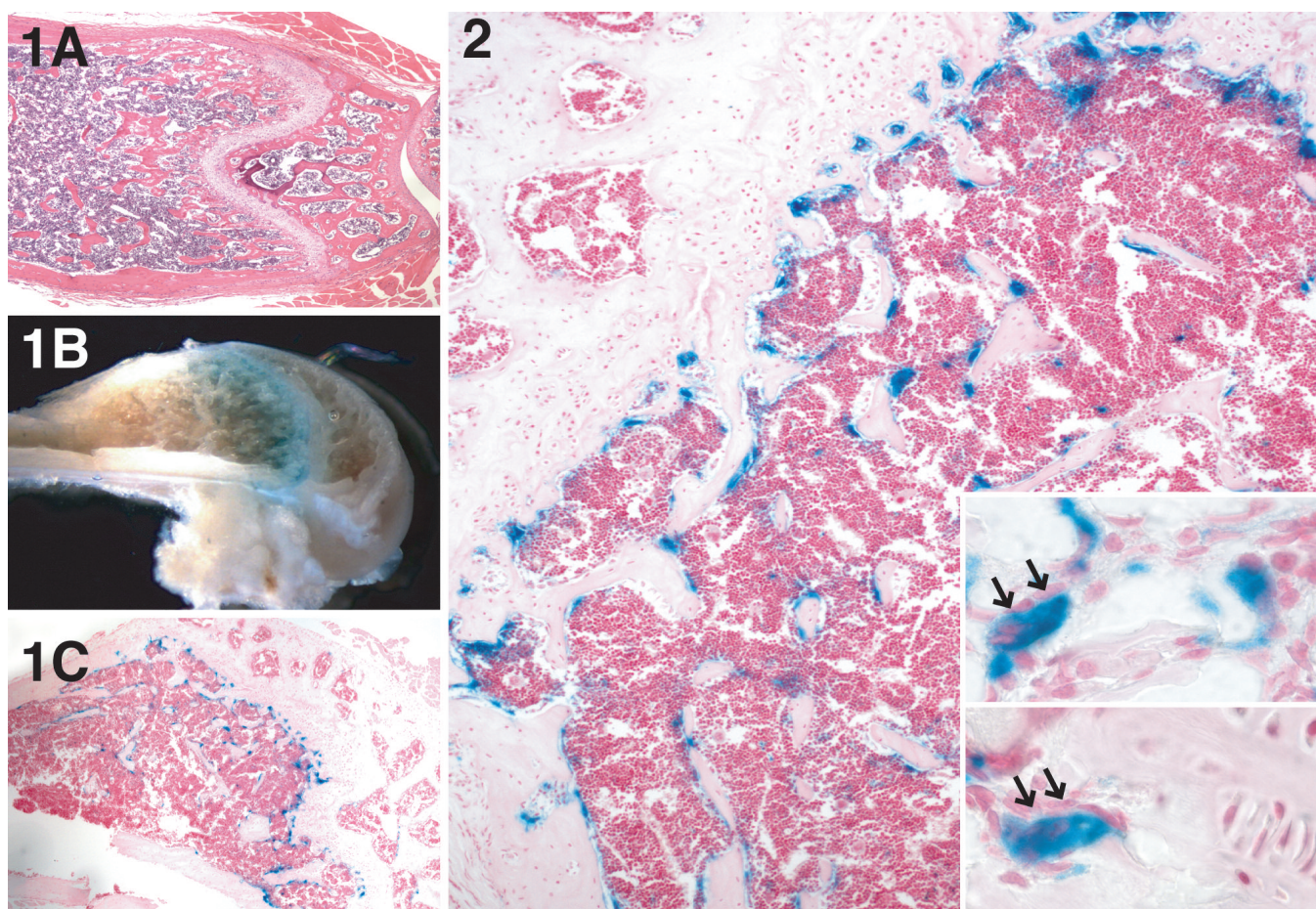


Fig. 1. X-gal stains bone marrow osteoclasts in wild type mice. **A.** Hematoxylin and eosin (H&E) stained wild-type (C57bl/6) bone marrow. Femur epiphyseal growth plates in experimental mice of typical age are those of a growing long bone. Therefore, the typical columnar chondrocytes as well as the resorption zone of enchondral ossification with abundant osteoclasts will always be present when bone marrow of these animals is examined. Obviously, there is also abundant trabecular bone in the distal femur. x 400. **B.** Whole mount preparation of the X-gal stained distal femur of wild type (C57bl/6) mice. Upon gross examination, macroscopic staining in the area of the epiphyseal growth plate after X-Gal staining was observed. Osteoclasts are typically located at the epiphyseal ossification front, where they phagocytose apoptosing chondrocytes. **C.** Paraffin section of wild type (C57bl/6) bone marrow, X-gal stained. Counterstained with nuclear fast red (NFR). The location of stained cells along trabecular bone endosteal surfaces and in the epiphyseal resorption zone is typical for bone marrow osteoclasts. x 400

Fig. 2. X-gal stained bone marrow displays a pattern of blue stained cells, which fulfill all the morphological criteria of osteoclasts. Paraffin section of wild type (C57bl/6) bone marrow, stained with X-gal. Counterstained with NFR. x 100. Insets (arrows): note the location, multiple nuclei and shape of the cells. In the lower inset note the chondrocytes on the right. x 1,000

to be a comparably reliable method to stain for bone marrow osteoclasts both in paraffin and frozen sections. This was confirmed with sequential staining with X-gal and TRAP, which revealed doubly stained, multinucleated osteoclasts displaying the typical histomorphological features of this cell type (Fig. 3C and inset). Taken together, these data provide further evidence that the cells that stain positive with X-gal are osteoclasts.

Some researchers may use *lacZ* reporter mouse lines such as ROSA26R, which express β -galactosidase following Cre-mediated recombination and thus they may want to assess β -galactosidase activity in doubly transgenic offspring as early as one to three days after

birth. Therefore, we also assessed endogenous mammalian β -gal expression in newborn mouse pups' long bones by staining with X-gal. Figure 4 shows a time course of these results for mice of one to six days of age. We consistently observed that osteoclasts stain positive with X-gal staining solution not only in adult mice, but also in newborn mouse pups up to one week of age (Fig 4A-C and insets). Although cell-specific expression of *lacZ* has successfully been used to evaluate the distribution of *Gpc3*-expressing chondrocytes in mouse embryos (Viviano et al. 2005), our results suggest that β -gal expressing osteoclasts can be misleading when it comes to analyzing genes with unknown expression patterns in murine bone marrow

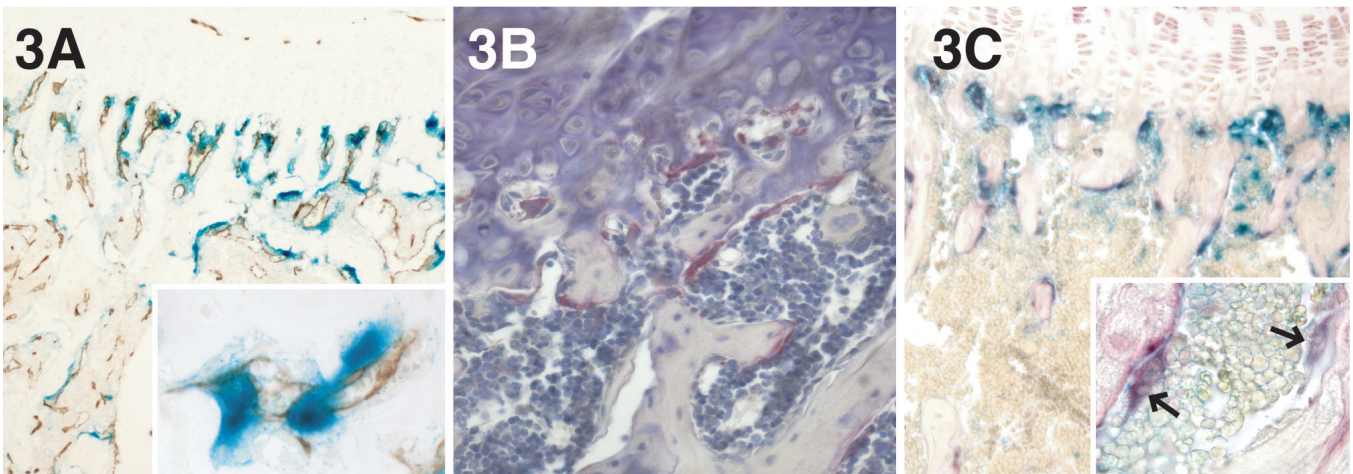


Fig. 3. X-gal staining procedure definitively stains bone marrow osteoclasts. **A.** Whole mount X-gal stained bone can safely be decalcified, embedded in paraffin, and later be used for immunohistochemistry. Paraffin section of wild type (C57bl/6) bone marrow stained with X-gal and with anti-pan endothelial cell antigen antibody, clone MECA32, no counterstain, 200X. Inset: note the perivascular location of the multinucleated osteoclast in the resorption zone of the depicted epiphyseal plate, where apoptosing chondrocytes are resorbed. x 400. **B.** Wild type (C57bl/6) bone marrow stained for TRAP-activity, counterstained with hematoxylin. x 400. **C.** LacZ-positive cells in wild type bone marrow are also TRAP-positive. Paraffin section of wild type (C57bl/6) bone marrow stained first with X-gal and afterwards for TRAP activity, no counterstain. x 200. Inset (arrows): note the purple appearance of osteoclasts from doublestain of X-gal and TRAP, blue and red respectively. x 1,000

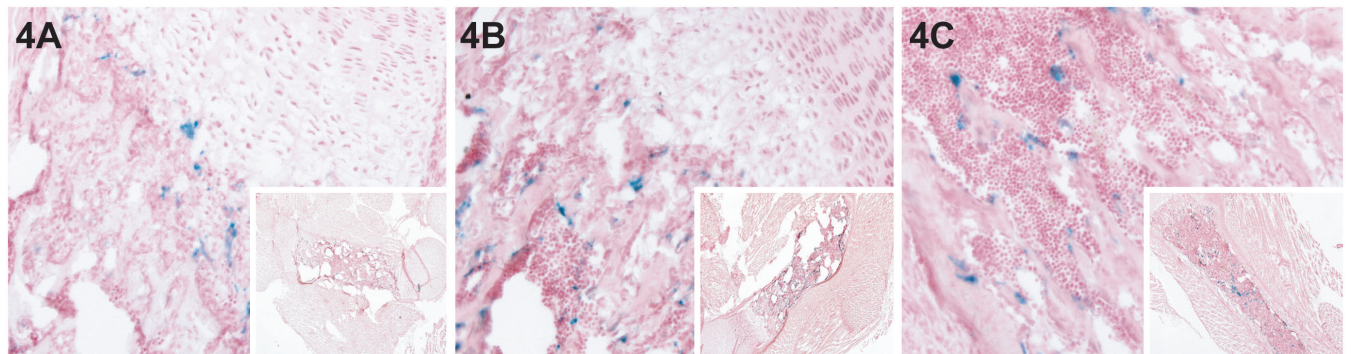


Fig. 4. Newborn mouse bones display osteoclast-associated background similar to adult osteoclasts. Femurs of mouse pups 1 (**A**), 3 (**B**), and 5 (**C**) days of age were harvested, fixed, frozen, cryosectioned, and stained with X-gal. Similar to adult bone marrow, newborn mouse tissue displayed abundant osteoclasts along the endosteum and on the proximal side of the growth plate. x 400, inset x 40

after birth.

We sought to determine whether alteration of experimental conditions would affect the detection of endogenous β -gal expression. Specifically, we examined whether time of exposure to substrate, pH, mode of euthanasia, mouse strain, or bone chosen for examination might play a role in the visualization of endogenous mammalian β -gal. Our results demonstrate that endogenous β -gal activity is detected even when we reduced time in substrate to barely acceptable limits of 6 hours and also when we altered pH by using pH of 7.0, 7.5, and 8.0. The use of a commercially available β -gal staining set (pH 7.5, Roche Diagnostics) reportedly reduces mammalian β -galactosidase to a minimum (Duffield et al., 2005). However, bone marrow osteoclasts still stained positive when we used the kit (data not shown). Because endogenous β -galactosidase activity has been suggested as a marker of senescence or stress, different methods of euthanasia (CO_2 -asphyxiation, cervical dislocation, injection anaesthetic overdose) were compared and shown to be without influence on the staining results (data not shown). In addition to femurs, tibiae, sterna, and vertebrae were stained for β -gal activity and similar staining of osteoclasts was observed for all bones examined. Finally, we used different strains and obtained similar results for C57bl/6, FVB, and CD1 wild type mice (data not shown).

Discussion

This study demonstrates for the first time that in murine bone marrow, from day 1 post partum, there is endogenous β -gal enzymatic activity along endosteal surfaces and particularly along the epiphyseal growth plates resulting in strong background staining. The results demonstrate that even with modifications of the technique such as paraffin vs. frozen sections, changes in time of X-gal reaction, and alterations in pH, the endogenous β -gal activity remained. In fact, these data indicate that the X-gal staining procedure is as reliable a staining method for osteoclasts in mouse bone marrow as TRAP staining and histomorphological analysis.

Taken together, these data suggest that when β -gal-containing transgenic cells are analyzed and described in murine bone-marrow – be it in the examination of transgenic animals, in stem cell transplantation studies or in studies where the Cre-loxP-Method is used and X-gal staining is used as a readout – osteoclasts can be mistaken for transgenic cells expressing the *E. coli* β -gal gene thereby complicating the interpretation of results. This is of particular importance in bone marrow related experiments focusing on hematopoietic stem and progenitor cells, which are mainly located along endosteal surfaces and may occur in an infrequent number. Therefore, these data beg the question as to whether careful controls have been performed when β -gal positive transgenic cells or animals are used for studying the bone marrow. Careful examination must be

undertaken to prove that the observed β -gal positive cells are in fact not osteoclasts staining “false-positive” due to endogenous β -gal activity. This discrimination can be performed via a variety of methods including analyses of H&E, double-staining with immunohistochemistry for known surface markers, TRAP staining, and, most importantly, a rigorous examination of wild-type controls or littermate control animals. In addition, other reporter approaches such as GFP/CYFP and/or luciferase can be used in conjunction to verify β -gal based results.

The sensitivity of any experimental assay is critically dependent on the amount of background detected. We sought to reduce background staining by altering our signal to noise ratio via several methods. Previous reports of effects of pH and length of exposure to substrate on X-gal staining induced us to attempt to deactivate endogenous osteoclast-associated β -gal activity by altering our protocol in respect to pH and exposure to substrate (Weiss et al., 1999). The lysosomal β -gal enzyme reportedly has activity at an optimal pH of about 4 and senescent cells are active at roughly pH 6. The β -gal enzyme of the lacZ reporter gene works at pH 7 or in slightly alkaline conditions, therefore this discriminator has been widely used in soft tissue studies such that endogenous β -gal activity is not easily misinterpreted as transgene expression or vice versa. Surprisingly however, in our studies in murine bone we found that altering pH to slightly alkaline conditions did not circumvent the problem of strong endogenous staining of β -gal in osteoclasts without compromising the signal beyond the point of detection. In addition, we found that reducing time of exposure to substrate to minimum acceptable times did not reduce the endogenous β -gal staining in bone marrow osteoclasts.

It has been suggested by several groups that use of various inhibitors of endogenous β -gal can be an effective method of decreasing background and increasing signal to noise ratio. In particular, D-galactose has been suggested as an effective differential background suppressor (Hendriks et al., 1994). We did not try this in our study but think it might be an intriguing next set of experiments; although, we suspect that the endogenous β -gal activity in osteoclasts may be robust enough to overcome suppression by this reagent. Another group used heat pretreatment at 50°C to reduce endogenous β -gal activity in cell lines (Young et al., 1993). We did not test this in our study but think it to be worthy of further investigation.

We conclude that the lacZ reporter gene should only be used in the bone marrow when appropriate wild type controls are used and critical examination using histological and immunohistological techniques are employed to ensure that osteoclasts are not mistaken for target cells expressing the lacZ transgene. Rigorous analysis of data is of critical importance in bone marrow related experiments focusing on hematopoietic stem and progenitor cells and should be included in all future reports.

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