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ARTICLE

Systemic and acute administration of parathyroid hormone-related peptide(1–36) stimulates endogenous beta cell proliferation while preserving function in adult mice

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Abstract
Aims/hypothesis A major focus in the treatment of diabetes is to identify factors that stimulate endogenous beta cell growth while preserving function. The first 36 amino acids of parathyroid hormone-related protein (PTHrP) are sufficient to enhance proliferation and function in rodent and human beta cells in vitro. This study examined whether acute and systemic administration of the amino-terminal PTHrP(1–36) peptide can achieve similar effects in rodent beta cells in vivo.
Methods Adult male mice were injected with 40, 80 or 160 μg of PTHrP(1–36) per kg body weight or with vehicle for 25 days. Glucose and beta cell homeostasis, as well as expression of differentiation markers and cell cycle genes were analysed.
Results All three doses of PTHrP(1–36) significantly enhanced beta cell proliferation in vivo at day 25, with 160 μg/kg PTHrP(1–36) increasing proliferation as early as day 5. Importantly, the two higher doses of PTHrP(1–36) caused a significant 30% expansion of beta cell mass, with a short-term improvement in glucose tolerance. PTHrP(1–36) did not cause hypercalcaemia, or change islet number, beta cell size, beta cell death or expression of differentiation markers. Analysis of islet G1/S cell cycle proteins revealed that chronic overabundance of PTHrP(1–139) in the beta cell significantly increased the cell cycle activator cyclin D2 and decreased levels of cyclin-dependent kinase 4 inhibitor ($p16^{Ink4a}$ [*Ink4a* also known as *Cdkn2a*]), but acute treatment with PTHrP(1–36) did not.
Conclusions/interpretation Acute and systemic administration of PTHrP(1–36) increases rodent beta cell proliferation and mass without negatively affecting function or survival. These findings highlight the future potential therapeutic effectiveness of this peptide under diabetes-related pathophysiological conditions.

Keywords Amino-terminal parathyroid hormone-related protein · Beta cell proliferation · Cyclin D2 · In vivo · $p16^{INK4a}$

Abbreviations

GLP-1	Glucagon-like polypeptide-1
INK	Inhibitor of kinase
IPGTT	Intraperitoneal glucose tolerance test
Maf	v-Maf musculoaponeurotic fibrosarcoma oncogene family
$p16^{Ink4a}$	Cyclin-dependent kinase 4 inhibitor
P-40	Peptide at 40 μg/kg body weight
P-80	Peptide at 80 μg/kg body weight
P-160	Peptide at 160 μg/kg body weight
PCNA	Proliferating cell nuclear antigen
PKC	Protein kinase C
PTH	Parathyroid hormone
PTHR1	PTH receptor-1
PTHrP	Parathyroid hormone-related protein
RIP	Rat insulin promoter

Introduction

Diabetes results from a loss of functional beta cells. Therefore a major goal in the treatment of diabetes is to

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find ways to induce endogenous beta cell regeneration without negatively affecting beta cell function. Proliferation is an important mechanism for normal postnatal growth and for regeneration after specific injury in rodent beta cells [1–3]. Importantly, beta cell proliferation has also been documented in humans, albeit less frequently [4, 5], providing a rational basis to search for molecules that can acutely enhance pancreatic beta cell replication and/or function in vivo. We examined the potential of parathyroid hormone (PTH)-related protein (PTHrP) in this context.

PTHrP is a 139 (rodent) or 173 (human) amino acid protein, which was first discovered as a tumour peptide that causes humoral hypercalcaemia of malignancy. It has since been shown to be produced in every tissue in the body, both during development and in adult life. Its receptor, the G-protein-coupled seven transmembrane PTH receptor-1 (PTHR1), which recognises the amino-terminal regions of PTHrP and its related member PTH, is also produced in the same or adjacent cells as PTHrP [6]. PTHrP/PTHR1 signalling is critical for the development and differentiation of numerous tissues, and for the sustenance of life itself, as mutations in this pathway lead to embryonic/neonatal death in rodents and humans [6–9].

PTHrP is a promising candidate for the promotion of endogenous beta cell proliferation for several reasons. PTHrP and its receptor, PTHR1, are both normally produced in rodent and human beta cells [10–14]. Indeed, several studies have now shown that PTHrP can enhance beta cell function and proliferation in vitro in human and rodent beta cells [13–17]. Of particular relevance is the finding that the first 36 amino acids of PTHrP are sufficient to manifest its salutary effects on beta cell proliferation and function in vitro [13–17]. Furthermore, we have found that full-length PTHrP also enhances beta cell growth and function in vivo, when produced under the rat insulin promoter (RIP) in transgenic mice [12, 15, 18, 19]. RIP-*PTHrP* (also known as *Pthlh*) transgenic mice are hypoglycaemic and hyperinsulinaemic, and have improved glucose tolerance, increased beta cell proliferation and enhanced beta cell mass. Finally, PTHrP(1–36) peptide is currently being used for its anabolic effects on bone in human clinical studies, demonstrating its probable safety as a potential therapeutic agent [20, 21].

Based on these studies, we hypothesised that PTHrP(1–36) has the potential to enhance endogenous beta cell proliferation and/or function in vivo, when administered acutely in mice. Indeed, as demonstrated here, PTHrP(1–36) augmented beta cell proliferation as early as the 5th day of treatment, continuing to do so until day 25. This resulted in a significant 30% increase in beta cell mass with the two higher doses of PTHrP(1–36), without negatively affecting plasma glucose levels or beta cell differentiation, size and turnover. Although PTHrP(1–36) significantly improved glucose tolerance at the beginning, glucose tolerance returned completely to baseline levels by the end of the treatment, suggesting a potential desensitisation of the functional effect of PTHrP as the treatment progresses. Analysis of the cell cycle molecules regulated by PTHrP showed a significant increase in abundance of the activator cyclin D2 and a decrease in the cyclin-dependent kinase 4 inhibitor ($p16^{Ink4a}$ [*Ink4a* also known as *Cdkn2a*]), in transgenic mouse islets overproducing PTHrP(1–139) in the beta cell; this did not occur in the islets of mice treated acutely and systemically with PTHrP(1–36). Thus, systemic administration of PTHrP(1–36) peptide can acutely induce beta cell replication and mass with short-term improvement in glucose tolerance in normal adult mice, identifying this peptide as a potential agent for beta cell regeneration.

Methods

Experimental design Balb/C male mice were injected s.c. with PTHrP(1–36) peptide at 40 (P-40 mice), 80 (P-80 mice) or 160 (P-160 mice) μg/kg body weight or vehicle (control). Injections were once daily for 5 days per week for 25 days, after which pancreases or islets were removed. PTHrP(1–36) was synthesised and assayed for activity as described previously [20, 21]. RIP-*PTHrP* transgenic mice bred on to a CD1 background were generated as described previously [18]. All studies were performed with the approval of, and in accordance with, guidelines established by the University of Pittsburgh Institutional Animal Care and Use Committee. For further details on experimental design, see electronic supplementary material (ESM) Methods.

Glucose homeostasis and islet isolation Blood glucose was measured 5 days per week for 25 days using a portable glucometer (Medisense, Bedford, MA, USA). An intraperitoneal glucose tolerance test (IPGTT) was performed on days 9 and 23 in mice that had been fasted for 16 to 18 h. These mice were injected i.p. with 2 g glucose per kg body weight [12, 15]. Islets that had been isolated for gene expression analysis from control and P-160 mice at day 5 or 25, and from 3- to 6-month-old RIP-*PTHrP* transgenic and normal mice were immediately handpicked in RPMI medium with 5.5 mmol/l glucose and 1% (vol./vol.) FBS [15].

Plasma calcium Plasma was obtained from retro-orbital bleeds on days 8 and 25, and plasma calcium measured using a blood gas analyser (Rapid Lab 348; Bayer, Leverkusen, Germany).

Pancreatic histomorphometry and beta cell homeostasis Histomorphometric analysis was performed on two to

three insulin-stained pancreatic sections per animal to quantify the number of islets or ratio of beta cell area to pancreatic area using the Image J program (NIH) [15]. Beta cell size, death and proliferation were quantified respectively as: (1) insulin-positive area per total number of insulin-positive cells (728±36 beta cells/mouse); (2) percentage of condensed beta cell nuclei after co-staining for insulin and propidium iodide [12], or TUNEL (Promega, Madison, WI, USA) (1,043±79 or 1,298±174 beta cells/mouse); and (3) percentage of BrdU-positive (Amersham Pharmacia Biotech, Piscataway, NJ, USA) or proliferating cell nuclear antigen (PCNA)-positive (Santa Cruz Biotechnology, Santa Cruz, CA, USA) beta cells (1,538±54 beta cells/mouse). Details on above, see also ESM Methods

RNA and protein analysis RNA from islets of control and P-160 mice at day 25, or of 3- to 6-month-old RIP-*PTHrP* transgenic and normal mice was processed and analysed on a real-time PCR system (ABI 7300; Life Technologies, Carlsbad, CA, USA). Whole-islet extracts were analysed by immunoblot and quantitative densitometry was performed using the Image J program [15]. Primers, primary antibodies and additional details are in ESM Table 1 and ESM Methods.

Statistical analysis Data are expressed as the mean±SE. Statistical significance, considered to be given at $p \leq 0.05$, was determined by unpaired two-tailed Student's *t* test or a one-way ANOVA with Dunnett's post hoc test.

Results

Effect of PTHrP(1–36) administration on body weight and plasma calcium PTHrP(1–36) was systemically administered by s.c. injection in 8-week-old male Balb/C mice at three different doses, 40, 80 and 160 μg/kg body weight or vehicle as control, for 5 days per week for 25 days (Fig. 1a). Body weight, plasma calcium, glucose and IPGTT, as well as beta cell proliferation, mass, size and death were analysed at specific times during the treatment (Fig. 1a). There was no change in the average body weight of the PTHrP(1–36)-injected mice, P-40, P-80 and P-160, compared with controls during the 25 days of treatment (Fig. 1b). As PTHrP has been discovered to be a factor that causes humoral hypercalcaemia of malignancy, we measured plasma calcium levels in these mice at early (day 8) and late (day 25) time points, and saw no significant increase in the average plasma calcium level, even at the highest dose of PTHrP(1–36) used (Fig. 1c).

PTHrP(1–36) increased beta cell mass without affecting islet number, beta cell size or beta cell death After 25 days of treatment, beta cell mass was measured in insulin-stained pancreatic sections from all four groups of mice. There were no significant changes in the pancreatic weights of these mice (Fig. 2a). PTHrP(1–36) caused a significant 29% increase in beta cell mass at the 80 and 160 μg/kg doses, but not at the 40 μg/kg dose relative to controls (Fig. 2b).

We then determined whether the increase in beta cell mass with PTHrP(1–36) resulted from changes in islet number, beta cell size or beta cell turnover. PTHrP(1–36) did not significantly change the total number of islets per pancreatic area (Fig. 3a) or the number of singlet and doublet insulin-positive cells in the exocrine tissue and ducts of the pancreases, which are presumed to represent newly formed islets (Fig. 3b). Beta cell hypertrophy did not contribute to the PTHrP(1–36)-induced increase in beta cell mass, as beta cell size was not significantly changed in any

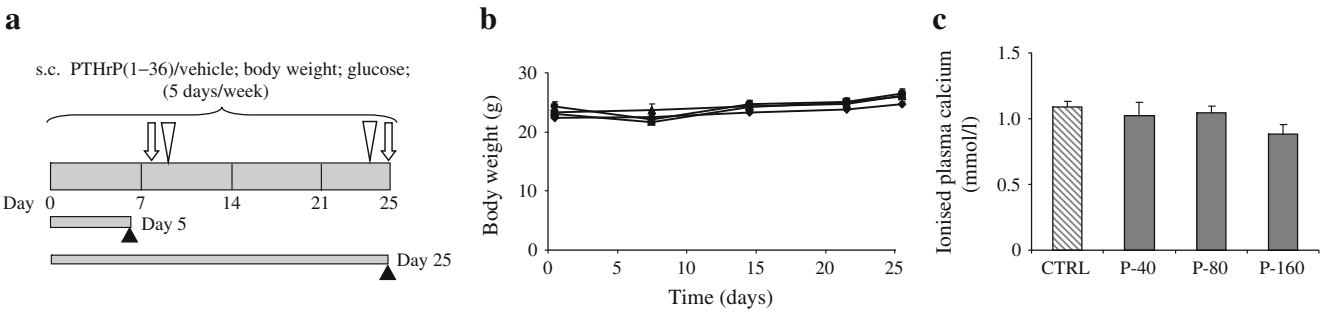

Fig. 1 Effect of systemic administration of PTHrP(1-36) on body weight and plasma calcium. **a** Schematic representation of the experimental design used in Balb/C male mice. The top grey bar represents the duration of the experiment (25 days). Mice were weighed, injected s.c. with vehicle or three different doses of PTHrP (1–36), and blood glucose was measured 5 days/week for the duration of the experiment. The white arrows mark days 8 and 25 when plasma calcium was measured. White triangles mark days 9 and 23 when IPGTT was done. The black triangles (days 5 and 25) represent administration of BrdU and killing of mice for removal of pancreases or islets. **b** Average body weight in the four groups of mice receiving vehicle (controls, diamonds), or PTHrP(1–36) at 40 (triangles), 80 (squares) or 160 (circles) μg/kg body weight over the 25-day period; n=6–7 mice/group. **c** Average plasma calcium levels measured at days 8 and 25 in the four groups of mice receiving vehicle (CTRL), or PTHrP(1-36) at 40 (P-40), 80 (P-80) or 160 (P-160) μg/kg body weight; n=4 mice/group

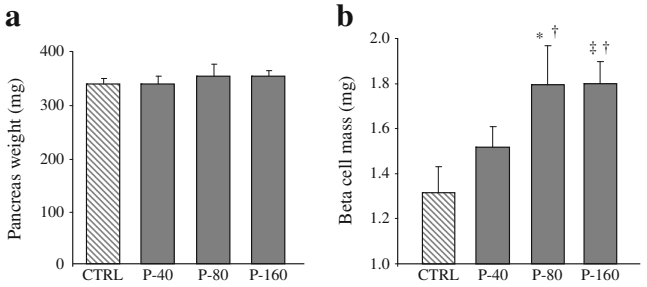

Fig. 2 Systemic administration of PTHrP(1–36) increases beta cell mass. **a** Pancreatic weight and **(b)** quantification of beta cell mass in insulin-stained pancreatic sections from control (CTRL) (n=9), P-40 (n=6), P-80 (n=6) and P-160 (n=8) mice after 25 days of treatment. *p<0.05 and ‡p<0.007 vs control by Student's t test; †p<0.05 vs control by one-way ANOVA

of the PTHrP(1–36)-treated groups vs controls (Fig. 3c). Finally, we measured beta cell death using two different methods: co-staining for insulin and propidium iodide (Fig. 3d), or TUNEL. The percentage of condensed beta cell nuclei, representing cell death, was similar in the four groups of mice (Fig. 3e), as was the percentage of TUNEL-positive beta cells in control and P-160 mice (Fig. 3f). This suggests that PTHrP(1–36) does not affect beta cell turnover under basal conditions.

All three doses of PTHrP(1–36) enhanced beta cell proliferation To determine whether changes in beta cell proliferation contributed to the PTHrP(1–36)-mediated increase in beta cell mass, the percentage of BrdU-positive beta cell nuclei was quantified after 25 days of PTHrP(1–36) treatment. There was a significant 1.7-fold to twofold increase in beta cell proliferation with all three doses of PTHrP(1–36) relative to controls (Fig. 4a). This was also confirmed using a different method to measure beta cell proliferation, namely co-staining with PCNA and insulin, which also showed a 1.6-fold increase in P-80 vs control mice (Fig. 4b).

PTHrP(1–36) stimulates beta cell proliferation after only 5 days of treatment To determine whether a progressive increase in beta cell proliferation could be responsible for the increase in beta cell mass, we measured beta cell replication at an earlier time point in P-160 mice. Mice injected with vehicle or 160 μg PTHrP(1–36) were analysed for beta cell proliferation at day 5, either after a single dose of BrdU injected 6 h prior to removal of the pancreases (Fig. 5a, b), or after giving BrdU in the drinking water for the previous 5 days (Fig. 5c, d). The two different methods of BrdU treatment provide information about beta cell replication occurring on day 5 vs cumulative proliferation over 5 days respectively. Using the single-dose BrdU injection approach, the percentage of BrdU-positive beta cells was significantly increased by about twofold in P-160 mice compared with controls (Fig. 5a, b, e), indicating an

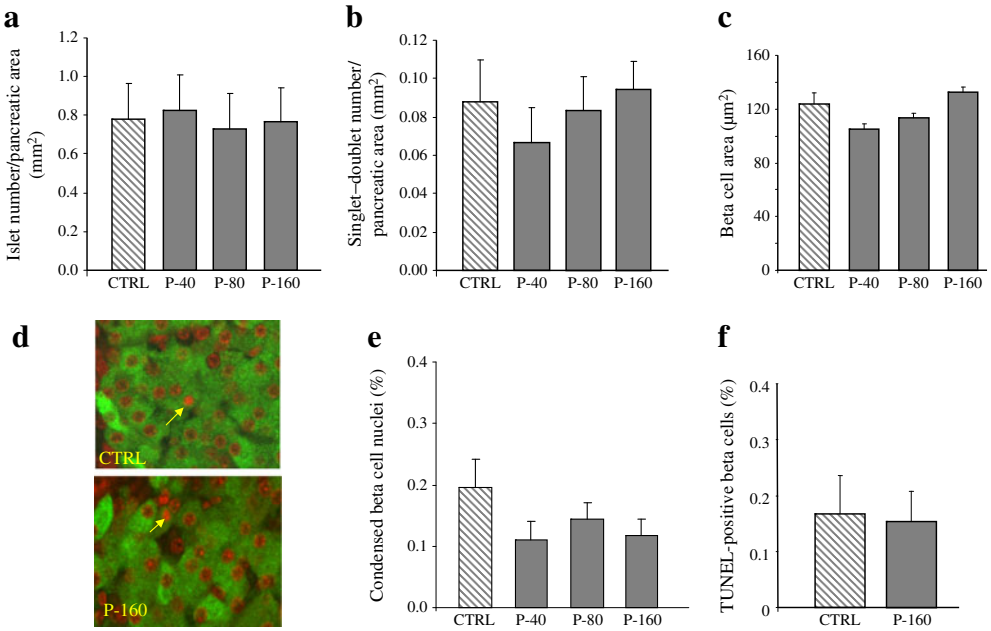

Fig. 3 PTHrP(1–36) treatment does not cause a significant change in islet number, beta cell size or beta cell turnover. **a** Total number of islets/pancreatic area, **(b)** number of insulin-positive singlet or doublet cells/pancreatic area in the exocrine tissue or ducts of the pancreas, and **(c)** average beta cell area in control (CTRL), P-40, P-80 and P-160 mice; n=4–5 mice/group. **d** Pancreas sections from CTRL and P-160 mice stained for insulin (green) and propidium iodide (red), with arrows identifying the infrequent condensed beta cell nuclei. **e** Quantification of the percentage of condensed beta cell nuclei in the four groups of mice; n=6–7 mice/group. **f** Quantification of the percentage of TUNEL-positive beta cell nuclei in control and P-160 mice; n=4 mice/group

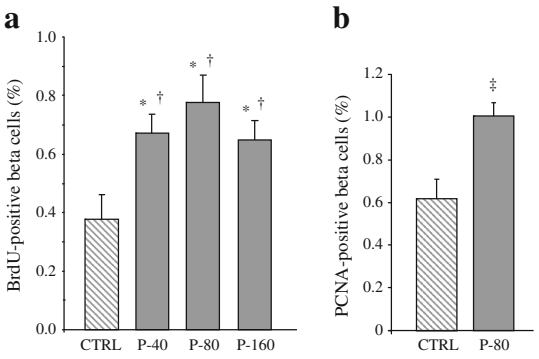

Fig. 4 PTHrP(1–36) stimulates beta cell proliferation after 25 days of treatment. Beta cell proliferation was measured at day 25 as **(a)** percentage of BrdU-positive beta cell nuclei in control (CTRL), P-40, P-80 and P-160 mice, and **(b)** as percentage of PCNA-positive beta cell nuclei in groups as shown; n=6–7 mice/group. *p<0.05 and ‡p<0.02 vs control by Student's t test; †$p \leq 0.05$ vs control by one-way ANOVA

induction of proliferation on day 5 by PTHrP(1–36). As expected, the cumulative proliferation over 5 days in control mice receiving BrdU in their drinking water was significantly greater than in the single-dose BrdU control mice (Fig. 5a, c, e). Here, too, the P-160 mice showed a significant twofold increase in proliferation compared with control mice in the group treated for 5 days with BrdU in their drinking water (Fig. 5c–e). The amount of water consumed by the control and P-160 mice receiving BrdU in drinking water was similar (not shown). Finally, proliferation quantified by PCNA-positive beta cells was also significantly increased after 5 days of PTHrP(1–36) treatment in P-160 mice compared with controls (Fig. 5f).

Effect of PTHrP(1–36) on glucose homeostasis and beta cell differentiation Glucose homeostasis, evaluated as weekly random blood glucose (Fig. 6a) or fasting plasma glucose at days 9 and 23 (0 min time point in IPGTT experiments; Fig. 6b, d), did not differ significantly among the four groups of mice. However, glucose tolerance was significantly and dose-dependently improved at day 9 following treatment with PTHrP(1–36) in the P-80 and P-160 groups vs controls (Fig. 6b, c). Surprisingly, the improvement in glucose tolerance observed at day 9 completely disappeared at day 23 in all three PTHrP(1–36)-treated groups (Fig. 6d).

To determine whether PTHrP(1–36)-induced beta cell proliferation caused changes in expression of genes regulating the function or differentiation of the beta cell, we examined the transcriptional profile of these genes in islets isolated from control or P-160 mice at day 25. There was no significant change in expression of genes encoding islet hormones (*insulin*, *somatostatin*), glucose sensors (*Glut2*, *glucokinase*, *Kir6.2*) or transcription factors important for beta cell differentiation and function (*pancreatic and duodenal homeobox 1* [*Pdx1*], *v-maf musculoaponeurotic fibrosarcoma oncogene family [Maf]*, *protein A [MafA]*, *Maf protein B*, *neurogenic differentiation[Neurod]*, *NK6 homeobox 1 [Nkx6.1]*) (Fig. 6e). This suggests that the enhanced beta cell proliferation induced by PTHrP(1–36) treatment does not negatively affect beta cell function or differentiation.

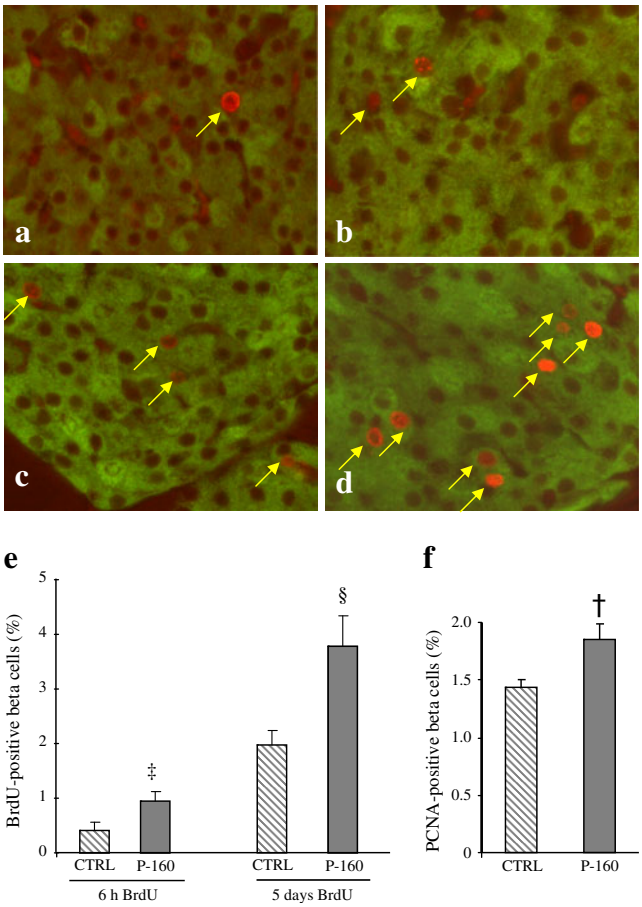

Fig. 5 PTHrP(1–36) stimulates beta cell proliferation by day 5 of treatment. The effect on beta cell proliferation was measured after 5 days of 160 μg/kg PTHrP(1–36) treatment. **a–d** Pancreatic sections stained for insulin (green) and BrdU (red), with BrdU-positive beta cells indicated by arrows. Sections were from control (**a, c**) and P-160 (**b, d**) mice injected with a single dose of BrdU 6 h (**a, b**) or given BrdU in drinking water for 5 days (**c, d**) prior to killing. **e** Quantification of beta cell proliferation as percentage of BrdU-positive beta cells in control (CTRL) and P-160 mice given BrdU as indicated (n=3–4 mice/group), and **(f)** as percentage of PCNA-positive beta cells in mice as shown. As PCNA is an endogenous marker of proliferation, mice from the 6 h and 5 day groups were combined for this analysis; n=7–8 mice/group. †p<0.02, ‡p<0.04, §p<0.008 vs corresponding controls by Student's t test

PTHrP modulates expression of the G1/S cell cycle regulators, cyclin D2 and p16^{Ink4a}, in islets We next examined whether PTHrP regulates expression of the cell cycle molecules in the G1/S phase. There was no significant change in mRNA expression of the genes encoding cyclins (*cyclin D1* to *D3, A1* and *E2*), *cyclin-*

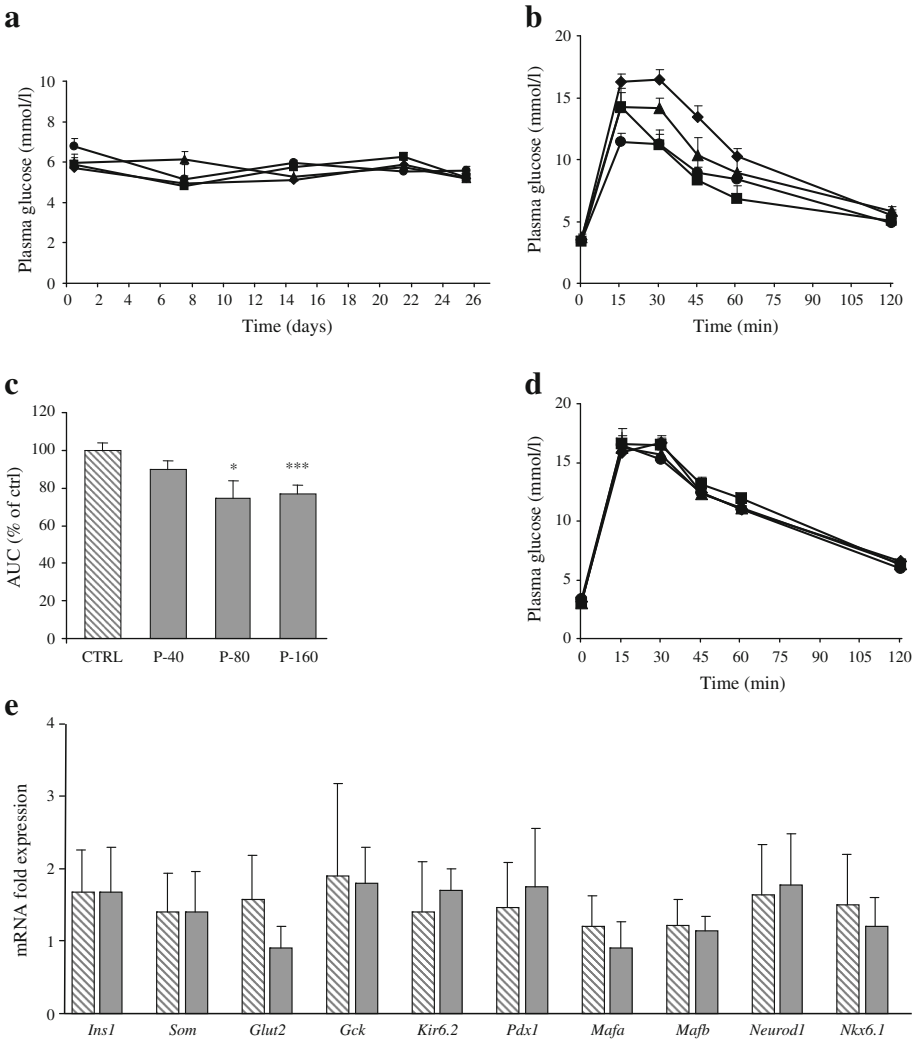

Fig. 6 Effect of PTHrP(1–36) on glucose homeostasis and beta cell differentiation. **a** Weekly blood glucose, **b** IPGTT on day 9 and **(c)** AUC of the glucose response on day 9 showed a significant improvement in the response at the two higher doses, i.e. P-80 and P-160, of PTHrP(1–36); n=6–17 mice/group; *p<0.05 and ***p< 0.001 vs control (CTRL) by Student's t test. **d** IPGTT on day 23 of treatment showed no difference between the four groups of mice; n=6–7 mice/group. **a, b, d** Diamonds, control; triangles, P-40; squares, P-80; circles, P-160. **e** Expression of differentiation markers by real-time PCR in islets from control (hatched bars) and P-160 (grey bars) mice after 25 days of treatment. PCR cycles for each gene were compared with actin as internal control; n= 4 mice/group, samples done in duplicate. *Som*, also known as *Sst*; *Glut2*, also known as *Slc2a2*; *Kir6.2*, also known as *Kcnj11*

dependent kinases 2, 4 and *6*, or the inhibitors $p21^{Cip1}$ (*Cip1* also known as *Cdkn1a*) and $p27^{Kip1}$ (*Kip1* also known as *Cdkn1b*) in islets of P-160 mice relative to controls after 25 days of treatment (Fig. 7a). To determine whether PTHrP regulates these molecules at the protein level, we performed western blot analysis on islet extracts. Since this approach requires a much larger number of islets, we used islets from RIP-*PTHrP* transgenic mice and normal littermates, as the supply of these islets was abundant relative to islets from the PTHrP(1–36)-injected mice and the PTHrP(1–139) produced in the islets of RIP-*PTHrP* transgenic mice also induces beta cell proliferation [15]. There was no change in abundance of the G1/S cell cycle activators cyclin D1, D3 and E, or of cyclin-dependent kinases 2 and 4, or of the inhibitors, $p18^{Ink4c}$ (*Ink4c* also known as *Cdkn2c*), p19, $p21^{Cip1}$ and $p27^{Kip1}$, and $p57^{Kip2}$ (*Kip2* also known as *Cdkn1c*) in transgenic vs normal mouse islets (Fig. 7b). In contrast, the G1/S cell cycle activator cyclin D2 (Fig. 7b, c) was significantly upregulated and the inhibitor $p16^{Ink4a}$ (Fig. 7b, d) was significantly downregulated, both by approximately two-fold in islets of RIP-*PTHrP* transgenic vs normal mice. To determine whether acute PTHrP(1–36) treatment led to similar changes in abundance, islets were isolated from control and P-160 mice after 5 days of treatment, when a significant increase in beta cell proliferation was observed in P-160 mice (Fig. 5). Analysis of cyclin D2 (Fig. 7e) and $p16^{Ink4a}$ (Fig. 7f) proteins showed no significant difference in expression between control and P-160 mice, suggesting that PTHrP(1–139) overproduction and PTHrP(1–36) administration enhance beta cell replication by different mechanisms.

Discussion

This study demonstrates that acute and systemic administration of amino-terminal PTHrP(1–36) peptide in rodents can stimulate beta cell proliferation and enhance beta cell mass in vivo, without negatively affecting beta cell function

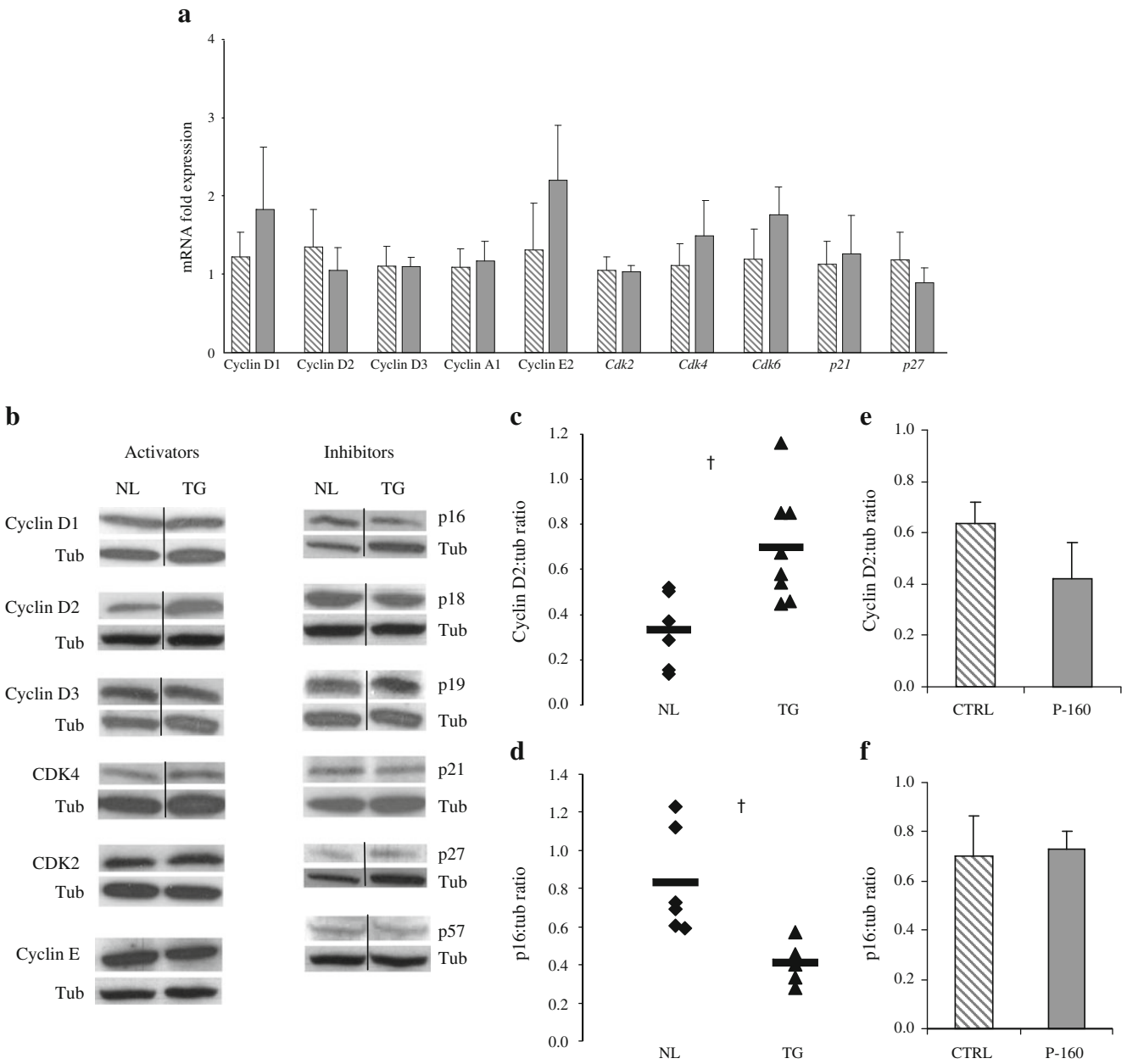

Fig. 7 The G1/S cell cycle activator, cyclin D2, is upregulated and the inhibitor, p16^{Ink4a}, is downregulated in RIP-*PTHrP* transgenic, but not in P-160 islets. **a** Expression of G1/S cell cycle regulators by real-time PCR in islets from control (hatched bars) and P-160 (grey bars) mice after 25 days of treatment. PCR cycles for each gene were compared with actin as an internal control; n=3–4 mice/group, with samples evaluated in duplicate. **b** Representative western blot analysis of the G1/S cell cycle activators and inhibitors in islets isolated from RIP-*PTHrP* transgenic (TG) and normal (NL) mice using tubulin (Tub) as internal control. Vertical lines divide samples from different regions on the same gel. CDK2/4, cyclin-dependent kinase 2/4. **c** Quantification of the ratio of cyclin D2: tubulin and **(d)** p16^{Ink4a} : tubulin protein showing the individual ratios from each islet sample. The horizontal line depicts the mean. Of all the cell cycle proteins analysed **(b)** (n=5–9 islet preparations/group), only cyclin D2 was significantly more abundant and p16^{Ink4a} significantly less abundant in RIP-*PTHrP* transgenic islets compared with normal islets; $^{\dagger}p<0.02$. **e** Quantification of the ratio of cyclin D2 : tubulin and **(f)** p16^{Ink4a}: tubulin protein in islets isolated from control (CTRL) and P-160 mice after 5 days of treatment showed no difference in abundance of these proteins; n=5–6 islet preparations/group

or survival. Previous studies have shown that transgenic production of the full-length PTHrP(1–139) in beta cells of RIP-*PTHrP* transgenic mice results in an increase in beta cell mass, proliferation and islet number, and in improved glucose homeostasis in these mice [12, 15, 18, 19]. In addition, in vitro studies have shown that the amino-terminal PTHrP(1–36) peptide is sufficient to enhance not only proliferation, in rodent insulinoma cell lines and primary

human beta cells, but also function, increasing insulin mRNA and protein, and augmenting glucose-stimulated insulin secretion [13–17]. Based on these data, we examined whether PTHrP(1–36) peptide could induce similar effects in vivo, as a first step towards determining its therapeutic potential for the treatment of diabetes.

The major novel aspects of this study compared with previous in vivo studies using RIP-*PTHrP* transgenic mice are: (1) PTHrP is administered systemically rather than being expressed endogenously in the beta cell; (2) PTHrP treatment is acute, for a short time, rather than via continuous expression in the beta cell; (3) PTHrP is given to adult mice rather than being produced from conception of the beta cell in transgenic mice; and (4) only the amino-terminal PTHrP(1–36) peptide is being administered, rather than the entire PTHrP(1–139) protein being produced in transgenic mice.

Our choice of doses and duration of PTHrP(1–36) treatment was based on previous work showing that amino-terminal PTHrP and PTH have anabolic effects on bone using a similar treatment regimen [22, 23]. Indeed, we found that systemic administration of all three PTHrP(1–36) doses increased endogenous beta cell proliferation at 4 weeks, as well as in the early phase of treatment for the highest 160 μg/kg dose. Importantly, the two higher doses of PTHrP(1–36) caused a significant increase in beta cell mass. RIP-*PTHrP* transgenic mice exhibit a progressive increase in their beta cell mass, with no significant change at 1 week, and a significant twofold increase by 2 to 3 months, which further increases to threefold at 1 year [19]. It is therefore likely that prolonged systemic administration of PTHrP(1–36) beyond 1 month could result in a similar progressive increase in beta cell mass, an issue that will be addressed in future studies. The enhanced beta cell mass in the PTHrP(1–36)-treated mice is likely to have been due to the increase in beta cell replication and not to changes in beta cell size, turnover or islet number, as none of these variables changed significantly in these mice. This was somewhat different from our observations in RIP-*PTHrP* transgenic mice, which showed a significant twofold increase in islet number at 2 months of age [18, 19]. This difference could stem from the fact that in the transgenic mice, PTHrP is probably produced early in embryonic life when the insulin promoter is first activated in the beta cell and, being a secretory protein, could potentially influence differentiation of neighbouring progenitor cells. In the current study, PTHrP(1–36) was injected once a day into adult mice and is therefore less likely to have had effects on islet development or number.

Although some studies have shown that PTHrP stimulates beta cell proliferation [13, 15, 16], very little is known about the effect of PTHrP on regulation of the G1/S cell cycle molecules at the transcriptional and protein levels in rodent islets. We found a significant increase in cyclin D2 protein and a decrease in the inhibitor $p16^{Ink4a}$ protein, in islets from RIP-*PTHrP* transgenic mice with overproduction of PTHrP(1–139) in beta cells vs normal littermates. PTHrP has been shown to modulate a number of cell cycle molecules. Thus it inhibits production of the cyclin-dependent kinase-interacting protein/kinase-inhibitory protein and inhibitor of kinase (INK) family of cell cycle inhibitors that includes $p15^{Ink4b}$ (*Ink4b* also known as *Cdkn2b*), $p27^{Kip1}$ and $p57^{Kip2}$, as well as inducing transcription of cyclin D1 in different cell types in which it enhances proliferation, including chondrocytes, early osteoblastic cells and vascular smooth muscle cells [24–28]. Surprisingly, the increase in cyclin D2 and decrease in $p16^{Ink4a}$ abundance were not observed in islets isolated from P-160 mice after 5 days of PTHrP(1–36) treatment relative to control mice. This differential regulation of the cell cycle molecules in PTHrP(1–139) transgenic vs PTHrP (1–36)-treated islets could be the result of several factors including the following: (1) the effect of chronic PTHrP production vs acute treatment; (2) the effect of full-length PTHrP(1–139) and/or other PTHrP fragments vs only the amino-terminal PTHrP(1–36) peptide; (3) the effect of endogenous production vs exogenous treatment; and (4) possible local variations in effective concentrations of the protein. A recent report that is relevant to our findings found that mice lacking the nuclear localisation sequence and C-terminus of PTHrP (amino acids 85–139), but producing the amino-terminus PTHrP peptide showed decreased levels of cyclin D and increased levels of $p16^{Ink4a}$ in bone cells and embryonic fibroblasts [29], implying that the C-terminus region of PTHrP is required for the regulation of these cell cycle molecules. We observed the mirror effect of this in beta cells over-producing full-length PTHrP in vivo, but not in islets treated with only the amino-terminal PTHrP(1–36) peptide, again suggesting that the C-terminus region of PTHrP is important in the regulation of cyclin D2 and $p16^{Ink4a}$. The question of whether the proliferative effects of PTHrP on rodent beta cells are mediated through the observed increase in cyclin D2 and/or decrease in $p16^{Ink4a}$ needs further verification.

RIP-*PTHrP* transgenic mice display hypoglycaemia and hyperinsulinaemia under fasting and non-fasting conditions, as well as improved glucose tolerance [15, 18, 19]. Systemic and acute administration of PTHrP(1–36) for 25 days did not alter plasma glucose in this study. However, there was a differential effect on glucose tolerance in the PTHrP-treated mice during the early vs the late phase of treatment. The significant improvement in glucose tolerance seen with the two higher doses of PTHrP(1–36) at day 9 was not observed at day 23, despite a 30% increase in

beta cell mass in the P-80 and P-160 mice. The return of glucose tolerance to baseline values in these mice at day 23 is unlikely to be due to a negative impact of PTHrP(1–36) on beta cell function, as several studies have shown that PTHrP(1–36) increases insulin expression and enhances insulin secretion in rodent and human beta cells, similarly to full-length PTHrP [13, 15–17]. Thus, the short-term improvement in IPGTT vs the long-lasting effect on proliferation is more likely to be due to a signal-specific differential desensitisation/downregulation of the PTHR1. PTHrP is known to enhance beta cell function and proliferation through two distinct signalling pathways, namely activation of cyclic AMP with inhibition of the c-Jun N-terminal kinase pathway [17], and activation of the phosphatidylinositol 3-kinase and atypical protein kinase C (PKC) ζ pathway [15] respectively. In this context, there is evidence in osteoblasts that the desensitisation of PTHR1 is signalling pathway-specific, with a more pronounced effect on the cAMP/protein kinase-A pathway than on the phospholipase-C/PKC pathway [30]. Similarly, it is possible that the beta cell also displays preferential attenuation of the cAMP, but not of the atypical PKCζ signalling pathway downstream of the PTHR1, leading to more short-lived effects of PTHrP(1–36) on glucose homeostasis vs beta cell proliferation. Importantly, there was no overall negative impact of PTHrP(1–36) treatment on glucose homeostasis in these mice.

The current studies raise numerous interesting questions. What would be the effect of higher doses of PTHrP(1–36) injected for varying durations on glucose and beta cell homeostasis? What would be the effect of treating mice with PTH instead of PTHrP, or with mutant peptides that activate specific signalling pathways [31]? It seems plausible that increasing the dose and/or duration of PTHrP(1–36) treatment would be likely to further enhance beta cell growth, based on the progressive increase in beta cell mass observed with age in RIP-*PTHrP* transgenic mice [19]. However, it is possible that long-term treatment (for 3–6 months) with PTHrP(1–36) injected on a daily basis may not induce any further increase in beta cell mass, similarly to its effects in bone, where intermittent treatment is anabolic, but continuous or longer term treatment is catabolic or does not cause further increases in bone mass [22, 32]. Based on this, it may be more beneficial to treat mice less frequently (1–2 times per week) on a long-term basis to induce further increases in beta cell mass and perhaps improve glucose tolerance.

Another question is whether long-term systemic PTHrP (1–36) treatment could cause tumours or hypercalcaemia in mice? The doses of PTHrP(1–36) in this study did not cause hypercalcaemia over 25 days, nor did daily injections of PTHrP(1–36) in rats for 6 months [33] or overabundance of PTHrP in the beta cells of RIP-*PTHrP* transgenic mice [19]. Furthermore, we have not detected insulinomas in RIP-*PTHrP* mice even after life-long (~2 years) production of PTHrP in their beta cells (R. C. Vasavada, unpublished observations). However, treatment duration and dose of the related PTH(1–34) peptide were found to be important determinants in the induction of bone tumours in rats [34]. Therefore, it is critical to find the right dose and duration of PTHrP(1–36) treatment to balance the positive effects of PTHrP on the beta cell and its potential negative systemic effects.

While a number of growth factors, signalling and cell cycle molecules have the capacity to enhance rodent beta cell proliferation when expressed transgenically in the beta cell in vivo [35–37], only very few have this effect when administered systemically and acutely in vivo in non-diabetic rodents. In this regard, glucagon-like polypeptide-1 (GLP-1) and its analogues, as well as prolactin constitute two peptide families that induce rodent beta cell proliferation in vivo either when injected or infused continuously [38–40]. GLP-1 and its analogues are currently commercially available drugs for the treatment of diabetes [41, 42]. Like GLP-1, PTHrP(1–36) (1) systemically enhances endogenous rodent beta cell proliferation and mass, (2) acts through a seven transmembrane G-protein-coupled receptor activating many signalling pathways including the cyclic AMP pathway [17], (3) is safe to use in humans as demonstrated through clinical trials of its use in the treatment of osteoporosis [20, 21] and (4) therefore has the potential to be used in the future treatment of diabetes. Other growth factors such as gastrin, epidermal growth factor and islet neogenesis-associated protein have also been shown to increase beta cell mass when administered systemically in rodents [43–46]. However, these growth factors increase beta cell mass through differentiation of new beta cells from non-beta cells, rather than by enhancing proliferation of pre-existing beta cells, as observed in our study. Therefore, it would be interesting to examine the effects of systemically administering PTHrP (1–36) in combination with one or more of these other growth factors to determine whether we can further augment beta cell mass through activation of differentiation and proliferation of beta cells.

In conclusion, this study clearly shows that the amino-terminal PTHrP(1–36) peptide is capable of enhancing rodent beta cell proliferation and mass in vivo, without negatively affecting beta cell function or survival, when administered acutely and systemically in normal adult mice. Future studies will determine whether this peptide can be used to induce endogenous beta cell regeneration in models of type 1 and type 2 diabetes or obesity. Given that clinical studies are currently ongoing to determine the ideal dose and treatment regimen of this peptide for the treatment of osteoporosis [20, 21], there is a good chance that PTHrP(1–36) could be a promising and safe therapeutic agent for diabetes.

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Duality of interest A.F. Stewart is a member of Osteotrophin LLC. The other authors declare that there is no duality of interest associated with this manuscript.

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