Greater Bone Formation of Y2 Knockout Mice Is Associated with Increased Osteoprogenitor Numbers and Altered Y1 Receptor Expression

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Germ line or hypothalamus-specific deletion of Y2 receptors in mice results in a doubling of trabecular bone volume. However, the specific mechanism by which deletion of Y2 receptors increases bone mass has not yet been identified. Here we show that cultured adherent bone marrow stromal cells from Y2−/− mice also demonstrate increased mineralization in vitro. Isolation of two populations of progenitor cell types, an immature mesenchymal stem cell population and a more highly differentiated population of progenitor cells, revealed a greater number of the progenitor cells within the bone of Y2−/− mice. Analysis of Y receptor transcripts in cultured stromal cells from wild-type mice revealed high levels of Y1 but not Y2, Y4, Y5, or Y6 receptor mRNA. Interestingly, germ line Y2 receptor deletion causes Y1 receptor down-regulation in stromal cells and bone tissue possibly due to the lack of feedback inhibition of NPY release and subsequent overstimulation of Y1 receptors. Furthermore, deletion of Y1 receptors resulted in increased bone mineral density in mice. Together, these findings indicate that the greater number of mesenchymal progenitors and the altered Y1 receptor expression within bone cells in the absence of Y2 receptors are a likely mechanism for the greater bone mineralization in vivo and in vitro, opening up potential new treatment avenues for osteoporosis.

A role for the neuropeptide Y (NPY)4 receptor system in the regulation of bone formation was first revealed with the demonstration that germ line deletion of Y2 receptors resulted in increased bone formation in the distal femur of mice. This increase in bone formation was due to elevated osteoblast activity and resulted in a 2-fold greater trabecular bone volume and significantly elevated cortical bone mass compared with wild-type mice (1, 2). Interestingly, conditional deletion of Y2 receptors solely from the hypothalamus of adult mice produced a comparable increase in bone volume within just 5 weeks, demonstrating this to be a potent and centrally mediated pathway (1, 2). However, the mechanism by which ablation of either germ line or hypothalamic Y2 receptors stimulates osteoblast activity to increase bone formation is not yet known.

The control of bone remodeling has been traditionally thought to be regulated primarily by endocrine systems and by locally acting factors such as cytokines and growth factors. However, this view is gradually changing with increasing evidence that neuronal factors are also able to modify the activity of bone cells. The presence of nerve fibers and neuronal factors within bone tissue has been demonstrated by a number of studies (3, 4). Furthermore, retrograde tracing studies have demonstrated that neuronal input into bone tissue has connections to the hypothalamus, consistent with the demonstration that conditional Y2 receptor deletion in the hypothalamus is capable of altering bone formation (1, 5).

NPY-immunoreactive fibers have been shown within bone marrow, periosteum, and bone tissue, preferentially associated with vascular elements, with some located around bone lining and marrow cells (3, 6–10). The direct modulation of bone cell activity by various neuronal factors has also been demonstrated in vitro. Peptides such as calcitonin gene-related peptide and substance P, as well as calcitonin gene-related peptide receptors and neurokinin-1 receptors are located on bone cells and have been demonstrated to modify bone cell function (11–15). Other neuropeptides in addition to NPY have also been identified within bone tissue. For example, the presence of vasoactive intestinal peptide receptors has been demonstrated on both these cell types and vasoactive intestinal peptide has been shown to regulate the activity of both osteoblasts and osteoclasts.
The bone-forming osteoblasts originate from mesenchymal stem cells (MSCs), a multipotential cell type that is also able to give rise to adipocytes and chondrocytes (22–24). The isolation and characterization of MSCs has proven to be difficult due to their rarity within bone, lack of knowledge of their localization within bone, and the lack of specific markers for their identification (25). Various methods have been utilized by different groups to isolate a homogenous population of multipotential mesenchymal progenitors from mice. For example, the immune-depletion of hematopoietic cells following plastic adhesion of stromal cells resulted in a population of cells that expressed stem cell antigen-1 (Sca-1), an antigen previously associated with hematopoietic cells, and were subsequently demonstrated to exhibit adipogenic, chondrogenic, and osteogenic potential (26, 27). Selection based on 5-flourouracil resistance and fluorescence-activated cell sorting (FACS) using wheat germ agglutinin and Sca-1 was used for the identification of a candidate osteoprogenitor cell population in mouse bone marrow (28). A method to isolate an immature stromal progenitor cell population from the tissue surrounding bone marrow by depletion of hematopoietic cells followed by cell sorting to isolate Sca-1⁺ cells was recently developed in one of our laboratories. This cell population exhibited properties of mesenchymal stem cells, being highly proliferative with a high capacity to form colony forming units (CFUs) and exhibiting multipotentiality, with the ability to differentiate down osteoblastic, adipogenic, and chondrocytic lineages (25). Furthermore, additional sorting of the Sca-1⁻ population of cells using an antibody recognizing CD51 eliminated CD51⁻ erythroid precursor cell types and yielded a more mature “osteoprogenitor” cell population.

Here we have investigated whether a change in the osteoprogenitor cell population may contribute to the greater bone formation and bone volume of the Y2 receptor-deficient models. We reasoned that a change in the release of factors may affect the number or the ability of bone-forming precursor cells to develop into mature mineral-producing osteoblasts. To test this we evaluated the ability of bone marrow stromal cells from Y2⁻/⁻ mice to produce mineral in vitro. We also assessed the number of mesenchymal and progenitor cells in these mice. Furthermore, we investigated whether Y receptors are expressed in this stromal cell population and whether an alteration in Y receptor expression may also constitute a mechanism by which deletion of Y2 receptors leads to increased bone formation in vivo. The results revealed that down-regulation of local Y1 receptor signaling may provide a mechanism for the greater bone formation of the Y2⁻/⁻ model.

**EXPERIMENTAL PROCEDURES**

Animal experiments were approved by the Garvan/St. Vincent’s Animal Experimentation Ethics Committee and were conducted in accordance with relevant guidelines and regulations.

**Generation of Y1⁻/⁻ and Y2⁻/⁻ Mutant Mice**—Germ line deletion of Y1 and Y2 receptor genes (Npy1r and Npy2r, respectively) was achieved as previously described (29, 30), by crossing Y1 and Y2 receptor-floxed mice (Y1lox/lox and Y2lox/lox) with oocyte-specific Cre-recombinase-expressing C57BL/6J mice (31), resulting in the removal of the entire coding region of the Y1 or Y2 gene.

**Isolation of Bone Marrow Stromal Cells**—To isolate plastic-adherent bone marrow stromal cells, 5- to 9-week-old male wild-type and germ line Y2⁻/⁻ mice were sacrificed by cervical dislocation, and marrow was flushed from femurs and tibias with α-minimal essential medium containing 10% fetal bovine serum, 2 mm-L-glutamine, 2.2g/liter sodium bicarbonate, 0.017 M HEPES, 100 IU/ml, 100 µg/ml penicillin/streptomycin, and 34,000 IU/ml, 34 mg/liter gentamycin (Control media). Cells were plated at a density of 1.9 × 10⁶ cells/cm² in 50-cm² plastic tissue culture plates (BD Biosciences Labware, Franklin Lakes, NJ) and maintained at 37 °C with 5% humidified CO₂. The non-adherent cell population was removed after 72 h by a medium change. Cells were trypsinized 4 days later (0.25% trypsin containing 0.53 mM EDTA) and re-plated at 6.6 × 10⁴ cells/cm² in control media. Cells were changed into differentiation media 2 days later.

**Differentiation of Bone Marrow Stromal Cell Cultures**—Differentiation into adipocytes and mineral-producing osteoblasts was achieved by using adipogenic (control α-minimal essential medium media with 5 µg/ml insulin and 10 nM dexamethasone) or osteogenic media (control α-minimal essential medium media with 50 mg/liter ascobic acid and 10 µM β-glycerol phosphate), respectively.

**Staining of Bone Marrow Stromal Cells for Assessment of Osteoblast and Adipocyte Differentiation**—Cells were fixed in 2% paraformaldehyde for 10 min at room temperature. Osteoblast differentiation and mineralization of extracellular matrix were visualized by von Kossa staining with 2% silver nitrate under UV light for 30 min. The extent of mineralization was assessed using the Leica QWin imaging system (Leica Microsystems, Heerbrugg, Switzerland). Formation of adipocytes was visualized using Oil Red-O (12% Oil Red-O, 60% isoproponol). The extent of adipogenic differentiation was assessed by counting the number of cells containing well stained oil droplets in 10 random low power visual fields using the QWin imaging system.

**RNA Extraction and Reverse Transcription-PCR Analysis of Cultured Bone Marrow Stromal Cells**—RNA was isolated from cultures of bone marrow stromal cells in 12-well plates using TRIzol® reagent as per the manufacturer’s instructions. reverse transcription-PCR was performed with TaqDNA polymerase (Roche Applied Science) using 1 µl of cDNA synthesized from 1 µg of total RNA with oligo(dT)₅₀ by using the SuperScript III First-Strand Synthesis System for reverse transcription-PCR (Invitrogen). The specific primers and annealing temperatures
used along with the resultant product size obtained are as follows: mY1 receptor (323 bp, 55 °C, forward: 5′-CTCGCTGCTTCTCATGGACGG-3′, reverse: 5′-GGGAAATATATATTTGTTAAGTAG-3′), mY2 receptor (520 bp, 60 °C, forward: 5′-TTCCAGATGGTTTTCTCAAGTCTG-3′, reverse: 5′-GGTGCTAGACTCACATTGGAC-3′), mY4 receptor (367 bp, 56 °C, forward: 5′-TCTACAGACAGTGGAGCCGAG-3′, reverse: 5′-GTAGGTTGCTGTCATTTGGAC-3′), mY5 receptor (204 bp, 60 °C, forward: 5′-GGGCTCTATACATTGTTAGTCTCCTTGGGG-3′, reverse: 5′-CATGGGCTTGCCGAACATTCACGTATCC-3′), mY6 receptor (347 bp, 56 °C, forward: 5′-GGAGGAGTTGATATTGTCAG-3′, reverse: 5′-GGTGGTGGCTTCTTTGCACTGCTTG-3′), and mouse glyceraldehyde-3-phosphate (269 bp, 57 °C, forward: 5′-ACTTTGTCAAGCTC-3′, reverse: 5′-GTTGTGGCTTCTTTGCACTGCTTG-3′). RT-PCR reactions were performed for the number of cycles indicated with denaturing at 94 °C and extension at 72 °C.

Quantitative Real-time PCR—Expression of the osteoblast-specific markers alkaline phosphatase and osteocalcin and the adipocyte marker peroxisome proliferator-activated receptor-γ were analyzed by quantitative real-time PCR using the TaqMan Universal PCR master mix (Applied Biosystems, Foster City, CA) and a sequence detection system (ABI Prism 7900 HT Sequence Detection System and Software, Applied Biosystems) with fluorescence-labeled probes (reporter fluorescent dye tyramide, acceptor fluorescent dye VIC at the 5′-end and quencher fluorescent dye tetramethylrhodamine at the 3′-end). The specific primers and probes used are as follow for β-actin (sense: 5′-GGACCTGACGGA-CTACCTCATG-3′, antisense: 5′-TCTTTGTGATGTCACGC-ACGATT-3′, probe: 5′-CCTGACGAGCCGTGGCTACA-GCTTC-3′), osteocalcin (sense: 5′-GGAGGCAATAAGG-TAGTGACA-3′, antisense: 5′-ACAAGCGAGATTGCAAGTGCAAG-3′), and alkaline phosphatase (sense: 5′-GGACTGTG-ACTCGGATAACGAGAT-3′, antisense: 5′-ACATCGTCTG-TGTTCTTCGGGTGAC-3′, probe: 5′-CGGCCACCATGAT-CAGTCGATATC-3′). Primers and probes for peroxisome proliferator-activated receptor-γ were analyzed using an inventory kit from Applied Biosystems. To control variability in amplification due to differences in starting mRNA concentrations, β-actin was used as an internal standard. The relative expression of target mRNA was computed from the target Ct values and the expression of target mRNA was computed from the target Ct values and the geometric mean of two reference genes (GAPDH and RPLP0). The fold change in gene expression was calculated using the formula 2-ΔΔCt, where ΔCt = Ct (target) - Ct (reference) and ΔΔCt = ΔCt (sample) - ΔCt (control).

Isolation of Mesenchymal Stem and Osteoprogenitor Cells—Femurs, tibia, and iliac crests from 8- to 12-week-old male mice were dissected and thoroughly cleaned of muscle and connective tissue, after which the outer surface of the bones was scraped to remove the periosteal surface. Long bones and iliac crests were crushed using a mortar and pestle in washing buffer (2% fetal bovine serum in PBS), then cut up finely in 3 mg/ml Worthington collagenase type 1. Bone fragments were then collagenase-digested for 45 min at 300 rpm at 37 °C. Following digestion, large fragments were removed by filtration (70-μm nylon mesh cell strainer, BD Biosciences). Cells were collected by centrifugation (1000 rpm, 4 min) and resuspended in 2 ml of washing buffer, and the viable cell number was counted using trypan blue.

Contaminating hematopoietic cells were removed by lineage depletion using magnetic activated cell sorting (MACS) microbeads. Cells were incubated with a mixture consisting of the purified antibodies against B220, Gr-1, Mac-1, CD4, CD8, CD3, CD5 (all 1:500 dilution), and TER119 (1:1000 dilution) (BD Pharmingen) diluted in washing buffer for 20 min at 4 °C. Excess antibody was removed by centrifugation, and the cell suspension was then incubated with MACS® goat anti-rat IgG magnetic microbeads (20 μl per 107 cells, Miltenyi Biotech, Bergisch- Gladbach, Germany) diluted in washing buffer for 15 min at 4 °C. The cell suspension containing the bound magnetic beads was then applied to a VarioMACS® separator with an LS MACS® Cell Separation Column (Miltenyi Biotech). Eluted negatively selected cells devoid of hematopoietic cells were collected, centrifuged at 1200 rpm for 4 min, and resuspended in 500 μl of washing buffer, and viable cells were counted using trypan blue.
To prepare for cell sorting for selection of MSCs and progenitor cells, cells (~3 x 10^6) were incubated for 20 min at 4 °C with 2.5 μl of each of the biotinylated or fluorescein isothiocyanate- or phycoerythrin-conjugated antibodies against the surface markers Sca-1, CD45 (BD Pharmingen), CD31, and CD51 (BD Biosciences). Appropriate controls (no antibody, isotype control using nonspecific IgG, fluorescein isothiocyanate, phycoerythrin, biotinylated, and all antibodies) were also set up by incubation of 0.5 μl of antibody with ~20,000 cells for each control group. The secondary fluorescein allophycocyanin-conjugated streptavidin (1:500 dilution, BD Biosciences) was then added to all tubes containing the biotinylated-CD51 antibody (CD51 control tube, all antibodies, and experimental samples), incubated for a further 15 min at 4 °C. 7-Amino-actinomycin D (1:300 dilution, BD Biosciences) was used to detect non-viable cells. Cells were then sorted using a FACS Vantage.

RESULTS

Greater Osteoprogenitor Number of Y2 Knockout Mice

Greater Mineralization and Adipocyte Formation in Cultured Stromal Cells from Germ Line Y2−/− Mice—To examine whether bone cells from germ line Y2−/− mice exhibit differences in proliferation and mineralization in vitro, we isolated bone marrow stromal cells from wild-type and germ line Y2−/− mice and cultured them under osteogenic and adipogenic conditions to induce differentiation down the osteogenic and adipogenic lineages, respectively. Comparable numbers of cells were isolated from flushed bone marrow from wild-type and Y2−/− mice (wild type, between 7.5 x 10^5 and 1.7 x 10^6 cells versus Y2−/−, between 8 and 9.8 x 10^5 cells obtained from four bones per mouse) and were then plated at equal density. Viable cell numbers as assessed by trypan blue staining did not differ significantly between mutant and wild-type cultures in non-differentiating control conditions over a week in culture (data not shown), suggesting stromal cells from the two genotypes did not differ in their rate of proliferation.

A time-dependent increase in the extent of mineralization was revealed in von Kossa-stained osteogenic cultures, with a more marked accumulation of mineral in cultures from Y2−/− mice compared with wild-type (Fig. 1A). These observations were confirmed by measurements of total mineral area by image analysis, with significantly greater mineralization measured in Y2−/− cultures at days 18 and 21 (Fig. 1B). The pattern of mineralization also appeared to be more evenly distributed in the Y2−/− cultures compared with wild-type cultures, in which mineral appeared to be more centrally localized (Fig. 1A), suggesting the presence of more mineral-producing osteoblastic cells throughout the Y2−/− cultures. Increases in expression of the osteoblast marker genes alkaline phosphatase and osteocalcin at days 3 and 9 in Y2−/− cultures supported these observations (Fig. 1C). Interestingly, under control culture conditions with no differentiation-inducing supplements in the media, von Kossa-positive mineral was observed in some wells from Y2−/− but not wild-type mice (data not shown). These findings together suggest an increased capacity of osteoblastic cells from Y2−/− mice to produce mineralized extracellular matrix in vitro.

Cells cultured under adipogenic conditions also showed elevated expression of peroxisome proliferator-activated receptor, a key regulator of adipocyte differentiation, in day 3 and day 9 Y2−/− cultures (Fig. 1C), as well as a time-dependent increase in adipocyte differentiation, evidenced by Oil Red O staining, with a significantly greater number of adipocytes on days 18 and 21 of culture in cells from Y2−/− compared with wild-type mice (Fig. 1, D and E). Thus, cultures of bone marrow cells from Y2−/− mice exhibited an enhanced capability to undergo both osteoblast and adipocyte differentiation under appropriate cell culture conditions.

Because proliferation rates of cultures from the two genotypes were not different, the increased osteoblast mineralization and adipocyte number in cultures from Y2−/− mice could be the result of intrinsic differences in the ability of progenitor cells to undergo osteoblast or adipocyte differentiation. Alternatively, because osteoblasts and adipocytes are derived from a common mesenchymal precursor, the differences observed in...
Greater Osteoprogenitor Number of Y2 Knockout Mice

A. **vitro** was increased in 19086 JOURNAL OF BIOLOGICAL CHEMISTRY within the bone of number of the common mesenchymal progenitors present differentiation capability may result from a difference in the populations were isolated from wild-type and Y2 knockout mice following collagenase digestion (wild-type, 4.9 ± 0.38 × 10^6 cells versus Y2-/-, 5.1 ± 0.38 × 10^6 cells total from 10 mice, average cell numbers from three separate experiments). Importantly, although cell numbers decreased markedly following depletion of hematopoietic cells, the cell yields remained similar between the two genotypes (wild-type, 3.4 ± 0.38 × 10^6 cells versus Y2-/-, 3.5 ± 0.38 × 10^6 cells total from 10 mice, average yield in three experiments). After further negative selection of CD45+ and CD31+ hematopoietic cells, the population was sorted based on the stem cell antigen Sca-1. Sca-1+ cells were gated and collected, whereas Sca-1- cells were sorted again and a Sca-1–CD51+ cell population was gated and collected. Previous studies have established that Sca-1+ cells are immature mesenchymal stem cells (25, 28, 33), whereas Sca-1–CD51+ cells represent a more mature mesenchymal progenitor cell type.5

Numbers of Sca-1+ MSCs were comparable between wild-type and Y2-/- mice, but there was a 2-fold greater number of Sca-1–CD51+ progenitor cells in bones from Y2-/- compared with wild-type mice (Fig. 2). These findings suggest that the greater mineralization and adipocyte formation observed in the initial bone marrow stromal cell cultures from Y2-/- mice was likely due to a greater proportion of mature mesenchymal progenitor cells present at the initial plating.

Gene array analysis of the different subpopulations of Sca-1 cells confirmed the up/down-regulation of bone-specific genes further supporting a role of these cells in increased bone formation (supplemental Table S1).

The CFU assay is an indication of the relative abundance of proliferating progenitor cells within an isolated population due to a greater proportion of mature mesenchymal progenitor cells present at the initial plating.

**FIGURE 1.** Stromal cells from Y2-/- mice exhibit greater mineralization and adipocyte differentiation in vitro. A, von Kossa staining of mineral in stromal cell cultures from wild-type and Y2-/- mice. B, quantification of mineral illustrates increased mineralization in Y2-/- stromal cells cultured in osteogenic differentiation media. C, the graphs show -fold difference of the bone and adipocyte marker genes alkaline phosphatase, osteocalcin, and peroxisome proliferator-activated receptor**C** media. D, adipocyte number was increased in Y2-/- stromal cells cultured in adipogenic media. E, Oil Red O staining of cultured stromal cells shows greater adipocyte number in Y2-/- cultures. *, p < 0.05; **, p < 0.01.

5 P. Simmons, unpublished data.
Available for differentiation down the distinct mesenchymal lineages. Cells within the colonies from Sca-1\(^+\) and Sca-1\(^+\)CD51\(^+\) cells were morphologically different, with a more fibroblastic appearance in the Sca-1\(^+\) cells and a more cuboidal morphology in Sca-1\(^+\)CD51\(^+\) cultures, consistent with the proposed role of the latter population as a more mature osteoprogenitor cell type (Fig. 3A). Furthermore, CFU number was significantly greater in Sca-1\(^+\) compared with Sca-1\(^+\)CD51\(^+\) cultures (\(p < 0.001\)), consistent with their proposed role as a more immature cell type. Importantly, however, there was no difference in CFU number or notable difference in the size of the individual colonies generated by either sorted cell type from wild-type and Y2\(^-/-\)/Y2\(^-/-\) mice (Fig. 3B and C), suggesting that the abundance of progenitor cells within these populations was comparable between genotypes.

Y Receptor Expression in Bone Marrow Stromal Cells—To explore the molecular mechanism by which progenitor cell number might be increased in bones from Y2\(^-/-\)/Y2\(^-/-\) mice, the mRNA expression of different Y receptor transcripts in bone marrow stromal cell cultures from wt and Y2\(^-/-\)/Y2\(^-/-\) mice on day 3 and day 9 of culture was investigated. Expression of all Y receptor transcripts was detected in RNA isolated from brain tissue of wild-type mice using reverse transcription-PCR (Fig. 4A, and data not shown), confirming the ability of our primers to detect expression of the different Y receptor subtypes. Expression of Y1 mRNA was abolished in brains of Y1\(^-/-\)/Y1\(^-/-\) knock-out mice (Fig. 4A). Y1 receptor expression was also demonstrated in bone marrow stromal cell cultures from wild-type mice but was all but abolished in cultures from Y2\(^-/-\)/Y2\(^-/-\) mice (Fig. 4, B and C). This pattern was consistently observed in control, osteogenic, and adipogenic culture conditions. Expression of Y2, Y4, Y5, and Y6 receptors was not detected in stromal cells from either genotype at these time points (supplemental Fig. S1 and data not shown).
Y1 receptor expression was also investigated in bone tissue using in situ hybridization. Y1 receptor gene transcripts were detected in osteoblasts on endocortical and trabecular bone surfaces within the distal metaphysis of femurs from wild-type but not Y1−/− mice (Fig. 5, A–D). Y1 receptor expression was also detected in osteoblastic cells of bones from Y2−/− mice but appeared significantly down-regulated relative to expression in wild-type mice (Fig. 5, E and F). These findings together with the above data provide clear evidence for the expression of Y1 receptors by osteoblastic cells within bone tissue and ex vivo.

Greater Bone Density in Y1−/− Mice—The presence of Y1 receptors on osteoblasts within bone tissue and their down-regulation following Y2 receptor deletion suggest a key role for local Y1 receptor signaling in the control of bone formation. To
investigate the effect of altered Y1 receptor signaling on bone mass in vivo, femora and tibia from germ line Y1–/– mice were assessed for changes in BMC and BMD. Consistent with an inhibitory role of Y1 signaling on bone formation, femoral BMD was significantly greater in Y1–/– mice compared with wild type, with a similar trend in femoral BMC (Fig. 6A). Similarly, in the tibiae, both BMC and BMD were significantly elevated in Y1–/– mice compared with wild type (Fig. 6B), demonstrating the ability of altered Y1 receptor signaling to alter bone formation.

**DISCUSSION**

The findings presented here demonstrate that adherent bone marrow stromal cells from Y2–/– mice have an enhanced ability to differentiate down osteoblastic and adipocytic lineages compared with wild-type stromal cells in vitro. Analysis of the population of mesenchymal progenitors within the bones of these mice revealed a 2-fold greater number of mesenchymal progenitor cells in Y2–/– compared with wild-type mice. After plating the immature Sca-1+ MSCs and the more mature Sca-1+CD51+ cells from the wild-type and Y2–/– mice at the same density, however, the number and sizes of colonies formed were similar between the two genotypes, indicating that these cells of the two mouse genotypes were comparable in their colony-forming ability and, therefore, most likely also in their differentiation potential. Together these findings suggest that a change in the size of a multipotential mesenchymal progenitor population may be a contributing mechanism for the greater bone mass of Y2–/– mice.

The greater mineralization seen in Y2–/– stromal cell cultures in vitro is consistent with the greater bone formation and bone volume of the Y2–/– model in vivo. In contrast, the enhanced adipocytic differentiation capacity was initially surprising, because the Y2–/– mice have a lean phenotype, with reduced white adipose mass compared with wild-type animals (34) and a trend for reduced numbers of marrow adipocytes (wild-type, 5.5 ± 1.0 versus Y2–/–, 2.3 ± 1.2 adipocytes per distal femur 5-μm sagittal section, p = 0.09). However, the greater number of mature multipotent progenitors in Y2–/– bones is consistent with an enhanced ability of cultured stromal cells from Y2–/– mice to differentiate down either the osteoblastic or the adipocytic lineage under appropriate culture conditions. Thus, unlike the consistent positive effects of Y2 receptor gene deletion on the osteogenic cell lineage in vitro and in vivo, the greater adipocytic differentiation in Y2–/– cells observed in vitro does not necessarily reflect the in vivo situation.

To date, there is no standard method for the isolation of murine mesenchymal stem or progenitor cells. Different approaches have yielded populations of cells with variability in their proliferative and differentiative capacities (24, 26–28, 33, 35–37). In the present study, a purified hematopoietic-depleted cell population was further divided into immature MSC and mature progenitor cells based on the presence or absence of Sca-1. Sca-1 is a cell surface glycoprotein commonly used as a marker for the isolation of hematopoietic stem cells from mouse bone marrow. Although the physiological role of Sca-1 in the hematopoietic lineage is incompletely understood, its presence appears to be required for normal hematopoietic stem cell activity and lineage fate, and possibly also for the appropriate self-renewal and homing ability of hematopoietic stem cells (38, 39). Importantly, however, Sca-1 is also expressed on non-hematopoietic bone marrow stromal cells, and mice lacking Sca-1 develop late-onset osteoporosis due to a deficiency in osteoprogenitor cells (40). Therefore, Sca-1 expression also appears to be required for the appropriate self-renewal of mesenchymal progenitors and identifies a population of immature mesenchymal cells with the ability to undergo osteoblast differentiation. The Sca-1+ cell population isolated from bone following depletion of hematopoietic cells, which represent an immature multipotent cell type, did not differ between Y2–/– mice and wild-type mice. The number of Sca-1–CD51+ mature progenitor cells, however, was 2-fold greater in bones from Y2–/– mice relative to wild type. The greater number of these more mature progenitor cells provides a likely mechanism by which mineralization and adipogenesis were increased in stromal cell cultures from Y2–/– mice compared with wild-type. These findings support the likelihood that an increase in the progenitor population may contribute to the increased bone formation and volume of Y2–/– mice in vivo.

Previous studies have demonstrated the presence of NPY-immunoreactive fibers in periosteum and bone marrow and associated with bone lining cells in vivo (3, 41). Also, administration of NPY inhibited the cAMP response to parathyroid hormone and norepinephrine in some osteoblastic cell lines in vitro (4, 42), suggesting the presence of functional NPY receptors on bone cells and a possible role for NPY in the regulation
osteoblast activity by modulation of connection to osteoblasts via sympathetic neurons, which alter ways (44). In the receptors or overlapping functions in different signaling pathways, indicative of cross-regulation between different Y brain regions has been observed in germ line Y receptor knock-in bone, with downstream consequences on osteoblast activity. or indirectly leads to a change in Y1 receptor mRNA expression between the Y2 and leptin pathways is still an open question. two pathways appear to have separate mechanisms, interaction and prevention of bone loss. For a much more detailed characterization of the role of Y1 receptors and their interaction with Y2 receptors in the control of bone formation, the reader is referred to the accompanying report (46).

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Greater Osteoprogenitor Number of Y2 Knockout Mice

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Supplementary table 1: Transcriptome profile analysis.

Total cellular RNA was prepared from 10x10^3 freshly isolated cells of each cell population obtained from 10 mice of each genotype, using RNeasy mini Columns (Qiagen) according to manufacturer’s instructions. RNA was then amplified and labeled using the Two-cycle Target Labeling and Control Reagents (Affymetrix, Santa Clara CA) according to the manufacturer’s instructions. 15µg of biotinylated amplified cRNA were hybridized onto Affymetrix GeneChip arrays MOE430 Plus-2.0 (Affymetrix). Arrays were scanned using Gene Array Scanner (Affymetrix). Affymetrix GCOS 0.0 was used for data acquisition and analysis. The comparative analysis of results obtained for the different cell population was performed using the Silicon Genetics GeneSpring GX 7.3.1 software (Agilent Technologies, Palo Alto CA), using combined data from two separate arrays with each array incorporating pooled cells from 10 animals. Affymetrix CEL files were normalized using the GC-RMA method. List of genes differentially expressed by at least two fold between cells isolated from wild type mice and from Y2^-/- mice were generated. The enrichment of the gene list with genes from a particular Gene Ontology (GO) category was calculated using the Gene Ontology Browser and sorted by p-value. GO categories with p-values >0.01 and containing 3 or less genes from the list were discarded.

Supplementary figure 1: RNA extraction and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) analysis of cultured bone marrow stromal cells.

RNA was isolated from cultures of BMSCs in 12-well plates using Trizol® Reagent and RT-PCR was performed as described in Experimental Procedures in the manuscript. The specific primers for amplification of mY1 and mY2 receptor mRNA and annealing temperatures used along with the resultant product size obtained were: mY1 receptor (323bp, 55°C) forward: 5' CTCGCTGGTTCTCATCGTGGAACGG-3', reverse: 5'-GCGAATGTATATCTTGAAGTAG-3', mY2 receptor (520bp, 60°C) forward: 5'-TCCTGGATTCCTCATCTGAG-3', reverse: 5'-GGTCCAGAGCAATGACTGTGTC-3'. PCR reactions were performed for the number of cycles indicated with denaturing at 94°C and extension at 72°C.
Figure legends – Supplementary tables and figures

Supplementary table 1
Lists of genes differentially expressed by at least 2-fold in sorted Sca\(^+\) and Sca\(^-\)CD51\(^+\) cell populations isolated from Y2\(^-\)/- and wild type mice, with potential to affect osteoblast or adipocyte differentiation. The transcriptome of the freshly isolated mesenchymal cells from the compact bone from the Y2\(^-\)/- and the wild type mice was performed using the Affymetrix platform, and the statistically significant gene ontology (GO) categories associated with each list was retrieved. No statistically significant difference was observed in GO categories related to bone metabolism.

Supplementary figure 1
mRNA for the Y2 receptor is not detectable in cultured bone marrow stromal cells isolated from wild type mice. However, Y1, but not Y2 receptor mRNA is expressed in stromal cell cultures from wild type mice at day 3 and to a lesser extend at day 9 of culture under control conditions. Neither Y1 nor Y2 receptor mRNA was detectable in stromal cell cultures from Y2\(^-\)/- mice. Number of PCR cycles and product size are indicated on figure. Data shows results for PCR performed with (+) and without (-) reverse transcription.
2-fold enriched in Sca-CD51+ Y2-/− cells relative to wild type
fold change  gene
17.4 & 21.1  leptin receptor
14.5  lipase, hepatic
14.5  estrogen receptor 1 (alpha)
13.8  adipocyte complement related protein (adipoq/adiponectin)
12.3  BMP-binding endothelial regulator
10.3  procollagen, type XIV, alpha 1
  8.5  lipoprotein lipase
  5.7  insulin-like growth factor binding protein 4
  5.4  interleukin 6
  4.7  insulin-like growth factor binding protein 4
  4.5  CCAAT/enhancer binding protein (C/EBP), alpha
  4.5  dentin matrix protein 1
  4.3  apolipoprotein E
  4.1  catenin beta
  3.3  bone gamma carboxyglutamate protein 1
  2.4  procollagen, type IX, alpha 1
  2.2  procollagen, type IX, alpha 2
  2.2  insulin-like growth factor binding protein 3
  2.1  fibroblast growth factor receptor 1

2-fold enriched in Sca-CD51+ wild type cells relative to Y2-/−
  3.3  Kuppl-like factor 5
  2.5  procollagen, type XIII, alpha 1
  2.4  matrix metalloproteinase 9

2-fold enriched in Sca+ Y2-/− cells relative to wild type
  3.3  procollagen, type IX, alpha 1
  3.2  osteoclast stimulating factor 1
  3  dishevelled 2, dsh homolog (Drosophila)
  2.8  tumor necrosis factor (ligand) superfamily, member 6

2-fold enriched in Sca+ wild type cells relative to Y2-/−
  10.4  matrix metalloproteinase 13
  5.8  bone morphogenetic protein 6
  5.5  adipose differentiation related protein
  5.5  estrogen receptor 1 (alpha)
  4  catenin beta
  3.7  very low density lipoprotein receptor
  3.6  parathyroid hormone receptor 1
  3.3  interferon gamma receptor
  3.1  bone gamma carboxyglutamate protein 1
  2.8  dickkopf homolog 3 (Xenopus laevis)
  2.8  leptin receptor
  2.7  very low density lipoprotein receptor
  2.7  dickkopf homolog 2 (Xenopus laevis)
  2.6  bone morphogenetic protein 1
  2.6  lipoprotein lipase
  2.5  osteomodulin
  2.4  CCAAT/enhancer binding protein (C/EBP), alpha
  2.3  colony stimulating factor 1 (macrophage)
  2.1  collagen, type XXIV, alpha 1
  2.1  insulin-like growth factor binding protein 4
  2  fibroblast growth factor receptor 1
**wild type mice**

|           | Day 3 | Day 9 |
|-----------|-------|-------|
| GAPDH 269bp | +    | -     |
| Y1 323bp   | 22   |       |
| Y2 520bp   |      | 36    |

**Y2⁻/⁻ mice**

|           | Day 3 | Day 9 |
|-----------|-------|-------|
| GAPDH 269bp | +    | -     |
| Y1 323bp   | 22   |       |
| Y2 520bp   |      | 36    |

Supplementary Figure 1
Greater Bone Formation of Y2 Knockout Mice Is Associated with Increased Oste progenitor Numbers and Altered Y1 Receptor Expression

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