Aging Cell (2011) **10,** pp962–971 Doi: 10.1111/j.1474-9726.2011.00735.x

Age-related decline in osteoblastogenesis and 1α-hydroxylase/CYP27B1 in human mesenchymal stem cells: stimulation by parathyroid hormone

Shuo Geng,[1,2] Shuanhu Zhou[1] and Julie Glowacki[1]

[1]Department of Orthopedic Surgery, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA
[2]Department of Orthopedic Surgery, The First Affiliated Hospital of Harbin Medical University, Harbin Medical University, Harbin, Heilongjiang, China

Summary

With aging, there is a decline in bone mass and in osteoblast differentiation of human mesenchymal stem cells (hMSCs) *in vitro*. Osteoblastogenesis can be stimulated with 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] and, in some hMSCs, by the precursor 25-hydroxyvitamin D_3 ($25OHD_3$). CYP27B1/1α-hydroxylase activates $25OHD_3$ and, to a variable degree, hMSCs express CYP27B1. In this study, we tested the hypotheses (i) that age affects responsiveness to $25OHD_3$ and expression/activity of CYP27B1 in hMSCs and (ii) that parathyroid hormone (PTH) upregulates CYP27B1 in hMSCs, as it does in renal cells. There were age-related declines in osteoblastogenesis ($n = 8$, $P = 0.0286$) and in CYP27B1 gene expression ($n = 27$, $r = -0.498$; $P = 0.008$) in hMSCs. Unlike hMSCs from young subjects (≤50 years), hMSCs from older subjects (≥55 years) were resistant to $25OHD_3$ stimulation of osteoblastogenesis. PTH1-34 (100 nM) provided hMSCs with responsiveness to $25OHD_3$ ($P = 0.0313$, Wilcoxon matched pairs test) and with two episodes of increased $1,25(OH)_2D_3$ synthesis, of cAMP response element binding protein (CREB) activation, and of CYP27B1 upregulation. Both increases in CYP27B1 expression by PTH were obliterated by CREB-siRNA or KG-501 (which specifically inhibits the downstream binding of activated CREB). Only the second period of CREB signaling was diminished by AG1024, an inhibitor of insulin-like growth factor-I receptor kinase. Thus, PTH stimulated hMSCs from elders with responsiveness to $25OHD_3$ by upregulating expression/activity of CYP27B1 and did so through CREB and IGF-I pathways.

Key words: CYP27B1; marrow stromal cells; mesenchymal stem cells; PTH; signaling; vitamin D.

Introduction

In humans, peak bone mass is attained during the third decade of life. With advancing age, there is a decline in bone mass and an increase in fracture risk (Hui *et al.*, 1988). Human bone marrow contains cells, called human mesenchymal stem cells or marrow stromal cells (hMSCs), that are progenitors of several lineages, including osteoblasts, chondrocytes, and adipocytes (Prockop, 1997; Pittenger *et al.*, 1999). We and others, however, showed that there is an age-related decline in osteoblast potential in hMSCs (D'Ippolito *et al.*, 1999; Mueller & Glowacki, 2001).

In vitro, the differentiation of hMSCs to osteoblasts is enhanced by 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$], the activated form of vitamin D_3 (Liu *et al.*, 1999). We recently reported that osteoblast differentiation was also stimulated by 25-hydroxyvitamin D_3 ($25OHD_3$) in some hMSCs (Zhou *et al.*, 2010). That led to the discovery that hMSCs have the capacity to enzymatically activate $25OHD_3$ to $1,25(OH)_2D_3$ with CYP27B1 (the gene that encodes the enzyme 1α-hydroxylase) (Zhou *et al.*, 2010). We reported that the constitutive level of expression of CYP27B1 in hMSCs was related to the vitamin D status of the subject from whom the cells were obtained and can be upregulated *in vitro* by the substrate $25OHD_3$ as well as by insulin-like growth factor-I (IGF-I) (Zhou *et al.*, 2010), but effects of age were not determined. Subsequently, we reported that experimental reduction of CYP27B1 by ketoconazole or CYP27B1-siRNA in hMSCs from young subjects prevented the osteoblastogenic response to $25OHD_3$ (Geng *et al.*, 2011). Those data provided evidence that 1α-hydroxylation is required for prodifferentiation effects of $25OHD_3$. Thus, one goal of this study was to assess the effects of age on the expression/activity of CYP27B1 and on the stimulation of osteoblast differentiation by $25OHD_3$.

Parathyroid hormone (PTH) peptides have been used clinically as osteoanabolic therapies for osteoporosis and fracture prevention (Neer *et al.*, 2001; Lane & Silverman, 2010). *In vivo* and *in vitro* evidence indicates that PTH induces IGF-I (Canalis *et al.*, 1989; Pfeilschifter *et al.*, 1995; Watson *et al.*, 1995; Shinoda *et al.*, 2010). We determined that PTH peptides upregulated both IGF-I and IGF-II in hMSCs (Zhou *et al.*, 2011) and that rhIGF-I induced CYP27B1 expression and 1α-hydroxylase activity in hMSCs (Zhou *et al.*, 2010). Recently, Jilka *et al.* (2010) showed that PTH has greater bone anabolic effects in older mice because in addition to its stimulation of bone formation, it antagonized the age-associated increase in oxidative stress and adverse effects on birth and survival of osteoblasts. Further, PTH *in vitro* (50 nM) protected osteoblasts from acute oxidative-stress-related effects. We recently demonstrated by genetic and pharmacological means that some effects of age on hMSCs were reproduced by experimental blocking of PTH signaling (Zhou, 2011). In addition, PTH is the major stimulus for renal production of $1,25(OH)_2D_3$ (Haussler *et al.*, 1976; Brenza *et al.*, 1998; Brenza & DeLuca, 2000). This reasoning suggested the possibility that PTH could restore functions of human MSCs.

In this study, we tested the hypotheses (i) that age affects responsiveness to $25OHD_3$ and expression/activity of CYP27B1 in hMSCs and (ii) that PTH could stimulate hMSCs from older subjects with responsiveness to $25OHD_3$ by upregulating the expression/activity of CYP27B1, as it does in renal cells. Further, we sought to identify the intermediary roles of CREB and IGF-I and to determine whether the effects of age on vitamin D metabolism in hMSCs could be corrected with PTH.

Correspondence
Julie Glowacki, PhD, Department of Orthopedic Surgery, Brigham and Women's Hospital, 75 Francis Street, Boston, MA 02115, USA. Tel.: +1(617) 732 5397; fax: +1(617) 732 6937; e-mail: jglowacki@rics.bwh.harvard.edu

Accepted for publication *22 July 2011*

© 2011 The Authors
Aging Cell © 2011 Blackwell Publishing Ltd/Anatomical Society of Great Britain and Ireland

Results

Age-related decline in osteoblastogenesis and CYP27B1 gene expression in hMSCs

As a test of reproducibility of previous findings, we evaluated osteoblast potential in hMSCs from four young (≤ 50 years, mean age 36 ± 14 years) and four older (≥ 55 years, mean age 74 ± 4 years) subjects. After 7 days in osteoblastogenic medium, the mean level of alkaline phosphatase enzymatic activity (ALP activity, Fig. 1A) in hMSCs from older subjects (57 ± 17 μmol min^{-1} g^{-1} protein) was 23% of that for hMSCs from young subjects (253 ± 35 μmol min^{-1} g^{-1} protein, $P = 0.0286$, Mann–Whitney test).

A larger cohort of hMSCs obtained from 27 subjects (41–87 years, mean age 57 ± 12 years) was used to determine the effect of age on constitutive expression of CYP27B1/1α-hydroxylase. There was an inverse correlation between CYP27B1 expression and age ($r = -0.498$; $P = 0.008$; Fig. 1B). The mean level of expression of CYP27B1 in the older group (≥55 years, $n = 15$, $P = 0.007$) was 56% of that for the younger group (≤ 50 years, $n = 12$, Fig. 1B inset). Another series of hMSCs was available from 14 subjects (64 ± 12 years) for whom serum 25OHD levels had been determined. Trios of young and old hMSCs from subjects known to be vitamin D-sufficient (serum 25OHD > 32 ng mL^{-1}) were selected for further studies (Fig. 1C). There was lower constitutive expression of CYP27B1 in hMSCs from the older than from the young subjects, with similar expression of 24-hydroxylase/CYP24A1, parathyroid hormone receptor type 1 (PTHR1), and vitamin D receptor (VDR).

PTH1-34 stimulated the effects of $25OHD_3$ on osteoblastogenesis in hMSCs from elders

In hMSCs from young subjects (≤ 50 years), osteoblast differentiation, as assessed by osteoblast signature genes, bone sialoprotein (BSP), and alkaline phosphatase (ALP), was stimulated by $25OHD_3$, $1,25(OH)_2D_3$, PTH1-34, and the combination treatment (Fig. 2A). In contrast, hMSCs from elders (≥ 55 years) were refractory to $25OHD_3$ and were stimulated by $1,25(OH)_2D_3$, PTH1-34, and the combination treatment. Three samples from elders were not responsive to $25OHD_3$ (Fig. 2B; ALP/glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 0.95 ± 0.12, fold relative to control), compared with three samples from young subjects (1.95 ± 0.40, $P = 0.05$). With the numbers available, the effects of $1,25(OH)_2D_3$ ($P = 0.40$) and combination treatments ($P > 0.999$) were statistically similar for the two age groups. For the hMSCs from elders, the combination of PTH and 25OHD resulted in greater ALP expression (2.74 ± 0.50) than for 25OH alone (0.95 ± 0.12, $P = 0.050$). Results for BSP expression were similar.

Osteoblast differentiation was also quantified by ALP enzymatic activity 7 days after transfer to osteoblastogenic medium (Fig. 2C). The ALP activity in hMSCs from an older subject (83 Y) was 48% of that for hMSCs from a young subject (42 Y) ($P < 0.0001$). In hMSCs from a young subject (42 Y), there was equivalent stimulation of osteoblast differentiation by $25OHD_3$ (1.50-fold, compared with control level, $P < 0.001$) and by $1,25(OH)_2D_3$ (1.63-fold, $P < 0.001$). In striking contrast, hMSCs (83 Y) were not stimulated by $25OHD_3$ (1.03-fold), yet they were stimulated by $1,25(OH)_2D_3$ (1.51-fold, $P < 0.001$). PTH1-34 stimulated osteoblast differentiation in both specimens (42 Y: 1.82-fold, $P < 0.001$; and 83 Y: 1.79-fold, $P < 0.001$). Pretreatment of the hMSCs (42 Y) with PTH1-34 stimulated differentiation with $25OHD_3$ (2.22-fold, $P < 0.001$) and with $1,25(OH)_2D_3$ (2.45-fold, $P < 0.001$). There was 48% more ALP with $25OHD_3$ and PTH pretreatment than without ($P < 0.001$), and there was

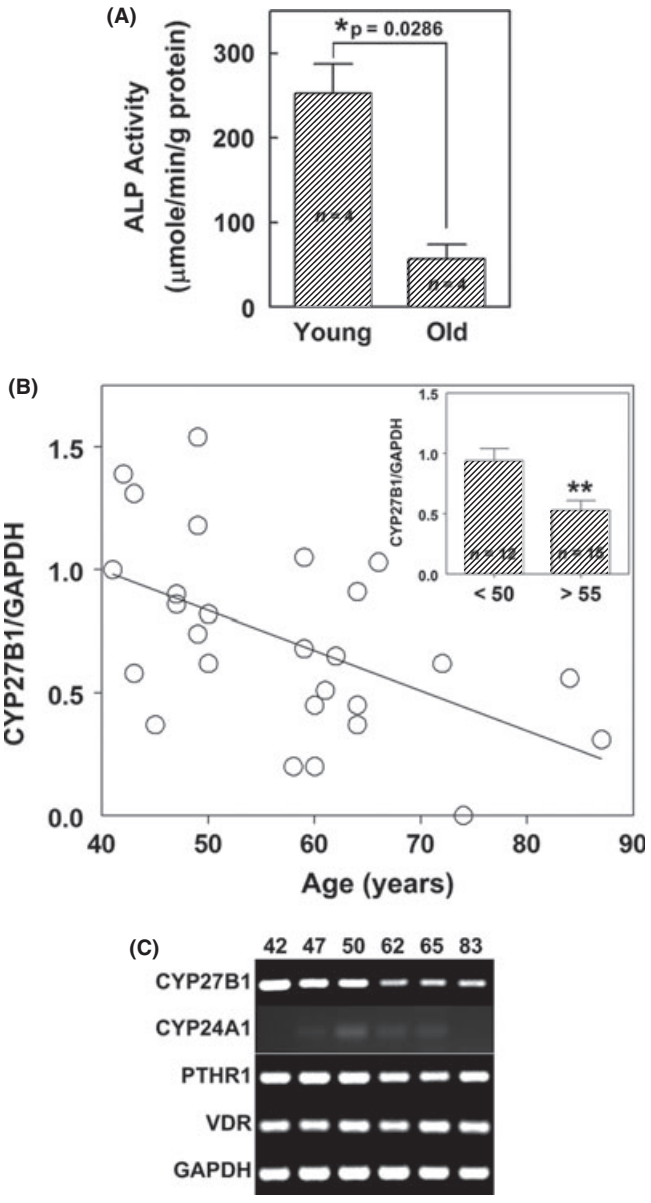


Fig. 1 Age-related decline in osteoblast potential and in constitutive expression of CYP27B1 in human mesenchymal stem cells (hMSCs). (A) The development of alkaline phosphatase enzymatic activity in hMSCs from four young (< 50 years, mean age 36 ± 14 years) and four old (> 55 years, mean age 74 ± 4 years) subjects was determined. The hMSCs from young and old subjects were incubated in 1% fetal bovine serum-heat inactivated osteogenic medium for 7 days. Results are expressed as mean ± SEM (*$P = 0.0286$, *Mann–Whitney test*). (B) The constitutive expression of CYP27B1 in hMSCs from 27 subjects (from 41 to 87 years, mean age 57 ± 12 years) was correlated with age (Spearman $r = -0.498$; $P = 0.008$). (Inset) the expression of CYP27B1 in hMSCs from young (< 50 years, $n = 12$) and old (> 55 years, $n = 15$) subjects was determined, with each value normalized to GAPDH. Results are expressed as mean ± SEM (*t*-test, **$P = 0.007$). (C) Gel electrophoretogram shows RT–PCR products of CYP27B1, CYP24A1, PTHR1, VDR, and GAPDH in six hMSCs from three young (42, 47, 50 years of age) and three old (62, 65, 83 years of age) subjects.

50% more ALP with $1,25(OH)_2D_3$ and PTH pretreatment than without ($P < 0.001$). It was striking that ALP in hMSCs (83 Y) was stimulated by $25OHD_3$ after pretreatment with PTH1-34 (2.23-fold, $P < 0.001$), with a magnitude equivalent to stimulation by $1,25(OH)_2D_3$ (2.44-fold,

© 2011 The Authors
Aging Cell © 2011 Blackwell Publishing Ltd/Anatomical Society of Great Britain and Ireland

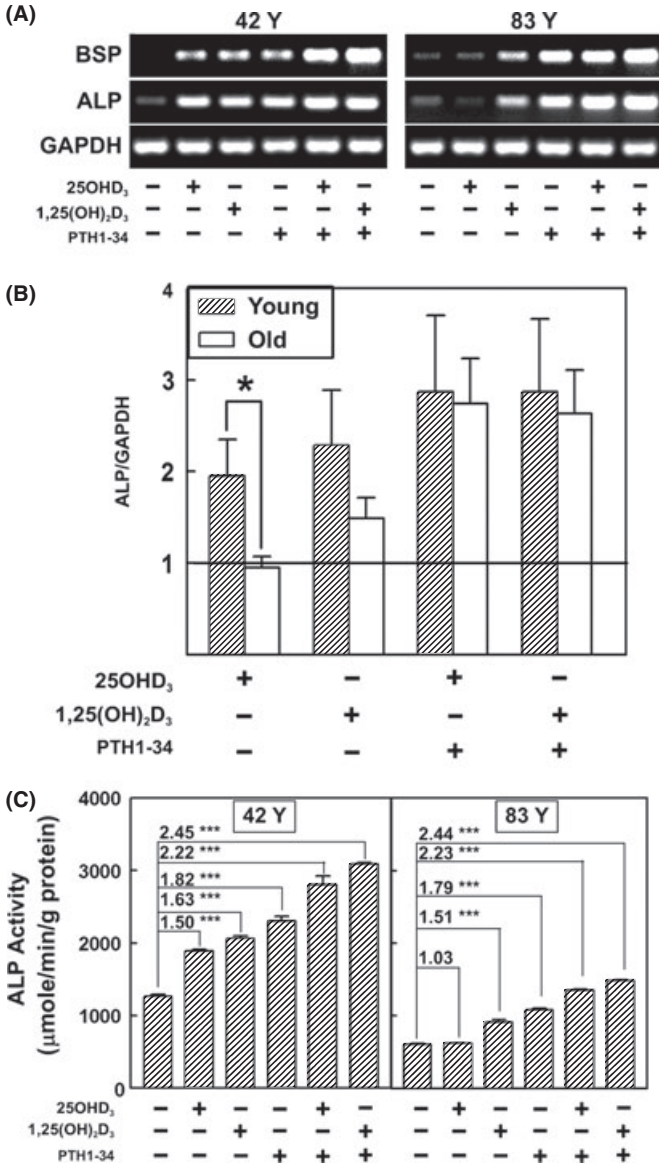


Fig. 2 Age-related effects of vitamin D metabolites and parathyroid hormone (PTH) on osteoblast differentiation. (A) Gel electrophoretograms of RT–PCR products for representative human mesenchymal stem cells (hMSCs) from a 42-year-old (42 Y) and an 83-year-old subject (83 Y) show bone sialoprotein (BSP), alkaline phosphatase (ALP), and GAPDH in the absence or presence of 25-hydroxyvitamin D_3 ($25OHD_3$) (10 nM), $1,25(OH)_2D_3$ (10 nM), PTH1-34 (100 nM) or preincubated with PTH1-34 for 12 h followed by $25OHD_3$ or $1,25(OH)_2D_3$ treatment in osteoblastogenic medium (1% fetal bovine serum-heat inactivated [FBS-HI]) for 3 days. (B) Comparison of expression of alkaline phosphatase (ALP/GAPDH) in hMSCs from young ($n = 3$) and old ($n = 3$) subjects in the absence or presence of $25OHD_3$ (10 nM), $1,25(OH)_2D_3$ (10 nM) or preincubated with PTH1-34 for 12 h followed by $25OHD_3$ or $1,25(OH)_2D_3$ treatment in osteoblastogenic medium (1% FBS-HI) for 3 days. Results are expressed as mean ± SEM for each treatment compared with control (*$P = 0.05$). (C) Alkaline phosphatase enzymatic activity (ALP activity, three replicate wells) was measured after 7 days in osteoblastogenic medium (1% FBS-HI) in two hMSCs (42 Y and 83 Y) in the absence or presence of $25OHD_3$ (10 nM), $1,25(OH)_2D_3$ (10 nM), PTH1-34 (100 nM) or preincubated with PTH1-34 for 12 h followed by $25OHD_3$ or $1,25(OH)_2D_3$ treatment. Results are expressed as mean ± SEM. The fold differences in treatments compared with corresponding control level (in 0.1% BSA in 1% FBS-HI osteogenic medium) are shown (ANOVA, ***$P < 0.001$).

$P < 0.001$). In these cells, there was 118% more ALP stimulated by $25OHD_3$ with PTH pretreatment than without ($P < 0.001$), and there was 62% more ALP stimulated by $1,25(OH)_2D_3$ with PTH pretreatment than without ($P < 0.001$).

In addition, osteoblast differentiation with combined treatments was greater than with PTH1-34 alone. With hMSCs (42 Y), ALP levels with PTH1-34 and $25OHD_3$ (122%, $P < 0.001$) and with PTH1-34 and $1,25(OH)_2D_3$ (134%, $P < 0.001$) were greater than PTH1-34 alone. Likewise, with hMSCs (83 Y), PTH1-34 with $25OHD_3$ (125%, $P < 0.001$) and PTH1-34 with $1,25(OH)_2D_3$ (136%, $P < 0.001$) were greater than PTH1-34 alone. For the hMSCs from the elder (83 Y), the combined PTH and $25OHD_3$ treatment resulted in ALP activity (1350 ± 17 μmol min^{-1} g^{-1} protein) similar to control level of hMSCs (42 Y) (1262 ± 25 μmol min^{-1} g^{-1} protein, 0.1% BSA in 1% fetal bovine serum-heat inactivated (FBS-HI) osteogenic medium).

These unexpected results indicate that PTH pretreatment provided hMSCs from older subjects with responsiveness to $25OHD_3$. These cells were used in subsequent experiments to determine the mechanism by which PTH 'restores' responsiveness to $25OHD_3$ in hMSCs.

PTH1-34 upregulated 1α-hydroxylase activity and expression in hMSCs from elders

We tested the effects of pretreatment with PTH1-34 on 1α-hydroxylase enzymatic activity (*in vitro* synthesis of $1,25(OH)_2D_3$) in hMSCs from young and old subjects. In baseline conditions, there was greater synthesis of $1,25(OH)_2D_3$ in hMSCs (42 Y: 4320 ± 146 $fmol^{-1}$ mg^{-1} protein per hour) than in hMSCs from an older subject (83 Y: 1593 ± 52, $P = 0.0011$, *t*-test with Welch correction, Fig. 3A). Pretreatment with 100 nM PTH1-34 for 1–12 h had little effect on the synthesis of $1,25(OH)_2D_3$ in hMSCs (42 Y), but for hMSCs (83 Y), there was 63% more at 4 h ($P < 0.001$) and 251% more at 12 h ($P < 0.001$, Fig. 3A). Synthesis of $1,25(OH)_2D_3$ in hMSCs (83 Y) after 12 h exposure to PTH (5589 ± 162 fmol mg^{-1} protein per hour) was similar to that synthesized by hMSCs from a younger subject (42 Y; 4407 ± 181, $P = 0.0011$, *t*-test with Welch correction, Fig. 3A). There was the appearance of two episodes of stimulation, with an early (4–6 h) and a later (12 h) greater increase in the synthesis of $1,25(OH)_2D_3$. In hMSCs (83 Y), there was a dose-dependent increase in PTH stimulation of synthesis of $1,25(OH)_2D_3$ (Fig. 3B). Analysis of gene expression showed that there was a dose-dependent effect of PTH on the expression of CYP27B1 in hMSCs (83 Y) (Fig. 3C). The effect of 100 nM PTH to increase the expression of CYP27B1 was reproduced in two additional hMSCs (Fig. 3C; 62 Y and 65 Y). Subsequent studies were carried out to determine the mechanisms by which PTH upregulates CYP27B1 in hMSCs from older subjects.

Two episodes of PTH upregulation of CYP27B1/1α-hydroxylase and CREB phosphorylation

Similar to the appearance of two periods of PTH stimulation of $1,25(OH)_2D_3$ synthesis, there were two increases in CYP27B1 gene expression, one peaking at 1–2 h and one at 6–8 h (Fig. 4A), and two periods of increases in CYP27B1 protein levels, at 2 and 8–12 h (Fig. 4B). We investigated whether there were distinct mechanisms by which PTH had two episodes of upregulation of CYP27B1. The kinetics of phosphorylation of the PTH signaling molecule, the nuclear transcription factor, and cAMP response element binding protein (CREB) were assessed. Similar to effects on its target gene CYP27B1, PTH1-34 stimulated two increases in CREB phosphorylation, one at 2 h and the other at 6–8 h.

© 2011 The Authors
Aging Cell © 2011 Blackwell Publishing Ltd/Anatomical Society of Great Britain and Ireland

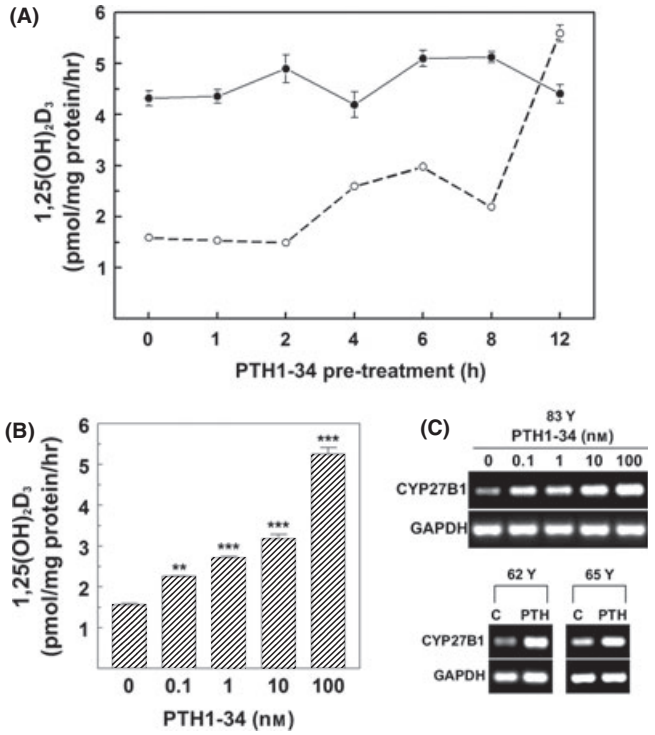
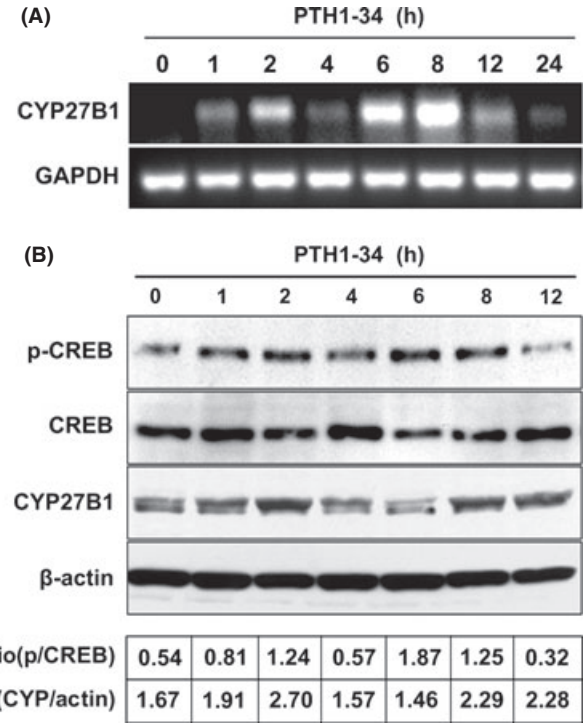


Fig. 3 Dose- and time-dependent effects of PTH1-34 on vitamin D 1α-hydroxylase activity and expression in human mesenchymal stem cells (hMSCs). (A) Comparison time-course of PTH1-34 effects on $1,25(OH)_2D_3$ production in hMSCs from two subjects (42 Y and 83 Y). Cells were treated with 100 nM PTH1-34 for 0, 1, 2, 4, 6, 8, and 12 h in serum-free α-MEM. For the synthesis of $1,25(OH)_2D_3$, they were changed to serum-free α-MEM supplemented with 1% ITS^{+1}, 10 μM 1,2-dianilinoethane (*N*,*N*′-diphenylethylene-diamine) with or without 1000 nM 25-hydroxyvitamin D_3 ($25OHD_3$) for another 24 h. Cellular $1,25(OH)_2D_3$ production was determined by EIA. Results are presented as mean ± SEM (three replicate wells). (B) Dose response of PTH1-34 on $1,25(OH)_2D_3$ production in hMSCs (83 Y). Cells were treated with 0, 0.1, 1, 10, or 100 nM PTH1-34 for 12 h in serum-free α-MEM, then changed to serum-free α-MEM supplemented with 1% ITS^{+1}, 10 μM 1,2-dianilinoethane (*N*,*N*′-diphenylethylene-diamine) with or without 1000 nM $25OHD_3$ for another 24 h. Cellular $1,25(OH)_2D_3$ production was determined by EIA. Results are presented as mean ± SEM (three replicate wells, ANOVA, $^{**}P < 0.01$; $^{***}P < 0.001$). (C) Gel electrophoretograms show RT–PCR products of CYP27B1 and GAPDH in hMSCs (83 Y) with 0, 0.1, 1, 10, or 100 nM PTH1-34 and hMSCs from 62- and 65-year-old subjects (62 Y and 65 Y) with 100 nM PTH1-34 for 8 h in standard growth medium (10% fetal bovine serum-heat inactivated).



Ratio(p/CREB)	0.54	0.81	1.24	0.57	1.87	1.25	0.32
Ratio(CYP/actin)	1.67	1.91	2.70	1.57	1.46	2.29	2.28

Fig. 4 Role of cAMP response element binding protein (CREB) signaling in mediating parathyroid hormone (PTH) upregulation of CYP27B1/1α-hydroxylase. (A) Gel electrophoretogram shows RT–PCR products of CYP27B1 and GAPDH in human mesenchymal stem cells (hMSCs) from 83-year-old subject after pretreatment with 100 nM PTH1-34 for 0, 1, 2, 4, 6, 8, 12, and 24 h in standard growth medium (10% fetal bovine serum-heat inactivated [FBS-HI]). Results are representative of three independent experiments. (B) Western immunoblot shows phospho-CREB (p-CREB), total CREB (CREB), CYP27B1, and β-actin in hMSCs from 83-year-old subject after 0, 1, 2, 4, 6, 8, and 12 h with 100 nM PTH1-34 in standard growth medium (10% FBS-HI). The ratios of p-CREB relative to total CREB (p/CREB) and of CYP27B1 relative to β-actin (CYP/actin ratio) are shown beneath the image of immunoblot. Results are representative of three independent experiments.

CREB signaling was required for PTH upregulation of CYP27B1/1α-hydroxylase

The hMSCs from an older subject (83 Y) were engineered to have reduced constitutive expression of CREB. There were no noticeable differences in cell density or appearance of control cells (electroporated with PBS, C), cells treated with nonsilencing control siRNA (NC), and cells treated with 100 pmol of CREB-siRNA (Fig. 5A). Transient transfection of CREB-siRNA into hMSCs resulted in reduction in basal levels of phosphorylated CREB (p-CREB) and total CREB protein (ratio of p-CREB to total CREB, 1% of control) but did not affect insulin-like growth factor-I receptor (IGF-IR) or CYP27B1 expression (Fig. 5B). No effect was shown with a nonsilencing, scrambled siRNA sequence (lane NC in Fig. 5B). After transfection, hMSCs were treated with PTH1-34. At both 2 and 8 h, PTH stimulation of p-CREB and CYP27B1 was abrogated by CREB silencing. At 8 h, phosphorylation of IGF-IR (p-IGF-IR) and total IGF-IR were not affected (Fig. 5C). Similar results were obtained from a replicate experiment with cells from a 65-year-old subject (data not shown).

Other hMSCs were treated with KG-501, an inhibitor of the downstream binding of activated phospho-CREB to CBP/p300. KG-501 abrogated PTH1-34 upregulation of CYP27B1 at both 2 and 8 h and, as expected, had small effects on CREB phosphorylation (Fig. 5D).

PTH induced IGF-I and IGF-I signaling, and treatment with rhIGF-I upregulated CYP27B1/1α-hydroxylase

Because IGF-I is a known target of PTH, its role in upregulating CYP27B1 was investigated. PTH1-34 upregulated IGF-I expression at 6 h but had no effect at 2 h, distinct from PTH's induction of CYP27B1 gene expression at both 2 and 6 h (Fig 6A). There was a dose-dependent upregulation of IGF-I expression by PTH1-34, evaluated at 6 h (Fig. 6B).

We examined the direct effects of rhIGF-I on IGF-IR, CREB, and CYP27B1. Ten-minute exposure to rhIGF-I resulted in a rapid 3.1-fold increase in IGF-IR phosphorylation and a 1.8-fold increase in CREB phosphorylation (Fig 6C). There was a more gradual increase in CYP27B1 protein to 2.7-fold at 120 min (Fig. 6C). These results suggest that the second episode of CREB signaling stimulated by PTH1-34 may be mediated by IGF-I.

© 2011 The Authors
Aging Cell © 2011 Blackwell Publishing Ltd/Anatomical Society of Great Britain and Ireland

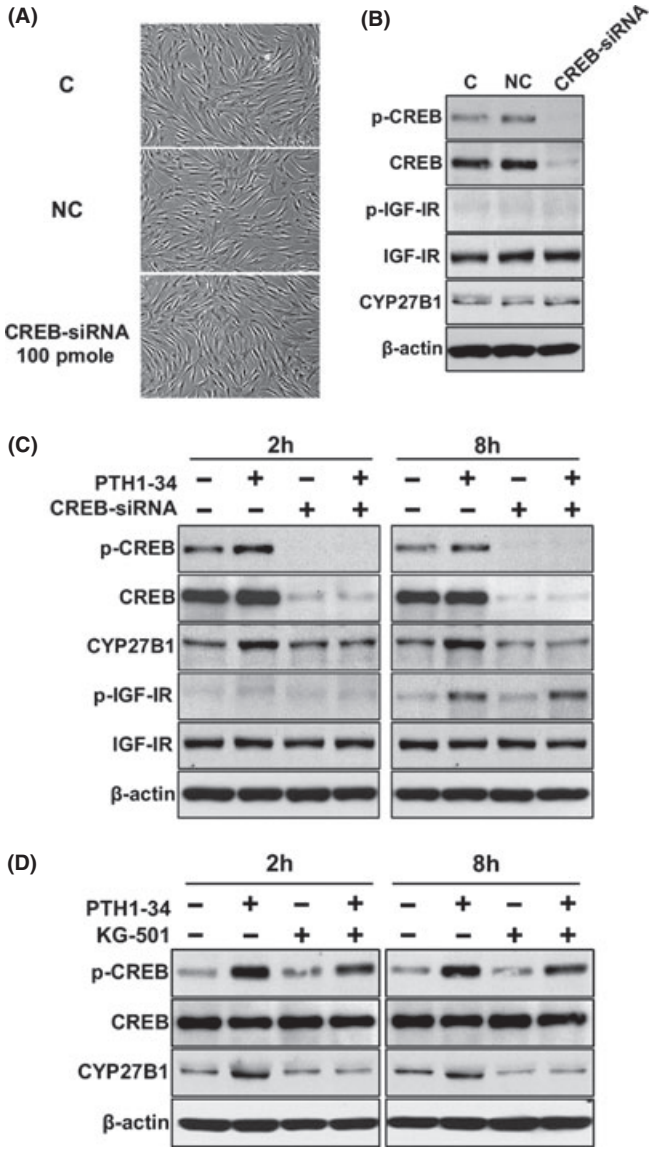


Fig. 5 Role of cAMP response element binding protein (CREB) signaling in mediating parathyroid hormone (PTH) upregulation of CYP27B1/1α-hydroxylase. Groups of human mesenchymal stem cells (hMSCs) were treated by electroporation with PBS (C, control), with nonsilencing control siRNA (NC), or with 100 pmol of CREB-siRNA (CREB-siRNA). (A) Photomicrographs show cultures of control (C), nonsilencing control (NC), and hMSCs transfected with 100 pmol of CREB-siRNA (CREB-siRNA 100 pmol) at 200 × magnification. (B) Western immunoblot shows p-CREB, CREB, phospho-IGF-IR (p-IGF-IR), total IGF-IR (IGF-IR), CYP27B1, and β-actin protein levels in controls and in transfected cells. (C) Western immunoblot shows p-CREB, CREB, CYP27B1, p-IGF-IR, IGF-IR, and β-actin in transfected hMSCs after 2 h or 8 h in the absence or presence of 100 nM PTH1-34. (D) Western immunoblot shows p-CREB, CREB, CYP27B1, and β-actin in hMSCs after 2 h or 8 h in the absence or presence of 100 nM PTH1-34 ± 30 μM KG-501. Human MSCs were incubated with the indicated concentration of KG-501 for 1 h before stimulation with PTH1-34. Results are representative of three independent experiments.

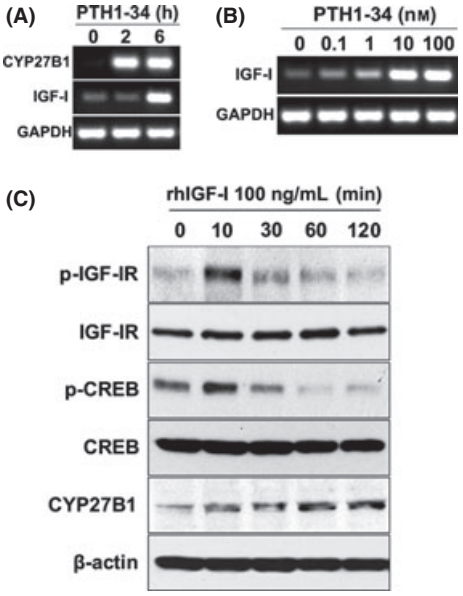


Fig. 6 Effects of parathyroid hormone (PTH) on IGF-I and of rhIGF-I on cAMP response element binding protein (CREB) and CYP27B1/1α-hydroxylase. (A) Gel electrophoretogram shows RT–PCR products of CYP27B1, IGF-I, and GAPDH in human mesenchymal stem cells (hMSCs) after 0, 2, and 6 h with 100 nM PTH1-34 in standard growth medium (10% fetal bovine serum-heat inactivated [FBS-HI]). (B) Gel electrophoretogram shows RT–PCR products of IGF-I and GAPDH in hMSCs after 6 h with 0, 0.1, 1, 10, or 100 nM PTH1-34 in standard growth medium (10% FBS-HI). (C) Western immunoblot shows p-IGF-IR, IGF-IR, p-CREB, CREB, CYP27B1, and β-actin in hMSCs after 0, 10, 30, 60, and 120 min with 100 ng mL^{-1} rhIGF-I in standard growth medium (10% FBS-HI). Results are representative of three independent experiments.

IGF-I signaling of CREB mediated PTH's second upregulation of CYP27B1/1α-hydroxylase

In the presence of AG1024, a specific inhibitor of IGF-IR kinase, IGF-I's rapid effects on IGF-IR phosphorylation and CREB activation were abrogated (Fig. 7A). In the presence of AG-1024, IGF-I's later upregulation of CYP27B1 (174%, relative to control) was blocked (112%, Fig. 7B).

In the presence of AG1024, PTH1-34's induction of IGF-IR phosphorylation at 8 h was abolished. AG1024 had no effect on PTH stimulation of IGF-IR phosphorylation, CREB phosphorylation, and CYP27B1 at 2 h, but abolished those effects of PTH at 8 h (Fig. 7C).

Discussion

The recent discovery that human marrow stromal cells, which include osteoblast progenitors, have the molecular machinery for regulated vitamin D metabolism (Zhou *et al.*, 2010) suggested that vitamin D metabolites may serve autocrine/paracrine roles in osteoblast differentiation. These studies provide new evidence that in hMSCs, there is an age-related decline in the expression of CYP27B1, the gene that encodes the vitamin D-activating 1α-hydroxylase. Diminished synthesis of $1,25(OH)_2D_3$ can explain the resistance of hMSCs from older subjects to $25OHD_3$ stimulation of osteoblast differentiation. This hypothesis is supported by our recent report that experimental silencing or inhibition of CYP27B1 in hMSCs from young subjects rendered them no longer responsive to $25OHD_3$ (Geng *et al.*, 2011). The studies herein present evidence that PTH1-34 stimulated CYP27B1 expression and enzymatic activity; this provided hMSCs from old subjects with responsiveness to $25OHD_3$. The effects of PTH were mediated directly by CREB signaling and indirectly by IGF-I signaling. Thus, the regulation of CYP27B1 by PTH in hMSCs is similar to PTH stimulation of CYP27B1 in renal cells (Haussler *et al.*, 1976; Brenza *et al.*, 1998; Brenza & DeLuca, 2000).

© 2011 The Authors
Aging Cell © 2011 Blackwell Publishing Ltd/Anatomical Society of Great Britain and Ireland

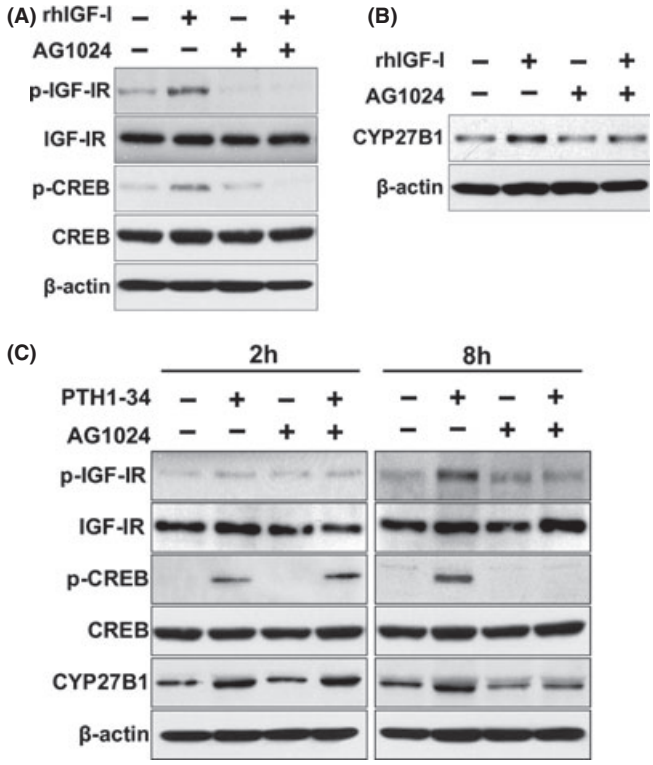


Fig. 7 Role of IGF-I signaling in mediating second episode of PTH1-34 upregulation of cAMP response element binding protein (CREB) and CYP27B1/1α-hydroxylase. Human MSCs were incubated with AG1024 for 1 h before stimulation with IGF-I or PTH1-34. (A) Western immunoblot shows p-IGF-IR, IGF-IR, p-CREB, CREB, and β-actin in human mesenchymal stem cells (hMSCs) after 10 min in the absence or presence of 100 ng mL^{-1} rhIGF-I ± 3 μM AG1024. (B) Western immunoblot shows CYP27B1 and β-actin in hMSCs from old subjects after 120 min in the absence or presence of 100 ng mL^{-1} rhIGF-I ± 3 μM AG1024. (C) Western immunoblot shows p-IGF-IR, IGF-IR, p-CREB, CREB, CYP27B1, and β-actin in hMSCs from old subjects after 2 h or 8 h in the absence or presence of 100 nM PTH1-34 ± 3 μM AG1024. Results are representative of three independent experiments.

A decline in the numbers of or differentiation potential of stem cell populations in adult organs could contribute to human age and age-related disease (Rao & Mattson, 2001; Carrington, 2005). A loss of progenitor cell functionality may in turn contribute to a number of age-related musculoskeletal pathologies such as osteoporosis, arthritis, and tendinosis (Stolzing & Scutt, 2006). Although there has been research to define the pathophysiology of bone loss associated with sex steroid deficiency and development of osteoporosis, there is less information about the mechanism(s) by which aging influences bone loss. Data in this report confirm other studies that show an age-related decline in osteoblast differentiation (Mueller & Glowacki, 2001; Zhou *et al.*, 2008). Data from studies with colony assays are variable, with some evidence for an age-related decline in colony number (D'Ippolito *et al.*, 1999; Nishida *et al.*, 1999), while others found no effects of age (Justesen *et al.*, 2002). Kassem & Marie, (2011) recently declared that 'impaired differentiation of MSC to osteoblasts may contribute to the age-related bone loss'. A better understanding of intrinsic age-related changes is needed to mitigate or avoid the loss of bone with age.

This study showed an age-related decline in CYP27B1 gene expression in hMSCs. Previously, we reported that the level of expression of CYP27B1 in hMSCs was related to the vitamin D status of the subject from whom the cells were obtained (Zhou *et al.*, 2010), but there was insufficient power to assess the influence of age. In a series of hMSCs from vitamin D-sufficient subjects evaluated herein, there was lower constitutive expression of CYP27B1 in the specimens from the older than from the young subjects. A larger study and multiple regression analysis will be needed to resolve the relative effects of age and serum 25OHD on constitutive expression of CYP27B1.

It is known that PTH is a major stimulus of renal CYP27B1 (Haussler *et al.*, 1976; Brenza *et al.*, 1998; Brenza & DeLuca, 2000) and that PTH1-34 positively regulates renal CYP27B1 gene expression through a PKA-dependent pathway (Murayama *et al.*, 1999). Thus, we tested whether PTH regulates CYP27B1 in hMSCs. Detecting lower expression of PTHR1 in hMSCs from older than from younger subjects in this series is consistent with our previous report of age-related declines in PTHR1 expression and signaling with 10 nM PTH1-34 (Zhou, 2011). In this project, however, a higher concentration (100 nM) of PTH1-34 was used and was shown to be effective in upregulating CYP27B1 in cells from elders. Compared with cells from young subjects, osteoblast differentiation of hMSCs from older subjects was resistant to stimulation by $25OHD_3$, but responsiveness to $25OHD_3$ became evident after pretreatment with PTH1-34. Stimulation of 1α-hydroxylation of $25OHD_3$ by PTH1-34 pretreatment explains the increase in osteoblast differentiation with the combined treatments. These data indicate that PTH1-34 'restored' hMSCs from old subjects with responsiveness to $25OHD_3$ by upregulation of CYP27B1 expression and enzymatic activity. Samadfam *et al.* (2008) recently showed that intermittently administered PTH increased bone density in 1α-hydroxylase$^{-/-}$ mice, but that there was a greater effect in mice with an active $1,25(OH)_2$D-synthesizing system. They concluded that PTH and vitamin D may interact to potentiate osteoblast differentiation. This concept is also supported by an analysis of factors associated with heterogeneity in skeletal response to clinical PTH therapy for osteoporosis (Sellmeyer *et al.*, 2007). Of all variables tested, only the change in serum $1,25(OH)_2$D explained larger gains in bone density in response to PTH.

Kinetic analysis of synthesis of $1,25(OH)_2D_3$ in hMSCs from an older subject revealed two waves of stimulation by PTH1-34, such that $1,25(OH)_2D_3$ production following 12-h exposure to PTH1-34 was similar to the level synthesized by hMSCs from a young subject. The levels of synthesis of $1,25(OH)_2D_3$ by these cells were similar to those reported for osteoblast-like cells (Atkins *et al.*, 2007). Our studies do not shed light on whether $1,25(OH)_2$D that is synthesized in marrow enters the circulation.

To determine the mechanisms by which PTH1-34 stimulated two episodes of increased CYP27B1 gene expression and protein levels, we monitored CREB activation, a well-characterized pathway for PTH action (Johannessen *et al.*, 2004). Upon binding to its receptor, PTH1-34 induces gene expression by its second messenger cAMP-activating protein kinase A (PKA), which subsequently phosphorylates CREB at Ser133. That phosphorylation alters the affinity of the transactivation domain of CREB to the acceptor domain of the CREB-binding protein (CBP) and p300 and eventually results in enhancing transcription of cAMP response element (CRE)-dependent genes. In C-21 human kidney cells, three CRE-like sequences were identified within the PTH-sensitive area of the CYP27B1 promoter; their deletion reduced induction by 50–95% (Flanagan *et al.*, 2003). Thus, CYP27B1 is a CRE-dependent gene in kidney cells. We used two experimental approaches to determine the role(s) of CREB in PTH upregulation of CYP27B1 in hMSCs: targeted CREB silencing and the use of small molecule inhibitor of CREB signaling. In a previous analysis of the age-related decline in PTH signaling in hMSCs, we showed that CREB-siRNA completely obliterated the PTH stimulation of osteoblast differentiation that was typical for hMSCs from young subjects (Zhou, 2011). On the other hand, in this study with hMSCs from older subjects,

© 2011 The Authors
Aging Cell © 2011 Blackwell Publishing Ltd/Anatomical Society of Great Britain and Ireland

transfection with CREB-siRNA blocked PTH1-34 upregulation of CYP27B1 at both 2 and 8 h. The compound KG-501, which disrupts the downstream interaction between phospho-CREB and CBP/p300, abrogated PTH1-34 upregulation of CYP27B1 at both time periods. It is noted, however, that KG-501 also interferes with the interaction with NF-κB (Best *et al.*, 2004). Our studies indicate that intact CREB signaling is necessary for PTH stimulation of osteoblast differentiation (Zhou, 2011) and, as shown herein, for PTH upregulation of CYP27B1 that is required for responsiveness to $25OHD_3$. Thus, for these cellular effects of PTH, experimental inhibition of CREB signaling can be viewed as a model for the natural age-related decline in CREB signaling in hMSCs. The CREB pathway is evolutionarily conserved and regulates many diverse genes (Johannessen *et al.*, 2004) and is likely to be involved in aging. There is other literature on the central importance of CREB-related genes in aging, in lifespan prediction, and in mediating lifespan extension by dietary restriction (Zhang *et al.*, 2009). Recent research shows the critical role of the *Caenorhabditis elegans* orthologue of CREB in extension of longevity (Mair *et al.*, 2011). The hMSCs may be useful to test other strategies for rejuvenating CREB pathway status and bone formation.

Evidence from a variety of systems indicates that osteoanabolic actions of PTH are mediated through IGF-I (McCarthy *et al.*, 1989; Watson *et al.*, 1995; Bikle *et al.*, 2002; Shinoda *et al.*, 2010). We previously reported that PTH peptides upregulate both IGF-I and IGF-II in hMSCs (Zhou, 2011) and that rhIGF-I upregulates CYP27B1 expression and 1α-hydroxylase enzymatic activity in hMSCs from old subjects (Zhou *et al.*, 2010). AG1024 is a tyrphostin with specificity to inhibit the activation of the IGF-I receptor (Parrizas *et al.*, 1997). Evidence in this study shows that the second episode of CREB phosphorylation and upregulation of CYP27B1 at 6–8 h is mediated by IGF-I. Other *in vivo* and *in vitro* studies indicate that IGF-I regulates the renal production of $1,25(OH)_2D_3$ (Nesbitt & Drezner, 1993; Menaa *et al.*, 1995; Wong *et al.*, 2000; Gomez, 2006). Another important concept that has emerged recently is the requirement of IGF-I and its receptor for bone-building effects of PTH therapies (Bikle *et al.*, 2002; Yamaguchi *et al.*, 2005). Cao *et al.* (2007) showed in mice that aging is associated with skeletal resistance to IGF-I signaling. Furthermore, IGF activation of the IGF-IR is believed to stimulate CREB phosphorylation and regulate the expression of CRE-target genes for the growth and survival in malignant and normal cell types (Pugazhenthi *et al.*, 1999; Liu *et al.*, 2002; Zheng & Quirion, 2006; Kim *et al.*, 2010).

These studies begin to elucidate the role of IGF-I in PTH1-34 effects on differentiation of hMSCs, but more information is needed. The regulation of IGF-I is important for many cell functions; for example, in bone cultures, PTH stimulation of collagen synthesis requires IGF-I, whereas its mitogenic effects do not (Canalis *et al.*, 1989). Moreover, in osteoblastic cells, there is crosstalk whereby PTH1-34 potentiates the effects of $1,25(OH)_2D_3$ on induction of c/EBPβ and target genes (Dhawan *et al.*, 2005). Although the samples studied were obtained from enrolled subjects known to be vitamin D-sufficient, it is possible that factors other than age may contribute to the biology of the hMSCs.

In conclusion, there are age-related declines in osteoblastogenesis and expression/activity of CYP27B1 in hMSCs and an age-related resistance to $25OHD_3$. *In vitro*, PTH1-34 provided hMSCs from old subjects with responsiveness to $25OHD_3$ by upregulation of CYP27B1 expression and activity through CREB- and IGF-I-mediated pathways. The stimulation of CYP27B1 by PTH in hMSCs is similar to that in renal cells. Vitamin D sufficiency is thought important to ensure proper mineralization of bone, but these studies further indicate that PTH and vitamin D may interact to potentiate osteoblast differentiation in elders. It will be clinically important to determine the proper vitamin D status needed to optimize PTH osteoanabolic therapy, especially in elders.

Experimental procedures

Reagents

Recombinant human parathyroid hormone peptide 1-34 (PTH1-34) was purchased from Bachem Americas, Inc. (Torrance, CA, USA), and recombinant human insulin-like growth factor (rhIGF-I) was purchased from R&D Systems (Minneapolis, MN, USA); other reagents such as $25OHD_3$, $1,25(OH)_2D_3$, KG-501, and AG1024 were purchased from Sigma (St. Louis, MO, USA). PTH1-34 and rhIGF-I were prepared in 0.1% BSA; $25OHD_3$ and $1,25(OH)_2D_3$ were prepared in 0.1% ethanol; others were prepared as stock solutions at 10^{-3} M in DMSO and stored at –80°C.

Preparation of human marrow stromal cells

Bone marrow samples were obtained with IRB approval as femoral tissue discarded during primary hip arthroplasty for osteoarthritis. A set of subjects scheduled for hip arthroplasty was consented for research studies, including the measurement of serum 25OHD. Criteria for exclusion are rheumatoid arthritis, cancer, and other comorbid conditions that may influence skeletal metabolism, i.e., renal disease, alcoholism, active liver disease, malabsorption, hyperthyroidism, ankylosing spondylitis, aseptic necrosis, hyperparathyroidism, morbid obesity, and diabetes. Also excluded were patients who were taking medications that may influence skeletal metabolism (e.g., thyroid hormone, glucocorticoids, nonsteroidal anti-inflammatory drugs, bisphosphonates, and osteoanabolic drugs). Low-density marrow mononuclear cells were isolated by centrifugation on Ficoll/Histopaque 1077 (Sigma, St. Louis, MO, USA) (Zhou *et al.*, 2005). This procedure removes differentiated cells and enriches for undifferentiated, low-density marrow mononuclear cells that include a population of nonadherent hematopoietic cells and a fraction capable of adherence and differentiation into musculoskeletal cells. The nonadherent hematopoietic stem cells were rinsed away 24 h after seeding, and the adherent hMSCs were expanded in monolayer culture with standard growth medium, phenol red-free α-MEM, 10% FBS-HI, 100 U mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin (Invitrogen, Carlsbad, CA, USA). For osteoblastogenic differentiation experiments, we lowered the serum of standard osteogenic medium (10% FBS-HI) to 1% FBS-HI to reduce possible differences in proliferation and cell numbers for cells from young and old subjects (Zhou *et al.*, 2008). After transfection with siRNA, all media used were without 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin. We used constant seeding density and constant splitting protocol, regardless of the time to proliferate. Initially, 30×10^6 bone marrow mononuclear cells were seeded per 100-mm dish. We define passage 0 as when the initial hMSCs proliferate to approximately 80% confluence. In each experiment, standardized conditions were used: same passage (passages 2–3), identical seeding density, and identical reagents. This approach avoids changes in cell behaviors that are associated with prolonged culture, such as *in vitro* senescence or culture stress (Zhou *et al.*, 2008). Like other normal mammalian cells, when cultured for many passages, MSCs display what is termed '*in vitro* senescence,' i.e., decreased proliferation, replicative quiescence, enlargement, increase in SA-β-gal activity, and erosion of telomeres (Fehrer & Lepperdinger, 2005; Sethe *et al.*, 2006). Further, there is a link between the accumulation of DNA damage and loss of multipotency of human MSCs with time of culture (Alves *et al.*, 2009). Cellular senescence can contribute to the physiological processes of normal organismal age (Jeyapalan & Sedivy, 2008). The results obtained herein, however, should reflect the effects of *in vivo* age because cells from young and old individuals were treated the same way and evaluated upon isolation or at early passage.

© 2011 The Authors
Aging Cell © 2011 Blackwell Publishing Ltd/Anatomical Society of Great Britain and Ireland

RNA isolation and RT–PCR

Total RNA was isolated from human MSCs with TRIZOL reagent (Invitrogen). For RT–PCR, 2 μg of total RNA was reverse-transcribed into cDNA with SuperScript II (Invitrogen), following the manufacturer's instructions. Concentrations of cDNA and amplification conditions were optimized for each gene product to reflect the exponential phase of amplification. One-twentieth of the cDNA was used in each 50-μL PCR (30–40 cycles of 94°C for 1 min, 55–60°C for 1 min, and 72°C for 2 min) as described (Zhou *et al.*, 2005). Gene-specific primer pairs for CYP27B1 (Zhou *et al.*, 2010), CYP24A1 (Zhou *et al.*, 2010), PTHR1 (Winn *et al.*, 1999), VDR (Zhou *et al.*, 2010), ALP (Zhou *et al.*, 2008), BSP (Zhou *et al.*, 2008), IGF-I (Zhou *et al.*, 2010), and GAPDH (Zhou *et al.*, 2008) were used for amplification. Polymerase chain reaction products were separated by agarose gel electrophoresis and were quantified by densitometry of captured gel images using KODAK Gel Logic 200 Imaging System and KODAK Molecular Imaging Software, following the manufacturer's instructions (KODAK, Molecular Imaging Systems, New Haven, CT, USA). Data were expressed by normalizing the densitometric units to GAPDH (internal control) and, in some cases, as treated relative to control.

Alkaline phosphatase enzymatic activity assay

For ALP enzymatic activity assay, the concentration of serum in standard osteogenic medium was reduced to 1% FBS-HI to minimize possible subsequent differences in proliferation that could confound interpretation of effects of vitamin D metabolites on osteoblastogenesis (Gregory *et al.*, 2005; Zhou *et al.*, 2008). ALP enzyme activity was measured spectrophotometrically, as previously described (Zhou *et al.*, 2008). Protein concentration was determined using the BCA system (Thermo Fisher Scientific Inc., Rockford, IL, USA). The ALP enzyme activity was expressed as $\mu mol\ min^{-1}\ g^{-1}$ protein and calculated as the fold change (treated relative to control). The standard curves are linear, but values differ from experiment to experiment; we always compare the results within an experiment because of the limitation of the assay. For the pretreatment experiments, 100 nM PTH1-34 was added to cells 12 h prior to 10 nM $25OHD_3$ or $1,25(OH)_2D_3$. Culture medium was changed every 2 days.

In vitro biosynthesis of 1, $25(OH)_2D_3$ by hMSCs

To generate detectable levels of $1,25(OH)_2D_3$, hMSCs were cultivated in 12-well plates until confluence, preincubated for duration of 0, 1, 2, 4, 6, 8, and 12 h with 100 nM PTH1-34 or for 12 h with or without 0.1, 1, 10, 100 nM PTH1-34 in serum-free α-MEM (Sigma, St. Louis, MO, USA), and then changed the medium to serum-free α-MEM supplemented with 1% ITS^{+1}, 10 μM 1, 2-dianilinoethane (*N*,*N*′-diphenylethylene-diamine) (Sigma, St. Louis, MO, USA) and treated with or without 1000 nM $25OHD_3$ for another 24 h. 1,2-Dianilinoethane was added into the cultures as an antioxidant as described (Zehnder *et al.*, 2002). Supernatants were harvested and stored at −20°C prior to analysis for $1,25(OH)_2D_3$ content. The 1, $25(OH)_2D_3$ levels in medium were quantitatively determined using a 1, $25(OH)_2D_3$ ELISA kit (Immunodiagnostic systems Ltd., Scottsdale, AZ, USA), according to the manufacturer's instructions. The hMSCs were lysed with a buffer containing 150 mM NaCl, 3 mM $NaHCO_3$, 0.1% Triton X-100, and a mixture of protease inhibitors (Roche Diagnostics, Indianapolis, IN, USA). Protein concentration was determined using the BCA system (Thermo Fisher Scientific Inc., Rockford, IL, USA). The CYP27B1 activity was expressed as biosynthesized $1,25(OH)_2D_3$ in medium per milligram protein per hour of $25OHD_3$ treatment (femtomoles per milligram protein per hour).

RNA interference with CREB-siRNA

Transient transfection of siRNA into hMSCs from old subjects were performed by electroporation with the Human MSC Nucleofector Kit® (Lonza/Amaxa Biosystems, Walkersville, MD, USA) with either CREB-siRNA (Stealth RNAi duplex siRNA; Invitrogen), nonsilencing control siRNA (a nonhomologous, scrambled sequence equivalent) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), or PBS according to the manufacturer's instructions and as previously described (Shen *et al.*, 2011; Zhou, 2011). In brief, hMSCs were harvested by trypsinization and resuspended at 10^6 cells in 100 μl of Nucleofector Solution with 100 pmol of CREB-siRNA. Electroporation was performed in Nucleofector II® device with program U-23 (Lonza/Amaxa Biosystems). Immediately after electroporation, the cells were transferred to 60-mm dishes in phenol red-free α-MEM, 10% FBS-HI. Some cells were collected at 80% confluence for Western immunoblot to determine the effect of CREB-siRNA. Twenty-four hours after electroporation, some cells were treated with either PTH1-34 (100 nM) or vehicle control (0.1% BSA) at 2 h or 8 h in standard growth medium (10% FBS-HI) for Western immunoblot.

Western immunoblot

Human MSCs were cultured in 100-mm dishes in standard growth medium (10% FBS-HI). At 80% confluence, the cells were treated with 100 nM PTH1-34 for different times. The whole-cell lysates were prepared with lysis buffer (150 mM NaCl, 3 mM $NaHCO_3$, 2 mM Na_3VO_4, 5 mM NaF, 0.1% Triton X-100, and a mixture of protease inhibitors, Roche Diagnostics, Indianapolis, IN, USA) and were homogenized with a pestle (Kontes) and centrifuged at 16 000 × *g* (Eppendorf centrifuge). Protein concentration was determined (BCA system; Thermo Fisher Scientific Inc., Rockford, IL, USA). The Western blotting was performed as previously described (Zhou *et al.*, 2004). In brief, proteins were resolved on 4–12% SDS-PAGE (NuPAGE Bis-Tris gel; Invitrogen) and transferred onto polyvinylidene fluoride membranes (PVDF; Amersham Biosciences, Piscataway, NJ, USA). The membranes were blocked with 5% nonfat milk in PBS buffer containing 0.1% Tween-20 (PBST) for 2–3 h at room temperature and incubated at 4°C for overnight with primary antibodies with 1:1000 Phospho-CREB (p-CREB), 1:3000 total CREB (CREB), 1:1000 phospho-IGF-I receptor (p-IGF-IR) and 1:1000 IGF-I receptor β (IGF-IR) (Cell signaling Technology, Beverly, MA, USA); 1:1000 CYP27B1 and 1:8000 β-actin (Santa Cruz Biotechnology). After removal of the unbound primary antibodies by three 5-min washes with PBST, the membranes were incubated with horseradish peroxidase–conjugated secondary antibodies (1:5000) for 1 h at room temperature and washed three times for 5 min with PBST. The antibody-associated protein bands were revealed with the ECL-plus Western blotting system (Amersham Biosciences).

Statistical analysis

All experiments were performed at least in triplicate. Group data are presented as mean values ± SEM. Unless otherwise indicated, quantitative data were analyzed by GraphPad Instat® (GraphPad Software, Inc., San Diego, CA, USA) with nonparametric tools, either the Mann–Whitney test for group comparisons or Spearman's correlation test, or with parametric tools, either group comparisons by two-tailed unpaired *t*-test with Welch correction or multiple comparisons by one-way ANOVA followed by Tukey's post hoc analysis. A value of $P < 0.05$ was considered significant.

© 2011 The Authors
Aging Cell © 2011 Blackwell Publishing Ltd/Anatomical Society of Great Britain and Ireland

Acknowledgments

This study was presented in part at the ASBMR annual meeting in Toronto, ON, Canada, 2010 and ASBMR Forum on Aging and Skeletal Health, Bethesda, MD, USA, 2011. The authors greatly appreciate Drs. Zhenggang Bi, Regina O'Sullivan, Shuichi Mizuno, and Ms. Sara Anderson for guidance and assistance. This work is based on a thesis by Shuo Geng for the MD/PhD degrees from Harbin Medical University, China. Shuo Geng was supported by the China Scholarship Council (CSC). This project was supported by NIH grants AG025015 and AG028114.

Author contributions

Shuo Geng, Shuanhu Zhou, and Julie Glowacki designed the studies, analyzed and interpreted the data. Shuo Geng acquired the data and drafted the manuscript. All authors critically edited each draft.

References

Alves H, Munoz-Najar U, De Wit J, Renard AJ, Hoeijmakers JH, Sedivy JM, Van Blitterswijk C, De Boer J (2009) A link between the accumulation of DNA damage and loss of multi-potency of human mesenchymal stromal cells. *J. Cell Mol. Med.* **14**, 2729–2738.

Atkins GJ, Anderson PH, Findlay DM, Welldon KJ, Vincent C, Zannettino AC, O'Loughlin PD, Morris HA (2007) Metabolism of vitamin D3 in human osteoblasts: evidence for autocrine and paracrine activities of 1α,25-dihydroxyvitamin D3. *Bone* **40**, 1517–1528.

Best JL, Amezcua CA, Mayr B, Flechner L, Murawsky CM, Emerson B, Zor T, Gardner KH, Montminy M (2004) Identification of small-molecule antagonists that inhibit an activator: coactivator interaction. *Proc. Natl Acad. Sci. USA* **101**, 17622–17627.

Bikle DD, Sakata T, Leary C, Elalieh H, Ginzinger D, Rosen CJ, Beamer W, Majumdar S, Halloran BP (2002) Insulin-like growth factor I is required for the anabolic actions of parathyroid hormone on mouse bone. *J. Bone Miner. Res.* **17**, 1570–1578.

Brenza HL, DeLuca HF (2000) Regulation of 25-hydroxyvitamin D3 1α-hydroxylase gene expression by parathyroid hormone and 1,25-dihydroxyvitamin D3. *Arch. Biochem. Biophys.* **381**, 143–152.

Brenza HL, Kimmel-Jehan C, Jehan F, Shinki T, Wakino S, Anazawa H, Suda T, DeLuca HF (1998) Parathyroid hormone activation of the 25-hydroxyvitamin D3-1alpha-hydroxylase gene promoter. *Proc. Natl Acad. Sci. USA* **95**, 1387–1391.

Canalis E, Centrella M, Burch W, McCarthy TL (1989) Insulin-like growth factor I mediates selective anabolic effects of parathyroid hormone in bone cultures. *J. Clin. Invest.* **83**, 60–65.

Cao JJ, Kurimoto P, Boudignon B, Rosen C, Lima F, Halloran BP (2007) Aging impairs IGF-I receptor activation and induces skeletal resistance to IGF-I. *J. Bone Miner. Res.* **22**, 1271–1279.

Carrington JL (2005) Aging bone and cartilage: cross-cutting issues. *Biochem. Biophys. Res. Commun.* **328**, 700–708.

Dhawan P, Peng X, Sutton AL, MacDonald PN, Croniger CM, Trautwein C, Centrella M, McCarthy TL, Christakos S (2005) Functional cooperation between CCAAT/enhancer-binding proteins and the vitamin D receptor in regulation of 25-hydroxyvitamin D3 24-hydroxylase. *Mol. Cell. Biol.* **25**, 472–487.

D'Ippolito G, Schiller PC, Ricordi C, Roos BA, Howard GA (1999) Age-related osteogenic potential of mesenchymal stromal stem cells from human vertebral bone marrow. *J. Bone Miner. Res.* **14**, 1115–1122.

Fehrer C, Lepperdinger G (2005) Mesenchymal stem cell aging. *Exp. Gerontol.* **40**, 926–930.

Flanagan JN, Wang L, Tangpricha V, Reichrath J, Chen TC, Holick MF (2003) Regulation of the 25-hydroxyvitamin D-1alpha-hydroxylase gene and its splice variant. *Recent Results Cancer Res.* **164**, 157–167.

Geng S, Zhou S, Glowacki J (2011) Effects of 25-hydroxyvitamin D3 on proliferation and osteoblast differentiation of human marrow stromal cells require CYP27B1/1α-hydroxylase. *J. Bone Miner. Res.* **26**, 1145–1153.

Gomez JM (2006) The role of insulin-like growth factor I components in the regulation of vitamin D. *Curr. Pharm. Biotechnol.* **7**, 125–132.

Gregory CA, Ylostalo J, Prockop DJ (2005) Adult bone marrow stem/progenitor cells (MSCs) are preconditioned by microenvironmental ''niches'' in culture: a two-stage hypothesis for regulation of MSC fate. *Sci. STKE* **2005**, pe37.

Haussler MR, Baylink DJ, Hughes MR, Brumbaugh PF, Wergedal JE, Shen FH, Nielsen RL, Counts SJ, Bursac KM, McCain TA (1976) The assay of 1alpha,25-dihydroxyvitamin D3: physiologic and pathologic modulation of circulating hormone levels. *Clin. Endocrinol. (Oxf).* **5**(Suppl), 151S–165S.

Hui SL, Slemenda CW, Johnston CC Jr (1988) Age and bone mass as predictors of fracture in a prospective study. *J. Clin. Invest.* **81**, 1804–1809.

Jeyapalan JC, Sedivy JM (2008) Cellular senescence and organismal aging. *Mech. Ageing Dev.* **129**, 467–474.

Jilka RL, Almeida M, Ambrogini E, Han L, Roberson PK, Weinstein RS, Manolagas SC (2010) Decreased oxidative stress and greater bone anabolism in the aged, when compared to the young, murine skeleton with parathyroid hormone administration. *Aging Cell* **9**, 851–867.

Johannessen M, Delghandi MP, Moens U (2004) What turns CREB on? *Cell. Signal.* **16**, 1211–1227.

Justesen J, Stenderup K, Eriksen EF, Kassem M (2002) Maintenance of osteoblastic and adipocytic differentiation potential with age and osteoporosis in human marrow stromal cell cultures. *Calcif. Tissue Int.* **71**, 36–44.

Kassem M, Marie PJ (2011) Senescence-associated intrinsic mechanisms of osteoblast dysfunctions. *Aging Cell* **10**, 191–197.

Kim HB, Kim WH, Han KL, Park JH, Lee J, Yeo J, Jung MH (2010) cAMP-response element binding protein (CREB) positively regulates mouse adiponectin gene expression in 3T3-L1 adipocytes. *Biochem. Biophys. Res. Commun.* **391**, 634–639.

Lane NE, Silverman SL (2010) Anabolic therapies. *Curr Osteoporos. Rep.* **8**, 23–27.

Liu P, Oyajobi BO, Russell RG, Scutt A (1999) Regulation of osteogenic differentiation of human bone marrow stromal cells: interaction between transforming growth factor-beta and 1,25(OH)(2) vitamin D(3) In vitro. *Calcif. Tissue Int.* **65**, 173–180.

Liu W, Chin-Chance C, Lee EJ, Lowe WL Jr (2002) Activation of phosphatidylinositol 3-kinase contributes to insulin-like growth factor I-mediated inhibition of pancreatic beta-cell death. *Endocrinology* **143**, 3802–3812.

Mair W, Morantte I, Rodrigues AP, Manning G, Montminy M, Shaw RJ, Dillin A (2011) Lifespan extension induced by AMPK and calcineurin is mediated by CRTC-1 and CREB. *Nature* **470**, 404–408.

McCarthy TL, Centrella M, Canalis E (1989) Parathyroid hormone enhances the transcript and polypeptide levels of insulin-like growth factor I in osteoblast-enriched cultures from fetal rat bone. *Endocrinology* **124**, 1247–1253.

Menaa C, Vrtovsnik F, Friedlander G, Corvol M, Garabedian M (1995) Insulin-like growth factor I, a unique calcium-dependent stimulator of 1,25-dihydroxyvitamin D3 production. Studies in cultured mouse kidney cells. *J. Chem. Biol.* **270**, 25461–25467.

Mueller SM, Glowacki J (2001) Age-related decline in the osteogenic potential of human bone marrow cells cultured in three-dimensional collagen sponges. *J. Cell. Biochem.* **82**, 583–590.

Murayama A, Takeyama K, Kitanaka S, Kodera Y, Kawaguchi Y, Hosoya T, Kato S (1999) Positive and negative regulations of the renal 25-hydroxyvitamin D3 1α-hydroxylase gene by parathyroid hormone, calcitonin, and 1alpha, 25(OH)2D3 in intact animals. *Endocrinology* **140**, 2224–2231.

Neer RM, Arnaud CD, Zanchetta JR, Prince R, Gaich GA, Reginster JY, Hodsman AB, Eriksen EF, Ish-Shalom S, Genant HK, Wang O, Mitlak BH (2001) Effect of parathyroid hormone (1-34) on fractures and bone mineral density in postmenopausal women with osteoporosis. *N. Engl. J. Med.* **344**, 1434–1441.

Nesbitt T, Drezner MK (1993) Insulin-like growth factor-I regulation of renal 25-hydroxyvitamin D-1-hydroxylase activity. *Endocrinology* **132**, 133–138.

Nishida S, Endo N, Yamagiwa H, Tanizawa T, Takahashi HE (1999) Number of osteoprogenitor cells in human bone marrow markedly decreases after skeletal maturation. *J. Bone Miner. Metab.* **17**, 171–177.

Parrizas M, Gazit A, Levitzki A, Wertheimer E, LeRoith D (1997) Specific inhibition of insulin-like growth factor-1 and insulin receptor tyrosine kinase activity and biological function by tyrphostins. *Endocrinology* **138**, 1427–1433.

Pfeilschifter J, Laukhuf F, Muller-Beckmann B, Blum WF, Pfister T, Ziegler R (1995) Parathyroid hormone increases the concentration of insulin-like growth factor-I and transforming growth factor beta 1 in rat bone. *J. Clin. Invest.* **96**, 767–774.

Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR (1999) Multilineage potential of adult human mesenchymal stem cells. *Science* **284**, 143–147.

© 2011 The Authors
Aging Cell © 2011 Blackwell Publishing Ltd/Anatomical Society of Great Britain and Ireland

Prockop DJ (1997) Marrow stromal cells as stem cells for nonhematopoietic tissues. *Science* **276**, 71–74.

Pugazhenthi S, Boras T, O'Connor D, Meintzer MK, Heidenreich KA, Reusch JE (1999) Insulin-like growth factor I-mediated activation of the transcription factor cAMP response element-binding protein in PC12 cells. Involvement of p38 mitogen-activated protein kinase-mediated pathway. *J. Chem. Biol.* **274**, 2829–2837.

Rao MS, Mattson MP (2001) Stem cells and aging: expanding the possibilities. *Mech. Ageing Dev.* **122**, 713–734.

Samadfam R, Xia Q, Miao D, Hendy GN, Goltzman D (2008) Exogenous PTH and endogenous 1,25-dihydroxyvitamin D are complementary in inducing an anabolic effect on bone. *J. Bone Miner. Res.* **23**, 1257–1266.

Sellmeyer DE, Black DM, Palermo L, Greenspan S, Ensrud K, Bilezikian J, Rosen CJ (2007) Heterogeneity in skeletal response to full-length parathyroid hormone in the treatment of osteoporosis. *Osteoporos. Int.* **18**, 973–979.

Sethe S, Scutt A, Stolzing A (2006) Aging of mesenchymal stem cells. *Ageing Res. Rev.* **5**, 91–116.

Shen L, Glowacki J, Zhou S (2011) Inhibition of adipocytogenesis by canonical WNT signaling in human mesenchymal stem cells. *Exp. Cell Res.* **317**, 1796–1803.

Shinoda Y, Kawaguchi H, Higashikawa A, Hirata M, Miura T, Saito T, Nakamura K, Chung UI, Ogata N (2010) Mechanisms underlying catabolic and anabolic functions of parathyroid hormone on bone by combination of culture systems of mouse cells. *J. Cell. Biochem.* **109**, 755–763.

Stolzing A, Scutt A (2006) Age-related impairment of mesenchymal progenitor cell function. *Aging Cell* **5**, 213–224.

Watson P, Lazowski D, Han V, Fraher L, Steer B, Hodsman A (1995) Parathyroid hormone restores bone mass and enhances osteoblast insulin-like growth factor I gene expression in ovariectomized rats. *Bone* **16**, 357–365.

Winn SR, Randolph G, Uludag H, Wong SC, Hair GA, Hollinger JO (1999) Establishing an immortalized human osteoprecursor cell line: OPC1. *J. Bone Miner. Res.* **14**, 1721–1733.

Wong MS, Tembe VA, Favus MJ (2000) Insulin-like growth factor-I stimulates renal 1, 25-dihydroxycholecalciferol synthesis in old rats fed a low calcium diet. *J. Nutr.* **130**, 1147–1152.

Yamaguchi M, Ogata N, Shinoda Y, Akune T, Kamekura S, Terauchi Y, Kadowaki T, Hoshi K, Chung UI, Nakamura K, Kawaguchi H (2005) Insulin receptor substrate-1 is required for bone anabolic function of parathyroid hormone in mice. *Endocrinology* **146**, 2620–2628.

Zehnder D, Bland R, Chana RS, Wheeler DC, Howie AJ, Williams MC, Stewart PM, Hewison M (2002) Synthesis of 1,25-dihydroxyvitamin D(3) by human endothelial cells is regulated by inflammatory cytokines: a novel autocrine determinant of vascular cell adhesion. *J. Am. Soc. Nephrol.* **13**, 621–629.

Zhang M, Poplawski M, Yen K, Cheng H, Bloss E, Zhu X, Patel H, Mobbs CV (2009) Role of CBP and SATB-1 in aging, dietary restriction, and insulin-like signaling. *PLoS Biol.* **7**, e1000245.

Zheng WH, Quirion R (2006) Insulin-like growth factor-1 (IGF-1) induces the activation/phosphorylation of Akt kinase and cAMP response element-binding protein (CREB) by activating different signaling pathways in PC12 cells. *BMC Neurosci.* **7**, 51.

Zhou S (2011) TGF-β regulates β-catenin signaling and osteoblast differentiation in human mesenchymal stem cells. *J. Cell. Biochem.* **112**, 1651–1660.

Zhou S, Eid K, Glowacki J (2004) Cooperation between TGF-β and Wnt pathways during chondrocyte and adipocyte differentiation of human marrow stromal cells. *J. Bone Miner. Res.* **19**, 463–470.

Zhou S, Lechpammer S, Greenberger JS, Glowacki J (2005) Hypoxia inhibition of adipocytogenesis in human bone marrow stromal cells requires transforming growth factor-β/Smad3 signaling. *J. Chem. Biol.* **280**, 22688–22696.

Zhou S, Greenberger JS, Epperly MW, Goff JP, Adler C, Leboff MS, Glowacki J (2008) Age-related intrinsic changes in human bone-marrow-derived mesenchymal stem cells and their differentiation to osteoblasts. *Aging Cell* **7**, 335–343.

Zhou S, LeBoff MS, Glowacki J (2010) Vitamin D metabolism and action in human bone marrow stromal cells. *Endocrinology* **151**, 14–22.

Zhou S, Bueno EM, Kim SW, Amato I, Shen L, Hahne J, Bleiberg I, Morley P, Glowacki J (2011). Effects of age on parathyroid hormone signaling in human marrow stromal cells. *Aging Cell*. 2011. Apr 25. doi: 10.1111/j.1474-9726.2011.00717.x. [Epub ahead of print].

© 2011 The Authors
Aging Cell © 2011 Blackwell Publishing Ltd/Anatomical Society of Great Britain and Ireland