

Critical Role for Y1 Receptors in Mesenchymal Progenitor Cell Differentiation and Osteoblast Activity

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Abstract

The neuropeptide Y (NPY) system has been implicated in the regulation of bone homeostasis and osteoblast activity, but the mechanism behind this is unclear. Here we show that Y1 receptor signaling is directly involved in the differentiation of mesenchymal progenitor cells isolated from bone tissue, as well as the activity of mature osteoblasts. Importantly, the mRNA levels of two key osteogenic transcription factors, *runx2* and *osterix*, as well as the adipogenic transcription factor *PPAR-γ*, were increased in long bones of *Y1*^{-/-} mice compared with wild-type mice. In vitro, bone marrow stromal cells (BMSCs) isolated from *Y1*^{-/-} mice formed a greater number of mineralized nodules under osteogenic conditions and a greater number of adipocytes under adipogenic conditions than controls. In addition, both the number and size of fibroblast colony-forming units formed in vitro by purified osteoprogenitor cells were increased in the absence of the Y1 receptors, suggestive of enhanced proliferation and osteogenesis. Furthermore, the ability of two specific populations of mesenchymal progenitor cells isolated from bone tissue, an immature mesenchymal stem cell population and a more committed osteoprogenitor cell population, to differentiate into osteoblasts and adipocytes in vitro was enhanced in the absence of Y1 receptor signaling. Finally, Y1 receptor deletion also enhanced the mineral-producing ability of mature osteoblasts, as shown by increased in vitro mineralization by BMSCs isolated from osteoblast-specific *Y1*^{-/-} mice. Together these data demonstrate that the NPY system, via the Y1 receptor, directly inhibits the differentiation of mesenchymal progenitor cells as well as the activity of mature osteoblasts, constituting a likely mechanism for the high-bone-mass phenotype evident in *Y1*^{-/-} mice. © 2010 American Society for Bone and Mineral Research

Introduction

Osteoporosis is a global public health problem, and the burden of osteoporotic fractures worldwide outranks several other chronic diseases, including many cancers, rheumatoid arthritis, and hypertensive heart disease.¹ Indeed, osteoporosis affects approximately one in three women and one in five men over the age of 50 years.² Management of osteoporosis traditionally has aimed to either reduce the risk of falls and therefore fractures or to reduce the rate of bone resorption with antiresorptive agents. Lately, focus has shifted toward attempting to increase the rate of bone formation with anabolic agents, thereby increasing bone mass and strength. Recent studies have highlighted the actions of neural signals in the control of bone formation, opening up possible therapeutic avenues for the development of effective anabolic treatments to enhance bone mass in osteoporosis.

It is now clearly established that, acting centrally, the neuropeptide Y (NPY) system, a classic neuronal regulator of energy homeostasis, plays a critical role in the regulation of bone mass through the modulation of osteoblast activity. Of the five known receptors for NPY, the Y1 and Y2 receptors so far have been the only ones shown to influence bone mass in mice.³ Germ-line deletion of Y1 or Y2 receptors produces similar anabolic responses in bone, resulting in a generalized increase in bone mass owing to stimulated osteoblast activity and an increased rate of bone formation.^{4, 5} However, the mechanism behind the action of the Y1 versus the Y2 receptor on bone differs: Conditional deletion of hypothalamic Y2 receptors recapitulates the phenotype of germ-line Y2 receptor knockout (*Y2*^{-/-}) mice,⁵ whereas bone homeostasis is unaltered by hypothalamus-specific deletion of the Y1 receptor.⁴ Y1 receptor expression has been demonstrated in osteoblastic cells lining endocortical and trabecular bone surfaces by in situ hybridization on femur sections and also by RT-PCR on RNA isolated from bone marrow stromal cell (BMSC) cultures⁶ and primary calvarial cultures,⁷ suggesting that the Y1 receptor may mediate effects on bone via direct actions on osteoblasts, whereas Y2 receptors could not be

detected on bone cells using these methods.

Several studies suggest that in addition to effects through central signaling, NPY is indeed capable of directly modulating bone cells. In vitro studies have shown that NPY can inhibit the cyclic adenosine monophosphate (cAMP) response to parathyroid hormone (PTH) and norepinephrine in osteoblastic cell lines,**8, 9** whereas more recently it was demonstrated that NPY can inhibit the formation of osteoclast-like cells induced by the addition of isoprenaline to BMSC cultures.**10** In addition, administration of NPY to BMSC and calvarial cultures isolated from wild-type mice markedly reduced cell numbers**4** and markers of osteoblast differentiation.**7** respectively. Interestingly, the inhibitory effect of NPY on cell number was absent in cells isolated from germ-line Y1 receptor knockout (*Y1*^{-/-}) mice,**4** indicating a direct effect of NPY on bone cells via the Y1 receptor. However, while Y1 signaling has been shown to affect the number of cells in BMSC cultures, it is unclear whether Y1 signaling also plays a direct role in the differentiation and mineral-producing ability of osteoblasts.

In order to investigate the role of Y1 receptors in the proliferation, differentiation, and mineral-producing ability of osteoblasts, we have investigated primary cells in culture isolated from wild-type and *Y1*^{-/-} mice. Bone-forming osteoblasts, along with adipocytes and chondrocytes, are derived from mesenchymal stem cells (MSCs).**11, 12** The study of MSCs has been hindered by their low abundance in vivo combined with a lack of specific cell surface markers to aid in their isolation. Therefore, since the 1970s, the ability of BMSCs to adhere to tissue-culture plastic has been used as a means of easily obtaining a population of cells that, under the appropriate culture conditions, can give rise to osteoblasts,**13** adipocytes,**14** chondrocytes,**15** and myoblasts.**16** Further studies have shown that under appropriate conditions, BMSCs are capable of forming new bone after in vivo transplantation (reviewed in ref. **17**). However, it should be noted that BMSC cultures are heterogeneous and have been shown to include hematopoietic pre-B cells, granulocytic, and monocytic precursors,**18** as well as macrophages and endothelial cells.**19, 20** It has been estimated that only 10% to 20% of the cells in a heterogeneous plastic-adherent marrow cell culture are mesenchymal cells capable of differentiating into osteoblasts and adipocytes.**18**

Several methods now have been developed to purify populations of MSCs from mouse bone marrow. Stem cell antigen 1 (Sca-1), a marker previously described on hematopoietic stem cells, also has been described on mesenchymal osteoprogenitor cells and is directly required for their self-renewal.**21–23** In addition, new evidence suggests that the majority of MSCs are not actually located within the bone marrow but rather within the bone tissue itself or, more specifically, within the vascular system of the bone.**24–26** Therefore, employing fluorescence-activated cell sorting (FACS) for Sca-1 on cells purified from murine bone tissue following a rigorous depletion of hematopoietic cells results in two populations enriched for mesenchymal progenitor cells: highly proliferative Sca-1⁺ MSCs, which have been shown to exhibit adipogenic, chondrogenic, and osteogenic potential, and Sca-1⁻CD51⁺ cells that are more committed toward the osteogenic lineage and hence referred to as *osteoprogenitor cells*.**6, 25, 27** The regulation of MSCs within the bone microenvironment is complex, involving numerous systemic and local factors. Therefore, the use of purified populations of mesenchymal progenitor cells from bone tissue avoids the complicating influence of multiple cell types present in the heterogeneous BMSC cultures.

Our observation that germ-line but not hypothalamus-specific Y1 receptor deletion has significant effects on bone formation,**4** combined with observations that the Y1 receptor is the only Y receptor present in bone cells,**6** led us to hypothesize that the Y1 receptor plays a direct, functional role in the differentiation of osteoblasts. Therefore, the aim of this study was to determine the effect of the lack of Y1 receptors on the proliferation and differentiation of osteoblastic cells in vitro using BMSC cultures as well as rare, but purified, populations of mesenchymal progenitor cells isolated from germ line *Y1*^{-/-} and wild-type mice. In addition, in order to assess whether the Y1 receptor is directly involved in regulating the activity of mature osteoblasts, we examined the mineral-producing ability of BMSC cultures isolated from conditional knockout mice lacking the Y1 receptor specifically from mature osteoblasts.

Materials and Methods

Mice

Germ-line *Y1*^{-/-} mice and NPY knockout (*NPY*^{-/-}) mice were generated as described previously.**28, 29** Mice with osteoblast-specific deletion of the Y1 receptor were developed using mice expressing *Cre* under the control of a 2.3-kb fragment of the rat $\alpha 1(I)$ -collagen promoter

(abbreviated as *2.3ColCre*).**30** By mating the *2.3ColCre* mice with the previously described *Y1lox/lox* mice,**28***Cre*-mediated recombination results in osteoblast-specific deletion of the entire coding region of the *Y1* gene. Male *2.3ColCre;Y1lox/lox* mice were bred with female *Y1lox/lox* mice to generate both *2.3ColCre;Y1lox/lox* mice and *Y1lox/lox* mice to be used as controls. Genotypes were determined by PCR using oligos to detect the *Cre* transgene (forward oligo: 5'-GTTTCACTGGTTATGCGG-3'; reverse oligo: 5'-AAGTGCCTTCTCTACACC-3') and oligos to detect the *Y1lox/lox* genotype (forward oligo: 5'-AGACGTCTCTGAAGCAGGCT-3'; reverse oligo: 5'-GCCAGAGATAGATTACAAAG-3'). PCR was performed with 40 cycles of 94°C for 1 minute, 55°C for 1 minute, and either 72°C for 20 seconds for the *Cre* oligos or 72°C for 45 seconds for the *Y1lox/lox* oligos.

All animal experiments were approved by the Garvan Institute/St Vincent's Hospital Animal Experimentation Ethics Committee and conducted in accordance with relevant guidelines and regulations.

Isolation of BMSCs

Plastic-adherent BMSCs were isolated from 5- to 9-week-old male or female mice as described previously.**6** Briefly, following killing by cervical dislocation, marrow was flushed from femurs and tibias with control medium, and cells were plated at a density of 1.9×10^6 cells/cm² in 50-cm² plastic tissue culture plates. If appropriate, 100 nM NPY (Auspep, Melbourne, VIC, Australia) was added at every medium change from this stage onward. The majority of nonadherent cells was removed by medium changes 3 and 5 days later and discarded. After 7 days in culture, cells were trypsinized, counted, and replated at 3×10^4 cells/cm² in 24-well plates in control medium with 100 nM NPY if appropriate. Cells were changed into differentiation medium 2 days later, and the medium then was changed three times per week until the cells were harvested or stained (see below for details). For proliferation analysis, cells were trypsinized at appropriate time points, and viable cell numbers were determined by trypan blue staining.

Isolation of mesenchymal progenitor cells

MSCs and osteoprogenitor cells were isolated from 12- to 18-week-old male mice as described previously with minor modifications.**6** Briefly, femurs, tibias, and iliac crests were dissected and thoroughly cleaned of muscle and connective tissue, after which the outer surfaces of the bones were scraped to remove the periosteal surface. Bone marrow was removed as the bones were crushed using a mortar and pestle in several changes of washing buffer [2% fetal bovine serum (FBS) in PBS]. The bones then were cut up finely in 3 mg/mL of Worthington collagenase type 1 (Worthington Biochemical Corporation, Freehold, NJ, USA) and incubated for 45 minutes at 300 rpm at 37°C. Following digestion, large fragments were removed by filtration (70- μ m nylon mesh cell strainer, BD Biosciences, San Jose, CA, USA). Cells were pelleted by centrifugation and resuspended in 2 mL of washing buffer, and viable cells were counted using trypan blue.

The remaining hematopoietic cells were removed by lineage depletion using magnetic-activated cell sorting (MACS) with purified antibodies against B220, Gr-1, Mac-1, CD3, and TER119 (Pharmingen, Franklin Lakes, NJ, USA) and MACS goat anti-rat IgG magnetic microbeads (Miltenyi Biotech, Bergisch-Gladbach, Germany). Negative selection was performed through a paramagnetic column (LS column, Miltenyi Biotech) according to the manufacturer's instructions.

Following depletion, MSCs and osteoprogenitor cells were selected by fluorescence-activated cell sorting (FACS). Cells were stained with biotinylated fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated antibodies against the surface markers Sca-1, CD45 (Pharmingen), CD31, and CD51 (BD Biosciences). The secondary fluorescein allophycocyanin (APC)-conjugated streptavidin (BD Biosciences) was used for the biotinylated antibody, and 7AAD (BD Biosciences) was used to detect nonviable cells. Cells were sorted using a FACS Vantage SE (BD Biosciences) with FACSDiva SE software Version 4.1.2 (BD Biosciences) using a nozzle size of 70 μ m at 4°C. The remaining contaminating CD31⁺ and CD45⁺ hematopoietic cells were excluded. The resulting cell population then was sorted based on Sca-1, with the Sca-1⁺ MSCs collected. CD51⁻ erythroid precursors were excluded from the Sca-1⁻ population, and Sca-1⁻CD51⁺ osteoprogenitor cells were collected.

Sorted MSCs and osteoprogenitor cells were plated at a density of 100 cells/cm² and cultured in control medium with the FBS concentration increased to 20% in standard culture

conditions of 37°C and 5% CO₂. Control medium consisted of α -minimum essential medium (α -MEM) containing 10% FBS, 2 mM L-glutamine, 2.2 g/L of sodium bicarbonate, 0.017 M HEPES, 100 μ g/mL of penicillin/streptomycin (all obtained from Invitrogen, Carlsbad, CA, USA) and 34 mg/L of gentamycin (Pfizer, New York, NY, USA).

Formation of colonies by MSCs and osteoprogenitor cells was assessed by evaluation of fibroblast colony-forming units (CFU-Fs) after 10 days in vitro, when individual colonies were easily identifiable. Cells were fixed with 4% paraformaldehyde (ProSciTech, Townsville, QLD, Australia), washed with PBS, then stained with filtered 1% toluidine blue (BDH Chemicals, West Chester, PA, USA) for 15 minutes at room temperature. Positively stained colonies containing more than 5 cells were counted in each experimental group.

The remaining cells were trypsinized to break up colonies and facilitate even growth. Once confluent, cells were passaged once more to increase numbers. After a total of approximately 3 weeks in culture, cells were trypsinized, counted, and replated at a density of 8.8×10^3 cells/cm² in 24-well plates in standard control medium. Cells were changed into differentiation medium 2 days later, and the medium was then changed three times per week until the cells were harvested or stained.

Osteogenic and adipogenic differentiation

Differentiation into mineral-producing osteoblasts was achieved by culturing the cells in osteogenic medium consisting of control medium supplemented with 50 mg/L of ascorbic acid (Sigma, St. Louis, MO, USA), 10 mM β -glycerophosphate (Sigma), and for MSCs and osteoprogenitor cells only, 100 ng/mL of bone morphogenetic protein 2 (BMP-2; Kamiya Biotech, Seattle, WA, USA). Mineralization of extracellular matrix was visualized by von Kossa staining with 2% silver nitrate (Sigma) under ultraviolet (UV) light for 30 minutes. Individual wells were photographed using a Leica dissecting microscope. The extent of mineralization in BMSC cultures was quantified using the Leica QWin imaging system (Leica Micro-Systems, Heerbrugg, Switzerland).

Adipocytic differentiation was achieved by culturing the cells in adipogenic medium consisting of control medium supplemented with 5 μ g/mL of insulin (Novo Nordisk, Bagsvaerd, Denmark) and 10 nM dexamethasone (Sigma). For the initial 3 days of adipocytic differentiation of BMSC cultures only, the adipogenic medium also was supplemented with 500 μ M isobutylmethylxanthine (IBMX; Sigma) and 100 μ M indomethacin (Sigma). To visualize adipocytes, cells were stained with oil red-O (12% oil red-O, 60% isopropanol; Sigma). Staining was photographed using a Leica dissecting microscope (Leica Micro-Systems). For MSCs and osteoprogenitor cells, oil red-O dye then was extracted with isopropanol, and its absorbance was measured at 510 nm. Absorbance per well was normalized to total cell number per well, as determined by cell counting following hematoxylin staining. For BMSCs, adipocyte formation was quantified by flow cytometry using Nile red (Sigma). After 7 days in adipocytic differentiation medium, cells were trypsinized, washed in PBS once, and fixed in 4% paraformaldehyde for 30 minutes at 4°C. The cells then were washed once and stained with 10 μ g/mL of Nile red for 45 minutes at 4°C. Nile red fluorescent emission then was measured between 565 and 604 nm using a FACS Vantage SE (BD Biosciences) with FACSDiva SE software Version 4.1.2 (BD Biosciences). BMSCs grown in control medium were used as a negative control.

RT-PCR

RNA extractions were carried out on MSCs and osteoprogenitor cells cultured in control medium at passage III using TRIzol reagent (Sigma) according to the manufacturer's instructions. RNA was quantified by spectrophotometry and cDNA synthesized from 1 μ g of total RNA with oligo(dT)₂₀ and random hexamers by using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). RT-PCR then was performed with TaqDNA (Roche Applied Science, Penzberg, Germany) using 1 μ l of cDNA per 25 μ l total volume. PCR reactions were performed for the number of cycles indicated with denaturing at 94°C and extension at 72°C. Mouse glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as a housekeeping gene to control for variations between samples. The specific primers and annealing temperatures used along with the resulting product sizes obtained are as detailed by Lundberg and colleagues⁶ and as follows for NPY: 160 bp, 58°C, forward: 5'-CAGAGGCACCCAGAGCAGACACC-3', and reverse: 5'-GGATTGTCCGGCTTGG AGGGGTAC-3'.

Quantitative real-time PCR

RNA was isolated from BMSCs after 6 to 7 days in control or osteogenic medium and from femurs and tibias isolated from 7- to 9-week-old male mice snap frozen in liquid nitrogen and homogenized in 2 mL of TRIzol reagent (Invitrogen, Carlsbad, CA, USA) using a Polytron homogenizer (Omni International, Kennesaw, GA, USA). All RNA extractions were performed by using TRIzol reagent according to the manufacturer's instructions. RNA samples were checked subsequently for consistent quality and quantified using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's instructions. Then 1 μ g of total RNA was taken for cDNA synthesis with oligo(dT)20 and random hexamers using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). Quantitative real-time PCR then was carried out using the TaqMan Universal PCR master mix, the ABI Prism 7900 HT Sequence Detection System, and inventoried kits containing primers and probes (all from Applied Biosystems). To control for variability in amplification owing to differences in starting mRNA concentrations, *GAPDH* was used as an internal standard. The relative expression of target mRNA was computed from the target *Ct* values and the *GAPDH* *Ct* value using the standard curve method (Applied Biosystems).

Statistical analysis

All data are expressed as means \pm SEM. Differences among groups of mice were assessed by ANOVA or repeated-measures ANOVA, followed by Fisher's post hoc comparisons if appropriate (StatView Version 4.5, Abacus Concepts, Inc., Berkeley, CA, USA). Statistical significance was defined as $p < .05$.

Results

Upregulation of key osteogenic transcription factors in long bones from *Y1*^{-/-} mice

To investigate whether altered osteoblastic differentiation may contribute to the high-bone-mass phenotype of the *Y1*^{-/-} mouse,⁴ we used quantitative real-time PCR to measure alterations in markers of osteoblastic differentiation in bones from *Y1*^{-/-} and wild-type mice. First, we measured the expression of alkaline phosphatase (ALP), a classic marker of osteoblast activity and thus bone formation. As shown in Fig. 1, *Y1*^{-/-} mice display a trend toward an increase in the expression of ALP compared with wild-type mice ($p = .06$), consistent with their high-bone-mass phenotype.⁴ More important, we investigated the expression of two key transcription factors, *runx2* and *osterix*, both of which are essential for osteogenesis, as demonstrated by a lack of bone formation in either *runx2*- or *osterix*-deficient mice.^{31, 32} The mRNA expression levels of both *runx2* and *osterix* were increased significantly in *Y1*^{-/-} mice (Fig. 1), demonstrating that osteogenesis is upregulated in vivo in the absence of Y1 signaling.

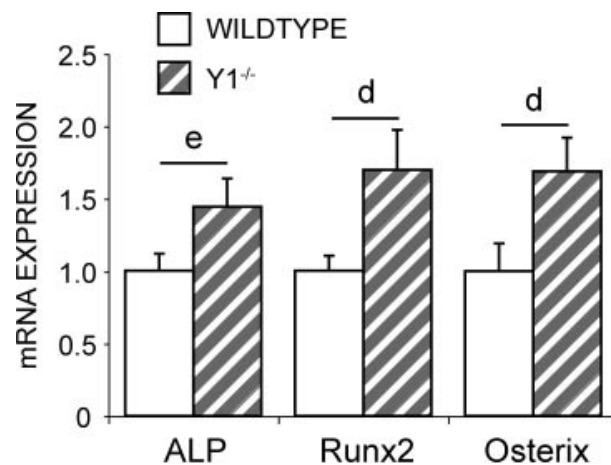


Fig. 1. Alkaline phosphatase (ALP), *runx2*, and *osterix* are upregulated in *Y1*^{-/-} bones. Quantitative real-time PCR analysis shows an increase in mRNA levels of *ALP*, *runx2*, and *osterix* in the long bones of 7- to 9-week-old male *Y1*^{-/-} mice compared with those of wild-type mice. Values shown are normalized to *GAPDH* levels and relative to wild-type expression. Data are means \pm SEM of 5 to 6 mice per genotype. ^d $p = .05$; ^e $p = .06$ as indicated.

Lack of the Y1 receptor alters in vitro mineralization by BMSCs

In order to further investigate the effect of Y1 receptor deficiency on osteogenic differentiation, we assessed the ability of BMSCs isolated from *Y1*^{-/-} mice to differentiate into mature, mineral-producing osteoblasts in vitro. Importantly, there was no difference with respect to cell number over the culture period between BMSCs isolated from wild-type or *Y1*^{-/-} mice (comparison between solid lines in Fig. 2A, B). However, the addition of NPY transiently reduced the number of BMSCs in cultures isolated from wild-type mice over this period (Fig. 2A) but had no effect on the number of BMSCs in cultures isolated from *Y1*^{-/-} mice over the same time period (Fig. 2B). Interestingly, the reduction in cell number caused by the addition of NPY was greater in BMSC cultures isolated from *NPY*^{-/-} mice (Fig. 2C, E), which also show an upregulation in Y1 receptor expression (Fig. 2D). No difference was observed in cell number over time between the BMSCs isolated from wild-type, *Y1*^{-/-}, or *NPY*^{-/-} mice without the addition of NPY (comparison between solid lines in Fig. 2A-C), suggesting that high levels of NPY may be required to have a noticeable effect on cell number in this culture system. However, these data show that the effect of NPY on BMSC number is mediated via the Y1 receptor, consistent with a direct role for the Y1 receptor in these cells.

We next investigated in vitro mineralization of BMSCs isolated from male *Y1*^{-/-} and wild-type mice under osteogenic conditions. Interestingly, despite a small but significant increase in mineralization on day 14, BMSCs from *Y1*^{-/-} mice produced significantly less mineral by days 25 and 28 of culture than BMSCs from wild-type mice (Fig. 3A). The increased mineral apposition rate (MAR) observed in germ-line *Y1*^{-/-} mice in vivo has been shown to be dependent on intact gonadal signaling in male but not female mice.³³ Therefore, in order to determine whether the decreased mineralization observed in BMSCs from male *Y1*^{-/-} mice was due to a similar gender-specific requirement for gonadal steroids or an in vitro-dependent effect in both sexes, we isolated BMSCs from female wild-type and *Y1*^{-/-} mice and investigated their mineralization in vitro. Consistent with the results obtained using male mice (Fig. 3A), BMSCs isolated from female *Y1*^{-/-} mice also formed significantly less mineral over time than their wild-type counterparts (data not shown), thereby indicating an in vitro-dependent effect.

Despite lower total mineralization, the pattern of mineralization in BMSC cultures isolated from *Y1*^{-/-} mice was more evenly distributed throughout the well than the centrally localized distribution seen in cultures from wild-type mice (Fig. 3B) and is similar to that previously demonstrated in BMSC cultures isolated from germ-line *Y2*^{-/-} mice.⁶ This observation was confirmed by a significant increase in the number of von Kossa-stained mineralized nodules per well in BMSC cultures from *Y1*^{-/-} mice compared with those from wild-type mice (Wild type 10.42 ± 1.45 nodules per well and *Y1*^{-/-} 16.65 ± 1.28 nodules per well on day 28, $p < .01$; means \pm SEM of 10 to 15 wells per genotype over three experiments). This finding suggests that BMSC cultures isolated from *Y1*^{-/-} mice contain a greater number of mineral-producing osteoblastic cells than BMSC cultures from wild-type mice.

Lack of the Y1 receptor increases in vitro adipocyte formation by BMSCs

In addition to osteoblasts, the mesenchymal progenitor cells present in BMSC cultures are capable of differentiating into several other cell types, including adipocytes.¹⁴ Y1³⁴ and Y2³⁵ receptors expressed on adipocytes have been shown to play a role in preadipocyte proliferation and differentiation. Therefore, we used BMSC cultures to examine whether the Y1 receptor also plays a role in the differentiation of adipocytes within the bone microenvironment. Interestingly, after 7 days in adipogenic conditions, significantly more adipocytes had formed in BMSC cultures isolated from *Y1*^{-/-} mice than in cultures from wild-type mice (Fig. 3C, D).

Together these data show that although the total amount of in vitro mineralization was suppressed (Fig. 3A), there was a significant increase in the number of mineralized nodules under osteogenic conditions (Fig. 3B) and a significant increase in the number of adipocytes under adipogenic conditions (Fig. 3C) in BMSC cultures isolated from *Y1*^{-/-} mice compared with those isolated from wild-type mice. This suggests that the number of mesenchymal progenitor cells within these BMSC cultures or their ability to differentiate into mineral-producing osteoblasts as well as adipocytes is increased in the absence of the Y1 receptor.

Mesenchymal progenitor cells: MSCs and osteoprogenitor cells

To investigate the effect of Y1 receptor deficiency on the ability of mesenchymal progenitor cells to differentiate in the absence of possible interference from other cell types in BMSC cultures, we isolated two purified populations of cells from *Y1*^{-/-} and wild-type mice and studied their in vitro proliferation and differentiation. Following depletion of hematopoietic cells from murine bone tissue, a highly proliferative Sca-1+ population of MSCs and a second population of Sca-1-CD51+ osteoprogenitor cells, which are more committed toward the osteogenic lineage, were collected and studied.

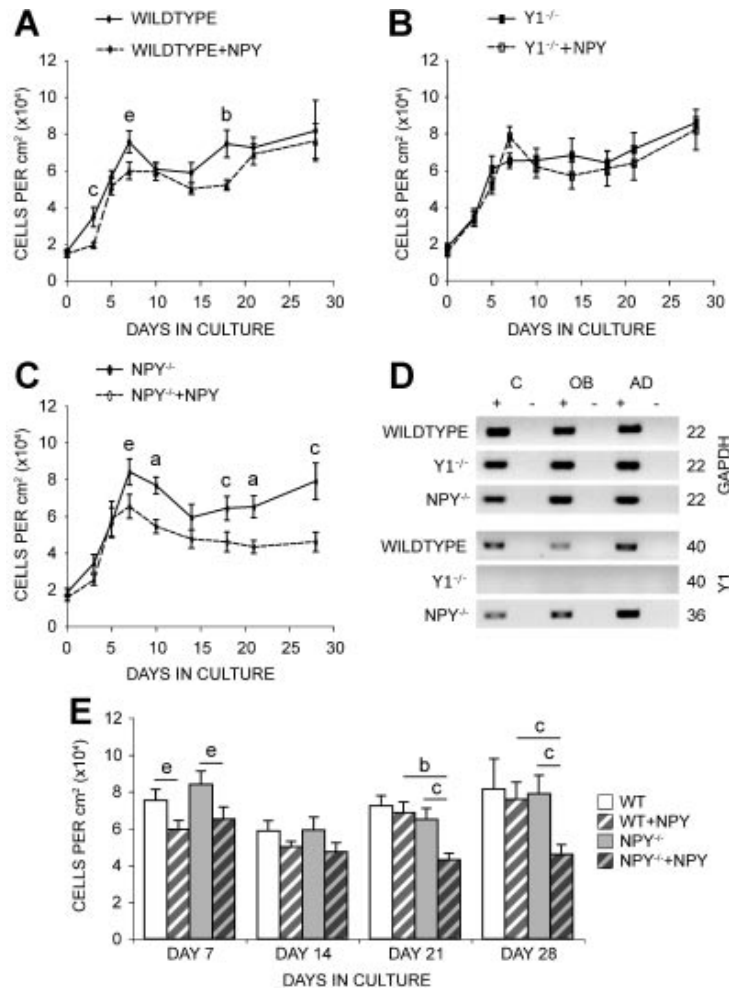


Fig. 2. Addition of NPY reduces cell number in BMSC cultures isolated from wild-type and *NPY*^{-/-} but not *Y1*^{-/-} mice. Numbers of BMSCs isolated from male wild-type (A), *Y1*^{-/-} (B), and *NPY*^{-/-} (C) mice at various time points until 28 days after replating. Comparison between numbers of BMSCs from wild-type and *NPY*^{-/-} mice at weekly time points is shown in panel E. Cells were cultured in control medium with or without 100 nM NPY, as indicated. Mean ± SEM of 10 to 12 wells per time point over three experiments are shown. Y1 receptor expression is upregulated in BMSCs from *NPY*^{-/-} compared with wild-type mice under control (C), osteoblastic (OB), and adipogenic (AD) conditions (D). Number of PCR cycles is indicated at right; data show results for PCR performed with (+) and without (-) reverse transcription on RNA isolated from cells after 6 to 7 days in differentiation media. ^a*p* < .001; ^b*p* < .01; ^c*p* < .05; ^e*p* = 0.06, as indicated.

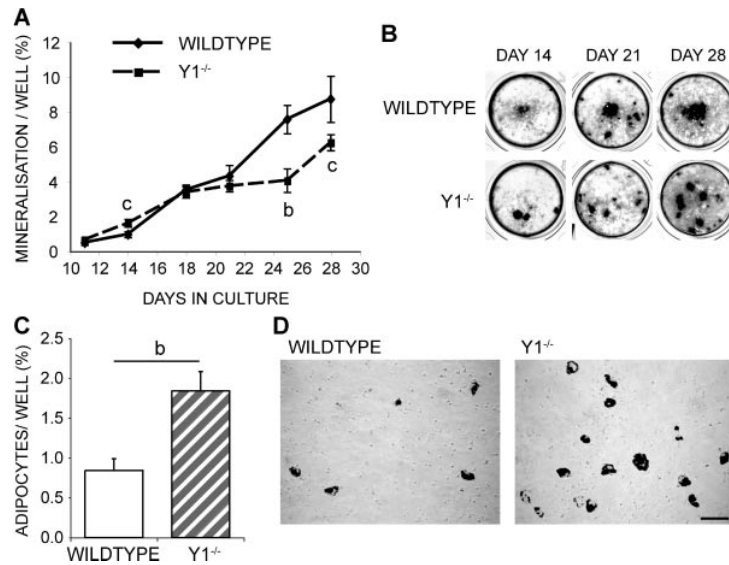


Fig. 3. BMSCs isolated from $Y1^{-/-}$ mice form a greater number of mineralized nodules despite less total mineralization under osteogenic conditions and a greater number of adipocytes under adipogenic conditions. Percentage of von Kossa⁺ mineralization per well over time (A) and representative images of von Kossa⁺ mineralization (B) in BMSC cultures isolated from male wild-type and $Y1^{-/-}$ mice under osteogenic conditions. Mean \pm SEM of 10 to 16 wells per time point over three experiments are shown. Percentage of Nile red⁺ adipocytes (C) and representative images of oil red O⁺ adipocytes (D) in BMSC cultures isolated from male wild-type and $Y1^{-/-}$ mice after 7 days in adipogenic conditions. Mean \pm SEM of five wells per genotype are shown. ^b $p < .01$; ^c $p < .05$ compared with wild type. Bar = 100 μ m.

Exclusive expression of the Y1 receptor in mesenchymal progenitor cells

To determine whether NPY-ergic signaling via the Y1 receptor could have direct effects on mesenchymal progenitor cells, it was essential to first establish whether Y1 receptors are expressed on these cells. RT-PCR analysis showed strong Y1 receptor expression in both MSCs and osteoprogenitor cells, with no expression in cells obtained from $Y1^{-/-}$ mice (Fig. 4A). In addition, no expression of Y2, Y4, Y5, or y6 receptors was observed in these cells when isolated from either wild-type or $Y1^{-/-}$ mice (Fig. 4A). Therefore, any direct effect of NPY-like peptides on the differentiation of mesenchymal progenitor cells in bone can be mediated only via the Y1 receptor.

Interestingly, whereas RT-PCR failed to detect convincing expression of NPY in either population of mesenchymal progenitor cells isolated from wild-type mice, MSCs and osteoprogenitor cells isolated from $Y1^{-/-}$ mice exhibited markedly upregulated NPY expression (Fig. 4A). A similar increase in NPY expression in the absence of the Y1 receptor was observed by quantitative real-time PCR in BMSCs isolated from $Y1^{-/-}$ compared with wild-type mice (Fig. 4B).

Lack of Y1 receptor increases the number and size of CFU-Fs formed by osteoprogenitor cells

To determine whether the increase in the number of mineralized nodules observed in BMSC cultures isolated from $Y1^{-/-}$ mice is due to differences in the number and/or proliferative potential of mesenchymal progenitor cells and whether this also may contribute to the bone anabolic phenotype of $Y1^{-/-}$ mice, we next investigated the number and proliferative ability of mesenchymal progenitor cells isolated from $Y1^{-/-}$ and wild-type mice. Isolation of Sca-1⁺ MSCs and Sca-1⁺CD51⁺ osteoprogenitor cells produced similar numbers from the bone of both $Y1^{-/-}$ and wild-type mice (Fig. 5A). After 10 days in vitro, the number and size of CFU-Fs was assessed as an indication of the relative abundance and activity of proliferating progenitor cells. The proliferative ability of Sca-1⁺ MSCs derived from $Y1^{-/-}$ mice was comparable with that derived from wild-type mice, as determined by the number (Fig. 5B) and mean size of CFU-Fs (Fig. 5C), as well as the total number of cells (Fig. 5D). In contrast, cultures of Sca-1⁺CD51⁺ osteoprogenitor cells isolated from $Y1^{-/-}$ mice formed a significantly greater number of CFU-Fs than cultures isolated from wild-type mice (Fig. 5B), suggesting an increase in the abundance of proliferating progenitor cells in the absence of the Y1 signaling. Additionally, the mean size of CFU-Fs formed by $Y1^{-/-}$ osteoprogenitor cells was significantly larger than that of their wild-type counterparts (Fig. 5C), leading to a significant increase in the total number of osteoprogenitor cells after 2 weeks in culture (Fig. 5D). These data indicate that lack of Y1 receptor signaling increases the

abundance as well as the proliferative ability of osteoprogenitor cells in the bone microenvironment.

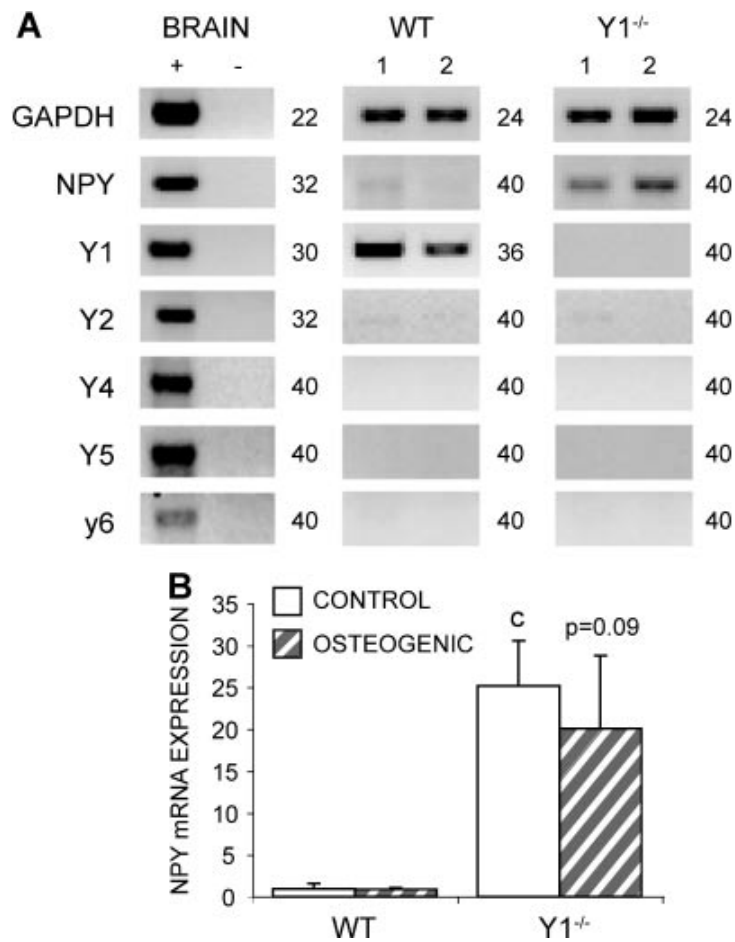


Fig. 4. NPY and Y1 receptor expression in MSCs, progenitor cells, and BMSCs in vitro. Y1 receptor, but not Y2, Y4, Y5, or y6 receptor, expression was detected in MSCs (1) and progenitor cells (2) isolated from wild-type mice (*middle panel*) but not Y1^{-/-} mice (*right panel*) by RT-PCR (A). Both cell types isolated from Y1^{-/-} mice show clear NPY expression, whereas their wild-type counterparts do not (A). PCR conditions were optimized on brain tissue from wild-type mice (*left panel*), and data show results for PCR performed with (+) and without (-) reverse transcription on RNA isolated from whole brain tissue. The number of PCR cycles is indicated to the right of each panel. Similarly, quantitative real-time PCR analysis shows an increase in NPY mRNA levels in BMSCs isolated from Y1^{-/-} compared with wild-type mice after 7 days in either control or osteogenic conditions (B). Values are normalized to GAPDH levels and relative to wild-type expression. Data are means ± SEM of three per group. *p < .05, as indicated.

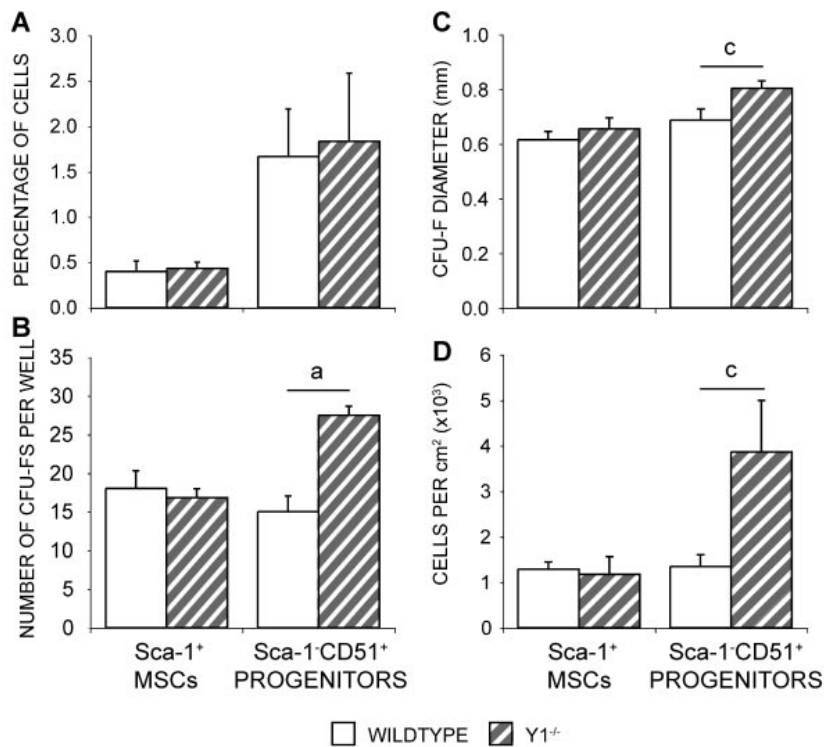


Fig. 5. $Y1^{-/-}$ progenitor cells form more and larger CFU-Fs than wild-type cells. Percentage of Sca-1⁺ MSCs and Sca-1⁻CD51⁺ progenitor cells obtained from $Y1^{-/-}$ and wild-type mice by FACS sorting immunodepleted bone cells (A). Number (B) and size (C) of CFU-Fs formed by these cells after 10 days in culture. Total number of these cells after 2 weeks in culture (D). Data are means \pm SEM of four experiments each using 10 or more mice per genotype (A), 12 wells per cell population over three experiments (B, C), and five flasks per cell population over three experiments (D). ^a $p < .0001$; ^c $p < .05$, as indicated.

Y1 receptor deficiency enhances differentiation of mesenchymal progenitor cells

The greater proliferative ability of $Y1^{-/-}$ osteoprogenitor cells in vitro led support to the hypothesis that Y1 receptors play a direct role in the regulation of these cells. In order to assess whether differentiation is also altered by the lack of Y1 signaling, we investigated the potential of both populations of mesenchymal progenitor cells from $Y1^{-/-}$ and wild-type mice to differentiate into adipocytes as well as osteoblasts.

Interestingly, in the Sca-1⁺ MSC cultures isolated from $Y1^{-/-}$ mice, spontaneous adipogenesis was evident after 20 days in culture in normal control medium (Fig. 6A). No spontaneous adipocyte differentiation was observed in the Sca-1⁻CD51⁺ osteoprogenitor cell cultures of either genotype, consistent with the fact that these cells are more committed toward the osteogenic lineage.⁶ To further investigate the adipogenic differentiation ability of $Y1^{-/-}$ MSCs, both MSCs and progenitor cells were cultured under adipogenic conditions. In wild-type cells, the formation of oil red-O⁺ adipocytes was again greater in the Sca-1⁺ MSC population than in the Sca-1⁻CD51⁺ osteoprogenitor cell population (Fig. 6B, C). However, both the Sca-1⁺ MSC and the Sca-1⁻CD51⁺ osteoprogenitor cell populations isolated from $Y1^{-/-}$ mice showed a greater differentiation into adipocytes than their wild-type counterparts (Fig. 6B, C), consistent with the data on MSCs described in Fig. 6A.

The enhanced ability of Y1-deficient MSCs to differentiate into adipocytes in vitro is consistent with the increased visceral adiposity observed in germ-line $Y1^{-/-}$ mice.^{36, 37} However, since adipogenesis in the bone marrow compartment is believed to be regulated by different mechanisms to adipogenesis in white adipose tissue depots, we used quantitative real-time PCR to investigate the levels of peroxisome proliferator-activated receptor γ (PPAR- γ), the main transcription factor regulating the differentiation of MSCs into adipocytes, in the long bones of wild-type and $Y1^{-/-}$ mice. Consistent with the increase in adipogenesis observed in vitro, Y1 receptor deficiency led to a twofold increase in the mRNA levels of PPAR- γ in bone tissue (Fig.

6D). These findings show not only that these populations of purified mesenchymal progenitor cells are capable of differentiation in vitro but also that Y1 deficiency in these cells affects their differentiation into adipocytes.

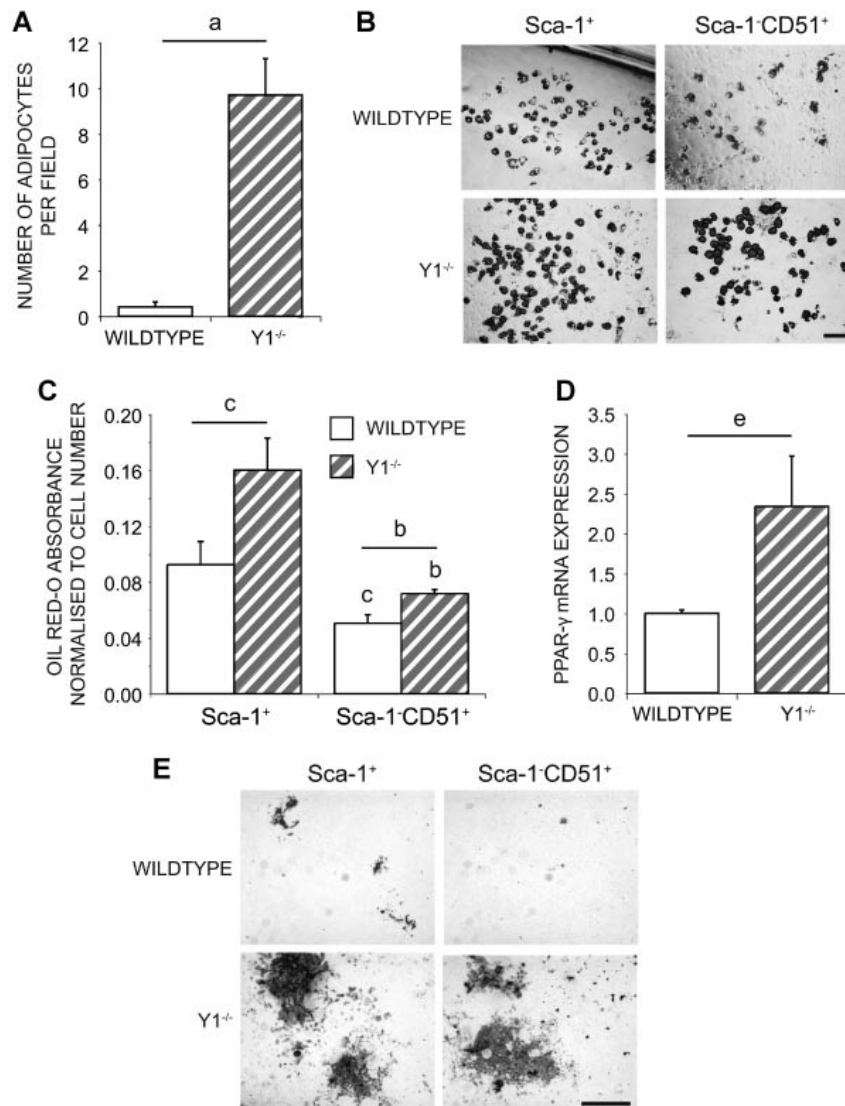


Fig. 6. $Y1^{-/-}$ MSCs and progenitor cells form more adipocytes under adipogenic conditions and more mineral under osteogenic conditions than wild-type cells. Number of adipocytes formed by $Sca-1^{+}$ MSCs isolated from male wild-type and $Y1^{-/-}$ mice after 20 days in culture in control conditions (A). Data shown are means \pm SEM of 10 random fields per genotype. The formation of adipocytes in cultures of $Sca-1^{+}$ MSCs and $Sca-1^{-}CD51^{+}$ progenitor cells isolated from male wild-type and $Y1^{-/-}$ mice after 3 weeks in adipogenic medium was visualised with oil red O staining (B) and quantified by normalizing the absorbance of the eluted stain to total cell number (C). Data are means \pm SEM of eight wells per group over two experiments. Quantitative real-time PCR analysis shows an increase in mRNA levels of $PPAR-\gamma$ in the long bones of 7- to 9-week-old male $Y1^{-/-}$ mice compared with those of wild-type mice (D). Values shown are normalized to $GAPDH$ levels and relative to wild-type expression. Data are means \pm SEM of five to six mice per genotype. Representative images of von Kossa⁺ mineralization by $Sca-1^{+}$ MSCs and $Sca-1^{-}CD51^{+}$ progenitor cells isolated from male wild-type and $Y1^{-/-}$ mice after 5 weeks in osteogenic medium are shown (E). Representative images from eight wells per group over two experiments are shown. Scale bar = 200 μ m (B) and 1 mm (E). ^a $p < .001$; ^b $p < .01$; ^c $p < .05$, ^d $p = .07$ compared with $Sca-1^{+}$ MSCs of the same genotype or as indicated.

We next assessed the ability of MSCs and osteoprogenitor cells from $Y1^{-/-}$ and wild-type mice to differentiate into mature, mineral-producing osteoblasts. Cells were cultured in the presence of BMP-2, a strong bone-inducing agent,³⁸ in addition to ascorbic acid and β -glycerophosphate, which are required to induce osteogenic differentiation in more mature stromal cells. After 5 weeks in osteogenic medium, the extent of mineralization, a measure of osteoblastic activity, was determined by von Kossa staining. In comparison with wild-type MSCs and osteoprogenitor cells, $Y1^{-/-}$ cells produced significantly more von Kossa-stained osteoid and mineral (Fig. 6E). Thus the differentiation into mineral-producing osteoblastic cells by mesenchymal progenitor cells isolated from the bone tissue of $Y1^{-/-}$ mice is enhanced compared

with those isolated from wild-type mice.

Selective deletion of Y1 receptors from mature osteoblasts enhances in vitro mineralization

Our data thus far suggest that Y1 receptor deficiency on mesenchymal progenitor cells can affect bone mass by influencing their proliferation and differentiation into mineral-producing osteoblasts. In addition to this mechanism, the high-bone-mass phenotype of *Y1*^{-/-} mice has been shown to be associated with an increased mineral apposition rate (MAR), suggesting that the activity of mature osteoblasts is increased in vivo.⁴ Therefore, in order to investigate whether the activity of mature osteoblasts is also enhanced in vitro in the absence of the Y1 receptor, the mineral-forming ability of BMSC cultures in which the Y1 receptor was missing solely from mature osteoblasts was assessed. To do this, we isolated and cultured BMSCs from *2.3ColCre;Y1lox/lox* mice, in which the Y1 receptor has been conditionally deleted in mature osteoblasts under control of the $\alpha 1(I)$ collagen promoter, and compared them with BMSCs isolated from wild-type littermates. As shown in Fig. 7, in the specific absence of osteoblastic Y1 receptor signaling, BMSCs from male and female *2.3ColCre;Y1lox/lox* mice produce significantly more mineral in vitro than those isolated from wild-type littermates. This finding is consistent with the enhanced osteoblast activity observed in germ-line *Y1*^{-/-} mice in vivo⁴ and shows that the activity of mature osteoblasts is increased in the absence of Y1 receptors. Furthermore, it suggests that the suppression of mineralization observed in BMSC cultures isolated from germ-line *Y1*^{-/-} mice was due to maturation-dependent effects and/or a lack of Y1 receptors on nonosteoblastic cells within the heterogeneous BMSC cultures rather than a direct effect of Y1 deficiency on mature osteoblastic cells.

Discussion

This study reveals an important role for Y1 receptor signaling in the differentiation of BMSCs, MSCs, and osteoprogenitor cells as well as on the activity of mature osteoblasts in vitro. Although BMSCs isolated from both male and female germ-line *Y1*^{-/-} mice formed less mineral in culture, they formed a greater number of mineralized nodules, suggesting the existence of more osteoblastic colonies than in wild-type cultures. Indeed, purified osteoprogenitor cells isolated from *Y1*^{-/-} mice formed significantly more CFU-Fs than their wild-type counterparts. Combined with the fact that the CFU-Fs formed by the Y1-deficient osteoprogenitor cells also were significantly larger and total cell number was significantly increased, these data suggest that proliferation of osteoprogenitor cells is increased in the absence of signaling through the Y1 receptor. Furthermore, both MSCs and osteoprogenitor cells from *Y1*^{-/-} mice showed an enhanced ability to differentiate into both adipocytes and osteoblasts. Importantly, *Y1*^{-/-} MSCs even showed an early, spontaneous ability to form adipocytes without the presence of adipogenic inducing factors, consistent with increased levels of PPAR- γ in the long bones of Y1-deficient mice. Additionally, BMSCs isolated from mice lacking the Y1 receptor solely in mature osteoblasts exhibited enhanced mineralization in vitro, suggesting that the Y1 receptor is also involved in inhibiting mineral production by mature osteoblasts. Finally, we have now clearly established that the Y1 receptor is the only Y receptor expressed by MSCs and osteoprogenitor cells as well as by mature osteoblasts in situ. This indicates that any direct responses of these cell types to Y receptor agonists such as NPY are mediated solely through the Y1 receptor.

Despite the increased number of mineralized nodules in BMSC cultures isolated from germ-line *Y1*^{-/-} mice, the percentage of total mineralization per well was significantly less than that formed by wild-type BMSCs. This finding is in contrast to the increased mineral apposition rate observed in vivo in germ-line *Y1*^{-/-} mice.⁴ These data therefore suggest that in the absence of Y1 receptor signaling, the in vitro mineralization capacity of differentiated osteoblasts was diminished. In contrast, mineralization was enhanced in BMSC cultures from conditional Y1 knockout mice in which the Y1 receptor was missing solely from mature osteoblasts, consistent with the increased mineral apposition rate observed in *Y1*^{-/-} mice. The differing in vitro responses of osteoblasts from germ-line *Y1*^{-/-} versus conditional osteoblast-specific Y1-deficient mice indicate the likely involvement of maturation-dependent actions of NPY. Y1 expression has been shown to change with maturation of osteoblastic cells, increasing with expression of late-stage markers such as dentin matrix protein 1 (DMP1).⁷ Indeed, mechanical loading, a process restricted to terminally differentiated osteoblastic cells, reduced NPY expression, consistent with Y1 signaling occurring in a maturation-specific manner.

Additionally, it is also possible that the decreased total mineralization observed in BMSC cultures from germ line *Y1*^{-/-} mice may relate to indirect effects caused by the global deletion of

the Y1 receptor, such as deficiency of Y1 receptors from other cells types in the heterogeneous BMSC cultures. Loss of Y1 increased NPY expression in BMSCs, and a similar increase in NPY on other cell types such as hematopoietic cells acting via other Y receptors could affect the cross-talk between immune and skeletal cells, resulting in less mineralization. The lineage and maturation-stage specificity of the *2.3ColCre;Y1lox/lox* model alleviates such potential confounders and produces a clearer indication of the role of Y1 in mature osteoblastic cells. As such, the repressive action of NPY on osteoblast function described by the *2.3ColCre;Y1lox/lox* studies is consistent with known actions of NPY on osteoblasts in vitro^{4, 7} and in vivo.^{4, 39}

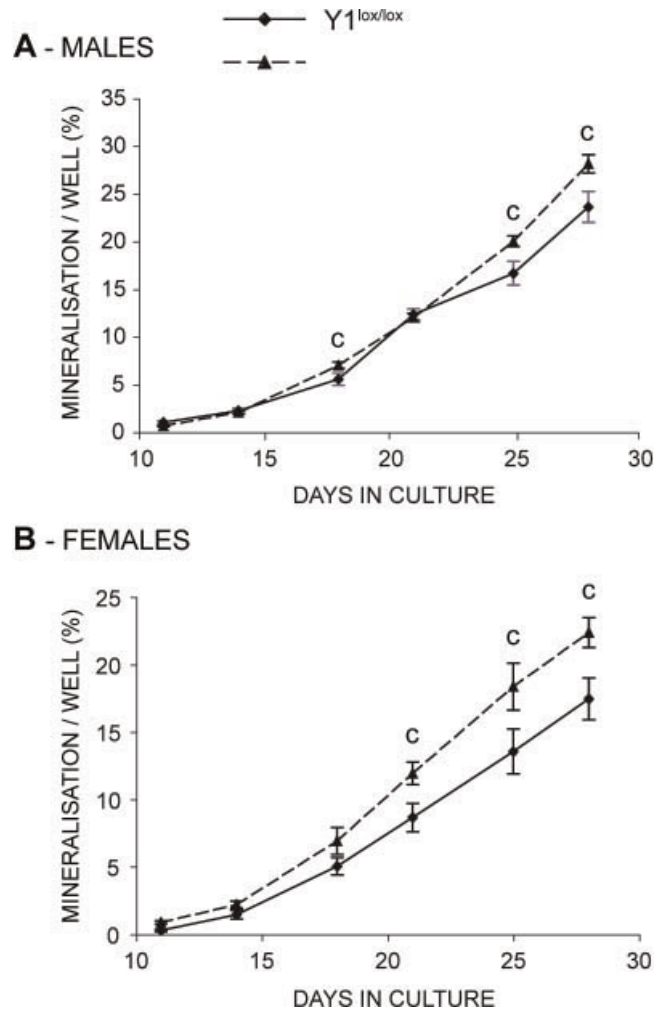


Fig. 7. In vitro mineralization is enhanced in BMSC cultures in the absence of mature osteoblastic Y1 receptors. Percentage of von Kossa⁺ mineralization per well over time in cultures of BMSCs isolated from male (A) and female (B) *2.3ColCre;Y1^{lox/lox}* and *Y1^{lox/lox}* mice under osteogenic conditions. Mean \pm SEM of 10 to 12 wells per time point over three experiments are shown. ^c $p < .05$ compared with *Y1^{lox/lox}*.

Cultures generated from single-cell suspensions of marrow have shown that BMSCs are clonogenic and grow from foci before becoming confluent.⁴⁰ Therefore, the increased number of mineralized nodules and adipocytes observed in BMSC cultures isolated from germ-line *Y1^{-/-}* mice suggests that either a greater proportion of mesenchymal progenitor cells were initially present in these cultures and/or that the differentiation of mesenchymal progenitor cells into osteoblasts and adipocytes is enhanced in the absence of Y1 receptor signaling. By isolating purified populations of MSCs and osteoprogenitor cells, we were able to demonstrate that the proliferation of osteoprogenitor cells was increased in the absence of the Y1 receptor and also that Y1 receptor deficiency enhanced the differentiation potential of both MSCs and

osteoprogenitor cells. Using similar cell isolation methods, it has been demonstrated that mice lacking the Y2 receptor have increased osteoprogenitor cell numbers.⁶ However, once plated in culture at an equal density to wild-type cells, they formed a similar number of CFU-Fs as their wild-type counterparts.⁶ Thus the findings presented here demonstrate that the Y1 and Y2 receptors play distinct roles in the regulation of bone metabolism.

In this study we have demonstrated that mRNA expression levels of *runx2* and *osterix*, two key transcription factors essential for osteoblastic differentiation, are increased in the long bones of *Y1*^{-/-} mice. Runx2 is now well established as the master osteogenic transcription factor, playing a critical role in inducing the expression of osteoblast-specific genes.⁴¹ It is believed to be one of the earliest master transcription factors involved in the differentiation of stem cells into osteoblasts, whereas osterix is thought to act mainly during the terminal differentiation of osteoblasts.³⁸ The observed increase in mRNA expression levels of these osteogenic transcription factors in bones from *Y1*^{-/-} mice suggests that there is a similar upregulation of osteoblastic differentiation in vivo to that seen in BMSCs, MSCs, and osteoprogenitor cells in vitro, probably contributing to the high-bone-mass phenotype of the *Y1*^{-/-} mice.⁴

Since osteoblastogenesis generally is believed to occur at the expense of adipogenesis and vice versa, the high bone mass of *Y1*^{-/-} mice would suggest a reduction in marrow adiposity. However, here we have demonstrated that Y1 receptor deficiency enhanced in vitro adipogenesis by mesenchymal progenitor cells purified from bone tissue. Furthermore, Y1 receptor deficiency also led to a twofold increase in mRNA levels of *PPAR-γ* in the long bones of male mice. These findings further advance those of a previous study by our group that investigated marrow adiposity in *Y1*^{-/-} mice by histomorphometric analysis and revealed a sex-specific difference in marrow adiposity whereby female *Y1*^{-/-} mice showed significant increases but male *Y1*^{-/-} mice showed significant reductions in marrow adipocyte number and volume.³³ However, both male and female *Y1*^{-/-} mice demonstrated a marked increase in marrow adiposity following gonadectomy relative to wild-type mice.³³ Interestingly, this increase was most evident in male *Y1*^{-/-} mice, and they also showed a greater loss of bone mass following gonadectomy than female *Y1*^{-/-} mice.³³ Thus enhanced adipogenesis at the expense of osteoblastogenesis may have contributed in part to the loss of bone mass. Together these data suggest that signaling through the Y1 receptor has two differential effects on mesenchymal differentiation in the bone microenvironment, first on osteoblastic differentiation and separately on adipocytic differentiation.

NPY signaling via the Y1 receptor has been implicated previously in the regulation of other stem cells, notably the proliferation of olfactory and hippocampal precursor cells. Investigation of olfactory neuronal precursor cells in mice with a targeted deletion of NPY revealed a reduction in neuronal precursor proliferation resulting in fewer olfactory neurons⁴² and indicated that NPY is required for the normal proliferation of olfactory neurospheres in vitro.⁴³ Similarly, NPY has been shown in mice to promote the proliferation of neuronal progenitor cells in the dentate gyrus of the hippocampus^{28, 44} and also in the subventricular zone.^{45, 46} Interestingly, in all cases, the Y receptor mediating these effects is the Y1 receptor. Our findings in the study of stem cells in the bone environment further advance these insights to show that Y1 may act as a master switch that may increase bone and fat formation via effects on MSCs.

Taken together the findings of this study clearly demonstrate the important role the NPY system, particularly the Y1 receptor, plays in the development, differentiation, and mineral-producing ability of osteoblastic cells as well as the production of adipocytes in the bone microenvironment. Knowledge of Y receptor involvement in regulating the differentiation of MSCs could lead to potential new therapies for the treatment and prevention of bone loss in osteoporosis.

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