

## Conditional Expression of a Gi-coupled Receptor in Osteoblasts Results in Trabecular Osteopenia

Abbreviated title: Gi-GPCR Signaling in Osteoblasts

J. Peng<sup>1</sup>, M. Bencsik<sup>1</sup>, A. Louie<sup>1</sup>, W. Lu<sup>1</sup>, S. Millard<sup>1</sup>, P. Nguyen<sup>1</sup>, A. Burghardt<sup>2</sup>, S. Majumdar<sup>2</sup>, T.J. Wronski<sup>3</sup>, B. Halloran<sup>1</sup>, B.R. Conklin<sup>4</sup>, R.A. Nissenson<sup>1</sup>

<sup>1</sup>Endocrine Research Unit, Veterans' Affairs Medical Center, and Departments of Medicine and Physiology, University of California, San Francisco, California, USA.

<sup>2</sup>Department of Radiology, University of California, San Francisco, California, USA.

<sup>3</sup>Department of Physiological Sciences, University of Florida, Gainesville, Florida, USA.

<sup>4</sup>Gladstone Institute of Cardiovascular Disease and Department of Medicine, University of California, San Francisco, California, USA.

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Robert A. Nissenson, Ph.D.  
Robert.Nissenson@ucsf.edu  
VA Medical Center (111 N)  
4150 Clement St.  
San Francisco, CA 94121  
Ph: 415-750-2089  
Fax: 415-750-6929

Jeffrey Peng jeffrey.peng@ucsf.edu  
Margaret Bencsik margaret.bencsik@ucsf.edu  
Alyssa Louie lyssabits@gmail.com  
Wei-Dar Lu weidar\_lu@yahoo.com  
Susan Millard susan.millard@ucsf.edu  
Phuong Nguyen phuongvi@stanford.edu  
Andrew Burghardt andrew.burghardt@radiology.ucsf.edu  
Sharmila Mujumdar sharmila.majumdar@radiology.ucsf.edu  
Thomas Wronski wronskit@mail.vetmed.ufl.edu  
Bernard Halloran bernard.halloran@ucsf.edu  
Bruce Conklin bconklin@gladstone.ucsf.edu

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## **Abstract**

G protein-coupled receptors (GPCRs) coupled to activation of Gs, such as the PTH1 receptor (PTH1R), have long been known to regulate skeletal function and homeostasis. However, the role of GPCRs coupled to other G proteins such as Gi is not well established. We used the tet-off system to regulate the expression of an activated Gi-coupled GPCR (“Ro1”) in osteoblasts *in vivo*. Skeletal phenotypes were assessed in mice expressing Ro1 from conception, from late stages of embryogenesis, and post-weaning. Long bones were assessed histologically and by micro-CT. Expression of Ro1 from conception resulted in neonatal lethality that was associated with reduced bone mineralization. Expression of Ro1 starting at late embryogenesis resulted in a severe trabecular bone deficit at 12 weeks of age (> 51% reduction in BV/TV in the proximal tibia compared to sex-matched control littermates; n = 11, p < 0.01). Ro1 expression for 8 weeks beginning at 4 weeks of age resulted in a > 20% reduction in trabecular BV/TV compared to sex-matched control littermates (n = 16, p < 0.01). Bone histomorphometry revealed that Ro1 expression is associated with reduced rates of bone formation and mineral apposition without a significant change in osteoblast or osteoclast surface. Our results indicate that signaling by a Gi-coupled GPCR in osteoblasts leads to osteopenia resulting from a reduction in trabecular bone formation. The severity of the phenotype is related to the timing and duration of Ro1 expression during growth and development. The skeletal phenotype in Ro1 mice bears some similarity to that produced by knockout of Gs- $\alpha$  expression in osteoblasts, and thus may be due at least in part to Gi-mediated inhibition of adenylyl cyclase.

## Introduction

Signaling by G-protein coupled receptors (GPCRs) plays a crucial role in regulating the functional activity of osteoblasts, but the precise actions of specific G protein signaling pathways are incompletely understood. The best studied osteoblast GPCR, the PTH1R, responds to PTH or PTHrP by activating two G proteins: Gs and Gq [1]. In mice, osteoblast-specific knockout of functional Gs-alpha results in neonatal lethality associated with a reduction in trabecular bone formation and reduced endocortical bone resorption [2]. This is congruent with reports that the ability to activate adenylyl cyclase (presumably via activation of Gs) is essential for the anabolic action of intermittent PTH in trabecular bone [3], and for the ability of continuous, high levels of PTH to promote osteoclastic bone resorption [4,5].

There is little available information on the role of osteoblast signaling by G proteins of the Gi class. GPCR-mediated activation of these G proteins is inhibited by pertussis toxin, and this agent is therefore frequently used as a tool to identify Gi-mediated effector responses. Interestingly, the *in vitro* actions of fluoride and strontium to promote proliferation of osteoblastic cells are inhibited by pertussis toxin [6,7]. This suggests that the *in vivo* anabolic actions of these agents could, at least in part, be mediated by stimulation of Gi signaling. Activation of the Gi-coupled apelin receptor also stimulates osteoblast proliferation *in vitro* [8]. Recently, deletion of the gene encoding the Gi-coupled CB2 cannabinoid receptor was shown to lead to high turnover osteoporosis [9]. Consistent with these studies, activation of Gi-coupled GPCRs can signal to anabolic pathways such as MAP kinase in other cellular contexts [10]. However, the classical effect of Gi activation is to inhibit the ability of Gs-coupled receptors to increase adenylyl cyclase activity, and this might be expected to oppose anabolic signaling in osteoblasts [3]. Clearly, *in vivo* studies directly assessing the effects of Gi-coupled GPCR signaling in osteoblasts are needed.

Recently, a new approach to ascribing physiological consequences to specific G protein signaling pathways has been developed. In this approach, GPCRs that are specific to a single G protein pathway are mutated to eliminate the response to naturally occurring agonists while retaining responsiveness to synthetic agonists. These mutated GPCRs are termed “RASSLs” (Receptors Activated Solely by Synthetic Ligands). In principle, targeting expression of the RASSL to a cell type of interest in transgenic mice would allow examination of a specific G protein signal by administration of the synthetic agonist. The prototype RASSL that we used in the present study is a mutated version of the Gi-coupled kappa-opioid receptor that responds to the synthetic agonist spiradoline [11]. This RASSL (termed “Ro1”), has been previously used to assess the role of Gi-coupled GPCR signaling in cardiac myocytes by targeted expression under the control of the D alpha-myosin heavy chain promoter. In those studies, Ro1 signaling was shown to mediate spiradoline-induced bradycardia [12] as well as to produce a constitutive phenotype of lethal cardiomyopathy [13].

In the present study, we have generated mice expressing the tetracycline transactivator, tTA, under control of the mouse 2.3-alpha type 1 collagen promoter, allowing osteoblast selective expression of Ro1 that can be readily suppressed by administration of the tetracycline analog,

doxycycline. This has allowed us to examine the skeletal effects of signaling by a Gi-linked GPCR in osteoblasts.

## **Materials and Methods**

*DNA construction and in vitro verification of transgene expression* – The Col I-2.3-tTA plasmid was constructed by cloning the 2.3 kb mouse alpha1-type I collagen promoter (Col I-2.3) [14] (provided by Dr. Gerard Karsenty) in place of the pCMV promoter upstream of the tTA sequence in the plasmid pUHG 15-1 [15]. Expression and functional doxycycline regulation was verified *in vitro* by co-transfecting ROS 17/2.8 rat osteosarcoma cells with the Col I-2.3-tTA plasmid and the plasmid pUHG 16-3 [12], which encodes a tet-off, tTA-driven, LacZ gene. Two days after transfection, cells were treated with or without doxycycline (2ng/ml) for 48 hours and then assayed for beta-galactosidase activity (Applied Biosystems, Galacto-Light Kit, Catalog #BL300G).

*Mice* – Mice harboring the tetO-Ro1 transgene have been previously described [12]. We have generated the transgenic mouse line that expresses the tetracycline transactivator (tTA) under regulatory control of the mouse 2.3 kb alpha1-type I collagen promoter. A purified Col I-2.3-tTA DNA fragment was prepared and used to microinject FVB/N oocytes. Injections were carried out at the Transgenic Core Facility of the UCSF-affiliated Gladstone Foundation, according to their standard methods. Founders expressing the transgene were identified by PCR from tail genomic DNA. After identification, they were sent to an AALAC-approved Transgenic Mouse facility at the VAMC-SF and backcrossed with wild-type FVB/N mice obtained from Jackson Labs. Mice were maintained on either standard mouse chow or a diet containing 200 mg/kg doxycycline (Bio-Serv catalog #S3888). With the exception of the animals in the “5-day protocol” described below, all animals were maintained on doxycycline diet until 4 weeks of age, at which time they were switched to the standard mouse diet for 8 weeks. All studies were approved by the San Francisco Veterans Affairs Institutional Animal Care and Use Committee.

*“5-day protocol”* – Female tetO-Ro1 mice were maintained on doxycycline for two weeks, and subsequently introduced to male Col I-2.3-tTA(139) mice. Females were then checked daily for the presence of sperm plugs. Five days following the detection of a sperm plug, the females were taken off the doxycycline diet and maintained on regular mouse chow thereafter. Progeny were maintained on regular mouse chow.

*Genotyping* – Mouse tail genomic DNA was purified using REDExtract-N-Amp Tissue PCR Kit (Sigma-Aldrich). The Col I-2.3-tTA transgene was identified using the primers, 5'- CCA GCC ACA CTC CAG TGA-3' and 5'-GTC GTA ATA ATG GCG GCA TA-3', resulting in a 930 bp fragment. The presence of the Ro1 transgene was determined using the primers, 5'-TGA TGA TGA TGT CGA CTC CC-3' and 5' GGC AAT GTA ACG GTC CAC-3', producing a 487 bp fragment. Genomic and plasmid DNA positive controls were included with each PCR reaction. PCR was performed with the following cycling profile: 94°C for 5 min., 16 cycles (94°C for 45 s, 65°C for 1 min., 72°C for 1.5 min.), 15 cycles (94°C for 45 s, 58°C for 1 min., 72 for 1.5 min.), and 72°C for 7 min.

*RNA extraction and qRT-PCR* – Tissue samples were isolated and kept frozen in liquid nitrogen until processing. Frozen tissues were pulverized using a bio-pulverizer (Biospec Products Inc.), followed by RNA extraction using Invitrogen's, Micro-to-Midi Total RNA Purification Kit (Catalog #12183-018). cDNA was synthesized using TaqMan Reverse Transcription Reagents (Applied Biosystems, Part No. N808-0234) and random hexamer primers, according to the recommendations of the manufacturer. SYBR Green (Applied Biosystems, Part No. 4309155) based gene amplification was measured using the ABI Prism 7900HT real-time thermocycler. Analysis was carried out using the SDS 2.0 and 2.1 software supplied with the machine. The sequences of the primer sets are as follows: 18S rRNA, 5' CGGCTACCACATCCAAGGAA 3' and 5' GCTGGAATTACCGCGGCT 3', Ro1, 5' GTGGACCGTTACATTGCCGT 3' and 5' GATGCCAGTAGCCAAATGCA 3'. All reactions were performed in triplicate, and target gene expression was normalized to 18S rRNA.

*Micro-CT* – Left tibiae from 12-week old mice were isolated and cleaned of adherent tissue. Prior to micro-CT analysis, bones were defatted by sequential extraction in ethanol and diethyl ether using a Soxhlet apparatus and dried overnight at 90°C. The tibiae were imaged using a Scanco Medical AG micro-CT apparatus as previously described [16]. Imaging of trabecular bone was carried out at the proximal metaphysis of the tibia. Imaging of cortical bone was carried out at the tibio-fibular junction. Data are presented as mean  $\pm$  SEM. Statistical significance was determined using a paired t-test between double transgenic animals and sex-matched littermate controls.

*$\beta$ -galactosidase staining* – Samples were frozen in liquid nitrogen and stored at -80°C until staining. Samples were then fixed in 2% PFA, 0.2% glutaraldehyde for 2 hours, followed by staining in LacZ staining solution (2mM MgCl<sub>2</sub>, 0.01 Sodium deoxycholate, 0.02% NP40, 5mM EGTA, 5mM K<sub>3</sub>Fe(CN)<sub>6</sub>, 5mM K<sub>4</sub>Fe(CN)<sub>6</sub>, 1mg/ml X-gal in PBS) for 4 hours. Samples were then post-fixed in PFA/glutaraldehyde solution, and embedded in paraffin and sectioned following standard procedure, and counterstained with eosin.

*Skeletal Staining* – Newborn pups were sacrificed and fixed in 99% ethanol overnight after skin and intestines had been removed. Samples were then transferred to acetone for 24 hours, and then stained in a solution of 0.015% Alcian Blue/0.005% Alizarin Red/5% acetic acid in ethanol at 37°C for 3 days. Samples were cleared with 1% KOH and stored in 80% glycerol in ethanol.

*Von Kossa Staining* – Samples were fixed in 4% paraformaldehyde overnight, and then stored in 70% ethanol until they were embedded in paraffin and sectioned under standard procedures. Paraffin embedded sections were deparaffinized and stained with 2% AgNO<sub>3</sub> under UV for 45 minutes. The samples were then treated with 5% sodium thiosulfate for 5 minutes and counterstained in hematoxylin.

*Serum Chemistry* – Blood samples were obtained from mice at the time of euthanasia and serum was isolated from each sample by centrifugation for 15 minutes at 3000 rpm. Serum samples were stored at -20°C. Serum pyridinoline levels were determined using Metra PYD kit (Quidel Corp., San Diego, CA) according to manufacturer's instructions. Serum osteocalcin was measured using BTI's Mouse Osteocalcin EIA Kit (Biomedical Tech. Inc., Stoughton, MA) according to manufacturer's instructions.

*Quantitative bone histomorphometry* – Femurs from 12-week old mice were isolated at sacrifice and fixed in 10% PBF overnight. After fixation, the femurs were dehydrated in graded ethanol and xylene, embedded in methylmethacrylate (MMA). Osteoblast and osteoclast surfaces in the distal femoral metaphysis were measured in Von Kossa/tetrachrome stained sections as previously described [17].

*Dynamic histomorphometry* – Mice were injected intraperitoneally with 20mg/kg calcein 7 days prior to sacrifice and 0.9mg/g xylenol orange 2 days prior to sacrifice. Tibias were isolated and fixed overnight in 4% PFA followed by 30% sucrose perfusion for 3 days. Bone specimens were frozen in OCT embedding medium and sectioned 5  $\mu$ m using a Leica CM 1900 Cryostat (D-69226; Leica, Inc., Nussloch, Germany) with a CryoJane Frozen Sectioning Kit (Instrumedics, Hackensack, NJ, USA) as previously described [18]. Images at 20x magnification were acquired with a Zeiss Axioplan 200 inverted microscope (Carl Zeiss MicroImaging, Inc. Thornwood, NY). Analyses were blinded and performed using an image analysis software (Bioquant Image Analysis Corporation, Nashville, TN) in the region 300  $\mu$ m from the lowest point on the growth plate extending one mm down the metaphysis. Statistical significance was determined using a paired t-test between double transgenic animals and sex-matched littermate controls.

*Bone marrow stromal cell (BMSC) cultures* – A previously described procedure [19] was used to isolate and culture BMSCs from 12 week old double transgenic mice that had been maintained continuously on doxycycline to prevent expression of the Ro1 transgene *in vivo*. Cells were cultured either in the presence of 2 ng/ml doxycycline (sufficient to repress Ro1 expression *in vitro*) or in the absence of doxycycline to allow Ro1 expression. Osteoblast differentiation and function were assessed as described [19]. The presence of doxycycline in the media had no effect on the differentiation or function of wt primary osteoblasts.

## Results

*Generation of Mice with Osteoblast-specific, tetracycline-regulated expression of the Gi coupled RASSL Ro1* – To assess the effects of signaling by a Gi-coupled receptor in osteoblasts, we targeted the Gi -RASSL Ro1 to osteoblasts in transgenic mice. Previously, it was reported that prolonged expression of Ro1 in cardiac myocytes resulted in >90% mortality [13], suggesting that Ro1 exhibits basal signaling in the absence of the synthetic agonist. To circumvent possible developmental effects of Ro1 expression in osteoblasts, we used the tetracycline regulatory system to permit controlled expression of the Ro1 gene. In studies of the cardiac effects of Ro1, the tet-off system allowed tight regulation of gene expression *in vivo* by administration of doxycycline [12,20,21]. Additionally, the tet-off system has been used to control transgene expression in osteoblasts, with no observed adverse effects of chronic administration of doxycycline [22]. We generated four transgenic mouse lines in which the tetracycline transactivator (tTA) is expressed under the mouse 2.3 kb alpha1, type I collagen (Col I-2.3) promoter, which has been demonstrated to be expressed selectively in osteoblasts and osteocytes [14,23].

The transgenic mouse lines expressed the tTA gene at various levels, with the highest expression seen in line “139” and the next highest in line “101” (Figure 1). The lines with the highest tTA expression, “139” and “101,” were then crossed with mice harboring a tTA-responsive Ro1 gene. qPCR analysis of RNA isolated from various tissues from 2 week old Ro1, Col I-2.3-tTA(101) reveal that bone-specific expression of Ro1 was achieved, as well as successful regulation with doxycycline administration (Figure 2). In animals maintained off doxycycline, expression of Ro1 is limited to long bones and calvariae, while Ro1 expression was almost completely suppressed in mice maintained on a diet that contained doxycycline. The tetO-Ro1 mice harbor a co-integrated tetO-LacZ gene, allowing for enzymatic assay of  $\beta$ -galactosidase in double transgenic mice to determine presumed sites of Ro1 expression (Figure 3A). As expected, the Col I-2.3 promoter is active selectively in osteoblasts and osteocytes within the tibia *in vivo*.

*Neonatal lethality* -Initial experiments were carried out without the use of doxycycline, allowing expression of the transgene in double transgenic mice throughout development. The most striking result is the paucity of viable double transgenic mice at weaning in Ro1 x Col I-2.3-tTA crosses maintained without doxycycline (Table 1). Ro1 x Col I-2.3-tTA(139) yielded 0 (of 203 total mice) viable double transgenic mice, whereas Ro1 x Col I-2.3-tTA(101) crosses yielded only 9 (of 127 total mice, 7.1%) surviving double transgenic mice. Surviving double transgenic mice were runted, with body weights approximately 50% that of their sex-matched littermate controls at weaning (data not shown).

We have examined mouse embryos from Ro1 x Col I-2.3-tTA(139) crosses from e11-e14. Normal appearing double transgenic embryos were present at roughly the expected Mendelian ratio, indicating that the lethality occurs later in embryogenesis or perinatally. Examination of newborn mice revealed that Ro1, Col I-2.3-tTA(139) pups appear grossly normal at birth but die within 2 hours due to apparent respiratory failure. Alizarin red/Alcian blue staining of newborn Ro1, Col I-2.3-tTA(139) mice reveals a moderate reduction in mineralized bone, especially evident in the ribs and vertebrae, as well as subtle alterations in the structure of craniofacial bones (Figure 3B). Histological studies of the proximal tibia confirm a reduction in trabecular

bone (Figure 3C). No abnormalities were evident in single transgenic mice harboring only the Col I-2.3-tTA or tetO-Ro1 transgene (data not shown).

*Inducibility of Ro1* -We next assessed the inducibility of Ro1 expression in double transgenic mice that had been maintained on doxycycline from conception to the time of weaning (4 weeks of age). Withdrawal of doxycycline at 4 weeks of age resulted in progressive expression of Ro1 in bone. Ro1 expression was readily detected after 1 week, and was maximal after 2 weeks following withdrawal from doxycycline (Figure 4).

*Skeletal effects of induced expression of Ro1* – Progeny of Ro1 x Col I-2.3-tTA(139) mice were maintained on doxycycline from conception until 4 weeks of age at which time they were switched to a doxycycline-free diet. 8 weeks later, mice were sacrificed, and micro-CT studies were carried out on the tibiae of control and double transgenic mice. As shown in Figure 5A, induced expression of Ro1 for 8 weeks resulted in a >20% reduction in trabecular bone volume (BV/TV) compared to sex-matched littermate controls. This was associated with decreased trabecular connectivity, decreased trabecular number, and increased trabecular separation. None of these changes were evident in double transgenic mice maintained continuously on doxycycline. A small decrease in trabecular thickness was also evident, but this was also seen in double transgenic mice maintained continuously on doxycycline and its significance is therefore uncertain. A small reduction in the thickness of cortical bone in the tibial diaphysis was also seen (Figure 5B). None of these effects was seen in double transgenic mice maintained continuously on doxycycline. The results indicate that 8 weeks of induced expression of Ro1 produces a significant decrease in the amount and quality of trabecular bone.

In order to assess the phenotype of mice in which Ro1 expression was initiated earlier in the course of development, we established an approach to rescue the Ro1, Col I-2.3-tTA mice from neonatal lethality. In this approach, we administered the doxycycline-containing diet to the pregnant mother for the first 5 days of pregnancy and then removed doxycycline from the diet. Due to the time required to clear doxycycline from the system, we would expect this method to result in the suppression of Ro1 for most of gestation. The pups were then maintained on a doxycycline-free diet until the time of sacrifice. Under this protocol (“5-day” protocol), the double transgenic mice were viable although consistently runted, with body weights less than half those of their sex-matched wild-type littermates at weaning, similar to the double transgenic mice of the “101” line (Figure 6). Mice that were allowed to express Ro1 throughout development via the “5-day” protocol showed osteopenia of greater severity than double transgenic mice maintained on doxycycline until weaning, with a 51% reduction in trabecular bone volume. These mice displayed a dramatic decrease in trabecular connectivity and number, and increased trabecular spacing (Figure 5A). Reconstruction of the micro-CT images highlights the striking loss of trabecular number and connectivity in mice expressing the Ro1 gene throughout postnatal life (Figure 7).

Dynamic and static histomorphometric measurements were taken on the bones of double transgenic animals to assess the effects of induced Ro1 expression on parameters of bone function. Dynamic histomorphometric measurements were taken at the metaphysis of the proximal tibiae of double transgenic mice at 8 weeks of age, 4 weeks after withdrawal of doxycycline (Table 2). Both male and female mice expressing Ro1 displayed a significant

reduction in bone formation rate and mineral apposition rate compared to sex-matched littermate controls. Static histomorphometric measurements were taken at the metaphysis of the distal femurs, and revealed no significant differences in osteoblast and osteoclast surfaces between double transgenic mice and sex-matched littermate controls.

Serum markers of bone formation and resorption were also examined in 8 week old double transgenic animals expressing Ro1 (Figure 8). Serum osteocalcin, a marker of bone formation, was significantly reduced in Ro1-expressing animals compared to sex-matched littermate controls. Expression of Ro1 also resulted in a decrease in serum pyridinoline, a marker of bone resorption.

To determine whether the inhibitory effect of Ro1 on osteoblast function was demonstrable outside of the bone microenvironment, we cultured bone marrow stromal cells (BMSCs) from double transgenic mice that had been maintained continuously on doxycycline to prevent the expression of Ro1. BMSCs were cultured for up to 28 days in either the presence or absence of 2 ng/ml doxycycline. The results in Figure 9 demonstrate that cells grown in the absence of doxycycline expressed readily detectable amounts of Ro1 at the mRNA and protein level, whereas little or no Ro1 was detected in cells grown in the presence of doxycycline. Expression of Ro1 in osteoblasts had no effect on the size or number of osteogenic colonies, assessed either by alkaline phosphatase or by Von Kossa staining. A variety of osteoblast marker genes in 21-day cultures were likewise unaffected by expression of Ro1, with the exception of the osteocalcin gene which was modestly suppressed.

## Discussion

The overall goal of the present study was to gain insight into the biological effects of Gi-coupled GPCR signaling in osteoblasts. We targeted expression of the Gi-activating RASSL, Ro1, to osteoblasts using the tet-off system under the control of mouse 2.3kb Col I promoter. qPCR analysis of Ro1 expression demonstrated skeletal expression that was efficiently suppressed by the administration of doxycycline in the diet. Since the tetO-Ro1 mice possess a co-integrated tetO-LacZ gene, staining for beta-galactosidase activity was used to confirm an osteoblast-specific pattern of expression in the bones of the double-transgenic mice. Our results indicate that the tet-off system is very well suited for the control of transgene expression in osteoblasts *in vivo*.

Unregulated expression of Ro1 in osteoblasts during embryogenesis produced deficient bone mineralization and early postnatal lethality. Unregulated expression of Ro1 driven by very high levels of tTA (mouse line 139) always produced lethality, whereas unregulated expression of Ro1 driven by lower levels of tTA (mouse line 101) occasionally resulted in surviving double-transgenic mice presumably due to a lower level of expression of the Ro1 transgene. Double transgenic pups appeared to die of respiratory failure within 2 hours of birth, but the precise cause of postnatal death is uncertain. The mice displayed a generalized reduction in bone mineralization, and this was confirmed by Von Kossa staining of the tibiae. Under-mineralization of the rib cage may have contributed to lethal respiratory failure.

As expected, lethality in Ro1, Col I-2.3-tTA double transgenic pups was rescued by administration of doxycycline to the mother throughout gestation. If the expression of Ro1 was delayed until post-weaning, the double transgenic mice expressing Ro1 for 8 weeks exhibited osteopenia, with a considerable reduction in trabecular bone volume. Micro-CT analysis of tibiae from animals expressing Ro1 in osteoblasts from birth ("5-day" protocol) revealed a similar effect of Ro1 on trabecular bone but the osteopenia was much more severe. Dynamic histomorphometric analysis of Ro1-expressing mice revealed a significant reduction in parameters of trabecular bone formation, suggesting that the reduction in their trabecular bone volume can be attributed to a decrease in bone formation. Lower levels of serum osteocalcin in these mice confirm the finding that Ro1 expression results in a decrease in bone formation. Osteoclast surfaces were not affected by Ro1 expression while bone resorption was decreased as assessed by serum pyridinoline levels. These results demonstrate that the primary cause of trabecular osteopenia produced by Gi-linked GPCR signaling in osteoblasts is decreased bone formation. Interestingly, expression of Ro1 produced little apparent effect on cortical bone. Cortical area was unaffected in mice with induced expression of Ro1, whereas cortical area was reduced in double transgenic mice on the "5-day" protocol presumably due to the generalized growth defect that was present.

We were surprised to find that expression of Ro1 in cultured BMSCs had little or no effect on the expression of osteoblast differentiation markers or the ability of the cells to produce a mineralized matrix. One possible explanation could be a requirement for something in the skeletal milieu *in vivo* to enhance the expression or signaling by Ro1. However, Ro1 appears to be well-expressed in differentiated BMSCs *in vitro*. Moreover, Ro1 is not known to respond to any endogenous ligands, and thus it is not clear how the *in vivo* milieu could enhance signaling

by Ro1. It is conceivable that the functional activity of Ro1 is only manifest in osteoblasts with activated Gs signaling. Such activation might be occurring *in vivo* but not in BMSCs *in vitro*. Another possible explanation is that the effects of Ro1 expression in maturing osteoblasts *in vivo* might be indirect. Differentiated cells in the osteoblast lineage are known to express paracrine factors that can influence the differentiation of early osteoblasts. Two examples are dkk-1 and SOST, both secreted inhibitors of canonical wnt signaling. It is possible that Ro1 signaling promotes the expression of these (or other) anti-osteogenic factors, resulting in suppression of early osteoblast differentiation. It would be difficult to document such an anti-osteogenic effect in culture in our *in vitro* studies since Ro1 expression does not occur until the BMSCs have undergone substantial maturation (allowing the 2.3 kb Col I promoter to become active) at which time few early osteoblasts are present.

The classical effect of Gi signaling is to antagonize adenylyl cyclase activation produced by Gs-coupled GPCRs [24]. Indeed, the skeletal phenotype of Ro1-expressing mice in the present study resembles in some respects the phenotype of mice with osteoblast-specific ablation of expression of the Gs-alpha gene [2]. Thus, mice lacking Gs-alpha in osteoblasts displayed marked osteopenia that was restricted to the trabecular compartment. Cortical thickness was increased, presumably due to the loss of the osteoclastogenic action of Gs/adenylyl cyclase signaling in osteoblasts. In the present study, mice expressing Ro1 also displayed significant loss of trabecular bone. In addition, bone resorption was suppressed as evidenced by reduced serum levels of pyridinoline, although little change in cortical bone was evident. It is possible that effects on cortical bone require a more severe dampening of Gs/adenylyl cyclase than that resulting from Ro1 expression. Indeed, constitutive Gi signaling by Ro1 appears to be weak, as we were unable to detect significant inhibition of Gs-dependent adenylyl cyclase by Ro1 either in BMSCs or in 293 cells transfected with Ro1 (data not shown). This was not surprising, as it is often difficult to document weak constitutive signaling by Gi-coupled receptors. Taken together, the results suggest that the phenotypic effects of Ro1 do not require strong inhibition of the adenylyl cyclase pathway in osteoblasts. Whether signaling pathways other than Gi contribute to the phenotypic effects of Ro1 remains an open and important question.

Previous results have suggested that endogenous Gs/adenylyl cyclase signaling in cells of the osteoblast lineage can serve to promote skeletal anabolism. The upstream components of this putative endogenous anabolic pathway are uncertain but may involve PTHrP-dependent activation of the PTHR. This is supported by the observation that the anabolic effect of intermittent PTH is at least in part due to PTHR-mediated activation of Gs/adenylyl cyclase [25]. Moreover, ablation of PTHrP expression by osteoblasts results in trabecular osteopenia [26]. Finally, constitutively-active PTHR targeted to osteoblasts produce an increase in trabecular bone and a decrease in cortical bone [27], whereas generalized deletion of the PTHR produces neonatal lethality and a reduction in trabecular bone formation [28]. The present results indicate that Gi-GPCR signaling in osteoblasts can produce inhibitory effects on bone formation. The balance between Gs and Gi signaling in cells of the osteoblast lineage appears to be one important determinant of skeletal anabolism *in vivo*.

In summary, the present results demonstrate the utility of RASSL technology for investigating the effects of Gi-coupled GPCR signaling in osteoblasts. The recent development of RASSLs for

Gs and Gq will permit studies on the skeletal effects of additional G protein signaling pathways in osteoblasts [29-31].

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## Figure Legends

**Figure 1: Relative tTA expression in Col I-2.3-tTA founder strains.** RNA was isolated from the long bones of 2-week-old Col I-2.3-tTA mice from each founder strain. The highest levels of tTA expression were observed in lines “139” and “101.” Data were obtained by qPCR analysis, and are normalized to 18S rRNA.

**Figure 2: Tissue specificity of the expression of the Ro1 gene in double transgenic mice.** RNA was isolated from the tissues indicated derived from 2-week-old Ro1,Col I-2.3-tTA(101) mice continuously maintained off doxycycline (LEFT) or on doxycycline (RIGHT). Data were obtained by qPCR analysis, and are normalized to 18S rRNA.

**Figure 3: Skeletal features in Ro1, Col I-2.3-tTA mice.** A) Beta-galactosidase activity in the proximal tibia of a wt and Ro1, Col I-2.3-tTA mouse maintained in the absence of doxycycline. The latter strain harbors a co-integrated tTA-driven LacZ gene for identification of sites of promoter activity. An additional higher magnification image is shown of staining in the Ro1, Col I-2.3-tTA tibia. B) Alizarin red/alcian blue staining of a newborn Ro1,Col I-2.3-tTA(139) mouse (off doxycycline) compared to a wt littermate. Note the reduced skeletal mineralization in the double transgenic mouse, especially evident in the ribs and vertebrae. Newborn double transgenic mice also display altered craniofacial morphology. C) Von Kossa stained section of the distal femur of a newborn Ro1,Col I-2.3-tTA(139) mouse (off doxycycline) compared to that of a wt littermate. Newborn double transgenic mice have reduced trabecular bone mineralization compared to wt. The growth plate appears to be grossly normal.

**Figure 4: Induction of Ro1 gene expression upon withdrawal of doxycycline from the diet of Ro1,Col I-2.3-tTA(139) double transgenic mice.** Expression of Ro1 was assessed in RNA isolated from the long bones of double transgenic mice that were maintained continuously on doxycycline until 4 weeks of age, then switched to doxycycline-free chow for the times indicated. Data are derived from qPCR analysis, and are normalized to 18S rRNA.

**Figure 5: Effect of osteoblast expression of Ro1 on the skeleton, assessed by micro-CT of the tibia.** Three groups of Ro1, Col I-2.3-tTA mice were studied. The first group was maintained continuously on doxycycline (“+ DOXY”, N=16). The second group was maintained on doxycycline until 4 weeks of age, then withdrawn from doxycycline (“INDUCED”, N=16). The third group had osteoblast expression of Ro1 continuously from birth using the “5 day protocol” described in Materials and Methods (“5 DAY”, N=11). All mice were sacrificed at 12 weeks of age and the tibiae harvested for micro-CT analysis. In each case, the results with double transgenic mice are compared with results obtained with sex-matched wt littermates maintained on the same regimen of doxycycline. An asterisk indicates a significant increase or decrease in the parameter relative to the value in the corresponding wt group ( $p < 0.05$ ), two asterisks ( $p < 0.01$ ). A) The proximal tibiae were used for assessment of the following parameters of trabecular bone: bone volume fraction (VOX-BV/TV); connectivity density (Conn-Dens.); trabecular number (DT-Tb.N\*); trabecular spacing (DT-Tb.Sp\*); and trabecular thickness (DT-

Tb.Th\*). B) Parameters of cortical bone were assessed from the tibial diaphysis: cortical thickness (Cort.Th.), and cross sectional area analyses (Cort. Area, Med.Area, Total.Area).

**Figure 6: Effects of continuous Ro1 Expression on body size.** Animals allowed to express Ro1 from birth, “5-day” protocol, were consistently runted with body weights at only half of their wild-type littermates.

**Figure 7: 3D micro-CT renderings of tibiae from control versus Ro1,Col I-2.3tTA(139) mice on the “5 day” protocol.** Representative images were selected from animals whose micro-CT parameters were closest to the mean for their representative group.

**Figure 8: Effects of Ro1 on serum markers of bone formation and resorption.** Serum osteocalcin, a marker for bone formation, was significantly reduced in 8-week old Ro1,Col I-2.3-tTA mice allowed to express Ro1 for 4 weeks (n=12, p<0.05). Serum pyridinoline, a marker for bone resorption, was also reduced in these mice (n=12, p<0.05). Results from double transgenic animals were compared with results from sex-matched wild-type littermates and significance determined using a paired t-test.

**Figure 9: Effects of Ro1 expression on primary bone marrow stromal cell cultures.** Stromal cells harvested from transgenic animals maintained on a doxycycline diet were cultured *in vitro* with and without doxycycline (+/- Doxy). A) Ro1 mRNA and protein expression were examined in 14, 21 and 28 day old cultures. In the absence of doxycycline, Ro1 RNA and FLAG-tagged Ro1 protein expression was evident at day 14 and greatly increased at the latter time points.  $\beta$ -galactosidase protein expression was similarly doxycycline regulatable. qPCR data was normalized to GAPDH and referenced to day 14 expression levels (n=4). A typical Western blot, for which protein loading was confirmed by Ponceau S staining, is shown. B) Cultures were stained for alkaline phosphatase activity at day14 or the extent of mineralization assessed by von Kossa staining at day 21. Quantification confirmed that the absence of doxycycline did not alter the number of stain positive colonies or the total stained area for either alkaline phosphatase (n=4) or von Kossa (n=8). C) The gene expression profile of markers of osteoblast identity and function was determined in 21 day old cultures. The absence of doxycycline resulted in a 25% decrease in the expression of osteocalcin (OC, p<0.001, n=7), without significant changes in expression of alkaline phosphatase (AP, n=7), Collagen TypeI (Col1, n=7), Runx2 (n=4), RANKL (n=4) or osteoprotegerin (OPG, n=4). Data were obtained by qPCR analysis, and normalized to GAPDH prior to calculating marker gene expression in the absence of doxycycline as a percentage of doxycycline treated controls.

## Table Legends

**Table 1:** Yield of double transgenic mice from crosses of Ro1 and two lines of Col I2.3-tTA. Ro1 mice were crossed with Col I-2.3-tTA lines “139” and “101.” The pups and their mothers were either never exposed to doxycycline or maintained continuously on doxycycline from time of conception.

**Table 2:** Bone histomorphometric measurements in Ro1 expressing mice and littermate controls. Ro1 expression was induced by doxycycline withdrawal at 4 weeks of age. Analyses of osteoblast surface (Ob.S/BS) and osteoclast surface (Oc.S/BS) were performed in the distal femur of 12 week old mice, male (n=13) and female (n=14). Measurements of percent mineralizing surface (MS/BS), mineral apposition rate (MAR), and bone formation rate (BFR) were performed on tibiae of 8-week old mice, male (n=4) and female (n=8). Mice were withdrawn from doxycycline at 4 weeks of age. An asterisk indicates a significant increase or decrease in the parameter relative to the value in the corresponding littermate control group ( $p < 0.05$ ).

Table 1

	Ro1 x Col I-2.3-tTA(139)		Ro1 x Col I-2.3-tTA(101)	
	OFF DOXY <i>n=80</i>	ON DOXY <i>n=280</i>	OFF DOXY <i>n=119</i>	ON DOXY <i>n=100</i>
<b>wild-type</b>	40.0%	25.9%	31.9%	24.0%
<b>Ro1</b>	26.3%	24.8%	30.3%	34.0%
<b>Col I-2.3-tTA</b>	33.8%	22.7%	31.1%	14.0%
<b>Ro1, Col I-2.3-tTA</b>	0.0%	26.6%	6.7%	28.0%

Table 2

Mice	Ob.S/BS (%)	Oc.S/BS (%)	MS/BS (%)	MAR (mcm/d)	BFR (mcm/d)
<b>Littermate controls, male</b>	4.60 ± 0.96	0.22 ± 0.04	73.77 ± 3.43	0.5 ± 0.02	36.46 ± 1.44
<b>Ro1, Col I-2.3-tTA, male</b>	4.49 ± 0.71	0.18 ± 0.04	67.08 ± 6.28	0.35 ± 0.04*	23.98 ± 3.83*
<b>Littermate controls, female</b>	18.68 ± 2.15	0.68 ± 0.10	82.65 ± 2.41	0.53 ± 0.04	44.32 ± 4.57
<b>Ro1, Col I-2.3-tTA, female</b>	15.37 ± 1.54	0.89 ± 0.18	75.27 ± 2.47*	0.426 ± 0.04*	31.88 ± 2.47*

Figure 1

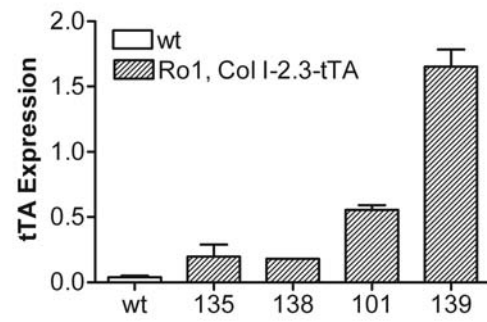


Figure 2

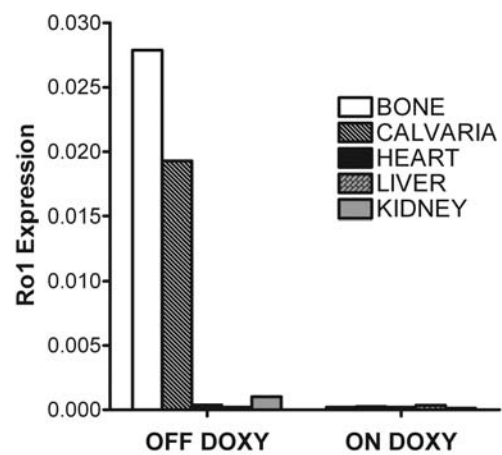


Figure 3

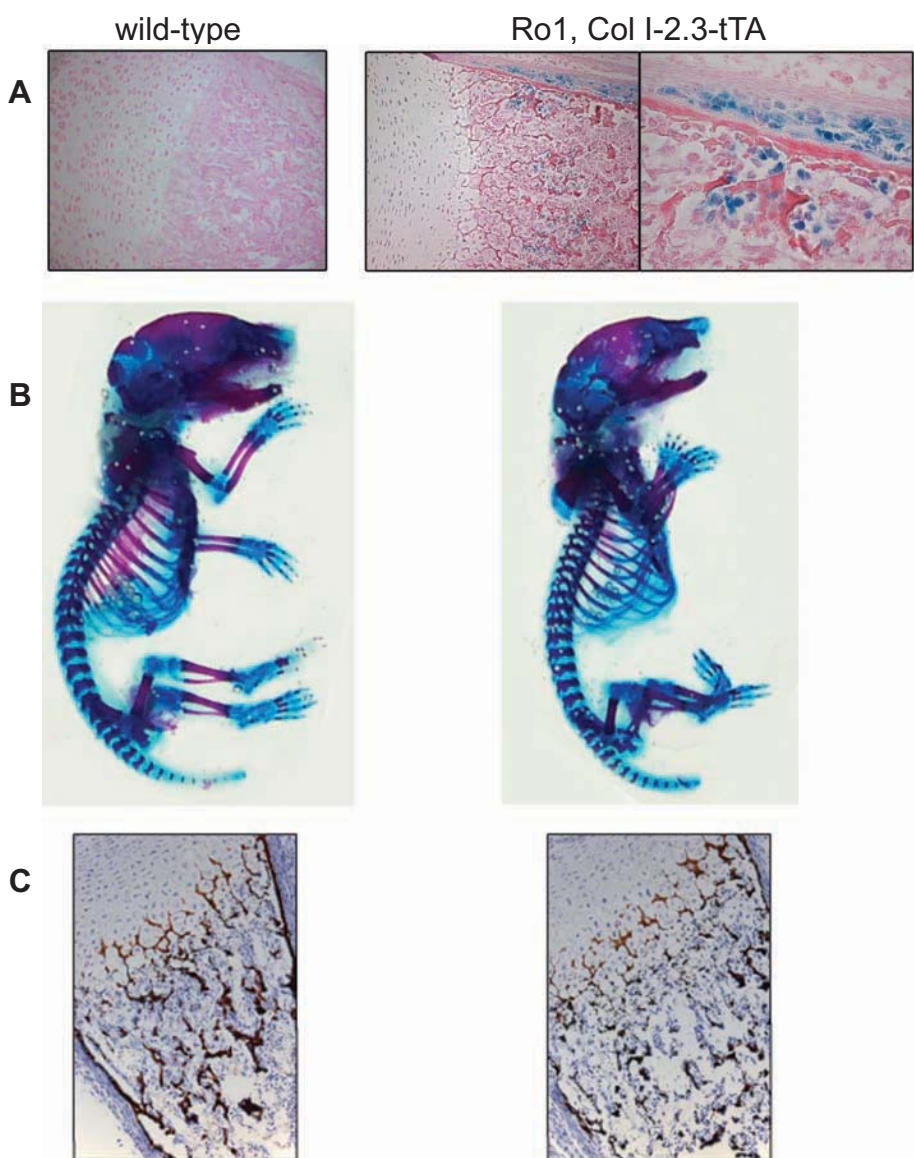


Figure 4

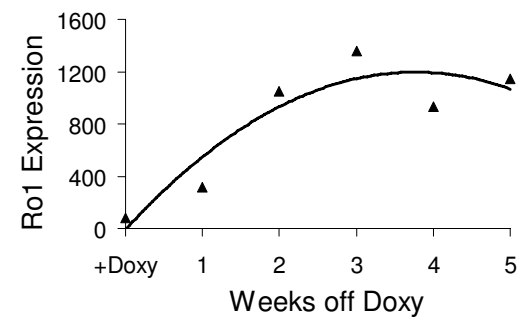
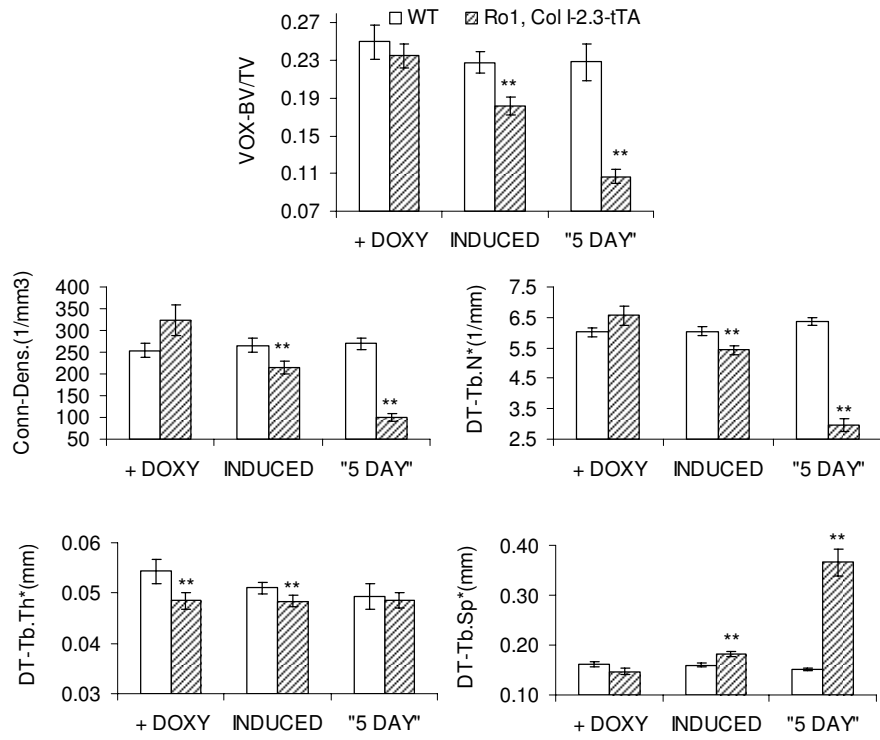


Figure 5

**A**



**B**

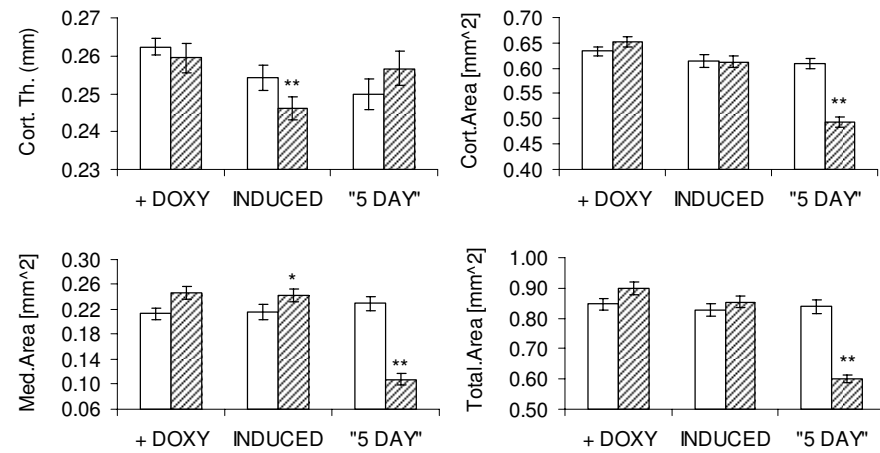
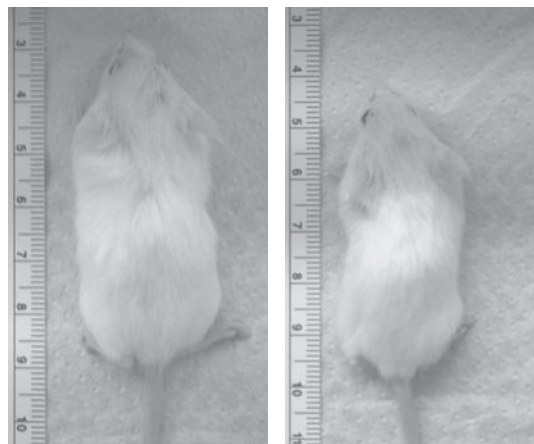


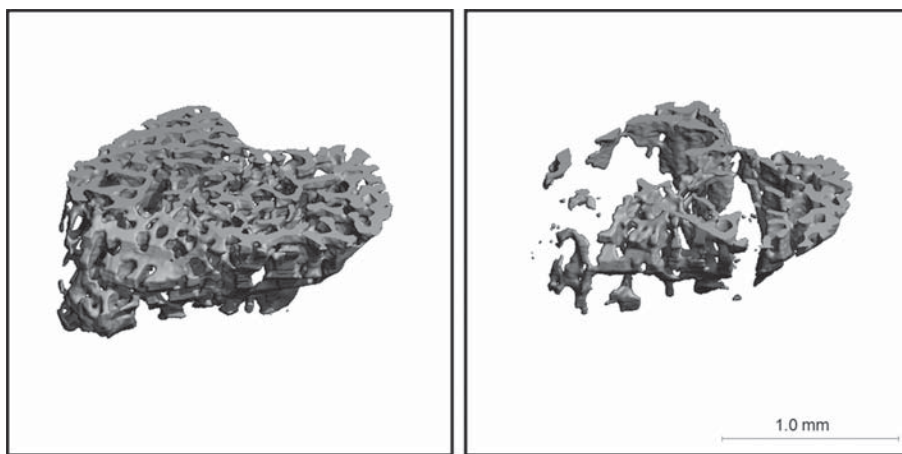
Figure 6



wild-type

Ro1, Col I-2.3-tTA  
"5-day"

Figure 7



wild-type

Ro1, Col I-2.3-tTA  
"5-day"

Figure 8

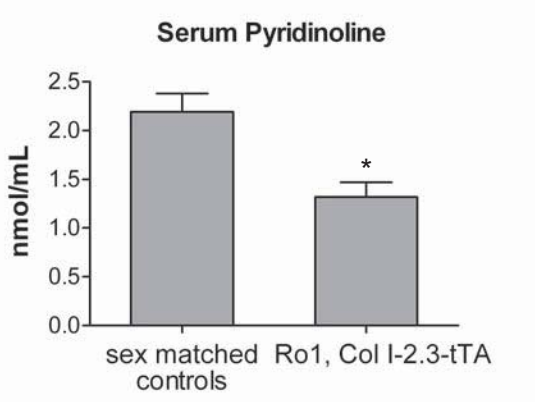
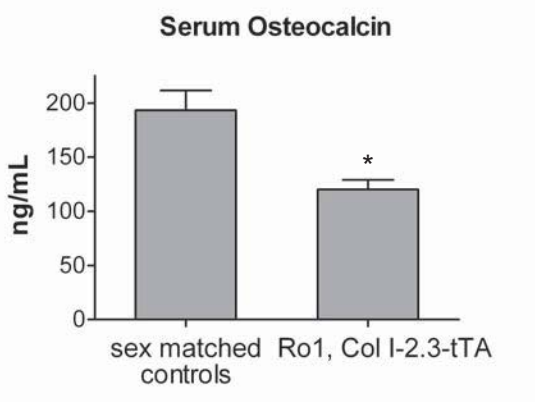


Figure 9

