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RESEARCH ARTICLE

Lysyl oxidase propeptide stimulates osteoblast and osteoclast differentiation and enhances PC3 and DU145 prostate cancer cell effects on bone in vivo

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Abstract Lysyl oxidase pro-enzyme is secreted by tumor cells and normal cells as a 50 kDa pro-enzyme into the extracellular environment where it is cleaved into the ~30 kDa mature enzyme (LOX) and 18 kDa pro-peptide (LOX-PP). Extracellular LOX enzyme activity is required for normal collagen and elastin extracellular cross-linking and maturation of the extracellular matrix. Extracellular LOX-PP acts as a tumor suppressor and can re-enter cells from the extracellular environment to induce its effects. The underlying hypothesis is that LOX-PP has the potential to promote bone cell differentiation, while inhibiting cancer cell effects in bone. Here we investigate the effect of LOX-PP on bone marrow cell proliferation and differentiation towards osteoblasts or osteoclasts, and LOX-PP modulation of prostate cancer cell conditioned media-induced alterations of proliferation and differentiation of bone marrow cells in vitro. Effects of overexpression of rLOX-PP in DU145 and PC3 prostate cancer cell lines on bone structure in vivo after intramedullary injections were determined. Data show that prostate cancer cell conditioned media inhibited osteoblast differentiation in bone marrow-derived cells, which was reversed by rLOX-PP treatment. Prostate cancer conditioned media stimulated osteoclast differentiation which was further enhanced by rLOX-PP treatment. rLOX-PP stimulated osteoclast differentiation by inhibiting OPG expression, up-regulating CCN2 expression, and increasing osteoclast fusion. In vivo studies indicate that rLOX-PP expression by PC3 cells implanted into the tibia of mice further enhanced PC3 cell ability to resorb bone, while rLOX-PP expression in DU145 cells resulted in non-significant increases in net bone formation. rLOX-PP enhances both osteoclast and osteoblast differentiation. rLOX-PP may serve to enhance coupling interactions between osteoclasts and osteoblasts helping to maintain a normal bone turnover in health, while contributing to bone abnormalities in disease.

Keywords Lysyl oxidase · Lysyl oxidase propeptide · Osteoblast · Osteoclast · Differentiation · Bone

Introduction

Lysyl oxidase (LOX) is an extracellular copper-dependent enzyme that plays a critical role in extracellular matrix biosynthesis (Uauy et al. 1998). It is synthesized and secreted as 50 kDa pro-lysyl oxidase (Pro-LOX) enzyme followed by extracellular enzymatic processing by procollagen C-proteinases yielding a 32 kDa mature and active LOX enzyme and the 18 kDa lysyl oxidase pro-peptide (LOX-PP) (Trackman et al. 1992; Uzel et al. 2001; Kessler et al. 1996; Panchenko et al. 1996). LOX is secreted by a variety of cells including normal osteoblast precursors, and tumor cells (Hong et al. 2004; Bais et al. 2012a). LOX-PP can be taken up by cells, and is therefore an autocrine and paracrine molecule. Studies have indicated that the *LOX* gene has tumor suppressor properties due to it is ability to reverse RAS-induced transformation of the NIH 3 T3 fibroblast cell line (Kenyon et al. 1991) while Palamakumbura *et al.* mapped the tumor inhibiting activity of *LOX* gene to the LOX-PP domain of the Pro-LOX protein (Palamakumbura et al. 2004). Data indicated that the 18 kDa LOX-PP inhibits RAS-dependent

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transformation as seen by its inhibition of cell proliferation assays, growth of cells in soft agar and PI3K/AKT and ERK1/2 MAP kinase signaling pathways.

LOX-PP treatment of Her-2/neu breast cancer cells inhibits tumor growth both in vivo and in vitro and rLOX-PP causes reversion of the invasive phenotype of Her-2/neu- driven cancer cells. LOX-PP suppresses PI3K/AKT, ERK1/2 MAP kinase pathways as well as the downstream NF-κB and cyclin D1 levels in breast, pancreatic, lung, prostate and oral cancer cell lines (Min et al. 2007; Palamakumbura et al. 2009; Wu et al. 2007). From these and other studies (Bais et al. 2012a; Bais et al. 2015; Sato et al. 2011; Sato et al. 2013). it is now understood that LOX-PP is an effective tumor suppressor and growth inhibitor and works by multiple mechanisms of action with a number of intracellular and extracellular targets. Mechanisms of cell uptake of rLOX-PP have recently been identified (Ozdener et al. 2015).

The effect of rLOX-PP treatment on normal MC3T3-E1 pre-osteoblasts showed that LOX-PP treatment inhibits serum and FGF-2 induced DNA synthesis and cell growth and inhibits FGF-2 induced phosphorylation of ERK1/2 and FRS2. rLOX-PP inhibited FGF-2 binding to cell layers in a dose-dependent manner (Vora et al. 2010a). In addition, LOX-PP treatment inhibited terminal differentiation of primary calvaria osteoblasts when used at early stages of culture with no apparent effect at late stages (Vora et al. 2010a).

The question evaluated here was to determine whether rLOX-PP could inhibit signaling or communication between tumor cells and bone cells based on its ability to interfere with tumor growth by a variety of mechanisms summarized above. This question was asked in the context of understanding a possible therapeutic strategy for addressing bone metastasis. Our expectation was that rLOX-PP secreted by either tumor cells or normal stromal cells or exogenous application of rLOX-PP would normalize cancer cell stimulated modulation of bone cells homeostasis. Data obtained instead identify a stimulatory role for rLOX-PP in both osteoblast and osteoclast differentiation in vitro, and exacerbation of tumor cell modification of bone in vivo.

Materials and methods

Cell lines and reagents MC3T3-E1 subclone 4 osteoblasts and androgen-refractory human prostate cancer cells (DU145 and PC3) were purchased from American Type Culture Collection (ATCC). Dulbecco's Modified Eagle's Medium (DMEM), α-MEM medium, phosphate-buffered saline (PBS), trypsin and antibiotics (Penn/Strep) were obtained from Invitrogen. F12K medium was purchased from ATCC and [^{3}H]thymidine was from DuPont NEN (Boston, MA). rLOX-PP was expressed in human TREX-293 cells and purified to homogeneity as described previously (Vora et al. 2010b). RNeasy Mini kits for RNA purification were from Qiagen. Real-time PCR TaqMan probes were from Applied Biosystems: β-Actin (Mm00607939_s1); TRAP (Mm00475698); RANKL (Mm01313943_m1); alkaline phosphatase (Mm00475831_m1); type I collagen (Mm00801666_g1); OPG (Mm00435452_m1), and CTGF (Mm01192931_g1), also known as CCN2. Chicken egg white lysozyme (L-6876-1G) was purchased from Sigma-Aldrich. TRAP staining of cultures employed the Acid Phosphatase Kit (387) from Sigma-Aldrich, while TRAP enzyme activity was measured using the TRAP Quantification in Culture Supernatant Kit (KT-008; Kamiya Biomedical). The Caspase-3 Assay Kit (556,485) to measure caspase 3 enzyme activity was purchased from BD Pharmingen. Recombinant murine RANKL (315–11) and recombinant murine soluble M-CSF (315–02) were obtained from Peprotech. Alizarin Red staining was performed using the Osteogenesis Assay Kit (ECM815) from Millipore. All solutions, TaqMan probes and instruments for real-time qPCR were from Applied Biosystems.

Cell culture and preparation of prostate cancer conditioned medium MC3T3-E1 cells were maintained in α-MEM medium supplemented with 10 % fetal bovine serum (FBS), 1 % nonessential amino acids, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C and 5 % CO_2 in a fully humidified incubator. All experiments were carried out with cells between passages 6–10. Cells were plated at 8×10^4 cells/well in 6-well plates. Two days after plating, cells were transferred to serum-free medium containing 0.1 % BSA for an additional 24 h and then treated as described in each experiment.

Preparation of marrow cell cultures Bone marrow stromal cell cultures (BMSCs) were generated from the femurs and tibia of adult 8–10 week old CD-1 male mice (Charles River Laboratories). After euthanasia hind limbs were aseptically removed and bones dissected free of soft tissues, marrow cavities were flushed, and the cell suspension was filtered through a 70 μm nylon strainer. Cells were plated at a density of 20×10^6 cells per well in 6-well culture plates and grown in medium containing α-MEM supplemented with 10 % fetal bovine serum (FBS), 1 % nonessential amino acids, 100 units/ml penicillin, and 100 μg/ml streptomycin and placed at 37 °C and 5 % CO_2 in a fully humidified incubator. Cells were left undisturbed for four days. On day four, half of the medium was replaced with fresh αMEM with 10 % FBS and penicillin and strptomycin. By Day 7 the cells were 70–80 % confluent and cultures were fed with osteoinductive medium consisting of αMEM supplemented with 10 % FBS, dexamethasone 10^{-8} M, L-ascorbic acid 70 ng/ml, and 8 mM β-glycerol phosphate (Bais et al. 2009; Edgar et al. 2007). Cultures were maintained with re-feeding every 48 h. The cells were then treated as described in each experiment.

Conditioned media preparation To prepare prostate cancer conditioned medium DU145 and PC3 cells were grown in 10 cm^2 cell culture plates in DMEM and F12K media respectively containing 10 % FBS, 100 units/ml penicillin and 100 μg/ml streptomycin. At 75–80 % confluence, cells were serum-depleted in α-MEM media containing 0.1 % BSA for 24 h. The medium was collected, centrifuged and filtered through 0.22 μm filters and used immediately in each experiment as described. To induce DNA synthesis in MC3T3 cells or BMSCs, 100 % prostate cancer conditioned medium that is serum free was used to treat the cells. To induce differentiation of bone marrow stromal cells, conditioned medium was prepared by mixing 50 % of the filtered serum-free prostate cancer conditioned medium (DU145 or PC3) and 50 % modified α-MEM medium containing 10 % fetal bovine serum (FBS), 1 % nonessential amino acids, 100 units/ml penicillin, and 100 μg/ml streptomycin and supplemented with 10 nM dexamethasone, 50 μg/ml ascorbic acid, and 8 mM β-glycerol phosphate. The resultant medium contained 5 % FBS.

DNA synthesis assay BMSCs or MC3T3 cells were cultured as described above and placed in non-conditioned serum-free medium containing 0.1 % BSA for 24 h. Cells were then treated with either non-conditioned medium, or DU145- or PC3 conditioned medium with or without indicated concentrations rLOX-PP or equivalent molar concentrations of lysozyme for 4, 6, 8 or 24 h. For the last 4 h of induction, [H]thymidine (4 μCi) was added to each well. At the end of the incubation period, cells were processed as previously described (Palamakumbura et al. 2009).

Alizarin red assay Bone marrow stromal cells were grown as described in Results for each respective experimental design. Cells were washed with PBS and fixed in 10 % formaldehyde at room temperature for 30 min. Wells were then washed thoroughly and stained with 1 % Alizarin Red solution added to each well and incubated at room temperature for at least 20 min. Excess dye was removed by washing with deionized until the washes were colorless. Wells were air-dried and photographed. The intensity of the Alizarin Red staining was quantified using the Versadoc 3000 imaging system with the Quantity One software (Bio-Rad). In addition, quantification of osteogenesis was performed by measuring the absorbance of eluted Alizarin Red dye at 540 nm with a micro-plate reader.

rLOX-PP cell treatment, RNA isolation and real-time qPCR MC3T3 cells were cultured as described above. On day 3 when the cells were 80 % confluent, they were placed in serum-free medium containing 0.1 % BSA for 24 h. Then they were treated with three different concentrations of rLOX-PP for 2, 4 or 6 h, followed by RNA isolation and RT-PCR (see below). BMSCs were cultured as described above. On day 8, the cells were placed in serum-free medium containing 0.1 % BSA for 24 h. Cells were then treated with either modified α-MEM medium (with 10 nM dexamethasone, 50 μg/ml ascorbic acid, 8 mM β-glycerol phosphate and 5 % serum to permit osteoblast differentiation and mineralization), 50 % DU145 conditioned medium plus 50 % modified α-MEM medium, or 50 % PC3 conditioned medium plus 50 % modified α-MEM medium with or without rLOX-PP. All treatment groups contained a final concentration of 5 % FBS in the growth media. Culture media were replaced every 48 h and the cells were grown for an additional 7 and 14 days after initiation of the conditioned media treatments. Cells were lysed using Qiagen buffer RLT supplemented with 1 % β-mercaptoethanol and passed through the QiaShredder columns. Purified total RNA was obtained using the RNeasy Mini kit. 1 μg of RNA from each sample was used in a reverse transcription reaction (30 μL) using random primers and Reverse Transcription Kit (Applied Biosystems), and then subjected to qPCR as we have previously described (Black and Trackman 2008).

TRAP staining and TRAP enzyme activity Bone marrow stromal cells were cultured as above in the presence or absence of 50 % DU145 or PC3 conditioned media or unconditioned media in the presence or absence of rLOX-PP or lysozyme as described above. Media were refreshed every 48 h for 3, 5 or 7 days. Cultures were then fixed in 10 % formaldehyde at room temperature for 30 min, and then washed with PBS. Cultures were then stained for TRAP using the TRAP Acid Phosphatase Kit (Sigma-Aldrich) according to the manufacturer's instructions. Digital images were obtained with an inverted microscope (Zeiss Axiovert 200) and four images from randomly selected non-overlapping areas in each well were recorded; and the experiment was performed in triplicate. Osteoclast data were expressed as the percentage of the surface area that is covered by osteoclasts to the total surface area of the culture dish. TRAP enzyme activity in the culture supernatant was measured using the colorimetric TRAP Quantification in Culture Supernatant Kit (Kamiya) according to the manufacturer's instructions.

Osteoclast differentiation in preparations of bone marrow-derived cells Cells were flushed from freshly isolated femurs and tibia of adult 8–10 week old CD-1 male mice (Charles River Laboratories). 5×10^6 Cells per well were plated in 6-well culture plates and grown in α-MEM medium supplemented with 10 % fetal bovine serum (FBS), 1 % nonessential amino acids, 100 units/ml penicillin, and 100 μg/ml streptomycin and placed at 37 °C and 5 % CO_2 in a fully humidified incubator. Osteoclast differentiation was induced with M-CSF, 50 ng/mL, added on day 0. On day 1, media were replaced with α-MEM supplemented with 5 % fetal bovine serum. Soluble M-CSF (50 ng/ml) and RANKL

(30 ng/ml) were added with or without rLOX-PP or lysozyme, as indicated. Culture media were changed every other day and the cells were grown for 3, 5 or 7 days. Cell plates were TRAP stained and their medium collected for TRAP enzyme activity or cell layers were subjected to RNA extraction and qPCR.

Analyses of osteoclast fusion In separate experiments the same protocol was followed to develop osteoclasts in the presence or absence of rLOX-PP. On Days 3, 5, and 7 of treatment cells were subjected to TRAP staining. Digital images of thirty randomly selected areas of each well were taken with an inverted microscope. In total 65 % of each well surface area was evaluated and the total number of osteoclasts in each treatment group was calculated. The number of nuclei in each osteoclast was determined by manual counting and the data divided into 6 groups: cells with 1–3, 4–10, 11–15, 16–25, 26–50 and over 50 nuclei/cell, respectively. The percentage of cells falling into each category was calculated.

Assessment of osteoclast apoptosis Osteoclasts were derived from bone marrow stromal cells as described above. The cells were grown for 3, 5 or 7 days with or without rLOX-PP (1 μg/ml, 4 μg/ml and 10 μg/ml) or lysozyme (0.8 μg/ml, 3.2 μg/ml, or 8 μg/ml). Growth medium was aspirated and cells were washed twice with PBS. Excess PBS removed by aspiration and the cells were suspended in cold Cell Lysis buffer (10 mM Tris-HCl; 10 mM NaH2PO4/NaHPO4 (pH 7.5); 130 mM NaCl; 1 % TritonR-X-100; 10 mM NaPPi) for 30 min on ice. Then for each reaction we added 5 μL of Caspase-3 Fluorogenic Substrate (Ac-DEVD-AMC) to a well containing 0.2 ml of 1X HEPES buffer (which is referred to as Protease Assay Buffer) into 96-well plate. 50 μL of cell lysates is added to each well/reaction. The reaction mixtures are incubated for 1 h at 37 °C. This was followed by measuring the amount of florescence liberated from Fluorogenic Substrate using a plate reader with an excitation wavelength of 380 nm and an emission wavelength range of 420–460 nm. Apoptotic cell lysates containing active caspase-3 yield a considerable emission as compared to non-apoptotic cell lysates. The readings of the Caspase-3 Fluorogenic Substrate without the cell lysates were subtracted and data analyzed for fold-changes compared to no rLOX-PP addition.

Intra-tibial injection of prostate cancer cells All experiments were performed as approved by Boston University Medical Center IACUC. PC3 and DU145 cells were infected with EF1α-luciferase lentivirus to facilitate the bioluminescence imaging in vivo. These cells were stably transduced with rLOX-PP expressing lentivirus particles EF1α-LOX-PP-myc-his-UBC-GFP (designated as rLOX-PP) and empty vector EF1α-Empty-UBC-GFP (designated as Empty), and rLOX-PP-myc-His_6 protein expression and secretion was verified (Bais et al. 2015). rLOX-PP and Empty cells (2.5×10^5 cells) were injected into the tibia of NCR nu/nu mice (Taconic Farms) as single cell suspensions in 25 μl serum free DMEM (Krishnan et al. 2013). DU145-empty/rLOX-PP (n = 6/condition) and PC3-Empty/rLOX-PP (n = 9/condition) were allowed to grow into the tibia for eight weeks and imaged at intervals with bioluminescence imaging. After eight weeks, tibiae were harvested, fixed and subjected to micro-CT analysis (Bais et al. 2009).

Bioluminescent imaging of DU145/PC3 cells injected into the tibia of mice All mice were imaged using an IVIS 200 system (Xenogen, Alameda, CA, USA) at weekly intervals. Anesthesia was administered in an induction chamber with 2.5 % isoflurane in 100 % oxygen at a flow rate of 1 L/min and with a 1.5 % mixture at 0.5 L/min maintained while the animal was in the IVIS imaging device. D-luciferin (150 μl of 30 mg/ml dissolved in PBS) was administered by intraperitoneal injection and bioluminescent images were obtained for 45 min. Peak signals were observed around 22 min. The data are reported as the photon flux (p/s) from a defined region of interest in tibia of DU145-Empty/rLOX-PP and PC3 Empty/rLOX-PP cells.

Micro computer assisted tomography (μCT) Tibiae were scanned at a resolution of 16 μm using a Scanco μCT 40 system (Scanco Medical, Basserdorf, Switzerland). A region of interest (ROI) was defined for the experimental samples in the longitudinal axis by measuring a fixed distance from the proximal growth plate of a bone from a non-experimental littermate. Measurements were initiated 100 slices to the distal side of this point in all experimental bones. A total of 500 slices distal to ROI were scanned per sample, and analyzed for BV/TV and after 3-D reconstruction (Bais et al. 2009; Bais et al. 2012b).

Statistics

Statistical tests were performed using two way ANOVA with Bonferroni correction Post Hoc analysis using Prism 5 Graphpad software to identify the differences between different treatment conditions. Within each treatment group one way ANOVA was carried out to evaluate the significant differences within the group. Statistical significance between two groups was established at the P value lower than 0.05. Standard deviation was calculated and plotted in all

experiments unless otherwise indicated. All experiments were carried out in triplicate.

Results

Prostate cancer conditioned medium stimulates BMSC DNA synthesis and is inhibited by rLOX-PP We first investigated whether prostate cancer conditioned medium treatment of bone cells would stimulate proliferation and whether rLOX-PP would inhibit these effects. We initially investigated the effect of prostate cancer conditioned medium on the proliferation of the MC3T3 osteoblast cell line in the presence or absence of rLOX-PP using a thymidine incorporation assay to measure changes in DNA synthesis. Data show that DU145 conditioned medium resulted in a 5-fold increase in MC3T3-E1 cell DNA synthesis (Fig. 1a). A dose-dependent decrease of the proliferative response was evident when DU145 conditioned medium treatment was accompanied with rLOX-PP. This inhibition was up to 50 % at 10 μg/ml rLOX-PP. rLOX-PP inhibited the low level of MC3T3 conditioned medium induced stimulation of DNA synthesis, while it had little effect on the non-conditioned medium treatment group (Fig. 1a). These data indicate that DU145 conditioned medium contains factors that induce a rapid proliferative response in the MC3T3 osteoblastic cell line that is inhibited by rLOX-PP. By contrast PC3 cell conditioned media had no stimulatory effect on MC3T3 cell proliferation (Fig. 1b and c).

We next sought to investigate if the effect of DU145 (osteoblastic) and PC3 (osteolytic) prostate cancer conditioned media on osteoblast DNA synthesis occurs in primary BMSCs. Cells were grown as indicated in Materials and Methods and serum-deprived cultures were then treated for 6 h with either DU145 conditioned medium, or PC3 conditioned medium in the presence or absence of increasing concentrations of rLOX-PP (0–10 μg/ml). An unconditioned serum-free medium control group was also assayed. The cell proliferative response was then determined by [^{3}H]thymidine incorporation. DU145 conditioned medium treatment stimulated DNA synthesis by 4-fold, and was inhibited 30–40 % by 10 μg/ml of rLOX-PP, and was dose-dependent (Fig. 1e). PC3 conditioned medium did not enhance DNA synthesis in BMSCs cultures. However

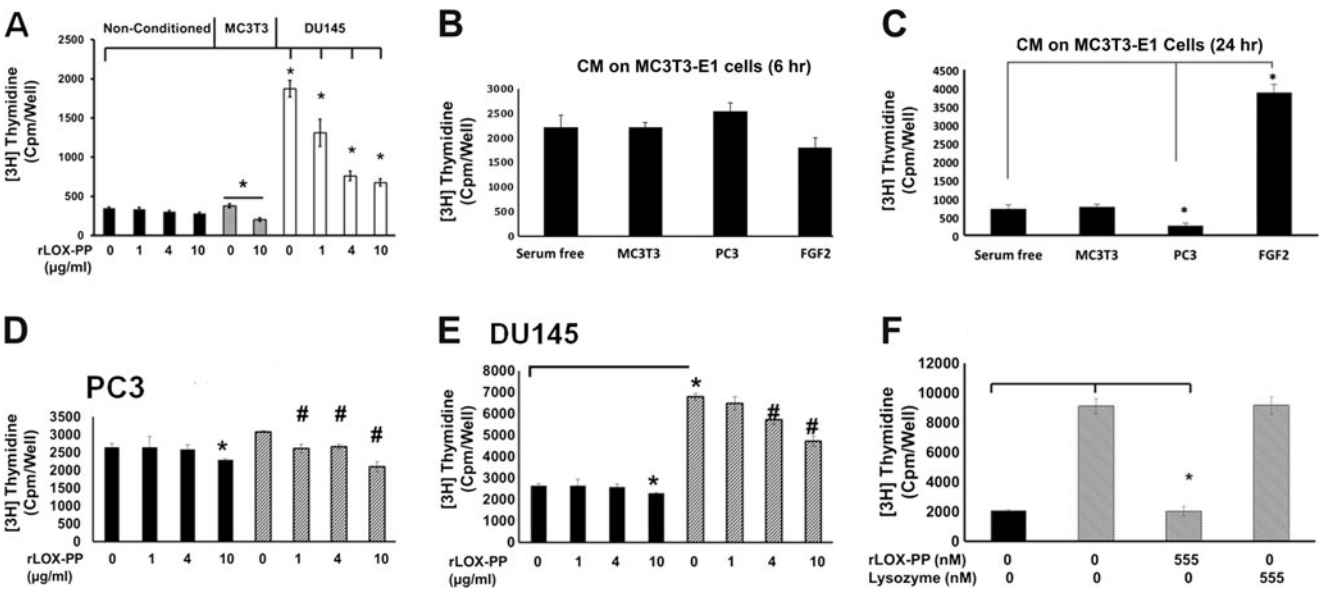

Fig. 1 rLOX-PP effects on prostate cancer cell conditioned media induced proliferation of **a-c** MC3T3-E1 osteoblasts, and BMSCs **d-f**, and effects of rLOX-PP are specific **f**: MC3T3 cultures were serum-depleted for 24 h and then **a** treated with DU145 conditioned medium for 8 h in the presence of rLOX-PP (0–10 μg/ml equivalent to 0–555 nM) **(white bars);** or non-conditioned serum-free α-MEM medium **(black bars)** or with MC3T3 cell conditioned serum-free α-MEM medium **(hatched bars)**. Data are cpm/well ± S.E.M. **b and c**. MC3T3 cells were treated with conditioned media from PC3 cells, or with positive control FGF-2 (1 ng/ml) for 6 or 24 h, and [^{3}H] -thymidine incorporation determined; (*, $p < 0.05$). BMSCs were placed in differentiation medium for 4 days. On Day 8 the cells were serum-depleted for 24 h and then treated with **d** PC3 or **e** DU145 conditioned medium (**hatched bars**) or non-conditioned medium plus rLOX-PP (0–10 μg/ml) (**black bars**), and subjected to [^{3}H]thymidine incorporation assays. Data are cpm/well ±S.E.M combined results from three experiments ($n = 3$, *, p 0.05; #, $p < 0.05$ compared to no rLOX-PP control). **f** MC3T3 cultures were serum-starved for 24 h and then induced to proliferate with DU145 conditioned medium (**hatched bars**) in the presence or absence of either rLOX-PP (10 μg/ml equivalent to 555 nM) or a molar equivalent of lysozyme (8 μg/ml). Non-conditioned serum-free α-MEM medium was used as a control group in this experiment (**black bar**). [^{3}H]thymidine incorporation was performed as indicated in Materials and Methods. Data are cpm/well ±S.D., performed once in triplicate ($n = 3$, *, p 0.05; #, $p < 0.05$ compared to no rLOX-PP control; one-way ANOVA).

adding rLOX-PP to PC3 conditioned medium resulted in ~25 % inhibition of DNA synthesis (Fig. 1d).

Effects of rLOX-PP treatment on bone cell proliferation are specific to this protein At this stage we investigated whether the inhibitory effects of rLOX-PP on the prostate cancer conditioned media-induced proliferation of osteoblasts and BMSCs is specific to LOX-PP, or is simply a function of its unusually high isoelectric point. Lysozyme has a high isoelectric point (9.2) and similar molecular weight (14.3 kDa) compared to rLOX-PP (18 kDa and an isoelectric point of 12) and was chosen as the negative control. MC3T3-E1 cells were grown as before and then treated with DU145 conditioned medium in the presence or absence of 10 μg/ml rLOX-PP (555 nM), or the molar equivalent of lysozyme (8 μg/ml), and [^{3}H]thymidine incorporation was then determined. Data (Fig. 1f) indicate that rLOX-PP reduced DNA synthesis to control non-stimulated levels in DU145 conditioned medium treated cells, while lysozyme had no inhibitory effect. Data indicate that effects of rLOX-PP are specific and that lysozyme can serve as a negative control to help establish the specificity of rLOX-PP.

Effects of rLOX-PP on prostate cancer cell conditioned medium inhibition of osteoblast differentiation in BMSC cultures The effect of prostate cancer conditioned medium treatment in the presence or absence of rLOX-PP on the terminal differentiation of primary bone marrow stromal cells toward osteoblasts was assessed by Alizarin Red staining. BMSCs were cultured and induced to differentiate as described in Materials and Methods in the presence of non-conditioned medium (control), or with conditioned medium from DU145 or PC3 cells in the presence or absence of rLOX-PP or lysozyme. The treatment media were replaced every 48 h, and cultures were grown under these conditions until day 28, followed by Alizarin Red staining. Images in Fig. 2a, (top row), indicate that the non-conditioned medium-treated control group had the highest degree of mineralization, followed by cells treated with DU145 conditioned medium, and cells treated with PC3 conditioned medium had the least amount of mineralization. rLOX-PP addition (Fig. 2a, second row) increased mineralization in all treatment groups when compared to the top row. This stimulation of mineralization was highest when rLOX-PP was added to PC3 conditioned medium, and quantitative analyses (Fig. 2b and c) indicate that rLOX-PP restored Alizarin Red staining to non-conditioned control medium levels. By contrast, lysozyme (Fig. 2a, third row) failed to stimulate Alizarin Red staining in any cultures. Data suggest that rLOX-PP can promote osteoblast differentiation in cultures of primary BMSCs, and that this stimulation is specific to rLOX-PP.

Regulation of osteoblast markers in primary BMSCs induced by conditioned media from DU145 and PC3 cells The stimulatory effects of rLOX-PP on osteoblast differentiation under conditions in Fig. 2a were next independently investigated by assessing the expression of mRNA levels of two key osteoblast differentiation markers: type I collagen, and alkaline phosphatase. Bone marrow stromal cells were grown as described for Fig. 2a and were permitted to differentiate for 7 or 14 days. Figure 2d indicates that DU145 conditioned medium alone stimulated COL1A1 mRNA levels on day 7, but reduced its expression on day 14, at which time cultures were mineralizing. rLOX-PP strongly up-regulated COL1A1 expression at both time points. Alkaline phosphatase was similarly up-regulated by rLOX-PP at both time points (Fig. 2e), consistent with its effects on promoting differentiation seen in Fig. 2a. PC3 conditioned media down-regulated COL1A1 mRNA levels (Fig. 2f), while it stimulated alkaline phosphatase levels at both time points (Fig. 2g). rLOX-PP stimulated both markers at both time points (Figs. 2e and g), again consistent with effect seen in Fig. 2a to stimulate differentiation in the presence of prostate cancer cell conditioned media. rLOX-PP stimulation of COL1A1 and alkaline phosphatase mRNA levels was of higher magnitudes in the osteoblastic cancer cell line DU145 compared to its effects in osteolytic PC3 cells (Fig. 2).

Prostate cancer conditioned medium stimulated osteoclast differentiation in BMSCs cultures which was further enhanced by rLOX-PP treatment In order to understand the relatively weak stimulation of osteoblast marker expressions by rLOX-PP in BMSCs in the presence of PC3 conditioned medium, effects of rLOX-PP on osteoclast development under the same culture conditions was next investigated. The notion was considered that if rLOX-PP stimulated osteoclast development from BMSCs under these conditions, then osteoblast differentiation and expression of osteoblast differentiation markers could be less robust in these cells compared to rLOX-PP effects in the presence of DU145 conditioned media. Initially, optimization of time of culture was established in order to detect osteoclasts by TRAP staining. BMSCs were treated with 50 % conditioned medium following the protocol outlined for experiments shown in Fig. 3a for 3, 5, or 7 days all in the absence of rLOX-PP. Cells were then fixed in 10 % formaldehyde, and stained for TRAP as indicated in Materials and Methods. Data indicate that prostate cancer conditioned medium treatment alone was capable of stimulating differentiation of mature osteoclasts in BMSC cultures even without the addition of exogenous RANKL and M-CSF (Fig. 3a). Osteoclast coverage was higher with PC3 conditioned medium treatment compared to DU145 conditioned medium which could be attributed to the

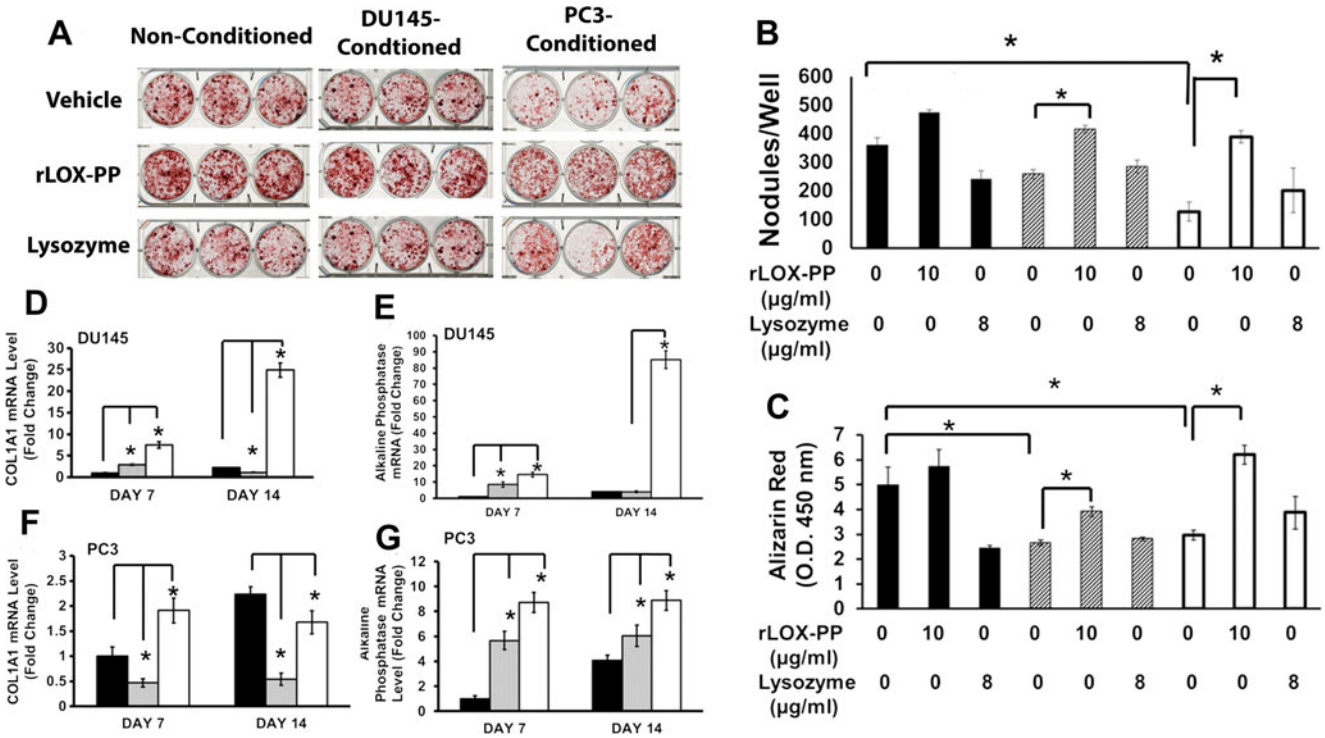

Fig. 2 Alizarin red staining of bone marrow stromal cells treated with prostate cancer conditioned medium in the presence or absence of rLOX-PP (**a** – **c**) and expression of markers (**d-g**)**:** BMSCs were plated and grown under osteoblast induction conditions as described in Fig. 1 and Materials and Methods. Cells were then treated with 50 % non-conditioned medium or 50 % DU145- or PC3 conditioned medium in the presence or absence of rLOX-PP (10 μg/ml) or lysozyme (8 μg/ml of medium) in 5 % serum and 10 nM dexamethasone, 50 μg/ml ascorbic acid, and 8 mM β-glycerol phosphate. The BMSCs were refed every 48 h with the same respective media for 28 days. Alizarin Red stained cultures **(a)**, and intensity of staining was measured using a Versadoc 3000 imaging system **(b);** absorbance determined after elution of Alizarin Red dye at 405 nm **(c);** black bars, non-conditioned media; hatched bars, DU145 conditioned media; white bars, PC3 conditioned media. Data are mean ± S.D. with $n = 3$, *, p 0.05. **(d-g)** Cells were grown for 7 or 14 days in respective differentiation medium, and relative RNA expressions determined from cells treated with non-conditioned medium (control, black bars), or 50 % DU145 or 50 % PC3 conditioned media in the absence (hatched bars) or presence (white bars) of rLOX-PP (10 μg/ml of medium equivalent to 555 nM). Fold changes in mRNA levels relative to the day 7 non-conditioned medium control all normalized to β-actin mRNA levels are shown **(d-g)**. Data are means ±S.D., $n = 3$, *,p 0.05 compared to the respective non-conditioned media control.

osteolytic phenotype of PC3 cells. We chose day 5 for subsequent experiments, since day 3 was too early to identify any changes among treatment groups, and day 7 was too late as most osteoclasts had undergone apoptosis (Fig. 3a).

The effect of rLOX-PP on DU145 and PC3 conditioned media stimulation of osteoclast development was next evaluated using the same experimental setup. Osteoclast differentiation data are expressed as the percentage of the well surface area that is covered by TRAP positive osteoclasts. Data indicate that 555 nM (10 μg/ml) rLOX-PP added to conditioned media from both DU145 and PC3 cultures stimulated osteoclast development of BMSCs over conditioned media alone by 2-fold, though far greater osteoclast development was observed in BMSCs treated with PC3 conditioned medium (Fig. 3b and c). By contrast lysozyme showed no ability to increase osteoclast development in the presence of conditioned media from either DU145 or PC3 cells, indicating that the ability of rLOX-PP to stimulate osteoclast development is unique to rLOX-PP. Non-conditioned media revealed no osteoclast development as expected.

rLOX-PP enhanced osteoclast differentiation in BMSC The effect of rLOX-PP treatment alone of BMSCs in the absence of prostate cancer cell conditioned media was then determined under the same experimental conditions as performed in Fig. 3a. rLOX-PP increased osteoclast covered surface area in a dose dependent manner in primary BMSC (Fig. 3b and 3c). These effects of rLOX-PP were not observed in cells treated with lysozyme, as expected.

Stimulation of osteoclast differentiation by rLOX-PP in the presence in RANKL and M-CSF in BMSCs rLOX-PP stimulation of osteoclast development under osteo-inductive conditions was unexpected because LOX-PP effects discovered so far have all been inhibitory. This finding raised the question regarding the mechanisms by which rLOX-PP would influence osteoclast development in bone marrow cells

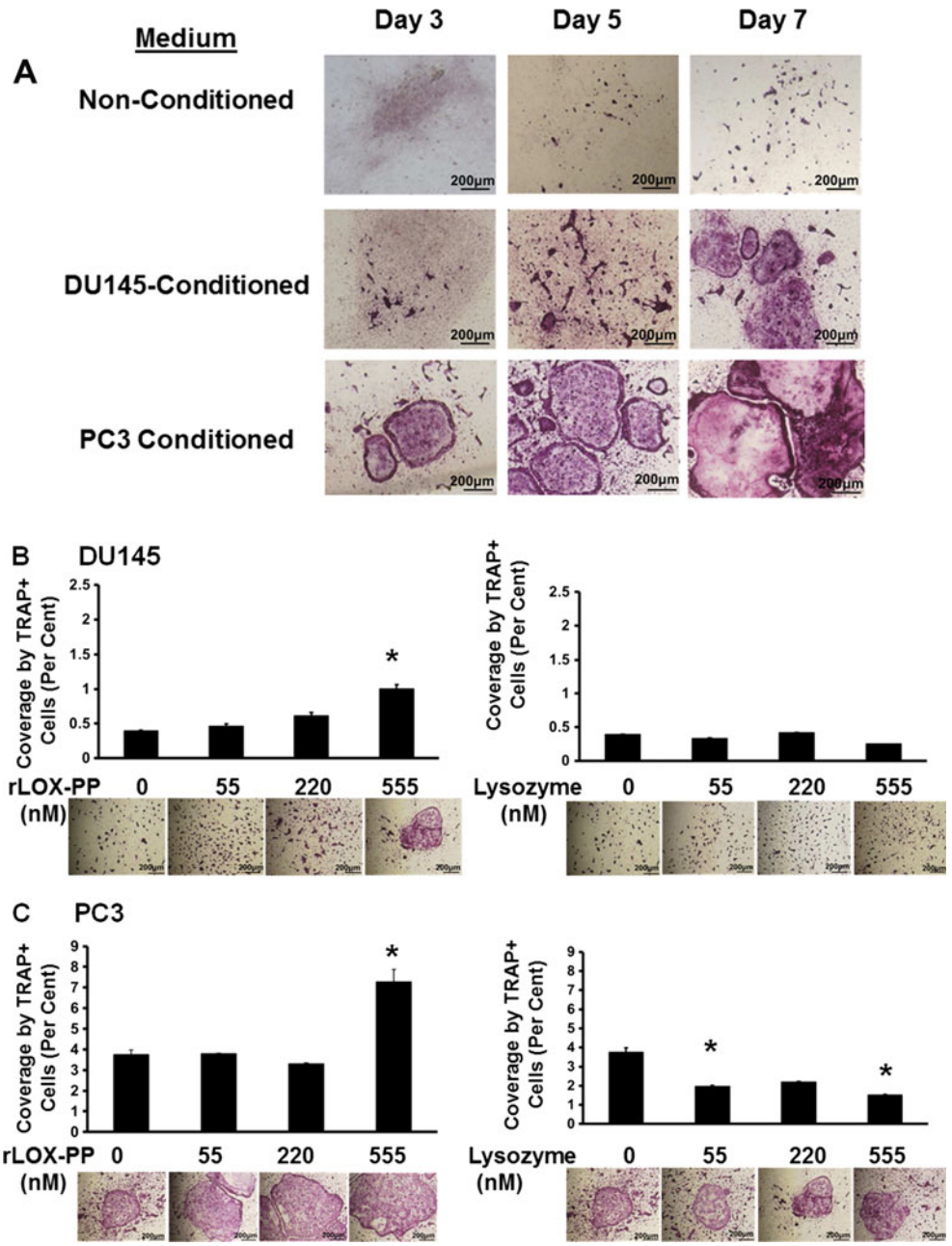

Fig. 3 Time course **a** and effects of DU145 **b** and PC3 **c** conditioned medium treatment on osteoclast development in BMSCs cultures: **a** BMSCs were plated and induced to undergo osteoblast differentiation as described in Materials and Methods. On day 8 cells were serum starved for 24 h. The control group was treated with 50 % non-conditioned serum free α-MEM medium or 50 % DU145 or PC3 cell conditioned media, with the remaining 50 % consisting of α-MEM containing 10 % FBS and osteogenic factors. Respective media were replaced every 48 h. Cultures were fixed in 10 % formaldehyde at room temperature for 30 min and stained for TRAP as described in Materials and Methods. **(b and c)** BMSCs were treated with DU145- **b** or PC3- **(c)** conditioned medium and increasing concentrations of either rLOX-PP or lysozyme (negative control). Cultures were fixed and TRAP stained (Materials and Methods) and digital images from each triplicate well were captured from four randomly selected areas with a Zeiss Axiovert 200 microscope 100X magnification. Data are expressed as the percentage of the well surface area that is covered by osteoclasts, and are compared to the respective non-treated controls (means +/− SD, $n = 3$, *, $p < 0.05$ compared to respective zero control). Statistical analysis was carried out using one way ANOVA. Representative images of the TRAP stained cells are shown below each data point (scale bars =220 µm).

cultured under conditions favoring osteoclast differentiation. Bone marrow cells were plated in 6-well plates (5×10^6 cells per well). The cells were grown in α-MEM supplemented with 10 % fetal bovine serum (FBS). Osteoclast differentiation was either not induced or not induced with macrophage colony-stimulating factor (M-CSF, 50 ng/ml) added at day 0 and RANKL (50 ng/ml) on day 1. rLOX-PP or equivalent molar concentrations of lysozyme were added on day 1.

TRAP enzyme activity in culture supernatants were assessed and compared to the vehicle control (no rLOX-PP or lysozyme). Qualitative analyses from TRAP stained cultures revealed that rLOX-PP treatment increased the osteoclast covered surface area in a dose dependent manner by day 5 while lysozyme did not (Fig. 4a and d). Quantitative analyses revealed that lysozyme did not increase TRAP activity in culture supernatants either in the absence or presence of M-CSF and RANKL (Fig. 4b and e), while rLOX-PP increased TRAP enzyme activity compared to respective controls (Fig. 4c and e). The total amount of TRAP activity was much higher in the presence of M-CSF plus RANKL, as expected (Fig. 4c and e). No TRAP activity was observed in culture supernatants on day 2 (left most bar with no addition of rLOX-PP or lysozyme, Fig. 4e), as expected. Data suggest that rLOX-PP stimulates osteoclast differentiation in BMSCs even in the presence of M-CSF and RANKL. Development of TRAP activity as a function of time of differentiation was investigated and data in Fig. 4f indicate that TRAP activity increases with time and peaks on day 5, and that rLOX-PP increases TRAP activity and is dose-dependent.

rLOX-PP regulates OPG, RANKL, CCN2 and TRAP expression under osteoclast promoting culture conditions

Bone marrow cells were grown under conditions favoring osteoclast development by supplementing media with M-CSF and RANKL as described above in the presence or absence of increasing concentrations of either rLOX-PP or equivalent concentrations of lysozyme. The relative mRNA expression levels of OPG, RANKL, TRAP, and CCN2 (also known as connective tissue growth factor, CTGF) at different treatment time points were compared to the untreated control group on day 3 of treatment. Earlier studies have reported that CCN2 stimulates osteoclast fusion (Nishida et al. 2011). Data

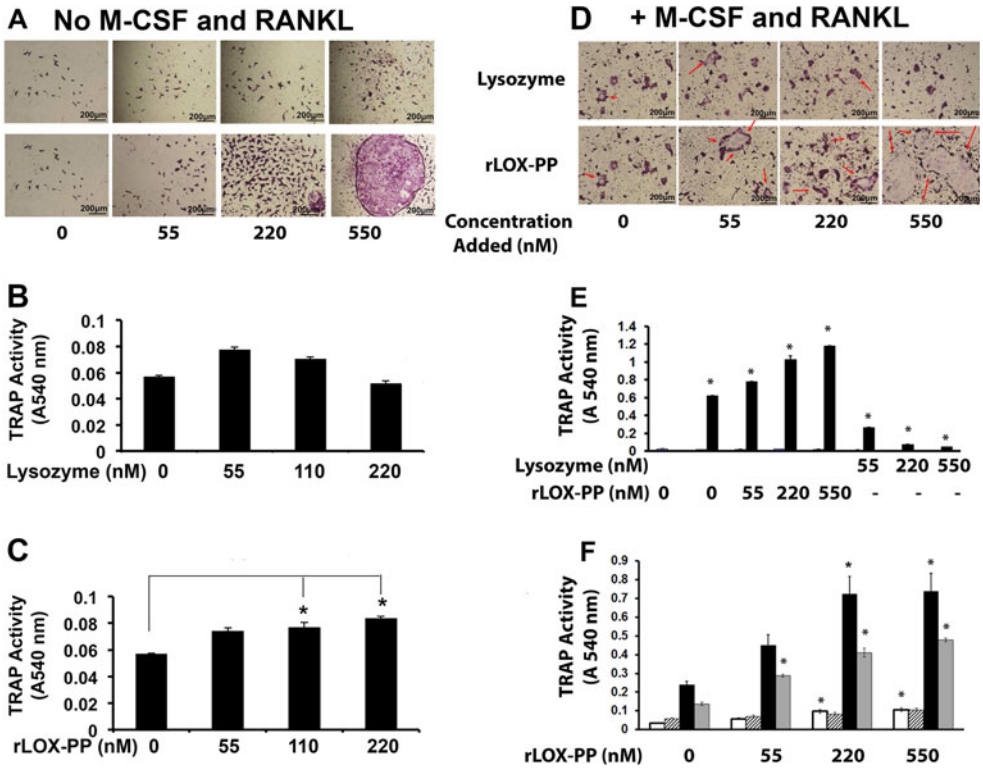

Fig. 4 Effect of rLOX-PP treatment on osteoclast development in the absence (**a-c**) or presence (**d-f**) of M-CSF and RANKL**:** BMSCs were plated in 6 well plates. At 80 % visual confluence they were treated with non-conditioned medium containing 5 % serum and osteogenic factors as described in Materials and Methods with increasing concentrations of rLOX-PP or lysozyme, or vehicle control (zero rLOX-PP or lysozyme). Cultures were fixed in 10 % formaldehyde and subjected to TRAP staining on Day 5. Digital images were collected and analyzed as in Fig. 3, scale bar, 200 μm **a**. Data in **(b** and **c)** are TRAP enzyme activity in the culture supernatant from the same cultures and absorbance measured at 540 nm +/− SD, *, $p < 0.05$, $n = 3$. **d** BMSCs were plated in 6-well plates in at 5×10^6 cells per well and grown in α-MEM supplemented with 10 % FBS. Osteoclast differentiation was induced with murine colony-stimulating factor M-CSF, 50 ng/mL and RANKL in the presence of increasing concentrations of either rLOX-PP or equivalent concentrations of lysozyme. BMSCs were fixed and TRAP-stained and representative images images are shown in **d**, scale bar, 200 μm. **e** TRAP enzyme activity in the culture supernatant from the same cultures was measured. Bars at the extreme left of Fig. 4e is TRAP activity from Day 2 culture supernatants in cells treated with M-CSF/RANKL but without rLOX-PP and indicates little or no TRAP activity at this time point as expected. In **f** the experiment was repeated as a function of both rLOX-PP concentration and time of culture. White bars, Day 3; hatched bars, Day 4; black bars, Day 5, gray bars, Day 7. Data are means ±S.D.; *, p 0.05 compared to no addition of rLOX-PP or lysozyme; $n = 3$ for each respective group or time point. Statistical analysis was carried out using one way ANOVA.

indicate that the expression of CCN2 increased in the untreated control cultures from day 3 to 5 and reached a plateau. rLOX-PP further increased CCN2 expression by 1.6, 1.5 and 2.6 fold respectively (Fig. 5a). rLOX-PP treatment strongly inhibited OPG expression (Fig. 5b), while TRAP mRNA levels were strongly induced (Fig. 5c). These findings suggest that one effect of rLOX-PP is to alter RANKL/OPG balance by down-regulating OPG followed by increased TRAP activity and possibly increased osteoclast fusion. Since RANKL was added in the growth medium, RANKL expression levels were not measured in this experiment. Taken together, data suggest that OPG is a major regulatory target of rLOX-PP which results in increased osteoclast differentiation and elevated TRAP activity.

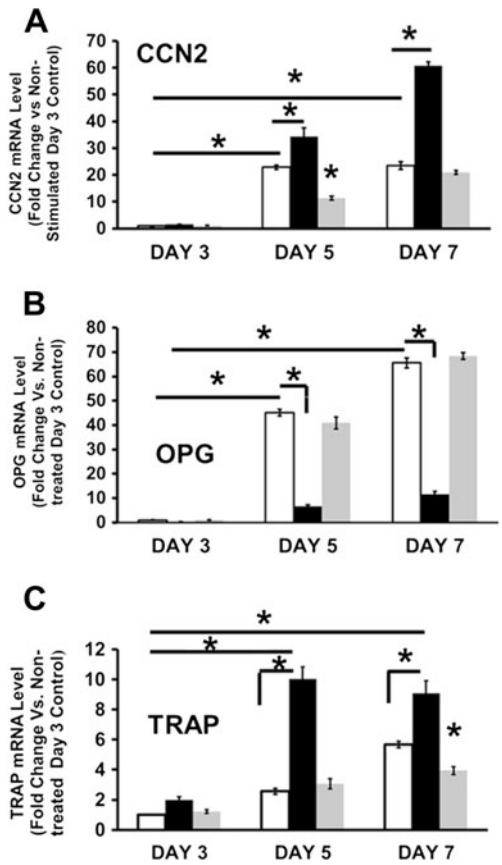

Fig. 5 Regulation of mRNA expression of osteoclast differentiation markers by rLOX-PP. BMSCs were plated in 6-well plates in a 5×10^6 cells per well and grown in α-MEM supplemented with 10 % fetal bovine serum (FBS). Osteoclast differentiation was induced with murine colony-stimulating factor (M-CSF, 50 ng/mL) and RANKL (30 ng/mL) in the presence or absence of rLOX-PP 550 nM (10 μg) or an equivalent molar concentration of lysozyme (8 μg/ml) as described in Materials and Methods. RNA was isolated at intervals and assessed by qPCR for the target markers and β-actin as the normalization control. The fold changes in expression of markers were relative to the Day 3 no treatment control group which consisted of only M-CSF/RANKL additions. Data represent means ± S.D., $n = 3$; $^*p$ 0.05; white bars, M-CSF/RANKL only; black bars, plus rLOX-PP; gray bars, plus lysozyme. Statistical analysis was carried out as described in Materials and Methods

rLOX-PP increases osteoclast fusion in BMSC cultures rLOX-PP-induced increases in osteoclast development could be accomplished by either altering their number or increasing their fusion or both. CCN2 is known as a factor which promotes both cell proliferation and osteoclast fusion, and was increased by rLOX-PP. To investigate effects of rLOX-PP on osteoclast fusion we cultured BMSCs as indicated above and osteoclast fusion was monitored as a function of time and rLOX-PP treatment as indicated in Materials and Methods. Data in Fig. 6a show that on Day 3 rLOX-PP slightly increased the percentage of TRAP positive cells containing 16–25 nuclei per cell compared to both the non-treated and lysozyme controls, while the great majority of TRAP positive cells contained 1–3 nuclei in all three groups. By Day 5 (Fig. 6b) nuclei per TRAP positive cells increased in all three groups, with a small but significant increase being caused by rLOX-PP compared to the non-treated control group. On Day 7 (Fig. 6c) a very substantial increase in TRAP positive cells containing higher numbers of nuclei was observed in cells treated with rLOX-PP compared to both control groups. Interestingly, the total number of cells per well was not strongly affected by rLOX-PP, though some increase in total cell numbers was observed on Day 3 and a relatively small decrease on Day 7 compared to respective controls at each time point (Fig. 6d). Taken together data suggest that rLOX-PP promotes fusion of osteoclasts. It appears that rLOX-PP treatment may initially modestly increase osteoclast numbers, and then stimulates fusion resulting in the development of markedly larger osteoclasts coming from fusion of several cells resulting in a lower number of total cells by Day 7.

Assessment of osteoclast apoptosis To investigate if rLOX-PP treatment would specifically stimulate osteoclast apoptosis in BMSCs cultures we differentiated osteoclast cells from bone marrow stromal cells following the protocol described previously. The cells were grown for 3, 5 or 7 days with or without rLOX-PP (1 μg/ml, 4 μg/ml and 10 μg/ml) or lysozyme (0.8 μg/ml, 3.2 μg/ml, or 8 μg/ml). Cells were extracted and assayed for caspase-3 activity as described in Materials and Methods. Data (Fig. 6e-g) indicate that caspase 3 enzyme activity was not altered by rLOX-PP on day 3 and 7 of treatment; however, on day 5 of treatment there was decrease in the caspase-3 activity with higher concentrations of rLOX-PP which was also seen in negative control group (lysozyme). Since alterations of caspase-3 activity were not specific for rLOX-PP, we conclude that the effect of rLOX-PP on osteoclast development and fusion cannot be attributed alterations in the degree of apoptosis of osteoclasts.

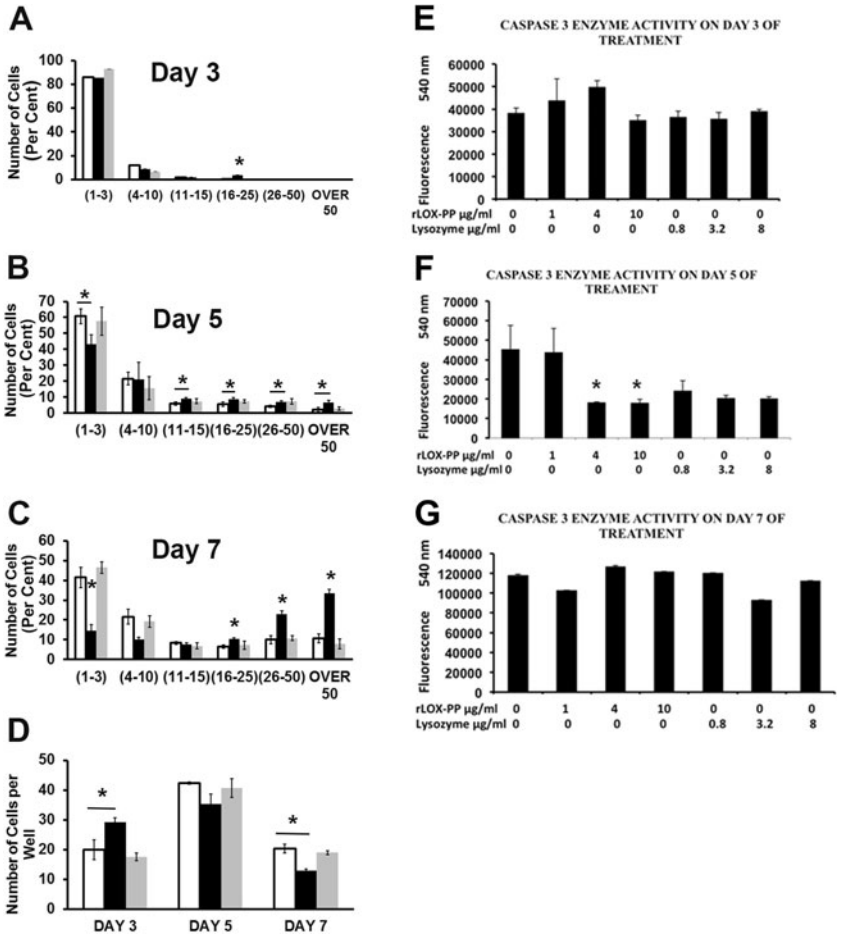

Fig. 6 Effect of rLOX-PP treatment of BMSCs on osteoclast number and fusion (**a-d**), and assessment of apoptosis (**e-g**): *Bone marrow* cells were induced with M-CSF (50 ng/mL) RANKL, (30 ng/mL) in the presence of either 550 nM (10 μg) rLOX- or 550 nM (8 μg) lysozyme. Culture media were changed every other day and rM-CSF and rRANKL were added to the growth medium throughout the experiment. The cells were grown for either 3, 5 or 7 days after initiation of rLOX-PP treatment. This was followed by TRAP staining of cell layers. Digital images of 20–36 randomly selected non-overlapping areas of each well were taken for analyses of TRAP staining with inverted microscope at 100X magnification. The total number of osteoclasts per well in each treatment group was calculated. The numbers of nuclei in each osteoclast were counted and the data were divided into 6 groups. The percentage of cells falling into each category was calculated and compared among different treatment conditions **(a-d).** White bars, cells treated with M-CSF and RANKL only; black bars, cells treated with M-CSF, RANKL and rLOX-PP; gray bars, cells treated with M-CSF, RANKL, and lysozyme. Data represent mean ± S.D., $n = 3$, *p 0.05. **(e-g)** Bone marrow cells were grown under conditions to promote osteoclast development in the presence or absence of rLOX-PP or lysozyme as in (A-D). Caspase-3 activity was measured in cell lysates. Data are means ±S.D., $n = 3$, *p 0.05 compared to no treatment controls

In summary, rLOX-PP was found to modulate differentiation of bone marrow stromal cells, with early stimulation of osteoclast development which occurred under both osteo-inductive and osteoclast-inductive conditions. This is followed by a late and somehow weaker stimulation of osteoblastic differentiation when bone marrow stromal cells were cultured under osteoinductive conditions. The mechanisms involved in osteoclastic stimulation include alteration of RANKL/OPG balance and stimulation of osteoclastic fusion.

In vivo effects on bone of rLOX-PP expressing prostate cancer cell lines We next wished to determine whether the ability of rLOX-PP to modulate effects of DU145 or PC3 cells in bone in vivo could be observed, and whether these effects would be consistent with in vitro studies presented above. The effect of rLOX-PP overexpression in prostate cancer cells on bones after intramedullary injections of rLOX-PP expressing and empty lentivirus transduced DU145 and PC3 cells into the tibia of immunodeficient mice was determined. For this purpose, we employed cells also expressing luciferase to permit in vivo imaging of growing cancer cells in bone (Materials and Methods). Data show that rLOX-PP expressing DU145 cells did not exhibit increased growth determined by bioluminescence imaging after luciferin injections (Fig 7a and b). Although a small increase in bone volume/total volume (BV/TV) was observed, the respective values for empty and rLOX-PP expressing cells of 0.1087 ± 0.015 and

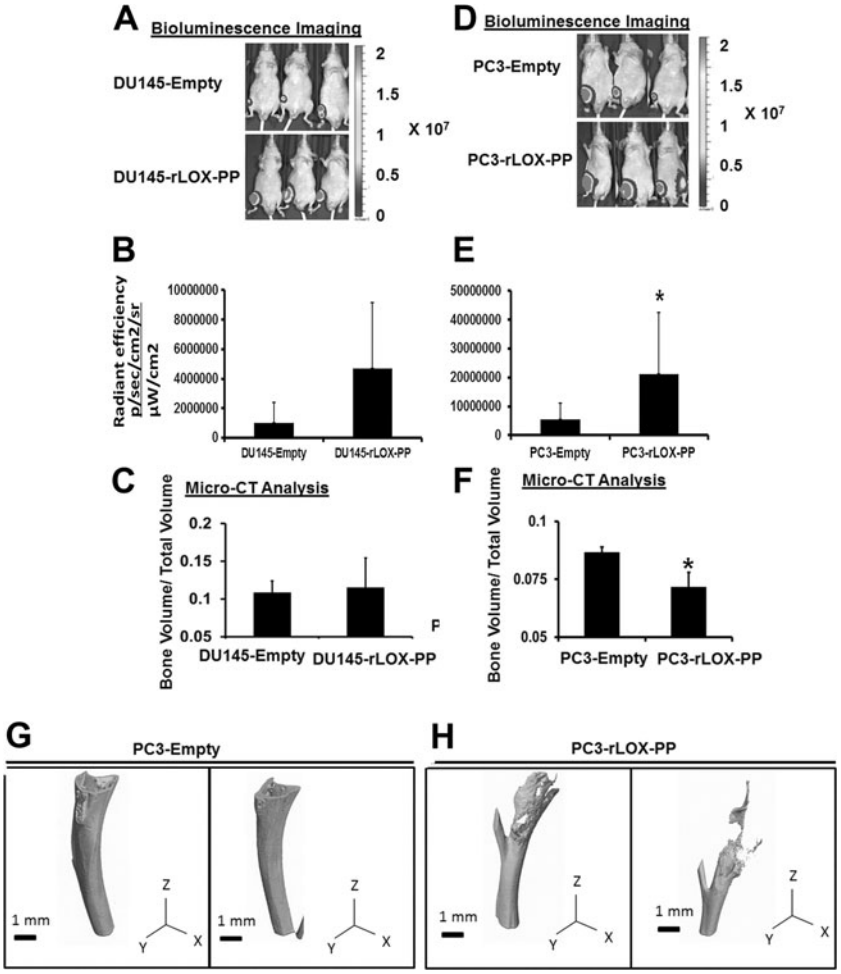

Fig. 7 Intra-tibial injection of DU145 and PC3 ectopically overexpressing rLOX-PP promotes growth and colonization leading to bone remodeling: Representative mice (n = 6/condition) showing bioluminescence imaging after 6 week intra-tibial injection of DU145-Empty or rLOX-PP cells **a**, and luciferase-bioluminescence +/− SD **b**. Luciferase expressing DU145 cells were infected by transduction of EF1α-LOX-PP-myc-his-UBC-GFP (designated as rLOX-PP) and empty vector EF1α-Empty-UBC-GFP (designated as Empty) lentivirus particles. These cells (2.5 X 10^5) were injected into the tibia of mice (n = 6) showing increase in tumor growth and colonization in the cells expressing rLOX-PP compared to Empty. **c** Micro-CT analysis of tibia shows no significant effect on bone volume/total volume in rLOX-PP expressing DU145 cells compared to empty controls. **d** Representative mice (n = 9/condition) showing bioluminescence imaging by IVIS after 8 weeks intra-tibial injection of PC3-Empty or rLOX-PP expressing cells, and **e** quantitation of bioluminescence by IVIS +/−SD, *, $p < 0.05$ compared to Empty expressing cells. **f** Micro-CT analysis of tibia shows decrease in the ratio of bone volume/total volume in rLOX-PP expressing group compared to empty; +/− SD; n = 9, * $p < 0.05$, analysis by one way ANOVA. Representative 3-D reconstruction images of tibia injected with PC3-Empty cells and **g**, and of tibia injected with PC3-rLOX-PP cells **h** at sacrifice on day 42.

0.1153 ± 0.038 were not significantly different ($p > 0.05$) (Fig. 7c). However, the same experiment performed with PC3 cells, bioluminescent imaging shows that rLOX-PP overexpression increased growth of PC3 cells in the bone microenvironment (Fig. 7e), and was accompanied by a very significant decrease in BV/TV (Fig. 7f). Three dimensional reconstruction of μCT images of PC3-injected bones further supports the finding of increased bone destruction in LOX-PP expressing PC3 cells (Fig. 7g and h). Thus, the activity of rLOX-PP to enhance osteoclast lineage mediated bone resorption occurs both in vitro and in at least one in vivo model, while rLOX-PP stimulation of osteoblast differentiation is more obvious in vitro. Taken together, data suggest that rLOX-PP can enhance osteoblast/osteoclast coupling by inhibiting OPG expression.

Discussion

Bone extracellular matrix is composed of mineralized and organic components of which type I collagen makes up 90 % of the organic matrix. Lysyl oxidase (LOX) enzyme, plays a central role in post translational modification of collagen and is required for inter- and intra fibrillar cross-link formation in collagen molecules which is essential for structural and functional integrity of extracellular matrices (Kagan and

Trackman 1991; Knott and Bailey 1998; Lees et al. 1990; Oxlund et al. 1995). Pro-LOX undergoes extracellular proteolytic processing by procollagen C-proteinases derived from the *BMP1*, *Tolloid-Like 1* (*Tll1*) and *Tolloid-like 2* (*TLL2*) genes to release the C-terminal anionic region which contains the active enzyme and an 18 kDa N-terminal arginine-rich region which makes up the lysyl oxidase propeptide (LOX-PP) (Trackman et al. 1992). In this study, we investigated the changes in bone stromal cell proliferation and differentiation in response to rLOX-PP treatment and its effects on prostate cancer cell conditioned media-induced alterations of proliferation and differentiation of BMSCs. The working model was that because rLOX-PP exhibits a variety of inhibitory activities in cancer cells, LOX-PP could potentially interfere with communication between cancer cells and stromal cells to disrupt and inhibit tumor growth in bone. Major novel findings here indicate that rLOX-PP can stimulate the differentiation of osteoclast progenitors in primary cultures of mouse BMSCs. In addition, data demonstrate that rLOX-PP inhibits proliferation of BMSCs stimulated by conditioned media from PC3 and DU145 prostate cancer cells, while it stimulates osteoblast and osteoclast differentiation. These effects of rLOX-PP are consistent with an earlier publication in which inhibition of proliferation of mineralizing primary rat osteoblasts was suggested to permit subsequent osteoblast differentiation. This suggestion was based on data in which rLOX-PP was seen to inhibit ultimate differentiation of calvaria osteoblasts only when applied to actively proliferating cells and not to cells which had already entered later stages of differentiation (Vora et al. 2010a). In the early study with calvaria osteoblasts, stimulation of osteoblast differentiation was not observed, by contrast to the data reported here performed with mouse BMSCs which contain a more complex mixture of cells including pluripotent mesenchymal stromal cells and hematopoietic cells. Thus, in a more complex mixture of cells which more closely mimics the cell types in vivo in endochondral bone, an activity of rLOX-PP to inhibit proliferation and enhance differentiation especially in the presence of prostate cancer conditioned media has now been demonstrated in both the context of osteoblast and osteoclast differentiation. These findings suggest that rLOX-PP influences cross-talk been coupling factors which regulate the balance between bone formation and bone resorption.

It is apparent that both DU145 and PC3 conditioned media inhibit osteoblast differentiation in BMSC cultures with effects of PC3 medium stronger than that from DU145. This is consistent with the fact that PC3 cells produce osteolytic bone lesions when injected into bones, while DU145 cells produce osteoblastic lesions. These conditioned media, therefore, are likely to influence the balance between osteoblast and osteoclast activity in different ways. Although the effects of rLOX-PP to reverse inhibitory effects of PC3 conditioned medium on osteoblast differentiation were very clear, rLOX-PP effects to stimulate osteoclast development in the presence or absence of PC3 conditioned media were even more dramatic. We speculate that interactions of rLOX-PP with endogenous osteoclast inhibitory factors contained in conditioned media may explain this outcome.

Until now, all activities of rLOX-PP identified have been inhibitory (Bais et al. 2012a; Palamakumbura et al. 2004; Min et al. 2007; Palamakumbura et al. 2009; Wu et al. 2007; Bais et al. 2015; Sato et al. 2011; Sato et al. 2013; Vora et al. 2010a; Jeay et al. 2003; Min et al. 2009; Min et al. 2010; Sanchez-Morgan et al. 2011; Yu et al. 2012; Zhao et al. 2009). The current report shows that rLOX-PP stimulates osteoclast development especially in the presence of M-CSF and RANKL. Osteoclast development is largely controlled by osteoblasts and the balance between RANKL and the decoy receptor OPG. The molecular mechanism of rLOX-PP is primarily to attenuate the production of an OPG most likely by developing osteoblasts. It is notable, however, that rLOX-PP stimulates CCN2 expressions in BMSC cultures, and so the possibility remains that LOX-PP may in some way increase the transcription of CCN2 by a specific cell type at a specific stage of differentiation. CCN2 stimulates osteoclast fusion, and proliferation in early osteoblast development, and has been reported to stimulate RANK receptor signaling, sequester OPG, and stimulate DC-STAMP expression and osteoclast fusion (Nishida et al. 2011; Aoyama et al. 2015).

Intramedullary injections of DU145 or PC3 cells and these cells expressing rLOX-PP independently support the finding that rLOX-PP can stimulate osteoclast development and bone resorption, while its effect to enhance the development of osteoblast lesions from DU145 cells was more limited in this model. Additional more complex regulatory interactions between LOX-PP, and the BMP pathways intersecting with extracellular BMP-2/4 decoy inhibitors chordin, noggin, and/or gremlin regulations can be imagined, but require considerable additional investigation.

The processes and mediators involved in osteoblast-osteoclast coupling seem likely to act as potential targets of prostate cancer derived factors and of rLOX-PP. Osteoclast-osteoblast coupling occurs through either cell-cell contact, diffusible paracrine factors and cell to bone matrix interactions. In addition to direct communication between osteoblasts and osteoclasts and factors secreted by these two cell lines it has been reported that other cells play a role in osteoblast-osteoclast coupling. Macrophages have been reported to play an important role during different steps in bone remodeling and found to be involved in local coupling process (Tran Van et al. 1982). Beside serving as precursor for osteoclasts, these cells maintain coupling during the remodeling cycle due to their anatomical location and their ability to promote optimal osteoblast mineralization (Pettit et al. 2008). Identifying the molecular and cellular targets of rLOX-PP and harnessing some of these interactions in therapeutic approaches to

address bone pathologies including metastasis is of considerable interest but requires more basic research to more fully understand LOX-PP modes of action in bone biology. An intricate balance between factors stimulating osteoblast differentiation and factors stimulating osteoclast differentiation dictate the fate of stromal cells. Our data indicate for the first time that LOX-PP enhances both osteoclast and osteoblast differentiation. Considering the temporal effect of LOX-PP on osteoblast and osteoclast proliferation and differentiation it is likely that LOX-PP enhances coupling interactions between osteoclasts and osteoblasts and functions to positively control a normal bone turnover phenotype which can be perturbed in a variety of bone pathologies.

It has recently been reported that active LOX emanating from tumor cells promotes the formation of pre-metastatic bone lesions in the absence of bone-associated tumor cells (Cox et al. 2015). The supporting evidence was based in part on administering conditioned media from pro-LOX expressing breast or colon cancer cells assumed to ultimately elaborate active LOX, and observing bone lesions. These conditioned media would also contain the LOX-PP domain either as part of enzymatically inactive pro-LOX or as free rLOX-PP. The current study, therefore, raises the notion that effects of the LOX-PP domain on bone reported here may contribute to the effects reported in (Cox et al. 2015). Further research into the mechanisms by which LOX-PP inhibits recently reported cancer metastasis (Ozdener et al. 2015) and effects seen here on bone are clearly required.

We and others have reported that rLOX-PP is a tumor growth inhibitor in models of non-mineralized tumor development (Bais et al. 2012a; Min et al. 2007; Bais et al. 2015; Ozdener et al. 2015; Min et al. 2009; Agra et al. 2013). and yet in the current study, evidence for rLOX-PP promotion of osteolytic lesion development in bone by PC3 prostate cancer cells is presented. These data point to the complexity of normal cell to cell communication which maintains tissue homeostasis in different contexts, perturbations of this balance caused in pathology, and effects of a signaling inhibitor such as rLOX-PP on these balances. So far, all soft tissue tumor studies have demonstrated that the variety of targets of rLOX-PP work together to result in a beneficial outcome. In mineralized tissue, which depends on an intricate balance between osteoblast and osteoclast activity and factors unique to bone, rLOX-PP shifts the balance towards resorption. We speculate that this effect is largely driven by LOX-PP-dependent regulation of factors most relevant to bone homeostasis as follows: increased expression of CCN2 which sequesters RANKL, and decreased expression of OPG, resulting in activated bone resorption. The primary target of rLOX-PP in this context is under investigation.

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