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Osteoblast specific Y1 receptor deletion enhances bone mass

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ABSTRACT

Neuropeptide Y, Y1 receptors are found in neuronal as well as bone tissue and Y1 signalling has been implicated in the regulation of bone mass. However, the contribution of Y1 receptors located in these different tissues, particularly that of the bone-specific Y1 receptors, to the regulation of bone homeostasis is unclear. Here we demonstrate that osteoblast-specific Y1 receptor deletion resulted in a marked increase in femoral cancellous bone volume, trabecular thickness and trabecular number. This is the result of elevated osteoblast activity as shown by increased mineral apposition rate and bone formation rate, and is associated with an upregulation in the mRNA expression levels of alkaline phosphatase, osteocalcin and dentin matrix protein-1. Furthermore, osteoblastic Y1 receptor deletion also led to increased mineral apposition rate on both the endocortical and the periosteal surfaces resulting in increased femoral diameter. Together these data demonstrate a direct role for the Y1 receptor on osteoblasts in the regulation of osteoblast activity and bone formation *in vivo* and suggest that targeting Y1 receptor signalling directly in the bone may have potential therapeutic implications for stimulating bone accrual in diseases such as osteoporosis.

Introduction

Osteoporosis is an increasingly common and debilitating condition creating major social and economic burdens worldwide. Whilst antiresorptive treatments can be successful in preventing progression of the condition, there is a pressing need for the development of effective anabolic agents capable of rebuilding the bone that has already been lost. This is particularly so in light of the fact that by the time osteoporosis is diagnosed as a result of fracture, considerable bone loss has usually already occurred [1,2]. A potential target for the development of bone anabolic agents is the neuropeptide Y (NPY) system, notably Y1 and Y2 receptors, which has been revealed as a powerful pathway in the control of bone mass through the regulation of osteoblast activity. Germline deletion of either Y1 or Y2 receptors in mice leads to a high bone mass phenotype associated with enhanced osteoblast activity [3,4]. Y2 receptors are predominantly expressed in the central nervous system where they are usually pre-synaptic [5,6]. Consistent with a central role of Y2 receptors in the regulation of bone, it has clearly been shown that the deletion of hypothalamic Y2 receptors is sufficient to replicate the high bone mass phenotype observed in germline Y2 receptor knockout ($Y2^{-/-}$) mice [4]. In contrast, hypothalamic deletion of Y1 receptors did not alter bone homeostasis [3], suggesting that non-hypothalamic Y1 receptors are important for the control of bone. Indeed, recent *in vitro* studies suggest that the Y1 receptor plays a peripheral, osteoblastic role in the regulation of bone formation [7].

Despite being first characterised as a post-synaptic receptor, the Y1 receptor can also be found pre-synaptically and has a wide distribution both in the central nervous system as well as in the periphery [8,9]. Germline Y1 receptor knockout ($Y1^{-/-}$) mice have a high bone mass phenotype with a generalised increase in bone formation on both cortical and cancellous surfaces [3]. In addition, osteoclast surface was increased, suggesting that bone resorption may also be elevated in these mice [3]. The deletion of both Y1 and Y2 receptors did not lead to an additive effect on bone thus suggesting that they may be acting at different points along the same pathway [3]. Y1 receptor, but not Y2 receptor, expression has been demonstrated by *in-situ* hybridisation in osteoblastic cells lining both cortical and trabecular bone surfaces [10], and by reverse transcription PCR in calvaria and long bone [11]. Moreover, we have recently shown that the Y1 receptor directly inhibits the differentiation of mesenchymal progenitor cells as well as the activity of mature osteoblasts *in vitro* [7]. Furthermore, identification of NPY expression in osteoblasts [12] and osteocytes [11] suggests the potential for local control of osteoblast activity by NPY via Y1 receptors. Indeed, the treatment of

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mouse calvarial osteoblasts with exogenous NPY resulted in an inhibition of osteoblast activity [11].

Given the clear expression of Y1 receptor mRNA in osteoblasts [10] combined with the increased mineral formation by Y1-deficient osteoblasts *in vitro* [7], we hypothesise that osteoblastic Y1 receptors play a direct regulatory role in bone metabolism. Therefore, we generated mice with selective deletion of the Y1 receptor in osteoblasts ($Y1^{lox/lox;Cre/+}$) by crossing $Y1^{lox/lox}$ mice with mice expressing *Cre* specifically in maturing osteoblasts under the control of a 2.3 kb fragment of the α1(I)-collagen promotor [13]. Investigating the *in vivo* bone phenotype of these $Y1^{lox/lox;Cre/+}$ mice allows us to determine the role of direct signalling via osteoblastic Y1 receptors on bone mass.

Materials and methods

Mice

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.

Mice with osteoblast-specific deletion of the Y1 receptor ($Y1^{lox/lox;Cre/+}$) were generated as previously described [7] by mating mice expressing *Cre* under the control of a 2.3 kb fragment of the rat α1(I)-collagen promoter with $Y1^{lox/lox}$ mice. *Cre*-mediated recombination resulted in osteoblast-specific deletion of the entire coding region of the Y1 gene. Male $Y1^{lox/lox;Cre/+}$ mice were bred with female $Y1^{lox/lox}$ mice to generate both $Y1^{lox/lox;Cre/+}$ mice and $Y1^{lox/lox}$ littermates to be used as controls. Genotypes were determined by PCR as previously described [7]. Male mice were used for all experiments reported here, at 16 weeks of age, an age where it is known that cancellous bone volume is at its peak before declining with age, and the mice were maintained on a mixed C57/BL6-129/SvJ background.

Tissue collection

Male $Y1^{lox/lox;Cre/+}$ and $Y1^{lox/lox}$ mice were injected with the fluorescent compound calcein (15 mg/kg; Sigma-Aldrich, St Louis, MO, USA) 3 and 10 days prior to tissue collection to enable subsequent calculation of bone formation rate. Whole body lean mass, fat mass, bone mineral content (BMC) and bone mineral density (BMD) were measured in mice anesthetised with isoflurane using a dedicated mouse dual X-ray absorptiometry (DXA) (Lunar Piximus II, GE Medical Systems, Madison WI) 3 days prior to tissue collection. BMD and BMC were also measured for the lumbar vertebrae with a sample area 75 pixels in length proximal from the lower lumbar. At 16 weeks of age, mice were culled between 13.00 and 16.00 hours by cervical dislocation and decapitation for collection of trunk blood. Serum was separated, immediately frozen and stored at −20 °C. Brains were immediately frozen on dry ice and stored at −80 °C. The white adipose tissue (WAT) depots (right side inguinal, retroperitoneal, epididymal (gonadal) and mesenteric), and skeletal muscle were removed, weighed, frozen on dry ice and stored at −80 °C. Femurs, tibias and caudal vertebrae were excised, fixed overnight in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) at 4 °C and then stored in 70% ethanol at 4 °C before undergoing processing.

Plasma assays

Serum glucose was measured using a glucose oxidase assay kit (Trace Scientific, Clayton, Victoria, Australia) and serum insulin was measured using an ELISA kit from Linco Research (St Charles, Missouri, USA). Serum corticosterone was measured using a radioimmunoassay detection kit from MP Biomedicals (Irvine, CA, USA), whilst serum insulin like growth factor (IGF)-1 was measured using a radioimmunoassay detection kit from Bioclone (Sydney, NSW, Australia).

Reverse-transcription PCR

Bone marrow stromal cells (BMSCs) were isolated, cultured and differentiated as previously described [7]. BMSCs cultured for 10 days in osteogenic media were used for RNA extractions. All RNA extractions on BMSCs and frozen tissue were carried out using TRIzol® reagent (Sigma) according to the manufacturer's instructions and reverse transcription-PCR was performed as previously described [7]. 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 resultant product sizes obtained are as detailed previously [10] and as follows for Cre: 300 bp, 55 °C, forward: 5′-GTTTCACTGGTTATGCGG-3′, reverse: 5'-AAGTGCCTTCTCTACACC-3′.

Quantitative real time-PCR

RNA was extracted from the mid-shaft of femurs and tibias isolated from 7- to 9-week-old male mice, snap frozen in liquid nitrogen and homogenised in 2 mL TRIzol® reagent using a Polytron homogeniser. All RNA extractions were performed by using TRIzol® reagent according to the manufacturer's instructions. RNA samples were subsequently checked for consistent quality and quantified using the Agilent 2100 Bioanalyser (Agilent Technologies) according to the manufacturer's instructions. 1 μg of total RNA was taken for cDNA synthesis with oligo(dT)20 and random hexamers using the SuperScript III First-Strand Synthesis System for reverse transcription-PCR (Invitrogen). Quantitative real-time PCR for Runx2, Osterix, alkaline phosphatase, osteocalcin and DMP-1 was then carried out using the TaqMan© Universal PCR master mix, ABI Prism 7900 HT Sequence Detection System and inventoried kits containing primers and probes (all from Applied Biosystems). Quantitative real-time PCR for RANK-ligand and OPG was carried out using the Lightcycler 480 Probes Mastermix with Universal Probe Library (UPL) probes (all from Roche) and the following primers: RANK-ligand forward: 5′-tgaagacacactacctgactcctg-3′, reverse: 5′-ccacaatgtgttgcagttcc-3′; OPG forward: 5′-ccacgcaaaagtgtggaata-3′, reverse: 5′-ggcaaactgtccac-caaaa-3′. To control for 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 β-actin Ct value using the standard curve method (Applied Biosystems).

Bone histomorphometry

Bone histomorphometry was carried out on 5 μm sagittal sections of the distal half of the right femur as previously described [14]. Briefly, sections were stained for mineralised bone and trabecular bone volume, trabecular thickness and trabecular number were calculated. In addition, fluorescence microscopy was used to calculate mineral apposition rate, mineralising surface and bone formation rate whilst osteoclast surface and osteoclast number were estimated using tartrate-resistant acid phosphatase-stained sections. Cortical mineral apposition rate was measured in an endosteal and a periosteal region both extending 1000 μm distal from the mid-point, as previously described [14].

Bone micro-computed tomography (micro-CT)

Following fixation, left femora were cleaned of muscle and analyses of the cortical bone were carried out using micro computed

tomography (micro-CT) with a Skyscan 1174 scanner and associated analysis software (Skyscan, Aartselaar, Belgium) as previously described [15]. Briefly, analyses of the cortical bone were carried out in 150 slices (1.07 mm) selected 750 slices (5.37 mm) proximally from the distal growth plate resulting in calculations of the following parameters: total tissue area, bone area, marrow area, endosteal perimeter, periosteal perimeter, cortical thickness, and polar moment of inertia (an index of strength).

Statistical analyses

All data are expressed as means ± SEM. Differences between knockout and wildtype mice were assessed by ANOVA. Statistical analyses were performed with SPSS for Mac OS X, version 17.0 (SPSS Inc., Chicago, IL, USA). For all statistical analyses, $p<0.05$ was accepted as being statistically significant, with $p<0.1$ accepted as showing a trend of change.

Results

Y1 receptor deletion is restricted to osteoblasts

The restriction of Y1 receptor deletion solely to osteoblastic cells of $Y1^{lox/lox;Cre/+}$ mice was confirmed by RT-PCR for *Cre* and Y1 receptor expression on RNA isolated from several tissues from $Y1^{lox/lox}$ and $Y1^{lox/lox;Cre/+}$ mice. As shown in Fig. 1, in $Y1^{lox/lox;Cre/+}$ mice which carry the *Cre* transgene, *Cre* mRNA expression was indeed restricted to bone tissue and isolated BMSCs and was not observed in other tissues examined namely brain, muscle or epididymal white adipose tissue. No *Cre* mRNA expression was observed in any tissue investigated from $Y1^{lox/lox}$ littermates. In addition, coincident with *Cre* expression, Y1 receptor expression was down-regulated in bone tissue and cultured BMSCs derived from $Y1^{lox/lox;Cre/+}$ mice (Fig. 1). Indeed, quantification of multiple analyses by densitometry revealed a 95% reduction in Y1 receptor expression in bone tissue from $Y1^{lox/lox;Cre/+}$ mice compared to their wildtype littermates. Y1 receptor expression was unaffected in other tissues examined, confirming the generation of mice with the Y1 receptor deleted solely in osteoblasts.

Osteoblast-specific Y1 receptor deletion enhances bone formation with no effect on adiposity

In addition to high bone mass, germline $Y1^{-/-}$ mice are characterised by increased adiposity and hyperinsulinemia, an obese phenotype that is exacerbated with advancing age [3,16,17]. Therefore, to examine whether osteoblast-specific deletion of the Y1 receptor could recapitulate the high bone mass phenotype of germline $Y1^{-/-}$ mice independent of other metabolic changes, we first measured body composition by DXA in $Y1^{lox/lox}$ and $Y1^{lox/lox;Cre/+}$ mice at 16 weeks of age, a similar age to that previously used for the analysis of the bone phenotype in germline $Y1^{-/-}$ mice [3,10]. Importantly, no differences were observed between $Y1^{lox/lox}$ and $Y1^{lox/lox;Cre/+}$ mice with respect to body weight, whole body lean tissue mass or fat mass (Table 1). Similar stature and adiposity between $Y1^{lox/lox;Cre/+}$ and $Y1^{lox/lox}$ mice was further confirmed by a lack of difference in femur length and the weight of individual white adipose tissue depots (Table 1 and data not shown). In addition, we found no significant effect of genotype on serum concentrations of insulin, glucose, corticosterone, or IGF-1 (Table 1). Importantly, however, whole body DXA analysis revealed a trend towards increased BMC and BMD in the $Y1^{lox/lox;Cre}$ mice compared to their wildtype littermates with a significant increase in BMC also evident at the lumbar vertebrae (Table 1), consistent with a role for Y1 receptors on osteoblasts in the inhibition of bone formation independently of any effects on adiposity or hormones such as corticosterone or IGF-1. Therefore, in order to further investigate the bone phenotype of these mice, we measured cancellous and cortical bone parameters by histomorphometry and micro computed tomography (micro-CT) in distal femurs isolated from 16 week old $Y1^{lox/lox;Cre/+}$ mice, as compared to their $Y1^{lox/lox}$ littermates.

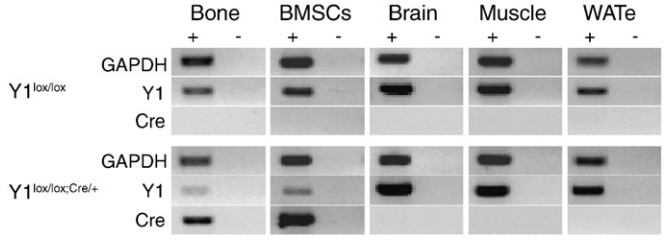

Fig. 1. Osteoblast-specific Y1 receptor deletion. Y1 receptor knockdown and *Cre* expression in bone tissue and BMSCs after 10 days in culture in osteogenic media but not in brain, muscle or epididymal white adipose tissue (WATe) derived from $Y1^{lox/lox;Cre/+}$ mice but not $Y1^{lox/lox}$ littermates. Data show results for PCR performed with (+) and without (−) reverse transcription. PCR was performed for 22–26 cycles for GAPDH, 32–26 cycles for Y1 receptor, and 36–40 cycles for *Cre* varying between tissue types but consistent across genotypes.

Cancellous bone

Histomorphometric analysis revealed that male $Y1^{lox/lox;Cre/+}$ mice have significantly greater (by 40%) cancellous bone volume in the distal femoral metaphysis compared to their wildtype littermates with significantly greater trabecular thickness and trabecular number (Fig. 2A–D). Similar to male germline $Y1^{-/-}$ mice [3], this was associated with an increase in osteoblast activity as shown by significantly enhanced MAR (Fig. 2E) and bone formation rate (Fig. 2F), without a change in the extent of bone undergoing bone formation (mineralising surface (Fig. 2G)). However, in contrast to germline $Y1^{-/-}$ mice, the activity of the osteoclastic lineage was similar between $Y1^{lox/lox;Cre/+}$ mice and their wildtype littermates in that there was no difference observed in osteoclast surface (Fig. 2H) or osteoclast number (Fig. 2I). Therefore, the enhanced cancellous bone volume observed in $Y1^{lox/lox;Cre/+}$ mice is entirely associated with enhanced bone formation with no change in bone resorption parameters.

Cortical bone

Micro-CT was used to examine the effect of osteoblast-specific Y1 receptor deletion on cortical bone. At the mid-femur, $Y1^{lox/lox;Cre/+}$ mice displayed a generalised increase in the diameter of the femur as shown by significant increases in total tissue area, marrow area, periosteal perimeter and endosteal perimeter (Fig. 3A–D). Despite

Table 1
Body composition, serum parameters and bone densitometry of $Y1^{lox/lox;Cre/+}$ mice.

	$Y1^{lox/lox}$	$Y1^{lox/lox;Cre/+}$
Body weight (g)	26.47 ± 0.50	26.77 ± 0.66
Lean mass (g)	20.55 ± 0.29	20.52 ± 0.45
Fat mass (g)	4.70 ± 0.29	4.54 ± 0.27
Femur length (mm)	15.99 ± 0.12	15.94 ± 0.14
Insulin (pM)	136.24 ± 29.88	165.90 ± 35.97
Glucose (mM)	10.95 ± 0.43	11.79 ± 0.49
Corticosterone (ng/mL)	89.42 ± 21.89	119.26 ± 23.08
IGF-1 (ng/mL)	215.34 ± 20.98	230.09 ± 16.70
Whole body BMC (g)	0.322 ± 0.006	0.341 ± 0.009 [c]
Whole body BMD (g/cm^2)	0.0500 ± 0.0006	0.0519 ± 0.0008 [b]
Lumbar BMC (g)	0.0285 ± 0.0005	0.0315 ± 0.0013 [a]
Lumbar BMD (g/cm^2)	0.0513 ± 0.0007	0.0528 ± 0.0020

Means ± SEM of 9–13 male mice per group shown.
[a] $p<0.05$.
[b] $p=0.06$.
[c] $p=0.09$ compared to $Y1^{lox/lox}$ mice.

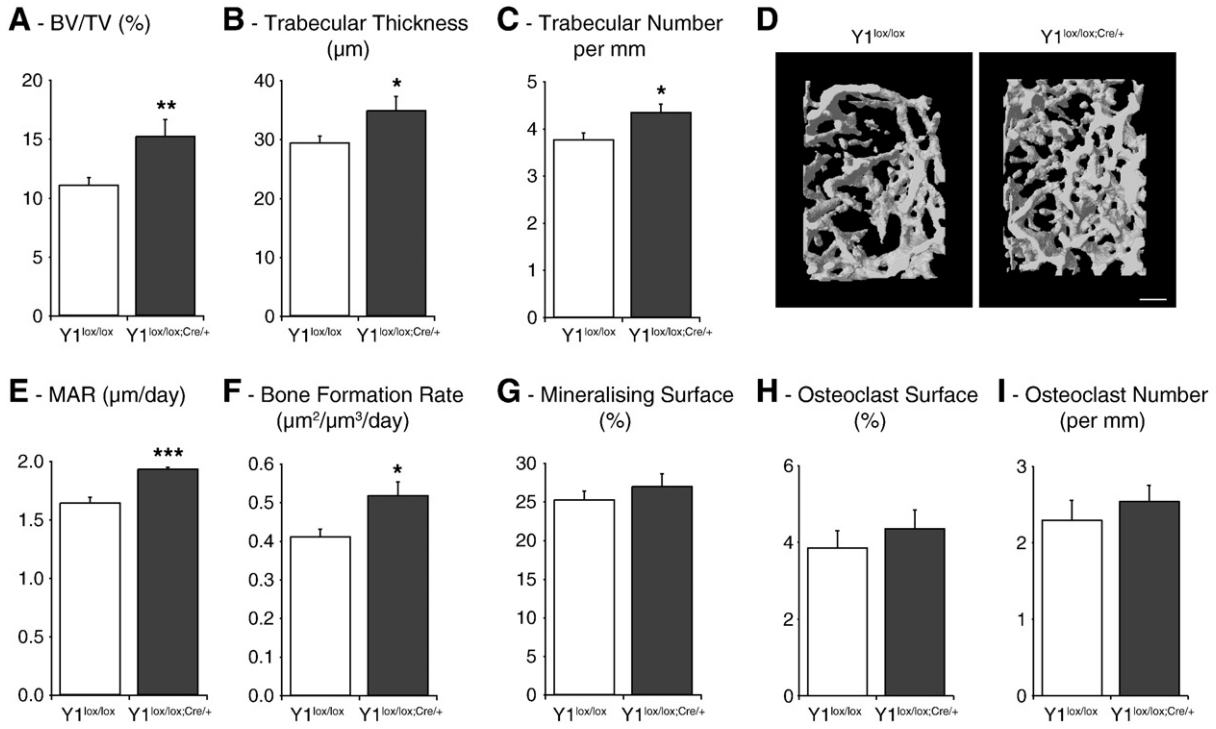

Fig. 2. Osteoblastic Y1 receptor deletion enhances cancellous bone mass and osteoblast activity. Histomorphometry reveals that $Y1^{lox/lox;Cre/+}$ mice show significantly greater cancellous bone volume (BV/TV) (A), trabecular thickness (B), and trabecular number (C) than their $Y1^{lox/lox}$ littermates, in keeping with 3-dimensional reconstructions of the distal femoral metaphysis (D). This increase is associated with significantly increased MAR (E) and bone formation rate (F) with no change in mineralising surface (G), osteoclast surface (H), or osteoclast number (I). Mean ± SEM of 6–13 male mice per group are shown. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ compared to $Y1^{lox/lox}$. Bar represents 0.2 mm (D).

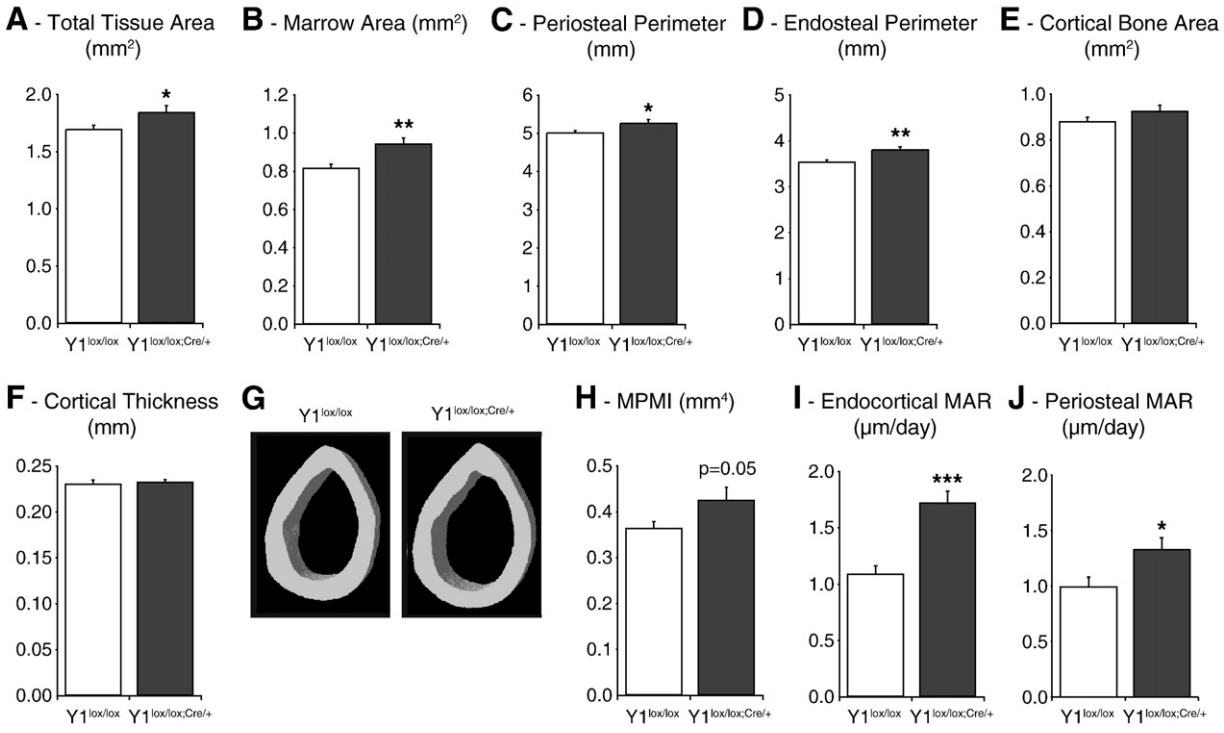

Fig. 3. Osteoblastic Y1 receptor deletion enhances cortical bone size and osteoblast activity. Micro-CT reveals that $Y1^{lox/lox;Cre/+}$ mice show greater total tissue area (A) at the mid-femoral diaphysis than their $Y1^{lox/lox}$ littermates with increased marrow area (B), periosteal perimeter (C), and endosteal perimeter (D) with no change in cortical bone area (E) or cortical thickness (F) as illustrated by representative 3-dimensional reconstructions (G). These changes are associated with increased mean polar moment of inertia (MPMI; H) and increased MAR on the endocortical (I) and periosteal (J) surfaces. Mean ± SEM of 6–13 male mice per group are shown. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ compared to $Y1^{lox/lox}$. Bar represents 0.2 mm (G).

no significant changes in cortical bone area or cortical thickness (Fig. 3E–F), the greater femur size in $Y1^{lox/lox;Cre/+}$ mice (Fig. 3G) was associated with increased polar moment of inertia, an index of bone strength (Fig. 3H).

To investigate the cellular basis for these differences, MAR on the endocortical and periosteal surfaces were examined. Interestingly, endocortical MAR was significantly elevated by approximately 60% in male $Y1^{lox/lox;Cre/+}$ mice compared to their wildtype littermates (Fig. 3I). Cortical osteoblast activity was also elevated on the periosteal surface as shown by a significant increase in periosteal MAR in the absence of osteoblastic Y1 receptors (Fig. 3J).

Together these data demonstrate that specific deletion of osteoblastic Y1 receptors enhances osteoblast activity leading to increases in both cortical and cancellous bone mass, with no effect on bone resorption, adiposity, circulating concentrations of the hormones insulin, IGF-1 or corticosterone, or body weight.

Mature osteoblastic markers are increased in the absence of osteoblastic Y1 receptors

To further analyse the functional role of Y1 receptors in mature osteoblastic cells, we isolated RNA from the long bones of $Y1^{lox/lox;Cre/+}$ mice and their wildtype littermates and analysed the expression of key osteoblastic markers. Consistent with a direct role for Y1 receptors in the differentiation of osteoprogenitor cells, it has previously been shown that the mRNA levels of two key osteogenic transcription factors, Runx2 and Osterix, are significantly upregulated in the bones of 7- to 9-week-old germline $Y1^{-/-}$ mice [7]. Interestingly, here we show no significant difference in the mRNA expression levels of either Runx2 or Osterix in the long bones of mice lacking Y1 receptors solely in mature osteoblasts at a similar age (Fig. 4), consistent with these transcription factors predominantly playing roles in early osteogenic differentiation. However, similar to germline $Y1^{-/-}$ mice [7] and in keeping with their high bone mass and anabolic phenotype, $Y1^{lox/lox;Cre/+}$ mice show a trend towards an increase in alkaline phosphatase mRNA expression compared to their wildtype littermates (Fig. 4). Importantly, $Y1^{lox/lox;Cre/+}$ mice also showed an upregulation in the mRNA expression levels of two mature osteoblastic markers, osteocalcin and dentin matrix protein (DMP)-1, compared to their wildtype controls, although this failed to reach significance in the case of osteocalcin ($p = 0.08$; Fig. 4).

Finally, no difference was observed between $Y1^{lox/lox;Cre/+}$ mice and their wildtype littermates in the mRNA expression levels of either RANK-ligand, OPG or the ratio of RANK-ligand to OPG (Fig. 4). These data are consistent with the histomorphometric data shown above and indicate that osteoblastic Y1 receptor deletion does not directly affect osteoclast activity.

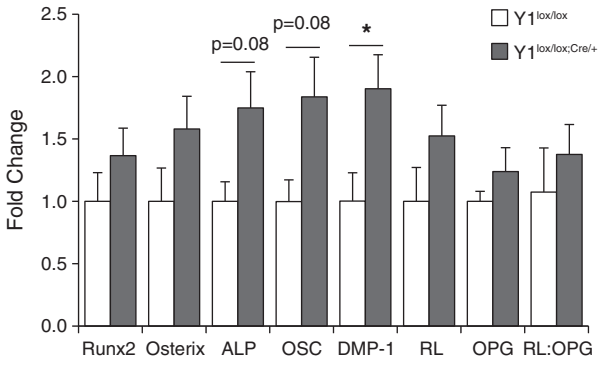

Fig. 4. Alkaline phosphatase (ALP), osteocalcin (OSC) and dentin matrix protein (DMP)-1 are up regulated in the absence of osteoblastic Y1 receptors. Quantitative real-time PCR analysis shows an increase in mRNA levels of ALP, OSC and DMP-1 but not Runx2, Osterix, RANK-ligand (RL), OPG or the RANK-ligand to OPG ratio (RL:OPG) in the long bones of 7- to 9-week-old male $Y1^{lox/lox;Cre/+}$ mice compared to those of their wildtype littermates. Values shown are normalised to β-actin levels and relative to wildtype expression. Data are means ± SEM of 5–8 mice per genotype. $^*p < 0.05$ as indicated.

Discussion

The findings presented here show for the first time that Y1 receptors expressed directly on osteoblasts play a critical role in the regulation of bone mass *in vivo*. The specific deletion of osteoblastic Y1 receptors in mice was sufficient to alter both osteoblast activity and bone mass. Specifically, $Y1^{lox/lox;Cre/+}$ mice showed increased cancellous bone volume, trabecular number and trabecular thickness in association with enhanced osteoblast activity as demonstrated by significantly increased MAR and bone formation rate despite no difference in mineralising surface. In addition, $Y1^{lox/lox;Cre/+}$ mice displayed enhanced endocortical and periosteal MAR with a trend towards increased cortical bone strength, as estimated by polar moment of inertia, associated with increased femoral diameter. Importantly, the changes observed in the bones of these mice occurred without any change in body weight, white adipose tissue mass, or circulating IGF-1, corticosterone, glucose or insulin levels. In keeping with the deletion of Y1 receptors being restricted to mature osteoblasts, key markers of mature osteoblast activity, alkaline phosphatase, osteocalcin and DMP-1, were all upregulated in $Y1^{lox/lox;Cre/+}$ mice with no change in the expression levels of transcription factors predominantly involved early in osteogenic differentiation, Runx2 and Osterix. Interestingly, these gene expression changes occur at a young age (7–9 weeks old) before an increase in bone mass can be detected by DXA analysis (data not shown). Taken together, these data suggest that osteoblastic Y1 receptors are primarily responsible for the Y1-mediated regulation of bone. Moreover, these data show that the NPY system can directly regulate osteoblast activity via Y1 receptors *in vivo*.

The high bone mass phenotype of $Y1^{lox/lox;Cre/+}$ mice shown here is similar to that previously described for germline $Y1^{-/-}$ mice [3]. However, the magnitude of change in the bones of $Y1^{lox/lox;Cre/+}$ mice compared to their wildtype controls was not as great as previously observed in germline $Y1^{-/-}$ mice. Whilst germline $Y1^{-/-}$ mice displayed a strong increase in cortical bone volume clearly evident by DXA analysis [10], the increase in cortical bone in $Y1^{lox/lox;Cre/+}$ mice was more subtle. We have previously shown that the Y1 receptor plays an inhibitory role in the differentiation of osteoblastic cells from mesenchymal progenitor cells *in vitro* [7]. As *Cre* expression and subsequent Y1 receptor deletion in the $Y1^{lox/lox;Cre/+}$ mice used in this study was restricted to mature osteoblasts, this suggests that enhanced osteoblast differentiation due to lack of Y1 receptors on less mature osteoblastic cells may also contribute to the high bone mass phenotype of germline $Y1^{-/-}$ mice. This is supported by the observation that the key transcription factors, Runx2 and Osterix, which predominantly play roles early in osteogenic differentiation are significantly upregulated in germline $Y1^{-/-}$ mice [7] but not in $Y1^{lox/lox;Cre/+}$ mice where Y1 deletion is restricted to mature osteoblasts. Although not significant, a slight increase in the expression levels of Runx2 and Osterix was still observed in $Y1^{lox/lox;Cre/+}$ mice, reflecting the fact that these factors can also play a role in more mature osteoblasts [18,19]. Additionally, it is also possible that indirect effects caused by the global deletion of the Y1 receptor, such as increased adiposity and hyperinsulinemia, also contribute to the higher bone mass phenotype of germline $Y1^{-/-}$ mice, due to–for example–greater weight bearing effects to stimulate bone mass [20] and effects of insulin to promote bone formation [21,22]. Importantly, here we show that without these potential compensatory factors, osteoblastic Y1 receptor deletion is sufficient to enhance bone mass without affecting body weight, adiposity or circulating hormone levels.

In addition to a generalised increase in bone formation, there is also a suggestion of altered bone resorption in germline $Y1^{-/-}$ mice as indicated by an increase in osteoclast surface [3]. Whether this is due

to a lack of direct action of Y1 receptors on bone cells is currently unknown. It is possible that the increase in osteoclast surface observed in germline $Y1^{-/-}$ mice is due to altered osteoblastic signalling essential in the control of osteoclast recruitment and activity [23]. Previous *in vitro* studies have shown that NPY can indeed alter osteoclastogenesis by affecting the production of cAMP, RANK-ligand and OPG in mouse bone marrow cells [24,25]. Thus, alterations in osteoblast behaviour due to the lack of Y1 receptors may be responsible for a change in osteoclast activity seen in germline $Y1^{-/-}$ mice. However, here we have shown that the deletion of Y1 receptors solely in mature osteoblasts enhanced osteoblast activity and bone mass without affecting osteoclast surface, osteoclast number or the expression levels of RANK-ligand, OPG or the RANK-ligand to OPG ratio. Consistent with the marked decrease in RANK-ligand expression during osteoblast maturation [26], these data suggest that the elevated activity of the osteoclast lineage observed in germline $Y1^{-/-}$ mice is not due to altered signalling from mature osteoblasts. The stimulation of the osteoclastic lineage in germline $Y1^{-/-}$ mice may instead result from signalling from less mature cells of the osteoblastic lineage, a direct effect of the lack of Y1 receptors on osteoclasts themselves or, as discussed above, an indirect effect caused by the global deletion of the Y1 receptor. Interestingly, it has been previously demonstrated that NPY signalling through the Y1 receptor plays a role in the normal function of macrophages, which, like osteoclasts, are derived from hematopoietic stem cells through the monocyte/macrophage lineage [27]. Therefore, it is conceivable that Y1 receptors are also expressed on osteoclasts where they can respond to NPY signalling and alter bone resorption, however further studies would be needed to support this hypothesis.

A peripheral, osteoblastic role for Y1 receptors in the regulation of bone is consistent with a growing understanding of the direct effects of neural signalling on bone cell activity. Adrenergic [28], glutamatergic [29] and cannabinoid [30] receptors are among those neural signals recently described as directly mediating changes in bone homeostasis. NPY-immunoreactive fibres [31–33] and NPY protein [34] have been shown in bone tissue and it has also been shown that osteoblasts and osteocytes express NPY [11,12] as well as the Y1 receptor [10]. Therefore, osteoblastic Y1 receptors may be mediating the effects of NPY produced from central, local as well as other peripheral sources.

It should also be noted that the gut hormone peptide YY (PYY), another ligand of the NPY system, binds to the Y1 receptor with equal affinity to NPY [35]. PYY has also been shown to play a role in bone homeostasis and may be involved peripherally in the regulation of bone formation through osteoblastic Y1 receptors. Elevated PYY levels have been shown to correlate with diminished BMD in women with anorexia nervosa, a condition that results in significant bone loss [36]. However, analysis of a PYY knockout line found that a lack of PYY led to an osteopenic phenotype with reductions in vertebral cancellous bone mass and bone strength [37]. Clearly, further investigations are needed to delineate the underlying mechanisms in the control of bone mass by PYY and to determine whether PYY regulates bone mass via central and/or peripheral pathways.

This novel model investigated in this study has enabled us to demonstrate a key role for osteoblastic Y1 receptors in the control of bone homeostasis. The results described here show that osteoblast activity was enhanced in the absence of osteoblastic Y1 receptors in male mice. This was sufficient to alter cancellous and cortical bone mass generating a bone phenotype similar to that of germline $Y1^{-/-}$ mice without the hyperinsulinemia and obesity associated with generalised Y1 receptor deficiency. Therefore, these data suggest that osteoblastic Y1 receptors may be a potentially important target for the development of therapeutic approaches to osteoporosis.

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