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Parathyroid hormone suppresses osteoblast apoptosis by augmenting DNA repair

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Abstract

Daily injection of parathyroid hormone (PTH) is a clinically approved treatment for osteoporosis. It suppresses apoptosis of bone forming osteoblasts although its exact anti-apoptotic mechanism(s) is incompletely understood. In this study, PTH treatment of cultured osteoblasts blocked the pro-apoptotic effects of serum withdrawal and nutrient deprivation; hydrogen peroxide induced oxidative stress, and UV irradiation. We hypothesized that PTH might suppress osteoblast apoptosis by enhancing DNA repair. Evidence is provided showing that post-confluent, non-proliferating osteoblasts treated with PTH exhibited a protein kinase A-mediated activation of two proteins that regulate DNA repair processes (proliferating cell nuclear antigen and forkhead box transcription factor 3a) as well as a suppression of the pro-apoptotic growth arrest and DNA damage protein 153. Additional proof of a connection between DNA damage and osteoblast apoptosis came from an unexpected finding whereby a majority of fixed PTH-treated osteoblasts scored weakly positive for Terminal Deoxynucleotidyl dUTP Nick-End Labeling (TUNEL), even though similar cultures were determined to be viable via a trypsin replating strategy. TUNEL identifies DNA excision repair, not just apoptotic DNA fragmentation, and the most likely explanation of these TUNEL results is that PTH's activation of DNA repair processes would permit nucleotide incorporation as a result of enhanced excision repair. This explanation was confirmed by an enhanced incorporation of bromodeoxyuridine in PTH-treated cells even though a majority of the cell population was determined to be non-replicating. An augmentation of DNA repair by PTH is an unreported finding, and provides an additional explanation for its anti-apoptotic mechanism(s).

Keywords

osteoblast; parathyroid hormone; apoptosis; DNA repair; DNA damage

Introduction

Intermittent parathyroid hormone (PTH)¹ therapy is a clinically approved treatment for osteoporosis. It has been proposed that osteoporosis, and other age-related diseases, are caused, in part, by apoptosis of bone-forming cells [1-3]. PTH is thought to improve osteoporosis by inducing anabolic responses in bone-forming cells that stimulate bone matrix production and

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suppress osteoblast apoptosis [2,4]. Indeed, several groups have reported a suppression of apoptosis in primary osteoblasts and in several osteoblastic cell lines treated with PTH in response to pro-apoptotic stresses [5,6]. PTH treatment of cultured osteoblasts has been shown to stabilize their mitochondrial functions thereby helping to suppress apoptosis [7]. Yet, a complete understanding of exactly how PTH suppresses osteoblast apoptosis remains to be determined.

PTH's binding to its cell surface receptor results in the production of cyclic adenosine monophosphate (cAMP), which then activates protein kinase A (PKA) [2]. cAMP/PKA has previously been reported to suppress apoptosis in multiple model systems including osteoblasts *in vitro* [2,8]. Hepatocytes treated with dibutyryl-cAMP suppressed markers for apoptosis, such as cleavage of the DNA repair protein Poly-ADP-ribose Polymerase. In addition, a selective inhibition of PKA with an A-kinase anchoring protein (AKAP) inhibitory peptide reversed this anti-apoptotic effect [9]. Other inhibitors of PKA (isoquinoline sulfonamide or H89) also enhanced apoptosis caused by DNA damaging radiation, but a protein kinase C (PKC) inhibitor failed to mimic PKA inhibition [10].

cAMP/PKA activation has also been correlated with an augmentation of DNA repair in systems other than bone [11-13]. When treated with cAMP analogs, hepatocytes derived from aged, calorie-restricted rats displayed a DNA repair response similar to that observed in hepatocytes derived from young, healthy animals after exposure to ultraviolet (UV) radiation [11]. In addition, most progeroid diseases which exhibit defects in nuclear DNA repair are associated with increases in apoptosis and bone loss [3,14,15]. As cell death has been hypothesized to play a role in bone loss, then a decreased DNA repair with a succeeding increase in apoptosis may contribute to osteoporosis [1,16]. Thus, we hypothesized that PTH might suppress osteoblastic cell death by enhancing DNA repair, and that this effect might be driven by an upstream activation of cAMP/PKA.

Proliferating cell nuclear antigen (PCNA) acts as a coordinating protein for DNA repair and is necessary, but not sufficient for repair to occur [17]. A genetic disruption of cAMP response element binding protein (CREB) expression in CHO cells results in decreased PCNA expression, an inhibition of DNA repair, and an increased sensitivity to radiation induced cell death [12]. Forkhead box (FOXO) transcription factors play an important role in the suppression of oxidative damage and the regulation of DNA repair protein expression in multiple model systems [18-20]. Also, FOXO activation is up-regulated by elevated cAMP which leads to a suppression of oxidative stress in other model systems such as fibroblasts [21]. Growth arrest and DNA damage protein 153 (Gadd153) is a protein up-regulated by excessive DNA-damage, which sensitizes cells to oxidative stress and enhances apoptosis in multiple cells types [22-24]. Indeed, Pereira *et al.* [25] recently demonstrated that over-expression of Gadd153 in mice caused bone loss due to enhanced osteoblast apoptosis. Thus, if PTH suppresses osteoblastic apoptosis via PKA activation leading to enhanced DNA repair, then we would expect (1) enhanced nuclear PCNA localization, (2) activation of FOXO, and (3) diminished Gadd153 expression.

¹Abbreviations used are: A-kinase anchoring protein (AKAP); bromodeoxyuridine (BrdU); cAMP response element binding protein (CREB); cyclic adenosine monophosphate (cAMP); 8-(p-Chlorophenylthio)-2'-O-methyladenosine-3', 5'-cyclic monophosphate (8-CPT-cAMP); Exchange protein directly activated by cAMP (Epac); epidermal growth factor (EGF); fluorescence-assisted cell sorting (FACS); fetal bovine serum (FBS); forkhead box transcription factor (FOXO); growth arrest and DNA damage protein 153 (Gadd153); hydrogen peroxide (H₂O₂); insulin-like growth factor-1 (IGF-1); (3-(4,5)-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT); nucleotide excision repair (NER); parathyroid hormone (PTH); parathyroid hormone related peptide (PTHrP); phosphate-buffered saline (PBS); phorbol 12-myristate 13-acetate (PMA); proliferating cell nuclear antigen (PCNA); protein kinase A (PKA); protein kinase C (PKC); terminal deoxynucleotidyl transferase (TdT); terminal deoxynucleotidyl transferase mediated dUTP nick-end-labeling (TUNEL); ultraviolet (UV).

Terminal deoxynucleotidyl transferase (TdT) mediated dUTP nick-end-labeling (TUNEL) is widely considered to be a “gold standard” assay for assessment of apoptosis, though a review of the literature reveals that there are exceptions to TUNEL's specificity [1]. For example, TUNEL has been shown to identify Okazaki fragments produced during S-phase replication as well as in gene transcription [26,27]. Relevant to our current study, it has been proposed that TUNEL may identify nucleotide excision repair (NER) in cardiac myocytes [28] and bacteria [26]. NER involves the creation of DNA breaks through an endonuclease-driven excision of damaged nucleotides creating 3'OH groups [28,29]. TdT, the enzyme used to label nicked DNA in the TUNEL reaction, recognizes 3'OH groups at nicked ends in DNA making it plausible for the TUNEL reaction to identify NER-induced DNA breaks in fixed cells [28].

To test our hypothesis, an osteoblastic cell line proven to elicit an anti-apoptotic response to PTH was exposed to several DNA-damaging agents, and then submitted to trypsinization and replating to verify its vitality. Portions of these cells were examined for a TUNEL-positive reaction, while other portions were used to measure cell viability and apoptosis. Assays for proteins involved in regulating DNA repair (PCNA and FOXO3a) were completed, and Western blots for the expression of the proapoptotic protein Gadd153 were run.

Experimental Procedures

Cell culture

Using a Cleveland Clinic Internal Review Board approved protocol human bone marrow-derived stromal cells were obtained from Dr. George Muschler's laboratory at the Cleveland Clinic. These cultures served as a source of normal human osteoprogenitor cells that exhibit osteogenic differentiation *in vitro* [30]. These cells were seeded (200 cells/mm²) in α MEM supplemented with 10% fetal bovine serum, 50 μ g/mL L-ascorbate, 5 mM β -glycerophosphate, 10 nM dexamethosone, gentamycin and amphotericin B. Cultures were used at 14 days of incubation; a time when the cells are beginning to exhibit osteogenic differentiation markers [30]. Given their early stage of osteogenic differentiation, these cells respond weakly to PTH to activate PKA but respond robustly to forskolin to activate PKA.

UMR106-01 cells were routinely passaged in T-75 culture flasks and cultured in Eagle's minimum essential medium, plus L-glutamine, nonessential amino acids, 20 mM HEPES (pH 7.2), 10% fetal bovine serum, gentamycin and amphotericin B. Cultures were seeded at 2.5×10^6 cells per flask in 30 ml of media and grown at 37°C in a humidified 5% CO₂ atmosphere with routine passaging every 3 days. Experiments were set up by briefly washing confluent T-75 cell layers with Hanks' balanced saline solution without Ca²⁺ or Mg²⁺ followed by trypsinization of the cells (10 ml of 0.05% trypsin plus 0.53 mM EDTA in Hanks' solution at 37°C for 10 min). Cells were counted by hemacytometer and plated at 1000 cells/mm² into individual 35 mm dishes (960 mm², 2 ml of growth medium).

This UMR cell line represents a fully mature, differentiated osteoblastic cell line [31]. Maintenance of an osteoblastic phenotype was monitored by testing for this osteosarcoma cell line's ability to express osteoblast specific markers including their ability to mineralize *in vitro*. Unless otherwise stated all data shown are representative of at least three trials. Tissue culture media were obtained from either Sigma or Cellgro/MediaTech; defined fetal bovine serum (FBS) was obtained from HyClone Laboratories. All culture ware was purchased from either Falcon/Becton Dickinson or Costar. Some cultures were pulse-labeled for 3 h at the indicated incubation times with medium supplemented with 10 μ M bromodeoxyuridine (BrdU, Sigma-Aldrich). Cultures were fixed and permeabilized as described below with the exception that cultures were digested with 100 U/ml DNase I (Sigma-Aldrich) at 37°C for 20 min, and then prepared for immunohistochemistry as described below. Anti-BrdU mouse monoclonal antibody was obtained from BD Biosciences. As a control, prolonged DNase I digestion (2 h)

eliminated the detection of BrdU labeled nuclei indicating the majority of BrdU incorporation was in DNA.

Ultraviolet (UV) irradiation was accomplished by placing cultures with their lids off into a pre-sterilized UV cross-linking oven (Stratalinker 1800, Stratagene). Cultures were UV irradiated (254 nm) for 30 sec at 150 J/m².

Apoptosis assays

Apoptosis was assessed by a Cell Death Detection ELISA kit (Roche Applied Science) that identifies the nuclear release of histone proteins in nucleosomes as a result of nuclear envelope breakdown after apoptotic death. This ELISA is based on the spectrophotometric detection of histone-associated DNA fragments in mono- and oligo-nucleosomes at 405 nm. The Quant-iTTM PicoGreen[®] dsDNA Assay (Invitrogen) was utilized to detect total dsDNA as a surrogate measure for measuring cell numbers. Cell viability was spectrophotometrically assessed via (3-(4,5)-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction using the MTT Live Cell assay (Sigma-Aldrich) at 540 nm. The APO-BRDUTM TUNEL Kit with Alexa Fluor[®] 488 anti-BrdU (BD Biosciences) is a two color staining method for labeling DNA breaks and total cellular DNA to detect apoptotic cells by flow cytometry or immunofluorescence. Flow cytometry TUNEL assessed all floating cells and debris from the medium combined with their corresponding adherent cells trypsinized off the culture plate. Cells were immediately fixed and stored in 70% ethanol and kept at -20° C until assayed. Cell cycle analysis was determined by DNA content via propidium iodide (PI) staining and flow cytometry (BD Biosciences).

PTH peptides

All PTH and PTHrP peptides [rat, bovine, or human Nle^{8,18}-Tyr³⁴-PTH-(1-34) amide, PTH-(1-31), Nle^{8,18}-Tyr³⁴ PTHrP (1-36), PTHrP-(1-34); PTHrP-(7-34) amide; and bovine Nle^{8,18}-Tyr³⁴-PTH-(3-34) amide] were obtained from Bachem. All peptides (1 mg/mL) were diluted in either 10 mM sodium acetate, pH 6.0, or in MilliQ water, sterile filtered using a Millipore GV4 device, aliquoted and frozen at -70° C. Multiple experiments were run to optimize the dosing of PTH/PTHrP. All data represent experiments with a single bolus dose of PTH or PTHrP at 20 nM. Unless stated otherwise, PTH treatments were done using PTH₁₋₃₄.

PKA activation & inhibition

The A-kinase anchoring proteins (AKAP) derived inhibitory peptide (InCELLectTM AKAP St-Ht31) is a steared, cell permeable form of the AKAP inhibitory peptide (Promega Corporation). The control peptide, St-Ht31P, is ineffective at disrupting PKA anchoring to AKAPs. 8-(p-Chlorophenylthio)-2'-O-methyladenosine-3', 5'-cyclic monophosphate (8-CPT-2'-O-Me-cAMP or 8-CPT-cAMP) (Biolog) is a novel cAMP analog that preferentially targets Exchange protein directly activated by cAMP (Epac) over PKA. 8-CPT-cAMP was diluted in water, aliquoted and stored at -20° C. 8-Bromo-cAMP analog (Sigma-Aldrich) was diluted in water, aliquoted and stored at -20° C. Prostaglandin-E₂ (PGE₂) (Sigma-Aldrich) was diluted out in serum free culture medium, aliquoted and stored at -70° C. Forskolin (Sigma-Aldrich), a direct activator of adenylate cyclase, was diluted in 100% ethanol and stored at -20° C. Phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich), an activator of protein kinase C, was diluted in dimethyl sulfoxide, aliquoted and stored at -20° C. Insulin and IGF-1 (Sigma) were diluted in 1 mM HCl, sterile filtered and stored at -20° C. DMSO and 30% hydrogen peroxide (H₂O₂) were both obtained from Sigma-Aldrich. Rat epidermal growth factor (EGF) was a generous gift from Dr. Ed Maytin (Dept. of Biomedical Engineering, Cleveland Clinic).

Western blot analysis

Western blot analyses were carried out after treating cells for the indicated time periods, placing them on wet ice, immediately washing them with ice cold PBS, and then lysing them as previously described [32]. Protein concentration was determined using the MicroBCA assay (Pierce). Portions of the cell lysates (20 µg protein) were separated on an SDS-polyacrylamide gel electrophoresis system under reducing conditions using linear 1.5 mm, 4-20% Laemmli mini-gels (Invitrogen/Novex). Proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore) at 25 V, and 125 mA for 2 h in a wet, mini-transblot apparatus using a transfer buffer (25 mM Tris, 192 mM glycine, 4 % methanol, pH 8.3). Membranes were blocked with 5% (w/v) ovalbumin (Sigma-Aldrich) in PBS and then exposed to various primary and secondary antibodies. Anti-rabbit, polyclonal phospho-FOXO3a antibody (p-Thr24/32, 90-95 kDa) and the anti-rabbit, monoclonal FOXO3a (78-82 kDa) were obtained from both Cell Signaling Technologies and Upstate/Millipore. The anti-rabbit, polyclonal Gadd153 antibody (29-30 kDa) was obtained from Santa Cruz Biotechnology. Activated Caspase 3 antibody which only detects its activated 17 kDa form was purchased from Abcam. Anti-mouse, monoclonal α -tubulin antibody (Sigma-Aldrich and Cell Signaling Technologies) was utilized as a loading control. Western blot signals were detected using the ECL-Plus Western blotting system and Hyblot X-ray film (Amersham Pharmacia Biotech). Films were scanned on a flatbed scanner (Mikrotek ScanMaker 8700).

Immunohistochemistry

PCNA, phospho-CREB and TUNEL staining were all performed by immunohistochemistry. Anti-rabbit, monoclonal phospho-CREB antibody (p-Ser133, 43 kDa) (Cell Signaling Technologies) was utilized in our studies as a downstream marker of cAMP/PKA activity. The anti-mouse, monoclonal antibody for PCNA (Dako and Cell Signaling Technologies) was used to detect nuclear PCNA levels. Immunofluorescent staining was performed on cells grown in 35 mm tissue culture dishes. Plating and treatment were identical to procedures for Western analysis. After treatment, medium was removed; culture dishes were placed on ice, washed once with ice cold PBS containing 1 mM AEBSF and 10 mM β -glycerophosphate. The cells were immediately fixed with 2% formaldehyde in PBS at room temperature for 1 h. Fixed cells were permeabilized with 0.1% Triton X-100 for 5 min, washed once with PBS, and blocked with 5% ovalbumin in PBS for 30 min. Cells were next incubated with primary antibody for 30 min. Cells were washed 3 times, 5 minutes per wash, with PBS followed by incubation with secondary antibody (Alexa488 or Alexa594, Molecular Probes) for 30 min. Prepared specimens were mounted with VectaShield (Vector Labs) and a glass coverslip. Specimens were imaged using an Olympus BX-50 microscope outfitted with a high resolution CCD camera (Polaroid DMC-2). For each set of experiments all untreated and PTH-treated microscope images were taken at identical exposure settings.

Statistical analysis

Analysis of variance (ANOVA) was implemented using SigmaStat software v3.5. Quantitative results are reported as the mean $\pm$ standard deviation. Unless stated otherwise, statistical significance was set at a p value of less than 0.05.

Results

PTH inhibits osteoblast apoptosis via cAMP/PKA activation

UMR osteoblast-like cells were subjected to serum withdrawal at 48 h of incubation, which is when they reach confluence and stop dividing [33]. These cultures were left in this serum-free medium for an additional 192 h to induce a nutrient deprived state. In response to this stress, a majority of untreated UMR osteoblasts by 96 h of incubation (or 48 h of serum-starvation)

lifted off of the culture dish as floating refractile bodies (Fig. 1a). In stark contrast, PTH-treated UMR osteoblasts remained adherent to the culture dish and exhibited fewer floating refractile bodies in the media when similarly stressed (Fig. 1b). Measurement of adherent cells left on the dish at 96 h as compared to those at 48 h showed that ~80% of the untreated cells have detached from the culture dish by this time (Fig. 1c, arrow), while a similar number of PTH-treated cells remained attached to the culture dish at 48 h and 96 h of incubation (Fig. 1c, arrowhead). MTT assays of adherent cells at 96 h indicated very few viable untreated cells, in contrast to a large number of PTH-treated cells assessed as viable (Fig. 1d).

Figure 1E shows that untreated cultures exposed to 48 h of serum withdrawal and nutrient deprivation exhibited a strong signal for nucleosome release into the medium indicating cell death via apoptosis. UMR cultures treated with PTH₁₋₈₄ or PTH₁₋₃₄, both of which contain full biological activity, resulted in a very low signal for nucleosome release and apoptosis after 48 h of metabolic stress (Fig. 1e). These findings indicate that PTH treatment potently inhibits osteoblast apoptosis in response to serum withdrawal and nutrient deprivation.

All PTH or parathyroid hormone related peptides (PTHrP) that activate PKA suppressed apoptosis in UMR osteoblast cultures initiated by serum withdrawal and nutrient deprivation (Fig. 1e). Notably, PTH₁₋₃₁, which is considered to be a more PKA-specific ligand than PTH₁₋₃₄ [34], suppressed apoptosis in UMR cultures. Conversely, all truncated PTH/PTHrP peptides that do not activate PKA, yet may activate other signaling pathways [34], did not suppress apoptosis in UMR cultures (Fig. 1e). Other agents that activate PKA, forskolin and prostaglandin E₂, suppressed apoptosis in UMR cultures mimicking the anti-apoptotic effects of PTH/PTHrP (Fig. 1e).

cAMP activates another downstream target called Epac, which is a guanine nucleotide-exchange factor for the small GTPases, Rap1 and Rap2 [35]. To verify that cAMP's activation of PKA rather than Epac/Rap results in suppression of apoptosis, UMR cultures were treated with either 8-Br-cAMP (potently activates PKA) or 8-CPT-cAMP (preferentially induces Epac and only weakly activates PKA) [35]. 8-Br-cAMP blocked apoptosis in cultures exposed to serum withdrawal and nutrient deprivation as effectively as PTH/PTHrP, while 8-CPT-cAMP had no discernable inhibition of osteoblast apoptosis (Fig. 1e). These findings suggest that cAMP's activation of PKA, over that of Epac/Rap, is primarily responsible for the suppression of apoptosis observed in this osteoblastic cell line.

Phorbol esters activate PKC, but do not activate PKA [38]. PMA treatment of UMR cultures had no measurable effect on apoptosis caused by serum withdrawal and nutrient deprivation (Fig. 1e). Insulin, insulin-like growth factor-1 (IGF-1) and epidermal growth factor (EGF) do not (or only minimally) activate PKA [32,37]. All of these agents had no measurable effect on the suppression of osteoblast apoptosis during serum withdrawal and nutrient deprivation (Fig. 1e) even though UMR cells exhibit receptors that respond to these factors [32]. Altogether, these combined findings suggest that an activation of cAMP/PKA is likely to be the upstream pathway that suppresses apoptosis in osteoblasts.

Specifically blocking PKA activation partially reverses the anti-apoptotic effects of PTH

AKAPs are scaffolding proteins that regulate the phosphorylation and precise subcellular localization of PKA [38]. An AKAP peptide inhibitor (InCELLect™ AKAP St-Ht31) competitively inhibits unoccupied native AKAP proteins for binding with PKA, and was used in an attempt to mitigate the anti-apoptotic effects of PTH/PTHrP on osteoblasts. AKAP-St-Ht31 reduced the signal intensity of phospho-CREB within the nucleus of UMR cells treated with PTH providing proof that it attenuates PKA downstream signaling (Fig. 2).

When used alone, 10 μ M AKAP-St-Ht31 did not affect apoptosis of UMR osteoblasts induced by nutrient deprivation and serum withdrawal, while it reversed the anti-apoptotic effects of PTH₁₋₃₄ to ~50% of the untreated control value (Fig. 2). AKAP inhibition of PTH's anti-apoptotic effects was maximal at 10 μ M, with higher doses showing no greater inhibition (data not shown). A control AKAP peptide (InCELLect™ AKAP St-Ht31P), which does not contain the proper amino acid sequence, had no discernable effect on apoptosis and did not affect the anti-apoptotic effect of PTH₁₋₃₄ (Fig. 2). These findings indicate that a highly specific PKA inhibitory peptide can attenuate the anti-apoptotic effects of PTH on osteoblasts, and suggests that an activation of PKA by PTH is an important upstream signaling step leading to the suppression of osteoblast apoptosis.

PTH treatment suppresses cell death yet induces nucleotide incorporation

UMR cells were cultured in the presence or absence of PTH₁₋₃₄ and 10% FBS for 72 h, and then switched to fresh serum-free medium for an additional 48 h incubation. After 48 h of serum withdrawal, untreated cells showed a strong positive outcome for the release of nucleosomes indicating widespread apoptosis (Fig. 3a). Untreated cultures displayed few adherent cells, many floating refractile bodies (Fig. 3e), and ample evidence of nuclear blebbing and condensation (Fig. 3g,i) characteristic of the terminal stages of apoptotic death. Also, untreated cultures displayed a low level of viability as assessed by MTT assay (Fig. 3b) and nearly 70% of the cell population scored positive by TUNEL flow cytometry (Fig. 3c).

PTH-treated cells at 48 h of serum withdrawal appeared fully adherent with minimal refractile bodies present (Fig. 3d), exhibited high MTT viability (Fig. 3b), and showed minimal nucleosome release (Fig. 3a). Unexpectedly, 70% of these PTH-treated cells scored positive by TUNEL flow cytometry (Fig. 3c), in contradiction to all other data indicating that PTH-treated cells were viable (Fig. 3a,b,d). In addition, despite a modest TUNEL positive immunostaining result (Fig. 3h), most PTH-treated cells displayed normal nuclear morphology (Fig. 3f). Remarkably, PTH-treated cells remained adherent and viable under these culture conditions for a few more days beyond the time point when they were processed for TUNEL staining (Fig. 1C, 192 h). Thus, the TUNEL-positive results for PTH-treated cells may not reflect an actual apoptotic process in its early stages.

Since the TUNEL assay is applied to fixed cells, we sought a direct test of nucleotide incorporation in live cells in the presence or absence of PTH. UMR cells were cultured as described above with the exception that after 48 h of serum starvation an aliquot of 100 μ M BrdU was added to half of the cultures to yield a final concentration of 10 μ M, and all cultures were incubated for another 3 h. Cells from unlabeled cultures were isolated by trypsin digestion and submitted to flow cytometry to determine their cell cycle status and proliferative index. Flow cytometry determined that 80% of untreated UMR cells were in G1, 4% in G2, while 16% were in S phase (Supplementary Fig. I). This same approach revealed that 91% of PTH-treated UMR cells were in G1, 4% in G2, and only 5% in S phase (Supplementary Fig. I). Thus, the majority of cells in both untreated and PTH-treated populations at this time were not actively replicating. BrdU labeled cultures were submitted to immunohistochemical detection of BrdU-labeled nuclei. This analysis revealed that untreated cell populations contained ~8% labeled nuclei, while PTH-treated cultures contained ~35% labeled nuclei (Fig. 4). Thus, at a time when the majority of PTH-treated cells were not replicating, there was an unexpectedly high level of nuclear BrdU incorporation. This suggested the possibility of enhanced DNA repair activity in PTH-treated UMR cells.

PTH treated cells resist apoptosis induced with DNA damaging agents

It has been reported that serum withdrawal can induce DNA damage [39]. As PTH was clearly suppressing apoptosis due to serum withdrawal, then a weak TUNEL positive signal might be

revealing the accumulation of DNA nicks in nuclei, not necessarily from apoptotic-driven DNA damage, but instead from DNA excision repair. If PTH is suppressing apoptosis by augmenting DNA repair, we hypothesized PTH-treated cells should resist apoptosis after direct DNA damage and exhibit TUNEL positive staining as a result of NER.

UMR cells were cultured in 10% FBS containing medium for 72 h in the presence or absence of PTH₁₋₃₄. They were then exposed to 400 μ M H₂O₂ for 4 h to induce DNA damage followed by a 24 h recovery period in fresh medium during which time cells would either die or effectively repair the DNA damage and resist apoptosis. Untreated cells displayed many round, floating, refractile bodies (Fig. 5b), elevated nucleosome release (Fig. 5c), and low cell viability via MTT assay (Fig. 5d). Additionally a majority of untreated UMR cells scored strongly positive for TUNEL under these test conditions (Supplementary Figure II) and exhibited an ample presence of activated caspase 3 by Western blot (Fig. 5e). Altogether these data indicate widespread apoptosis in untreated UMR cultures treated with H₂O₂.

Conversely, PTH-treated cells were adherent and displayed only a few floating, refractile bodies (Fig. 5a), very little nucleosome release (Fig. 5c), high cell viability via MTT assay (Fig. 5d), and an absence of activated caspase 3 by Western blot (Fig. 5e) suggesting that they were healthy and viable. Yet, a majority of PTH-treated cells exposed to H₂O₂-induced DNA damage stained weakly to moderately positive for TUNEL (less intense than apoptotic bodies) while exhibiting morphologically normal-appearing nuclei (Supplementary Figure II). These observations suggested the presence of nucleotide incorporation via TUNEL assay in H₂O₂-damaged, PTH-treated cells may be due to NER because subsequent caspase 3 activation, nuclear fragmentation and cellular disruption did not ensue.

After H₂O₂ treatment the number of PTH-treated cells in S-phase (8%) was almost identical to the percentage of cells in S-phase immediately prior to H₂O₂-induced DNA damage (Supplementary Fig. I). Based on these findings, there were far too few PTH-treated cells actively proliferating to account for the high TUNEL positive numbers observed by both immunohistochemistry and flow cytometry (Supplementary Figure II). Thus, enhanced TUNEL labeling of PTH-treated cells exposed to H₂O₂ would more likely be the result of a DNA repair response where purposeful DNA nicks are generated as a result of NER.

As a means to independently assess cell viability, UMR cells were trypsinized and replated after 4 h of H₂O₂ exposure and a 24 h recovery period to examine their ability to reattach, spread, and populate a new culture dish. The proportion of PTH-H₂O₂ treated cells that reattached to a new culture dish after trypsin release appeared to be quantitative (Fig. 5f) and demonstrated high viability by MTT assay (Fig. 5h). In contrast, almost none of the untreated cells reattached (Fig. 5g) and displayed low cell viability by MTT assay (Fig. 5h). Thus, PTH-treated UMR cells after H₂O₂ treatment were indeed viable.

To prove that these results were not unique to just UMR osteosarcoma cells, human bone marrow derived osteoprogenitors were treated with forskolin to activate PKA. Phase contrast imaging of live cultures showed that forskolin treated cells resisted cell death when challenged with 600 μ M H₂O₂ to induce DNA damage (Fig. 6d), while untreated cells exhibited widespread cell death when challenged with H₂O₂ (Fig. 6c). MTT assays of adherent cells after H₂O₂ challenge indicated a substantial decrease in the number of viable untreated cells, in contrast to a majority of forskolin-treated cells assessed as viable after H₂O₂ challenge (Fig. 6e). Interestingly, most of the PKA-activated cells exhibited weak to moderate TUNEL positive nuclear staining, but did not lift off the dish or exhibit widespread nuclear condensation indicative of apoptosis (Fig. 6f). Thus, normal cells within the osteogenic lineage after PKA activation resist apoptosis and seem to react in a manner similar to that exhibited by UMR cells.

UV irradiation induces DNA damage both through induction of oxygen radicals and by photon-directed DNA dimerization [40]. Data from visual inspection (Fig. 7b), nucleosome release assay (Fig. 7c), and cell viability by MTT assay (Fig. 7d) indicated that most untreated UMR osteoblasts died by apoptosis when exposed to acute UV irradiation. To determine how many viable cells remained after UV irradiation, untreated UMR cells were released from the culture dish by trypsin digestion, and then re-plated into a new culture dish. Very few irradiated, untreated cells survived this re-plating test (Fig. 7f), and they exhibited very little MTT cleavage activity (Fig. 7g). Thus, 150 J/m² of UV irradiation is lethal to untreated UMR osteoblasts.

In contrast, PTH-treated UMR cells exposed to acute UV irradiation appeared adherent with few floating, refractive bodies (Fig. 7a), exhibited little nucleosome release (Fig. 7c), and showed high cell viability via MTT assay (Fig. 7d). After trypsin-release and replating, a large number of the UV-irradiated PTH-treated cells were able to reattach and spread on a new culture surface (Fig. 7e), and exhibited ample cell viability by MTT assay (Fig. 7g). Nevertheless, 150 J/m² UV irradiation was observed to induce irreversible damage in some PTH-treated UMR cells as roughly one-third of the original cells were not recovered after trypsin release as determined by cell counting (compare Figs. 7a & 7e). These replated PTH-treated, UV-damaged cells also exhibited normal proliferation rates and eventually achieved confluency (data not shown). Thus, many of the PTH-treated UMR osteoblasts are viable and capable of resuming cell cycle progression even after exposure to a lethal dose of UV irradiation, which indicates that PTH treatment confers protection against UV-induced DNA damage and apoptosis. Our findings to this point suggest that PTH-treated cells exhibit augmented DNA repair responses.

PTH treatment of UMR osteoblasts suppresses a DNA-damage response gene (Gadd153) that induces apoptosis

Confluent UMR cells were treated with PTH₁₋₃₄ (or left untreated) for 48 h, and then stressed by exposure to H₂O₂ or to serum withdrawal. Western blot analysis for Gadd153 shown in Figure 8 demonstrates that the levels of Gadd153 in PTH-treated cells (lane 2) were lower than that of untreated cells (lane 1) in the absence of a pro-apoptotic stimulus. When untreated cells were exposed to either H₂O₂ (lane 5) or serum withdrawal (lane 6) treatments, Gadd153 protein expression greatly increased compared to untreated cells in the absence of such pro-apoptotic stimuli (lane 1). In contrast, PTH-treated cells exposed to either H₂O₂ (lane 3) or serum withdrawal (lane 4) exhibited relatively low Gadd153 protein expression levels compared to their untreated but stressed counterparts (lanes 5 and 6, respectively). Thus, the levels of Gadd153 are increased in untreated osteoblasts undergoing apoptosis caused by H₂O₂ or serum withdrawal, whereas prior PTH treatment suppressed both an enhancement of Gadd153 expression and an apoptotic outcome. If TUNEL staining of PTH-treated cells was due to apoptotic DNA cleavage then Gadd153 levels should also have been enhanced. This is not the case suggesting that PTH is suppressing lethal levels of DNA damage in UMR osteoblasts by either blocking DNA damaging reactions and/or augmenting DNA excision repair processes.

PTH treatment of UMR osteoblasts activates two proteins regulating DNA repair

PCNA has been shown to have an important role in DNA repair synthesis [28]. PCNA activity was examined in confluent UMR cultures treated with PTH₁₋₃₄ (or left untreated) for 48 h, and then stressed by exposure to H₂O₂. Untreated UMR cells exposed to H₂O₂ exhibited a relatively small number of nuclei that stained weakly positive for PCNA (Fig. 9a). By comparison, nuclear PCNA staining in PTH-treated cells exposed to H₂O₂ was greater both in staining intensity (Fig. 9b) and the number of nuclei that were scored positive (Fig. 9e). Thus, at a time when only 5-10% of PTH-treated cells were in S-phase (see Supplementary Fig. IB) over 60%

of them stained positive for nuclear PCNA. Similar enhancement in nuclear PCNA staining was also observed after DNA-damage due to UV irradiation (data not shown).

Western blot analyses showed that phosphorylation of a transcription factor that regulates the expression of DNA repair proteins (FOXO3a) on its repressive Thr24/32 residue was reduced by PTH treatment of UMR cells (Fig. 9f) suggesting an up-regulation of its activity [19]. Other agents that activate PKA also reduced FOXO3a phosphorylation similar to that of PTH treatment (data not shown). The low proliferative state of these cultures, an accumulation of PCNA in the nuclei of PTH-treated cells, and a decreased phosphorylation of FOXO3a on Thr24/32 would suggest that DNA excision repair processes are augmented by PTH's activation of cAMP/PKA.

Discussion

Bellido *et al.* [7] reported that PTH treatment of osteoblasts results in a PKA-mediated phosphorylation of Bad and an increase in the expression of Bcl-2. These combined responses would help suppress mitochondrial membrane breakdown, the release of cytochrome C and an activation of caspases. Altogether this would enhance an osteoblast's survival under metabolic stress. Yet these authors pointed out that the Bad/Bcl-2 axis is likely to be only part of a larger overall mechanistic process whereby PTH enhances osteoblast vitality and suppresses apoptosis. We hypothesized that PTH treatment of osteoblasts might also augment nuclear DNA excision repair mechanisms mediated through an upstream activation of PKA thereby leading to a more efficient DNA-damage repair process, better chromatin stability, and enhanced cell survival.

This hypothesis was tested by comparing the cellular and molecular responses of UMR osteoblasts with or without PTH treatment to extreme metabolic stress and direct DNA damage. Several key findings support this hypothesis. PTH treatment (and other PKA activating agents) enabled a majority of UMR osteoblasts exposed to lethal doses of DNA damaging agents to remain viable and reattach to a new culture substrate after trypsin-replating. PTH-treated osteoblasts after DNA damage exhibited very little evidence of apoptotic bodies, ample MTT reduction activity, and re-initiated normal replication activities when replated at lower densities, in stark contrast to the outcomes for untreated cells. In addition, PTH treatment decreased Gadd153 expression in UMR osteoblasts exposed to pro-apoptotic stimuli suggesting that they were able to effectively manage nuclear DNA stability. These findings indicate that PTH treatment conferred survival protection against direct DNA damaging methods.

In addition, PTH-treated cells under metabolic stress exhibited nuclear incorporation of BrdU at a time when a majority of the cells were not replicating. Together with PTH's enhancement of nuclear PCNA staining and decreased phosphorylation of FOXO3a on its repressive Thr24/32 residue, this would suggest the BrdU incorporation was more likely the result of DNA repair. Altogether, these findings offer another mechanistic explanation for why PTH treatment suppresses osteoblast apoptosis. Namely, PTH treatment of osteoblasts may enhance their DNA repair properties providing a more efficient mending of genomic DNA damage from external stressors thereby preventing the accrual of lethal levels of DNA damage that would initiate an apoptotic response. A limitation in this study's design is that it cannot distinguish between direct actions of PTH on osteoblasts that might enhance DNA repair versus PTH acting indirectly by modulating the expression of paracrine or autocrine anti-apoptotic factors that also may be able to enhance DNA repair reactions in osteoblasts. Indeed, PTH has been shown to upregulate a sustained production of PTHrP by osteoblasts [41]. Our results in Figure 1 indicate that PTHrP is as potent as PTH to suppress osteoblast apoptosis, and this type of

feedback mechanism is likely involved in sustaining the enhanced DNA repair observed for UMR cells treated with PTH.

Apoptosis is commonly induced by serum withdrawal and nutrient deprivation, and some groups consider that the induction of apoptosis by this method entails DNA damage. Driscoll *et al.* [39] observed that serum starvation induced apoptosis in lung cancer cells and attributed this increased apoptosis with decreased PCNA expression and decreased DNA repair. It has also been reported that serum starvation induces oxidative stress, and serum starvation-induced apoptosis can be abrogated with antioxidants [42,43]. Treatment of UMR osteoblasts with H₂O₂ yielded similar pro-apoptotic responses as with serum withdrawal and nutrient deprivation, and PTH treatment effectively blocked all of these pro-apoptotic stimuli. This suggests that most of the pro-apoptotic stimuli in our study may be causing DNA damage via the formation of oxygen radicals.

Like many anti-cancer therapies (*e.g.*, radiation and cisplatin), etoposide induces apoptosis by causing DNA nicks through augmented oxidative stress, inhibition of DNA synthesis and prevention of repair by its binding to topoisomerase II [23,44]. Jilka *et al.* [5] and Sowa *et al.* [6] reported that PTH inhibited etoposide-induced apoptosis in osteoblastic cells, including UMR cells, though there was no mention of augmented DNA repair. It is worth noting that Gadd153 is upregulated by etoposide and contributes to etoposide's pro-apoptotic effects [23,45]. Since the current study shows that PTH treatment suppresses apoptosis and down regulates Gadd153 expression in UMR osteoblasts, it seems reasonable to conclude that an augmentation of DNA repair by PTH might also explain its ability to suppress the pro-apoptotic effects of etoposide.

Interestingly, PTH-treated UMR osteoblasts exposed to DNA damaging agents stained weakly to moderately positive by TUNEL reaction even though prior to fixation they were determined to be viable. Since PTH-treated UMR cells displayed relatively normal cell cycle groupings during TUNEL flow cytometry (see Supplementary Figure II), the attachment of nucleotides to the 3'OH ends of fixed DNA via the TUNEL reaction in PTH-treated nuclei was more likely generated by NER-mediated nicking and not apoptotic cleavage. While many researchers agree that nuclear DNA repair occurs in most cell types, most do not recognize that TUNEL is selective, but not specific for apoptosis and may be used to reveal NER [1]. In this regard, both Kanoh *et al.* [28] and Rohwer & Azam [26] have proposed that TUNEL may identify DNA repair in cardiac myocytes and bacterial cells respectively. Also, Hegyi & Skepper [46] reported that TUNEL positive cardiac myocytes were simultaneously PCNA positive, and Bartunek *et al.* [47] reported that DNA repair proteins such as redox factor endonuclease, DNA-protein kinase, and PCNA were elevated in failing human myocardium even though these same cells did not show evidence for proliferation or apoptosis suggesting that DNA repair was likely occurring. Thus, we submit that PTH treatment of osteoblasts in the presence of DNA damaging agents might be suppressing apoptosis in part through an augmentation of NER in nuclear DNA. This in turn would likely lead to an increased number of 3'OH groups in fixed cells available for TUNEL staining.

The possibility of TUNEL recognizing NER induced DNA breaks may help to explain an apparent contradiction in recent bone literature. Bellido *et al.* [7] observed increasing numbers of *in situ* nick-end labeled osteoblast nuclei associated with progressively lower bone formation rates. Conversely, Lindsay *et al.* [48] observed an increasing number of TUNEL positive osteoblast nuclei correlated with increasingly higher bone formation rates in PTH-treated patient bone biopsies. Though it is counter-intuitive to expect that increasing cell death would lead to increasing amounts of new bone tissue, it can be reasoned if some of the TUNEL positive nuclei observed in these types of studies were actually osteoblasts engaged in NER-mediated DNA damage repair, then a positive correlation between TUNEL signal output and bone

formation rates might be possible. Thus, the outcomes from these two studies may not be contradictory, but rather reflect different levels of stringency in their respective nick-end labeling reactions whereby Lindsay *et al.* [48] counted nuclei exhibiting NER mediated DNA damage repair, while Bellido *et al.* [7] had excluded these from their final nuclear counts.

Herein we conclude that PTH treatment of cultured osteoblasts augments DNA excision repair mechanisms. This overall process is mediated through an upstream activation of PKA leading to a more efficient DNA-damage repair process and enhanced survival under extreme metabolic stress and direct DNA damage (see summary diagram in Fig 10). The overall mechanism involves enhanced activities of at least two proteins influencing DNA repair, PCNA and FOXO3a, and includes decreased expression of the pro-apoptotic protein Gadd153. Undoubtedly other DNA repair proteins will likely be involved in this overall survival process, and future investigations will focus on delineating a more complete panel of proteins associated with this DNA repair mechanism. Altogether, PTH's enhancement of DNA damage repair coupled with its ability to stabilize mitochondrial functions [7] may account for much of PTH's ability to suppress osteoblast apoptosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. PTH suppresses apoptosis induced by serum withdrawal and nutrient deprivation
 PTH treated cells were exposed to PTH₁₋₃₄ from the time of plating. **Panel a and b** show phase contrast images of an untreated and a PTH treated culture, respectively, at 96 h of incubation; scale bars are 50 microns. **Arrows** point to round, refractile bodies mentioned in the text. **Panel c** shows a graph of adherent cell DNA determined by a PicoGreen assay; **arrow** (no PTH) and **arrowhead** (+ PTH) point to the 96 h time point ($n = 6$ cultures from 3 trials; mean $\pm$ SD; $p < 0.05$ between PTH treated and untreated). **Panel d** shows the results from MTT assays analyzed at 96 h of incubation ($n = 6$ cultures from 3 trials; mean $\pm$ SD; $p < 0.05$ between PTH treated and untreated). The assay control represents cultures that had daily media exchanges. **Panel e** shows an assessment of apoptotic cell death by a nucleosome release ELISA at 96 h of

incubation (n = 6 cultures from 3 trials; mean $\pm$ SD; $p < 0.05$ between PKA activators versus control). Drug concentrations were as follows: 20 nM PTH/PTHrP peptides, 1 μ M PGE₂, 10 μ M forskolin, 300 μ M 8Br-cAMP, 300 μ M 8-CPT-cAMP, 5 μ g/ml insulin, 50 ng/ml IGF-1, 10 ng/ml EGF, and 100 nM PMA.

Figure 2. AKAP inhibitor peptide attenuates the anti-apoptotic effect of PTH

UMR cultures were treated with a PKA inhibitory peptide (InCELLect™ AKAP St-Ht31) in the simultaneous presence (or absence) of PTH at the time of plating, and cultures were then submitted to serum withdrawal and nutrient deprivation. Results depicted here are from 48 h time points after serum withdrawal (96 h of incubation). Left panels depict phospho-CREB immunohistochemistry results with respective nuclear stain (DAPI) of the same field of views in the right panels. Scale bars are 50 microns. Lower graph depicts apoptosis results determined by nucleosome release ELISA (n = 6 cultures from 3 trials; mean $\pm$ SD $p < 0.05$ between PTH + AKAP St-Ht31 versus PTH alone). AKAP St-Ht31P is an inactive control peptide.

Figure 3. Viable PTH-treated UMR cells display TUNEL staining after 48 h serum starvation
 Panel a shows a measure of apoptosis via nucleosome release ELISA (absorbance at 405 nm). Panel b shows an assessment of cell viability by MTT assay (absorbance at 570 nm). Panel c summarizes the results from flow cytometry sorting of TUNEL positive nuclei. All plotted data represent mean $\pm$ SD from 3 trials ($n = 6$ cultures; $p < 0.05$ between PTH treated and untreated cultures in panels a & b). Phase contrast images of PTH-treated (panel d) and untreated (panel e) cells after 48 h of serum starvation; white-outlined black arrows point to round, refractile bodies mentioned in the text; scale bars are 50 microns. Immunohistochemical TUNEL stained images for PTH-treated (panel h) and untreated (panel i) cells are shown along with their corresponding DAPI-stained images (panel f) and (panel g), respectively; scale bars are 25 microns. Relatively intact nuclei (white solid arrows) stain TUNEL-positive in PTH-treated cells, while untreated cells display ample TUNEL-positive apoptotic bodies (white-outlined black arrows).

Figure 4. PTH-treated UMR cells display significant nuclear incorporation of BrdU even though the cell population is not actively replicating

Half of the cultures were treated with PTH at the time of plating, and then all cultures were submitted to serum withdrawal and nutrient deprivation. After 48 h of serum withdrawal, cultures were pulsed labeled with BrdU, fixed and then immuno-stained for the presence of nuclear BrdU incorporation. Scale bars are 50 microns. The numbers of BrdU-labeled nuclei normalized to the total number of nuclei were counted within three 10X fields of view and are shown in the lower graph (mean $\pm$ SD; $p < 0.05$ between PTH treated and untreated cultures). Cell cycle analyses for untreated and PTH-treated cell populations are shown in Supplementary Figures IA and IB, respectively.

Figure 5. H₂O₂-stressed, PTH-treated UMR cells survive trypsinization and replating

UMR cells were incubated for 48 h in the presence of PTH₁₋₃₄ or left untreated. They were then exposed to 400 μ M H₂O₂ for 4 h followed by a 24 h recovery period. Panels a and b depict phase contrast images of PTH-treated and untreated cells 24 h after H₂O₂-stress, respectively. Panel c shows the results from nucleosome release ELISA (absorbance at 405 nm) after H₂O₂-stress for PTH-treated and untreated cells as a measure of apoptosis. Panel d depicts an assessment of cell viability after H₂O₂-stress measured by MTT assay (absorbance at 570 nm). Panel e shows Western blot results for activated caspase 3 (top, 17 kDa) and α -tubulin (bottom, 50 kDa) as a loading control; molecular weight markers are indicated to the right of the blots. Panels f and g show phase contrast images of PTH-treated and untreated cells stressed by H₂O₂ exposure 16 h after trypsin-replating, respectively. Panel h shows a measure of cell viability 16 h after trypsin-replating assessed by MTT assay (absorbance at 570 nm). For panels c, d, and h the results shown are from 3 independent trials (n = 6 cultures; mean $\pm$ SD; p < 0.05 between PTH treated and untreated). All scale bars are 50 microns.

Figure 6. Forskolin-treated normal human osteoprogenitor cells survive H_2O_2 -stress yet exhibit weak TUNEL staining

Human bone marrow derived stromal cells were cultured in medium to induce osteogenic differentiation for 14 days. Half of the cultures were treated with 10 μ M forskolin 48 h before H_2O_2 challenge. All cultures were then exposed to 600 μ M H_2O_2 for 4 h followed by a 24 h recovery period. Panels a and b depict phase contrast images of untreated and forskolin-treated cells before H_2O_2 -stress, respectively. Panels c and d depict phase contrast images of untreated and forskolin-treated cells after H_2O_2 -stress, respectively. *Black arrows* point to refractile bodies referred to in the text. Panel e depicts an assessment of cell viability before and after H_2O_2 -stress measured by MTT assay (absorbance at 570 nm). Immunohistochemical TUNEL stained image for forskolin-treated cells is shown along with its corresponding DAPI-stained image (panel f). A TUNEL-positive apoptotic body is indicated by a *white arrowhead*, an intact nucleus lacking TUNEL staining is indicated by a *white asterisk*, while other seemingly intact nuclei stain TUNEL-positive (*white arrows*). All scale bars are 50 microns.

Figure 7. PTH suppresses apoptosis induced by UV irradiation

UMR cells were PTH treated or left untreated (no PTH) for 48 h and then exposed to a 30 sec dose of 150 J/m^2 UV radiation. Panels a and b show phase contrast images of PTH-treated and untreated cells 24 h after UV exposure, respectively. Panel c depicts the results from assessments of apoptosis 24 h after UV exposure assessed by nucleosome release ELISA (absorbance at 405 nm). Panel d shows a measure of cell viability 24 h after UV exposure assessed by MTT assay (absorbance at 570 nm). Panels e and f depict phase contrast images of UV-damaged, PTH-treated and untreated cells 16 h after trypsin-replating, respectively. Panel g shows a measure of cell viability 16 h after trypsin-replating assessed by MTT assay (absorbance at 570 nm). For panels c, d, and g the results shown are from 2 independent trials ($n = 6$ cultures; mean $\pm$ SD; $p < 0.05$ between PTH treated and untreated). All scale bars are 50 microns.

Figure 8. Gadd153 expression is up-regulated by serum withdrawal or H₂O₂ treatment, and PTH suppresses this response

UMR cells were treated with PTH or left untreated for 48 h, and then exposed to pro-apoptotic stress by either a 4 h exposure to 400 μ M H₂O₂ or 48 h of serum-starvation. Western blotting was performed on total cell lysates for the Gadd153 protein with band intensities normalized to those of their α -tubulin loading controls (% *Untreated* represents the untreated ratio set as 100%). The results shown are from 3 independent trials (n = 6 cultures; mean $\pm$ SD; p < 0.05 between respective PTH treated and untreated corresponding groups).

Figure 9. PTH stimulates nuclear localization of PCNA and reduces FOXO3a phosphorylation on its repressive Thr24/32 residue

Immunofluorescent labeling for nuclear PCNA was performed after a 4 h exposure to 400 μ M H_2O_2 on untreated (panel a) and PTH-treated UMR cells (panel b). Panels c and d provide corresponding fields of view to those shown in panels a and b showing nuclear staining (DAPI) for untreated and PTH-treated cells, respectively. Scale bars are 50 microns. Panel e shows the calculated percentage of PCNA positive nuclei to total nuclei within 3 fields of view from 3 separate cultures (mean $\pm$ SD; $p < 0.05$ between PTH treated and untreated). Panel f shows the results from Western blotting for phosphorylation of FOXO3a on its repressive Thr24/32 residue using total cell lysates from untreated cells, 30-min treatment with 1 μ g/ml insulin (positive control for phospho-FOXO), and a 30-min treatment with PTH₁₋₃₄. Band intensities were normalized to total FOXO3a (% Untreated represents the untreated ratio set as 100%). The results shown are from 2 independent trials (n = 6 cultures; mean $\pm$ SD; $p < 0.05$ between PTH treated and untreated).

Figure 10. Summary of PTH's mechanism of augmenting DNA repair

After DNA damage, damaged nucleotides are enzymatically removed via DNA excision repair producing 3'OH groups that in fixed cells are targets for TdT incorporation of dUTP employed in the TUNEL reaction. In live cells, activated DNA repair proteins such as PCNA and FOXO3a augment endogenous polymerases to promote the incorporation of new nucleotides (e.g., BrdU) at these 3'OH groups thus effectively repairing the DNA damage and suppressing subsequent apoptosis. Our proposed mechanism suggests that PTH treatment of osteoblasts leads to an activation of PKA which then upregulates the DNA excision repair process and downstream DNA repair functionalities (e.g., PCNA and FOXO3a) (*gray shaded area*).