Osteoprotegerin-deficient mice develop early onset osteoporosis and arterial calcification

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Osteoprotegerin (OPG) is a secreted protein that inhibits osteoclast formation. In this study the physiological role of OPG is investigated by generating OPG−/− mice. Adolescent and adult OPG−/− mice exhibit a decrease in total bone density characterized by severe trabecular and cortical bone porosity, marked thinning of the parietal bones of the skull, and a high incidence of fractures. These findings demonstrate that OPG is a critical regulator of postnatal bone mass. Unexpectedly, OPG−/− mice also exhibit medial calcification of the aorta and renal arteries, suggesting that regulation of OPG, its signaling pathway, or its ligand(s) may play a role in the long observed association between osteoporosis and vascular calcification.

[Key Words: Osteoprotegerin; osteoporosis; knockout mice; arterial calcification]

Received February 9, 1998, revised version accepted March 19, 1998.

Development and maintenance of the mammalian skeleton involves the regulation and interaction of its component cell types (Erlebacher et al. 1995; Marks 1997). Major contributors to skeletal architecture include chondrocytes, which form cartilage; osteoblasts, which synthesize and deposit bone matrix; and osteoclasts, which resorb bone. Chondrocytes are derived from mesenchymal cells and function to generate an initial cartilage template required for endochondral bone formation. Osteoblasts, derived from mesenchymal osteoprogenitor cells, are located on the surface of bone where they synthesize, transport, and arrange the matrix proteins. Osteoclasts are derived from granulocyte-monocyte precursors present in the hematopoietic marrow (Mundy 1993a; Manolagas and Jilka 1995; Roodman 1996). After establishing a tight adherence to the bone surface, osteoclasts form resorption zones that are acidified by a specialized structure known as the ruffled border. The ruffled border functions as a secretary conduit in which secreted protons and acid proteases decalcify and then digest bone matrix. During the process of osteoclast-mediated resorption, it is thought that protein factors are elaborated that act as signaling molecules to initiate bone renewal by osteoblasts. Osteoblasts, in turn, can influence osteoclast function through the expression of soluble or membrane-bound regulators (Takahashi et al. 1988). The coupling between osteoblast and osteoclast functions is critical for skeletal modeling, remodeling, and repair (Mundy 1993b; Mundy et al. 1995).

Postmenopausal osteoporosis, the most common bone disease in the developed world, has been causally linked to estrogen loss (for review, see Pacini 1996). Postmenopausal bone loss can be attributed to loss of regulatory control exerted by estrogen on the production of cytokines and other factors that regulate osteoclast development. The resultant shift in the balance of osteoclast and osteoblast activity favors a net loss of bone mass, ultimately leading to osteoporosis.

Osteoporosis in human populations has been associated with a higher incidence of arterial calcification, a component of many atherosclerotic lesions (Banks et al. 1994; Parhami and Demer 1997; Parhami et al. 1997). Common factors may underlie the pathogenesis of these two diseases. Some arterial calcium mineral deposits appear identical to fully formed lamellar bone, including trabeculae, lacunae, and islands of marrow (Bunting 1906; Haust and Geer 1970). Furthermore, calcified arteries have been shown to express several bone matrix proteins, including collagen type I, matrix GLA protein, osteocalcin, osteonectin, and bone morphogenetic protein type 2 (Bostrom et al. 1993, 1995; Giachelli et al. 1993; O’Brien et al. 1995). These findings have led to speculation that arterial calcification is an organized, regulated process with cellular and molecular mechanisms similar to bone formation (Demer 1995; Parhami et al. 1996).

Osteoprotegerin (OPG), a recently identified member of the tumor necrosis factor receptor gene superfamily, is a secreted factor that inhibits osteoclast development.
both in vitro and in vivo (Simonet et al. 1997). Transgenic mice overexpressing OPG in the liver have high levels of OPG protein in their systemic circulation and exhibit a marked increase in bone density (osteopetrosis). OPG in normal mouse embryos has been localized within cartilage rudiments of developing bones, as well as in the small intestine and the muscular wall of several major arteries. We show here that OPG is a physiological regulator of normal postnatal bone mass by demonstrating that targeted deletion of OPG in mice results in severe, early-onset osteoporosis. Interestingly, loss of OPG also results in calcification of the aorta and renal arteries, sites of endogenous OPG expression in normal animals. These findings demonstrate that OPG plays an important role in regulating physiological bone formation in the postnatal animal and suggest an additional role for OPG in regulating pathological calcification of arteries.

Results

We have shown previously that systemic OPG administration or overexpression of OPG in mice results in a marked increase of trabecular bone within the long bones and vertebrae, with no detectable effects on the bones of the skull. Severely affected animals lack a clearly defined cortex because of the presence of non-remodelled trabecular bone. Although OPG levels have not been measurable in normal mice, OPG mRNA is detectable predominantly in liver and lung in adult normal mice, suggesting that low circulating levels of this protein may be important in aspects of skeletal metabolism. Therefore, to discern the physiological role and importance of OPG in normal skeletal development and other processes, the endogenous OPG gene was deleted. Data presented here show for the first time that endogenous OPG is not required for embryonic bone formation yet is essential for maintenance of postnatal trabecular and cortical bone mass throughout the skeleton, including the skull. Unexpectedly, loss of OPG results in marked calcification of the aorta and renal arteries.

The strategy for targeted deletion of the mouse OPG gene is outlined in Figure 1. OPG-deficient mice (OPG−/−) are viable and appear histologically normal at birth (data not shown). Analysis of 265 mice generated from OPG+/− hemizygous matings showed that the percentage of +/+ , +/− , and −/− mice was 29%, 53%, and 18%, respectively. The frequency of homozygous knockout mice was close to the expected Mendelian ratio of 25% during the first week of life. This number decreased to ~15% in litters genotyped at 3–4 weeks of age, indicating some mortality in young OPG−/− mice. Interestingly, ~10% of postnatal OPG−/− mice are runted during adolescence and adult life. The decrease in OPG−/− mouse viability observed in adolescence is associated with an increase in the incidence of vertebral or endochondral bone fractures (see below) in the first 2 weeks of life. Despite the presence of brittle bones during early adolescence, OPG−/− mice that survived past weaning age were fertile. Female OPG−/− mice carry litters to term and give birth to OPG−/− offspring that are histologically normal, indicating that endogenous and/or maternal OPG are not required for normal embryonic development. Both male and female OPG−/− mice have survived to 5–6 months of age. Otherwise, the mice appear normal and no hematological or biochemical abnormalities other than elevated alkaline phosphatase levels are observed. In contrast to the splenomegaly observed in OPG transgenic mice (Simonet et al. 1997), the spleens of OPG knockout mice appear normal.

A decrease in bone mineral density is first evident by radiography at 1 month of age in OPG−/− mice. Changes are more pronounced by 2 months of age in both males and females (Fig. 2), and become more severe as the mice

Figure 1. Gene targeting at the OPG locus. (A) Schematic representation of the genomic structure of the murine OPG locus. Restriction sites are indicated as follows: (RI) EcoRI; (X) XmnI; (P) PstI. Exons are indicated as black boxes and numbered by Roman numerals. Introns are shown as thin black lines. (B) The targeting vector used to disrupt the OPG gene. The PGK−neo cassette was placed in reverse orientation within exon 2, replacing the portion of exon 2 encoding the first 93 amino acids of the mature OPG protein. This strategy effectively eliminates the coding region for the first two cysteine-rich domains of OPG that are required for its activity and places a translation stop codon in-frame, preventing translation of any downstream OPG sequence. (C) The structure of a targeted OPG allele following recombination of the targeting vector at the OPG locus. (D) Southern blot analysis of PstI-digested genomic DNA from wild-type (+/+), heterozygous (+/−), and homozygous (−/−) OPG knockout mice. The wild-type allele is a 2.3-kb PstI fragment, and the targeted allele is a 3.0-kb PstI fragment. The small open box in A and C represents the DNA probe used to screen for recombinant ES cell clones. (E) Northern blot analysis of RNA from offspring derived from heterozygous matings. Ten micrograms of total RNA collected from liver was probed for OPG and β-actin expression. The absence of OPG expression in OPG−/− mice confirmed that OPG is a null allele.
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Figure 2. Radiographic analysis of the bones of OPG−/− and wild-type mice. (A,B) Radiographs of 2-month-old female wild-type and OPG−/− mice, respectively. (C,D) Radiographs of the leg, hemipelvis and vertebrae of 2-month-old female wild-type and OPG−/− mice respectively. OPG−/− mice were x-rayed adjacent to wild-type and heterozygous mice using the same x-ray film, to allow for direct comparison of bone density. The strongest phenotype is evident in the vertebrae and long bones. The cortical bone in the femur and pelvis are thinned, and the femoral growth plate is not visible. OPG−/− mice are not different from OPG+/− mice at this time point. (E) Gross anomalies of the skeleton are seen as early as 1 month after birth in the form of multiple fractures (arrows). (F) Radiograph of 6-month-old female wild-type mouse. (G) Radiograph of 5-month-old OPG−/− female mouse. Note severe deformity of the vertebral column due to the collapse of several vertebral bodies (arrow). This was confirmed by subsequent lateral radiographs of the spine.

Age. Gross morphological abnormalities are evident as early as 1 month after birth. Some OPG−/− mice exhibit multiple fractures (Fig. 2). Older mice have marked kyphosis of the spine, sometimes seen with fractures. Another common deformity is flattening of the distal articular region of the femur, indicating partial collapse or compression of the epiphysis. Heterozygous knockout mice exhibit normal radiological appearance.

Histologically, the lumbar vertebrae and metaphyseal regions of the humerus, femurs, and tibias are profoundly osteoporotic in the OPG−/− mice, with almost complete absence of trabeculae by 1–2 months of age (Fig. 3). Loss of cancellous bone and the onset of vertebral osteoporosis are evident within a week after birth and become more severe through adolescent life. Cortical shafts of the long bones showed increased porosity beginning at ∼1 month of age. The porosity is characterized by an increased number of vessels within the cortex. Numerous osteoclasts and osteoblasts are present on the bone surfaces adjacent to these vessels. Polarization microscopy revealed that cortical bone is composed chiefly of woven bone (Fig. 4C,D) and that osteocyte density is higher than normal (Fig. 4I). These histologic features are consistent with a high turnover state, and because woven bone is not as strong as lamellar bone, an increase in fracture incidence would be expected. The trabecular bones of the skull also show marked thinning and increased porosity (data not shown). The proximal epiphysis of the femur and humerus of OPG−/− mice exhibit resorption of subchondral bone and collapse of the joint surface, indicating mechanical damage at the end of the bone (Fig. 3H). Interestingly, OPG mRNA is detectable by in situ hybridization in adult articular chondrocytes (data not shown), suggesting that OPG has an effect on the structural integrity of the joint surfaces by maintenance of subchondral bone mass.

Quantification of bone density in 2- and 6-month-old OPG-deficient mice and their heterozygous and wild-type littermates was performed by peripheral quantitative computed tomography (pQCT) measurements (Beamer et al. 1996) of one tibia and by image analysis of Von Kossa-stained sections of the other tibia (Fig. 4E–G). At 2 months of age, trabecular bone density in the metaphysis and cortical bone density in the diaphysis are significantly reduced in OPG−/− mice relative to OPG+/+ and OPG+/− mice when assessed by pQCT. Trabecular bone density in the OPG−/− mice at 2 month of age is 273.11 ± 44 mg/cm³ versus 448.38 ± 65 mg/cm³ in OPG+/+ and 496.55 ± 58 mg/cm³ in OPG+/− mice, P < 0.005. Cortical bone density in the OPG−/− mice is 549.58 ± 41 mg/cm³ vs. 669.07 ± 43 mg/cm³ in OPG−/− and 676.21 ± 78 mg/cm³ in OPG+/− mice, P < 0.005. Interestingly, heterozygous (OPG+/−) mice exhibit a significant loss of total bone density relative to wild-type littermates by 6 months of age (487.6 ± 27 vs. 521.2 ± 29 mg/cm³, P < 0.05), indicating that loss of a single OPG allele results in significant loss of bone mass over a period of several months. Histomorphometric analysis shows that the trabecular area in the proximal tibia is markedly reduced in OPG-deficient animals, to <5% compared to ∼30% in controls (Fig. 4K). Again, heterozygotes have an intermediate level of trabecular area compared to OPG−/− and OPG+/+ mice, thereby confirming the changes seen using the pQCT method.

Osteoclast and osteoblast numbers were determined in decalcified hematoxylin and eosin (H & E) stained tibia and vertebrae (Fig. 4H,I). Osteoclast surface as a percent of total bone surface is increased in vertebrae and
both the trabecular and cortical regions of the tibiae (Fig. 4H). The highest osteoclast surface is observed in the tibial metaphysis of OPG−/− mice in which the number of osteoclasts per millimeter of tibial metaphyseal trabecular bone perimeter in 2-month-old OPG−/− mice is increased about threefold (4.90 ± 2.8 vs. 1.4 ± 0.6, P < 0.01). Osteoblast-lined bone perimeter as a percentage of total perimeter is also increased in the vertebrae and both the trabecular and cortical regions of the tibiae (Fig. 4I). Proportionally, the largest effect is seen in the tibial diaphysis; however, on the basis of absolute values the vertebrae have the highest percentage of bone surfaced by osteoblasts. Consistent with this increase of osteoblast activity seen histologically, serum alkaline phosphatase levels (which can be a marker of osteoblast activity) are elevated in both male and female OPG−/− mice from 2 weeks onward, likely reflecting the coupling between osteoclast-mediated resorption and osteoblast-mediated bone synthesis. At 2 months of age, OPG−/− mice have significantly elevated serum alkaline phosphatase values of 477.2 ± 200.9 IU/liter (n = 5, P < 0.0001) compared to 106.1 ± 39.6 IU/liter (n = 7) and 112.9 ± 15.7 IU/liter (n = 8) for the wild-type and heterozygous mice, respectively.

We demonstrated previously that OPG inhibits the terminal stages of osteoclast development in vitro and in vivo but does not effect the development of osteoclast precursors (Simonet et al. 1997). The levels of osteoclast

Figure 3. Histological evaluation of bone morphology in 7-day and 2-month-old female OPG−/− vs. OPG+/+ mice. (A) OPG+/+ femur; normal femur morphology. (B) OPG−/− femur; note minimal loss of the primary and secondary spongiosa. (C) OPG+/+ femur, cortical area; normal morphology. (D) OPG−/− femur, cortical area, note somewhat increased bone resorption and remodeling. (E) OPG+/+ vertebral body; normal morphology. (F) OPG−/− vertebral body; note osteoporosis characterized by loss of trabecular bone and thinning of the cortical bone. Magnification, 4× in A and B; 30× in C and D; 20× in E and F; H & E stained. (G) OPG+/+ femur; normal femur morphology. (H) OPG−/− femur; note marked loss of the primary and secondary spongiosa, and collapse of the normally rounded articular surface. (I) OPG+/+ femur, cortical area; normal morphology. (J) OPG−/− femur, cortical area; note severe cortical bone porosity with bone resorption and active remodeling. (K) OPG+/+ vertebral body; normal morphology. (L) OPG−/− vertebral body; note severe osteoporosis characterized by loss of trabecular bone and thinning of the cortical bone. Magnification, 4× in G and H; 15× in I and J; 10× in K and L; H & E stained.
precursors contained in OPG−/− or wild-type bone marrow and spleen cells were found to be similar when measured by an in vitro osteoclast-forming assay (Lacey et al. 1995; data not shown). This finding suggests that the osteoporosis in OPG−/− mice is most likely due to dysregulated osteoclast recruitment and activation in vivo rather than alterations in the number of osteoclast precursors.

Expression of OPG in mice has been localized within embryonic cartilage rudiments, focally adjacent to lymphatics in the small intestinal mucosa, and in large arteries (Fig. 5A, B) (Simonet et al. 1997). The gastrointestinal tract is normal in OPG−/− mice. The large arteries, including the aorta, however, exhibit some calcification as early as 2 weeks after birth and marked calcification by 2 months of age (Fig. 5), when it affected two-thirds of the OPG−/− animals examined (both males and females). The aortic calcification is mainly in the media and is associated with mild to moderate intimal and medial proliferation and partial aortic dissection in 2- to 6-month-old OPG−/−, OPG+/−, and OPG+/+ mice. Note normal morphology in the OPG+/+ mice (E), lower density in the metaphysis of the OPG−/− mice (F), and even further reduced density in the OPG−/− mice (G). Osteoclast surface as a percent of bone surface (OcS%BS) (H) and osteoblast surface as a percent of bone surface (ObS%BS) (I) are increased in the vertebrae, tibial metaphysis (Tibia MP) and the tibial diaphysis (Tibia DP) of the OPG−/− (solid bars) vs. the OPG+/+ (shaded bars) mice. (* Different from OPG+/+, P < 0.005. (J) Osteocyte number per mm² bone area (OtN/BAr) in the tibial diaphysis is increased in OPG−/− vs. the OPG+/+ mice, P < 0.05. (K) Quantitative representation of the trabecular bone density in the metaphyseal region of the tibia expressed as a percent of the total tissue area (BV%TV) in 6-month-old OPG+/+, OPG−/−, and OPG+/− mice. The trabecular bone density was markedly reduced in the proximal tibial metaphysis in the homozygous knockout mice, n = 6, vs. heterozygous mice, n = 9, and wild type mice, n = 7. ( * ) Significantly lower than wild type, P < 0.001.

Discussion

Osteoprotegerin inhibits osteoclast formation when administered in vivo to mice or when added to an in vitro osteoclast-forming assay (Simonet et al. 1997). Ablation
bones by weaning age. Many OPG−/− mice exhibit gross skeletal abnormalities from shortly after birth up to 5–6 months of age. Interestingly, OPG−/− mice also exhibit significant loss of trabecular bone by 6 months, suggesting that modest decreases in OPG expression lead to long-term declines in bone mineral density. It is tempting to speculate that OPG, its signaling pathway, or ligand(s) may be sensitive to estrogen regulation. Imbalances in this pathway may occur during postmenopausal periods associated with bone loss, potentially implicating a role for OPG in the pathogenesis of osteoporosis. The osteoporotic phenotype of OPG-deficient mice supports a potential utility for OPG in treating osteoporosis and other diseases of bone loss.

Compared to other rodent osteoporosis models reported in the literature, OPG−/− mice exhibit rapid postnatal bone loss and more severe bone porosity. Ablation of OPG from the mouse genome is the first targeted mutation to exhibit osteoporosis of this severity. Other engineered osteoporosis models involve overexpression of specific cytokines and growth factors. Ectopic overexpression of IL4 or TGFβ in the bone have been reported to result in osteopenia, both of which are likely mediated by primary effects on osteoblast activity (Lewis et al. 1993; Erlebacher and Derynck 1996). Overexpression of granulocyte colony-stimulating factor (GCSF) in transgenic mice leads to an osteoporosis-like phenotype attributable to an increase in osteoclast activity (Takahashi et al. 1996). Numerous gene mutations [macrophage CSF (MCSF), c-src and c-fos] have been associated with impaired osteoclast function, resulting in osteoporotic phenotypes in mice (Yoshida et al. 1990; Soriano et al. 1991; Wang et al. 1992). These mutations are associated with defects in osteoclast development or activation.

Some systemic and local factors that regulate development and interplay between osteoclasts and osteoblasts have been identified (Mundy 1993a,b; Erlebacher and Derynck 1996; Manolagas and Jilka 1995; Hughes et al. 1996; Pacifici 1996). IL1, IL3, IL4, IL6, and IL11 and several CSFs, including MCSF, have been implicated in the control of osteoclast development (Jilka et al. 1992; Lewis et al. 1993; Girasole et al. 1994). Systemic hormones, such as parathyroid hormone and Vitamin D3, and local factors like tumor necrosis factor, promote the development of osteoclasts through their ability to stimulate production of cytokines like IL6 and IL11 by osteoblasts or their precursors (Kitazawa et al. 1994; De La Mata et al. 1995; Uy et al. 1995). OPG is a secreted regulator of osteoclast-mediated bone resorption, and the results of the current gene knockout study clearly implicate OPG as a physiological regulator of osteoclast-mediated bone resorption postnatal bone growth and remodeling of the skeleton.

OPG−/− mice exhibit an unexpected increase in vascular calcification in the aorta and renal arteries. Most of the OPG−/− mice died or were moribund by 6 months of age due to complications associated with gross skeletal changes. The association of vascular calcification with osteoporosis in patient populations is a recognized phenomenon (Banks et al. 1994; Parhami and Demer 1997;
Materials and methods

Generation of OPG knockout mice

Murine OPG genomic clones were obtained from a 129 SVJ mouse genomic library in Lambda Fix II Vector (Stratagene, La Jolla, CA) by screening with a radiolabeled DNA fragment corresponding to nucleotides 90–1296 of the murine OPG cDNA (GenBank accession no. U94331). A targeting vector was constructed that contained a 1.1-kb short arm of homology and a 5.5-kb long arm of homology flanking a PGK-neo cassette. The PGK-neo cassette replaced a 279-bp region of exon 2 that coded for the first 93 amino acids of the mature OPG protein. A PGK- tk cassette was adjacent to the long arm. The targeting vector was electroporated into RW4 embryonic stem cells (Genome Systems, St. Louis, MO), which were selected in the presence of Gancyclovir and G418, or G418 only. Southern blot analysis using genomic DNA prepared from the 35 isolated cell colonies, identified three recombinant ES cell clones with a single targeted allele. Targeted cells were injected into fertilized blastocysts from C57BL/6 female mice. Chimeric male offspring were crossed with C57BL/6 or Swiss black females. Germ-line transmission of the mutated OPG locus was obtained with chimeras generated from all three targeted ES cell clones. Following heterozygous matings, homozygotes (OPG−/−) were identified and distinguished from heterozygotes (OPG+/−) and wild-type (OPG+/+) mice by Southern blot analysis of genomic DNA cut with PstI and probed with an EcoRI-Xmol OPG-specific probe that was used to confirm homologous recombination.

Histology and bone density measurements

Groups of OPG−/−, OPG+/−, and OPG+/+ mice were necropsied at embryonic day 18, and postnatal day 7, 14, 30, 60, and 180. Radiography was performed prior to the gross dissection on a Faxitron X-ray system (model 43855A, Faxitron X-ray Corp., Buffalo Grove, IL). Blood from the mice was analyzed for clinical chemistries and full hematology. Total body and major organs were weighed and all tissues fixed in formalin. Bone tissue was decalcified using a formic acid solution and embedded in paraffin. Enzyme histochemistry was performed to detect the expression of tartrate-resistant acid phosphatase activity (TRAP) that specifically stains osteoclasts red (Simonet et al. 1997). In situ hybridization using OPG riboprobes was performed as described previously (Simonet et al. 1997). Measurements and counts were done in a 1-mm² area distal to the growth plate in the tibia and vertebrae. The field of measurement started adjacent to the growth plate in the center of the narrow cavity, not including the cartilage or cortical bone. Osteoclast number and osteoblast surface were determined. Osteocyte number was determined in three measurement fields per sample at 20× magnification in the cortex of the tibial diaphysis and related to bone area. All measurements were made by tracing the section image onto a digitizing tablet with the aid of a camera lucida attachment on the microscope and Osteomasure (Osteometrics, Inc.) bone analysis software. Mineral density was determined from bones fixed in 70% ETOH at the proximal tibial metaphysis and tibial cortical shaft of OPG−/−, OPG+/−, and OPG+/+ mice by pQCT (XCT-960M, Norland Medical Systems, Ft. Atkinson, WI). Two 0.5-mm cross sections of bone, 1.5 and 2.0 mm from the proximal end of the tibia, were analyzed (XMICE 5.2, Stratec, Germany) to determine total and trabecular bone mineral density in the metaphysis. A single 0.5-mm cross section of bone, 4.0 mm from the proximal end of the tibia, was analyzed to determine the cortical bone density in the tibial diaphysis. In the metaphyseal measurement, total bone density and trabecular bone density (defined as the innermost 20% of the bone cross section) were determined, and an average value for both cross sections is reported. A soft tissue separation threshold of 1500 was used to define the boundary of the metaphyseal bone, and a separation threshold of 2000 was used to define cortical bone.
Matrix mineralization was determined in frozen sections of nondecalcified bone stained by the Von Kossa method. The mineralized matrix is black, whereas other tissues stain red. Bone density was determined in a region 1.0 x 1.5 mm midway between the cortices adjacent to the edge of the growth plate in the proximal tibia by automated image analysis (Metamorph, Universal Imaging Systems, West Chester, PA).

Acknowledgments

We thank Margaret DeRose for assistance in mapping OPG genomic clones, Marlesse Pisegna for assistance in genotyping, Laura Martin for injection of blastocysts, Kathy Christensen for colony maintenance, Dave Hill for pathology support, and Hosung Min for critical review of the manuscript.

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Genes Dev. 1998, 12:

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