# Low-dose PTH increases osteoblast activity via decreased *Mef2c/Sost* in senescent osteopenic mice

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## Abstract

Intermittent administration of parathyroid hormone (PTH) 1–34 at a standard dose has been shown to induce anabolic effects in bone. However, whether low-dose PTH promotes bone formation during senescence is unknown. To address this issue, we determined the effects of low-dose PTH and analysed the underlying mechanisms in prematurely senescent mice that display osteopenia. Treatment of 9-week-old *Samp6* mice for 6 weeks with PTH at a standard dose (100 μg/kg per day) increased vertebral and femoral bone mass and improved bone microarchitecture as a result of increased bone-forming surfaces and mineral apposition rate (MAR). At a tenfold lower dose (10 μg/kg per day), PTH increased axial bone volume and trabecular thickness, as detected by bone histomorphometry but not by micro-computed tomography analysis. This anabolic effect resulted from increased osteoblast activity, as reflected by increased serum N-terminal propeptide of type 1 procollagen (P1NP) levels and MAR, with unchanged bone-forming surface or osteoblast surface. Mechanistically, low-dose PTH increased the expression of osteoblast markers in bone marrow stromal cells and mature osteoblasts, which was associated with increased expression of the Wnt effector *Wisp1*. Moreover, low-dose PTH decreased the expression of the *Mef2c* transcription factor, resulting in decreased *Sost* expression in osteoblasts/osteocytes. These results indicate that PTH at a low dose is effective at promoting bone formation and increased bone volume in senescent osteopenic mice through increased osteoblast activity and modulation of specific Wnt effectors, which raises the potential therapeutic use of intermittent PTH at low dose to increase bone forming activity and bone mass in skeletal senescence.

**Key Words**
- low-dose PTH
- senescent mice
- osteoblast activity
- Wnt signalling

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## Introduction

Bone loss associated with ageing is characterised by decreased bone formation relative to bone resorption, resulting in altered bone microarchitecture, osteoporosis and increased risk of fractures (Khosla *et al.* 2011). The age-related decline in bone formation results from multiple intrinsic and extrinsic mechanisms that lead to decreased differentiation of bone marrow stromal cells (BMSCs) into osteoblasts and decreased osteoblast number and activity (Manolagas & Parfitt 2010, Kassem & Marie 2011). One important challenge is therefore to improve the number and functioning of osteoblasts in order to prevent the decrease in bone formation and bone mass in skeletal senescence. Intermittent parathyroid hormone (PTH) administration is the only proved strategy for inducing an anabolic effect that is

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available in clinics for the treatment of severe osteoporosis. This treatment has been shown to increase bone formation to a greater extent than bone resorption in animals and humans, resulting in improved bone mass and microarchitecture (Compston 2007). In adult mice, the anabolic effect of PTH on bone is mediated by increased numbers and activity of osteoblasts (Canalis *et al.* 2007, Jilka 2007). The PTH-induced increase in osteoblast number in adult mice is achieved by increased osteoblast differentiation, by converting quiescent lining cells to active osteoblasts and by reducing osteoblast apoptosis (Jilka 2007, Kim *et al.* 2012). The anabolic effect of PTH is mediated through direct and indirect mechanisms. PTH signals through G-protein-coupled type 1 PTH/PTH-related peptide receptor and subsequent activation of protein kinase A, protein kinase C and other pathways that control osteoblastogenesis (Jilka 2007). In addition, PTH interacts with fibroblast growth factor 2 (Fei *et al.* 2013) and insulin-like growth factor 1 signalling (Canalis *et al.* 1989) promoting bone formation, which shows that the anabolic effect of PTH is mediated in part through interaction with other signalling pathways.

Wnt signalling is an important regulator of osteoblast proliferation, differentiation and survival (Baron & Kneissel 2013). The Wnt canonical pathway involves Wnt binding to the co-receptors LRP5 and Frizzled, leading to the inhibition of glycogen synthase kinase 3β and decreased phosphorylation, stabilisation and subsequent translocation of β-catenin into the nucleus. This results in the binding of β-catenin to T-cell-specific transcription factor/lymphoid enhancer-binding factor 1 transcription factor and activation of target genes (Clevers 2006). The Wnt canonical pathway is negatively regulated by extracellular antagonists such as soluble Frizzled-related proteins (sFRPs), Dickkopf (DKK) and sclerostin that interact with Wnt proteins or Wnt signalling partners to antagonise Wnt signalling (Kawano & Kypta 2003, Bodine *et al.* 2004, Yao *et al.* 2010). Results from several studies have indicated that PTH may interact with Wnt signalling (Kulkarni *et al.* 2005). Specifically, PTH upregulates Wnt-responsive genes in osteoblastic cells (Qin *et al.* 2003, Tobimatsu *et al.* 2006), and PTH1R interacts with the co-receptor LRP6 to increase β-catenin (Wan *et al.* 2008). PTH may also promote Wnt/β-catenin signalling by inhibiting the Wnt antagonists sFRP1 and DKK1 (Bellido *et al.* 2005, Bodine *et al.* 2007, Guo *et al.* 2010). In addition, PTH decreases the expression of *Sost* in osteocytes (Bellido *et al.* 2005, Keller & Kneissel 2005). *Sost* encodes sclerostin, which binds to the Wnt co-receptors LRP4/5/6 and thereby antagonises Wnt/β-catenin signalling (van Bezooijen *et al.* 2004, Li *et al.* 2005).

To date, only a few studies have analysed the anabolic effect of PTH in the ageing skeleton (Knopp *et al.* 2005, Brennan *et al.* 2009, Jilka *et al.* 2010). Moreover, the effect of PTH 1–34 in osteopenic mice has been tested at standard doses ranging from 80 to 100 μg/kg per day, and it is not known whether PTH at a much lower dose (i.e. a tenfold lower dose) may be effective at promoting bone formation in senescent mice. To address this issue, we determined the skeletal response to low-dose PTH in prematurely senescent osteopenic mice and analysed the cellular and molecular mechanisms involved in this response. We show herein that intermittent PTH at a low dose is effective at increasing axial bone mass in prematurely senescent osteopenic mice by promoting osteoblast activity, through modulation of specific Wnt effectors.

## Materials and methods

### Animals and treatment

We used *Samp6* mice, a murine model of ageing in the P6 strain of senescence-accelerated mice which is characterised by decreased bone formation and low bone mass (Jilka *et al.* 1996, Clement-Lacroix *et al.* 2005). Seven-week-old *Samp6* mice (Harlan Laboratories, Derby, UK) were fed with mouse standard diet (A04 rat/mouse diet, SAFE, Augy, France) containing calcium (0.84%), phosphorus (0.57%) and vitamin D (1000 IU/kg), and were weighed once a week. Nine-week-old mice (eight mice per group) were treated with s.c. human PTH 1–34 at a high standard dose (100 μg/kg BW) or a low dose (10 μg/kg BW) (Sigma) or the vehicle, 5 days/week for 6 weeks. The rationale for choosing the low dose is that it is tenfold lower than the dose usually used in osteopenic mice (Knopp *et al.* 2005, Jilka *et al.* 2010, Hanyu *et al.* 2012, Kim *et al.* 2012). To label bone mineralisation fronts, control and treated mice were given tetracycline (20 mg/kg, Sigma) and calcein (10 mg/kg) by s.c. injection, respectively, at days 8 and 3 before being killed by injection of ketamine/xylazine. The protocol was conducted according to the guidelines of the Local Ethical Committee (ref. no. CEEALV/2011.11.01).

### P1NP analysis

The animals were killed and blood was collected and spun in order to recuperate the serum. Serum aliquots were frozen before analysis of N-terminal propeptide of type 1 procollagen (P1NP) levels, an established marker of bone formation, by ELISA (R&D Systems, Lille, France).

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### Bone microarchitecture and histomorphometry

The animals were killed and, lumbar vertebrae and right femurs (distal metaphysis) were obtained for analysis of micro- and macro-structures. The bones were scanned using a high-resolution micro-computed tomography (microCT) system (SkyScan 1172, MicroPhotonics, Allentown, PA, USA) and analysed using a 3D morphometry evaluation program (NRecon reconstruction program). For histomorphometric analysis, the bones were embedded in methylmethacrylate and 5 μm sections were stained with aniline blue to analyse structural parameters (osteoblast surface, bone volume, trabecular number and thickness), as described previously (Haÿ *et al.* 2009). TRAP staining was carried out to evaluate the number of active osteoclasts (Haÿ *et al.* 2009). Unstained sections (8 μm thick) were used to assess dynamic parameters (mineral apposition rate (MAR), double labelled surface and bone formation rate (BFR); (Parfitt *et al.* 1987).

### Quantitative PCR analysis

The animals were killed and the bone marrow was flushed from the tibia, yielding BMSCs and remaining bone containing mature osteoblasts and osteocytes. Total RNA was extracted from the two bone cell pools, and 1 μg of total RNA from each sample was reverse-transcribed using the Applied Biosystems Kit (High-Capacity cDNA RT Kit). The relative mRNA levels of osteoblast differentiation markers and Wnt effectors were evaluated by quantitative PCR analysis (LightCycler; Roche Applied Science) using a SYBR Green PCR Kit (ABGen, Courtabœuf, France) and specific primers (Haÿ *et al.* 2009, Andrews *et al.* 2012, Saidak *et al.* 2012). The signals were normalised to hypoxanthine phosphoribosyltransferase (HRPT) as an internal control.

### Statistical analysis

Values are presented as the mean ± S.E.M. of six to eight animal groups. Data were analysed with the unpaired two-tailed Student's *t*-test. A $P$ value $<0.05$ was considered statistically significant.

## Results

### Low-dose PTH increases bone volume in senescent mice

We first compared the effect of the two doses of PTH on trabecular bone mass and microarchitecture as evaluated by microCT analysis. As it might be expected, intermittent PTH at the standard dose increased vertebral bone mass and volume in *Samp6* mice (Fig. 1A and B). This effect resulted from increased trabecular thickness and number and decreased trabecular separation (Fig. 1C, D and E). Interestingly, the low dose of PTH was sufficient to increase trabecular bone thickness as evaluated by microCT analysis (Fig. 1C). To further investigate the anabolic effect of PTH at low doses, we performed a histomorphometric analysis of vertebral bone. We found that PTH at a low dose increased trabecular bone volume

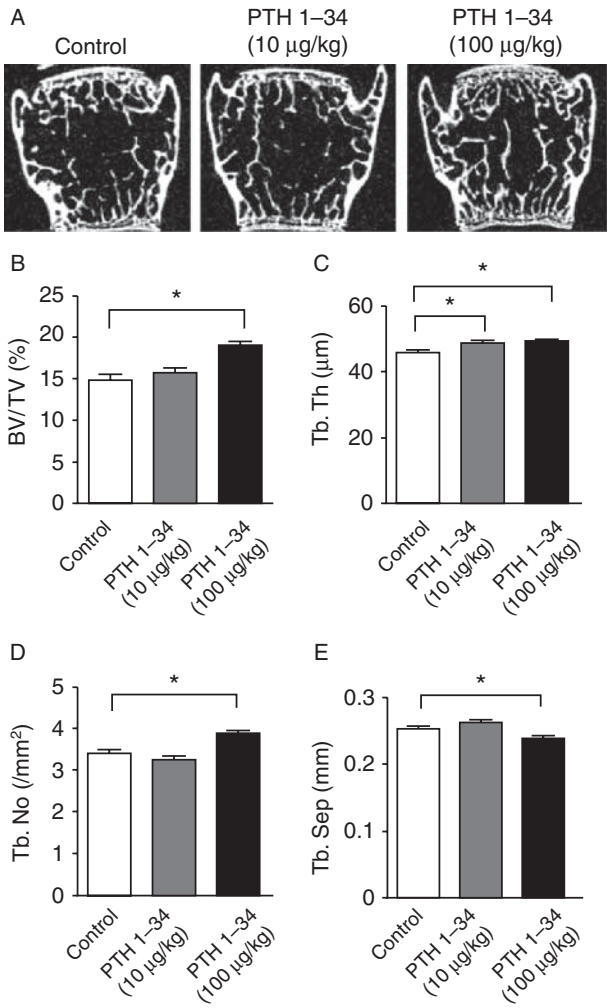


**Figure 1**
Distinct effects of standard and low-dose intermittent PTH on bone mass and microarchitecture in prematurely senescent osteopenic *Samp6* mice. Representative microCT images of lumbar vertebrae (A) showing that both doses of PTH increased bone mass in *Samp6* mice. (B, C, D and E) Analysis of bone microarchitecture parameters showing the distinct effects of PTH at the standard and low doses on bone volume (BV, expressed relative to trabecular volume (TV)), trabecular thickness (Tb. Th), trabecular number (Tb. No) and trabecular separation (Tb. Sep). Mean ± S.D. of six to eight mice. *Statistically significant ($P<0.05$).



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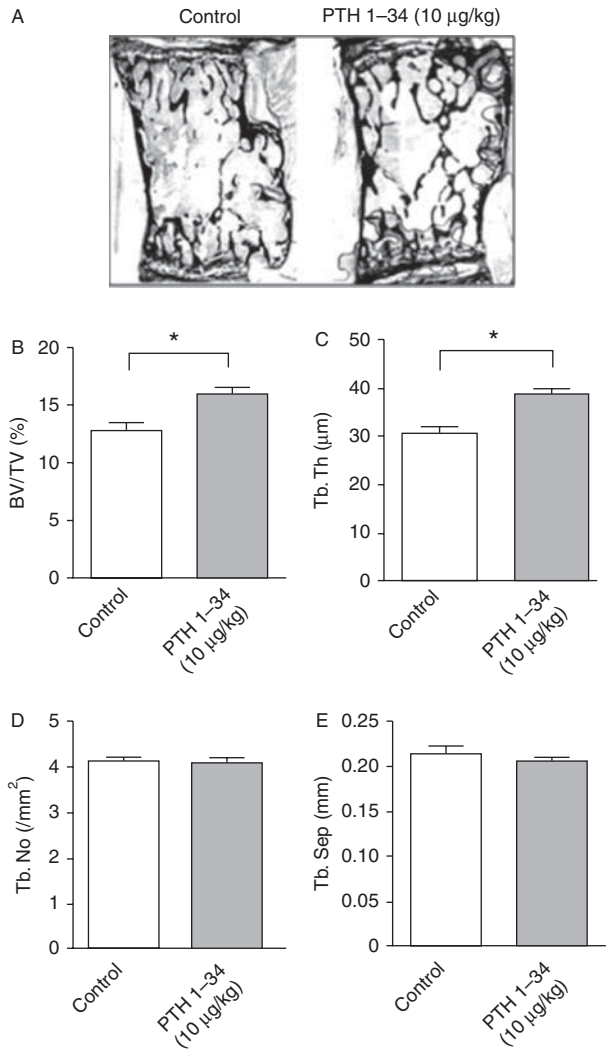


**Figure 2**
Histomorphometric analysis showing that intermittent low-dose PTH increased trabecular bone volume (A and B) and trabecular bone thickness (C) but not trabecular number (D) or separation (E) in vertebral bone in prematurely senescent osteopenic *Samp6* mice. Mean±S.D. of six to eight mice. *Statistically significant ($P<0.05$).

in vertebrae (Fig. 2A and B) as a result of increased trabecular bone thickness (Fig. 2C), with no significant change in trabecular number or separation (Fig. 2D and E). The positive effect on bone volume was restricted to the axial bone because the standard dose, but not the low dose, increased bone volume and trabecular thickness and number and decreased trabecular separation in the femur (Supplementary Fig. 1A, B, C and D, see section on supplementary data given at the end of this article). The increased axial bone volume induced by low-dose PTH was not related to changes in bone resorption, because the trabecular separation was not affected (Figs 1 and 2) and the number of $TRAP^+$ osteoclasts was unchanged (Supplementary Fig. 2A). These results indicate that PTH at a low dose is effective at increasing trabecular bone thickness and bone volume in the axial skeleton in senescent osteopenic *Samp6* mice.

### Low-dose PTH increases osteoblast function but not number in senescent mice

To determine whether the low-dose PTH increased bone mass by increasing bone formation, we analysed the levels of serum P1NP, an established marker of bone-forming activity. Both the standard-dose and the low-dose PTH increased serum P1NP levels, indicating an anabolic response at both doses (Fig. 3). To confirm that the low dose of PTH was efficient at promoting trabecular bone formation, histomorphometric analysis was performed in axial bone. PTH at low dose had no effect on the extent of double-labelled surface (Fig. 4A). Consistently, the osteoblast surface, which reflects osteoblast number, was unchanged (Supplementary Fig. 2B). In contrast, the low-dose PTH increased the bone MAR, which reflects the bone-forming activity (Fig. 4B) without change in the BFR (Fig. 4C). These effects were not restricted to axial bone because similar results were obtained in the femur (Supplementary Fig. 3, see section on supplementary data given at the end of this article). In contrast, the standard high-dose PTH increased bone MAR, double-labelled surface and BFR by twofold (Supplementary Fig. 3). These results indicate that PTH at a low dose is effective at increasing bone formation in senescent osteopenic mice by increasing the activity but not the number of active osteoblasts.

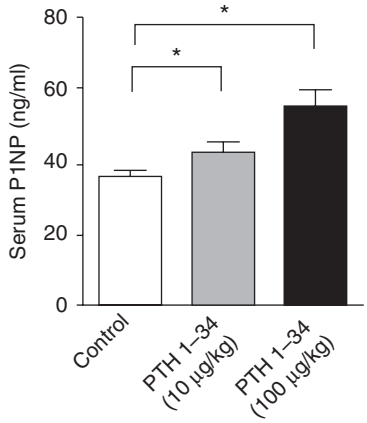


**Figure 3**
Intermittent PTH at low and standard doses increased serum P1NP levels in prematurely senescent osteopenic *Samp6* mice after 6 weeks of treatment. Mean±S.D. of six to eight mice. *Statistically significant ($P<0.05$).



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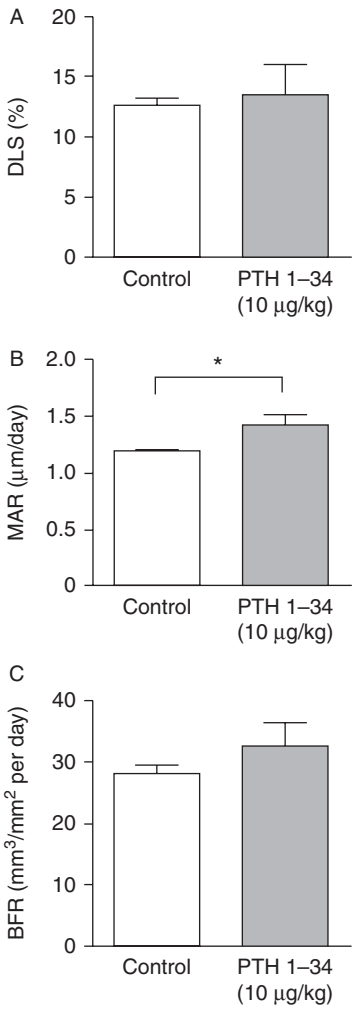


**Figure 4**
Intermittent low-dose PTH increased osteoblast activity but not bone forming surface in the vertebrae of prematurely senescent osteopenic *Samp6* mice. Low-dose PTH had no effect double-labelled surface (DLS) (A) or bone formation rate (BFR) (C) but increased the mineral apposition rate (MAR) (B). Mean ± S.D. of six to eight mice. *Statistically significant ($P<0.05$).

## Low-dose PTH increases functional osteoblast markers in senescent mice

To determine the mechanisms by which the low-dose PTH increased osteoblast activity, we performed a molecular analysis of genes that characterise osteoblast function in osteoprogenitor cells present in the bone marrow stroma. At a low dose, PTH was effective at increasing the expression of alkaline phosphatase (*Alp*) and type 1 collagen (*Col1a1*) and tended to increase the expression of osteocalcin (*Oc*), a marker of mature osteoblasts (Fig. 5A–C). In the bone-marrow-free tibia that contains mature osteoblasts and osteocytes, *Alp* and *Col1a1* levels were not affected by low-dose PTH, whereas *Oc* was markedly increased (Fig. 5A–C). These results indicate that PTH at low dose acted by promoting osteoblast function in *Samp6* mice.

## Low-dose PTH modulates specific Wnt effectors in senescent mice

We next determined the molecular mechanisms underlying the positive effect of the low-dose PTH on osteoblast activity in *Samp6* mice by determining the changes in direct Wnt effectors (Clevers 2006, Gordon & Nusse 2006). We first analysed the effect of low-dose PTH on

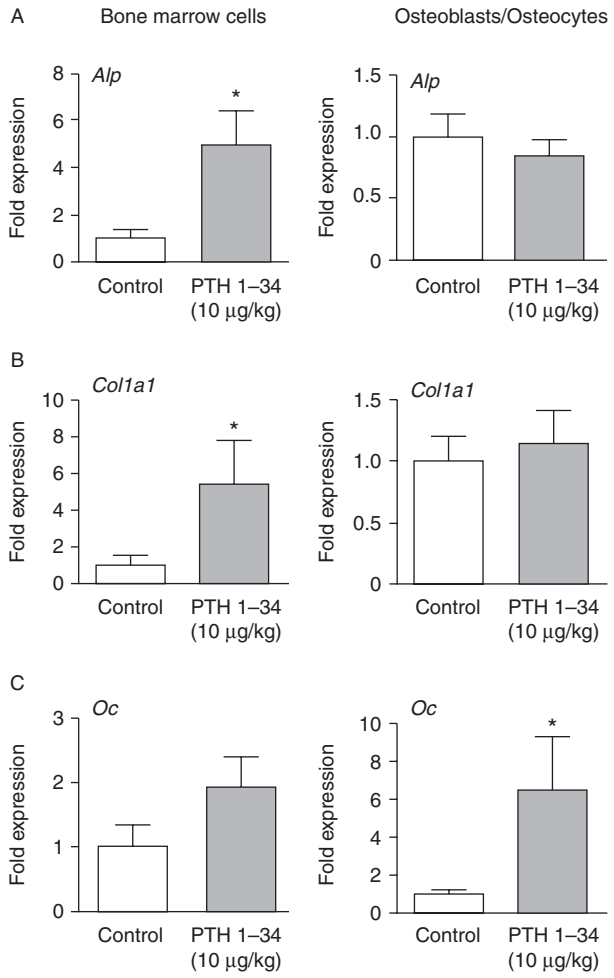


**Figure 5**
Intermittent low-dose PTH increased expression of the osteoblast differentiation markers *Alp* (A), *Col1a1* (B) and *Oc* (C) in prematurely senescent osteopenic *Samp6* mice. Quantitative RT-PCR analysis of osteoblast gene expression was carried out in the bone marrow stromal cells and osteoblasts/osteocytes extracted from long bones in control *Samp6* mice or mice treated with low-dose PTH for 6 weeks. The transcript levels were normalized to the values for HPRT. Mean ± S.D. of six to eight mice. *Statistically significant ($P<0.05$).



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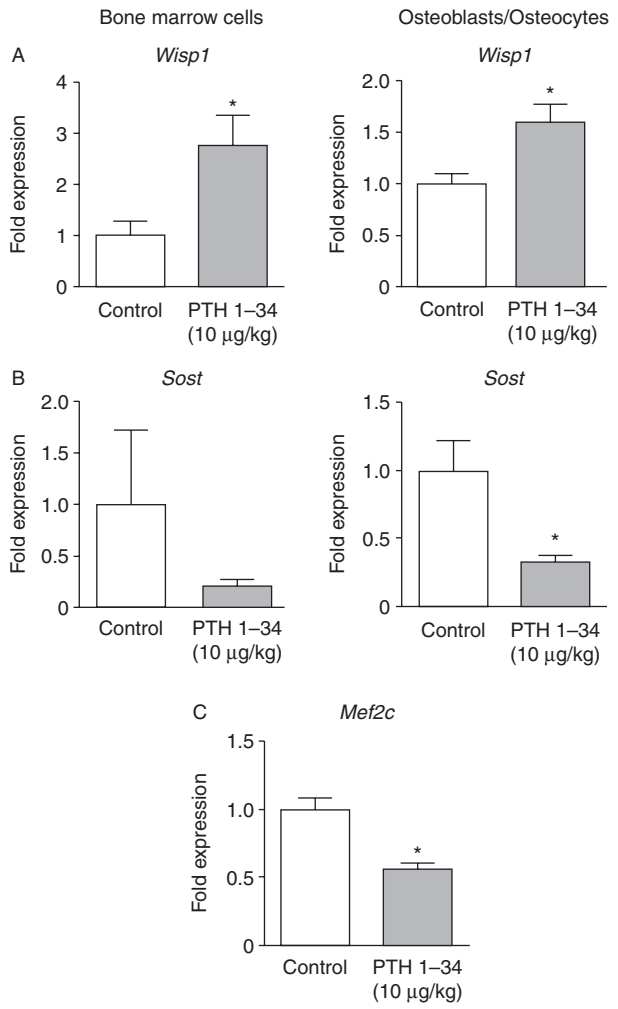


**Figure 6**
Intermittent low-dose PTH modulated Wnt signalling effectors *Wisp1* (A), *Sost* (B) and *Mef2c* (C) in prematurely senescent osteopenic *Samp6* mice. Quantitative RT-PCR analysis of Wnt effectors was carried out using bone marrow stromal cells and osteoblasts/osteocytes extracted from long bones (or pooled populations for *Mef2c*) of control *Samp6* mice or mice treated with PTH at a low dose for 6 weeks. The transcript levels were normalized to the values for HPRT. Mean ± S.D. of six to eight mice. *Statistically significant ($P<0.05$).

Wnt-induced secreted protein 1 (*Wisp1*) because it is a direct target of canonical Wnt signalling (Ono *et al.* 2011). The low-dose PTH increased *Wisp1* mRNA levels in both BMSCs and osteoblasts/osteocytes, indicating that Wnt signalling was activated in both osteoblast precursor cells and more mature osteoblasts/osteocytes (Fig. 6A).

Wnt signalling is negatively regulated by inhibitors such as DKK1 and sFRP1 (Bodine *et al.* 2004). To investigate whether low-dose PTH acts by downregulating these Wnt inhibitors, we analysed the changes in *Dkk1* and *sFrp1* gene expression in bones of *Samp6* mice. We found that low-dose PTH increased the expression of *Dkk1* in both BMSCs and osteoblasts/osteocytes (Supplementary Fig. 4A, see section on supplementary data given at the end of this article). In contrast, *sFrp1* mRNA levels remained unchanged (Supplementary Fig. 4B). This indicates that these Wnt antagonists were distinctly affected by low-dose PTH in *Samp6* mice. Importantly, we found that *Sost* mRNA levels tended to be reduced in BMSCs and were greatly reduced by PTH at a low dose in osteoblasts/osteocytes (Fig. 6B). To determine how the low-dose PTH may affect *Sost* expression in *Samp6* mice, we analysed the expression of *Mef2c* which upregulates *Sost* expression (Leupin *et al.* 2007, Kramer *et al.* 2010) and is regulated by PTH *in vitro* (Bonnet *et al.* 2012). We found that *Mef2c* in bones was decreased by low-dose PTH, which provides a mechanism by which low-dose PTH acts on *Sost* expression and promotes osteoblast activity in osteopenic senescent *Samp6* mice (Fig. 6C).

Overall, these results provide the first evidence that low-dose PTH treatment in *Samp6* mice is effective for increasing bone mass in the axial skeleton by increasing osteoblast activity, but not cell number, via upregulation of *Wisp1* and downregulation of *Mef2c/Sost* in bone (Fig. 7).

## Discussion

Intermittent PTH at a standard dose is known to produce an anabolic effect on bone in adult animals. However, it is not known whether a low dose (i.e. tenfold lower than the

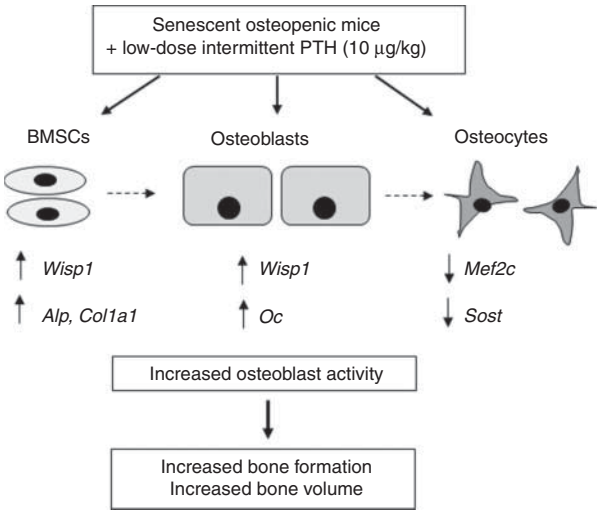


**Figure 7**
Proposed mechanism of the anabolic effect of low-dose PTH in prematurely senescent osteopenic *Samp6* mice. Low-dose PTH increased osteoblast activity, but not number, by modulating specific Wnt effectors, resulting in increased bone formation and axial bone volume in senescent osteopenic *Samp6* mice.



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usual dose used) is effective in osteopenic ageing mice. In this study, we showed that low-dose PTH is efficient at increasing bone formation and axial bone volume in senescent osteopenic mice, and that this anabolic effect results from increased osteoblast activity and modulation of specific Wnt effectors. First, we showed that PTH treatment at a low dose increased trabecular bone thickness and volume in axial bone in *Samp6* mice, demonstrating that a low dose of PTH is sufficient to augment bone mass in senescent osteopenic mice. This effect was evidenced by histomorphometric but not microCT analysis. This discrepancy is probably due to the distinct resolution between the two techniques. Second, the results indicate that the increased bone volume induced by low-dose PTH results from increased osteoblast activity, as shown by the increased PINP levels and MAR. In contrast, the osteoblast surface and double-labelled surfaces were unchanged, indicating that low-dose PTH acted by increasing osteoblast activity, but not the number of active osteoblasts, in *Samp6* mice. This is in contrast with the effect produced by PTH at a standard dose, which increased the double-labelled bone forming surface in *Samp6* mice (this study), as has been found in WT ageing mice (Jilka 2007). These results support the notion that PTH at a low dose is effective at increasing bone volume in senescent osteopenic mice through enhanced osteoblast activity.

In WT mice, the anabolic effect of PTH at a standard dose results from activation of multiple signals (Canalis *et al*. 2007, Jilka 2007). In prematurely senescent osteopenic mice low-dose PTH increased the expression of *Alp* and *Col1a1*, which are targets of Wnt signalling (Gaur *et al*. 2005, Bodine & Komm 2006). Consistent with this finding, low-dose PTH increased the expression of *Wisp1*, a direct target of Wnt signalling, as has been also previously reported for WT mice treated with a standard dose of PTH (Jilka *et al*. 2010). WISP1 is a member of the CCN family that is upregulated by β-catenin (Xu *et al*. 2000) and that positively regulates bone formation (French *et al*. 2004, Ono *et al*. 2011). Our findings that low-dose PTH increased *Wisp1* expression in both BMSCs and osteoblasts/osteocytes therefore reveals one mechanism for the anabolic effect of low-dose PTH in senescent mice. We also found that low-dose PTH increased the expression of *Dkk1* in BMSCs and osteoblasts/osteocytes. This is surprising because DKK1 antagonises canonical Wnt signalling *in vivo* by binding to Wnt ligands, leading to the attenuation of Wnt/receptor activation and bone formation (Bovolenta *et al*. 2008). The increased expression of *Dkk1* by low-dose PTH is likely to result secondarily from the activation of Wnt signalling which is known to increase *Dkk1* expression (Chamorro *et al*. 2005). This is consistent with a negative-feedback response restricting the exposure of bone cells to prolonged activation of Wnt signalling. The induction of *Dkk1* expression is unlikely to reduce the overall response to low-dose PTH in senescent *Samp6* mice because, although the suppression of Wnt signalling by DKK1 was found to attenuate PTH-mediated stromal cell response and new bone formation (Guo *et al*. 2010), targeted overexpression of *Dkk1* in osteoblasts does not impair the anabolic response to PTH in mice (Yao *et al*. 2011). In contrast to *Dkk1*, the level of *sFrp1*, a negative regulator of bone formation (Yao *et al*. 2010), was unchanged by low-dose PTH in *Samp6* mice. Results from previous studies have indicated that the bone anabolic effect of PTH is attenuated by either overexpression or deletion of *sFrp1* in mice (Bodine *et al*. 2007). The lack of change in *sFrp1* expression in PTH-treated *Samp6* senescent mice is thus unlikely to affect the anabolic effect of low-dose PTH in these mice.

Our finding that PTH at a low dose markedly decreased *Sost* levels in osteoblasts/osteocytes provides another mechanism for the anabolic effect of low-dose PTH in senescent *Samp6* mice. The decreased *Sost* level with a low-dose of PTH in *Samp6* mice is consistent with the effect of PTH at a standard dose in WT mice (Bellido *et al*. 2005, Keller & Kneissel 2005). *Sost* expression in osteocytes has been shown to be positively regulated by the transcription factor MEF2C (Leupin *et al*. 2007). Recent results have indicated that *Mef2c* invalidation results in reduced *Sost* expression and increased bone mass in mice (Kramer *et al*. 2010, 2012, Collette *et al*. 2012). In this study, we found that PTH at a low dose decreased *Mef2c* levels which was associated with decreased *Sost* levels, increased osteoblast activity and bone volume in senescent osteopenic *Samp6* mice. Our data thus support the notion that the anabolic response to low-dose PTH involves downregulation of *Mef2c* and subsequent reduction of *Sost*, leading to increased Wnt/β-catenin signalling, osteoblast activity and bone mass in senescent osteopenic mice (Fig. 7). The finding that PTH at low dose is able to modulate specific Wnt signalling effectors in *Samp6* mice is particularly important in the context of skeletal senescence because results from current studies indicate that bone ageing is associated with alterations in the local expression of Wnt ligands (Rauner *et al*. 2008) among other mechanisms (Manolagas & Parfitt 2010, Marie 2014). We previously reported that the anti-osteoporotic agent strontium ranelate promotes Wnt signalling in osteoblasts and



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thereby increases bone formation and bone mass in senescent osteopenic *Samp6* mice (Saidak *et al*. 2012). The results of the present study support the interest in targeting Wnt signalling for improving osteoblast activity and bone mass in skeletal ageing.

In summary, our results reveal that PTH at a low dose is effective at increasing bone volume in senescent osteopenic mice through activation of osteoblast activity and modulation of specific Wnt effectors, which supports the potential therapeutic interest in using intermittent PTH at a low dose for improving bone formation and bone mass in skeletal senescence.

**Supplementary data**

This is linked to the online version of the paper at http://dx.doi.org/10.1530/JOE-14-0249.

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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**Author contribution statement**

Z S and P J M designed the experiments, Z S, C L H, S A and C M performed the experiments and Z S and P J M wrote the manuscript.

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