

# Castration-induced bone loss triggers growth of disseminated prostate cancer cells in bone

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## Abstract

Up to 90% of patients with castrate-resistant prostate cancer develop bone metastases, and the majority of these men have received androgen deprivation therapy known to cause bone loss. Whether this treatment-induced change to the bone microenvironment affects disseminated tumour cells, potentially stimulating development of bone metastasis, remains to be determined. The objective of this study was to use an *in vivo* model mimicking androgen ablation to establish the effects of this intervention on disseminated prostate cancer cells in bone. We mimicked the effects of androgen deprivation on bone metastasis by castrating 12-week-old BALB/c nude mice that had disseminated, hormone-insensitive PC3 prostate cancer cells present in the long bones. Castration caused increased bone resorption and loss of bone volume, compared with sham operation. In addition, castration triggered growth of disseminated PC3 cells to form bone metastasis in 70% of animals. In contrast, only 10% of sham-operated animals had detectable long bone tumours. Weekly administration of 100 µg/kg zoledronic acid (ZOL) prevented castration-induced tumour growth in bone and increased bone volume, but did not eliminate the disseminated tumour cells. ZOL had no effect on tumour growth in the sham-operated animals, despite causing a significant increase in bone volume. This is the first demonstration that, in a model of prostate cancer bone metastasis, mimicking androgen ablation results in growth of disseminated tumour cells in bone through osteoclast-mediated mechanisms. We provide the first biological evidence supporting the administration of ZOL to prostate cancer patients at the time of androgen ablation to prevent subsequent relapse in bone.

## Key Words

- ▶ prostate cancer
- ▶ castration
- ▶ bone
- ▶ metastasis

*Endocrine-Related Cancer*  
(2014) 21, 769–781

## Introduction

Prostate cancer is the most common malignancy and the second most common cause of cancer death in men. In the USA, there were ~238 590 new prostate cancer diagnoses and more than 29 720 deaths in 2013 (American Society for Cancer 2013). The majority of these deaths was a result

of metastatic spread. Prostate cancer frequently metastasises to bone, especially to the axial skeleton, pelvis and long bones. Once this occurs, the cancer is considered incurable and treatment strategies change from curative to palliation and control of the disease.

At diagnosis, most prostate cancers express androgen receptors and are hormone dependent, with the presence of circulating androgens maintaining growth and survival of tumour cells both in the prostate and in metastases. Therefore, for patients with either locally advanced tumours, evidence of disease progression, or whose disease has become metastatic, androgen ablation treatment is commonly used. This involves surgical or pharmacological castration, thus reducing circulating levels of testosterone. Androgen deprivation causes decreased proliferation and increased apoptosis in prostate tumours and results in initial remission in around 70% of patients (Matsushima *et al.* 1999). However, this response is temporary and relapse will eventually occur with the tumour cells becoming castration resistant (Debes & Tindall 2002). Up to 90% of men with metastatic castration resistant prostate cancer develop bone metastases (Petrylak *et al.* 2004, Tannock *et al.* 2004).

For tumour cells to colonise bone, they must first be shed from the prostate into the circulation, home to the bone microenvironment, leave the circulation and then lodge in the specific 'niches' before proliferating. In prostate cancer, the perivascular niches of the bone marrow sinusoids are believed to be the sites of active metastases formation (van der Horst *et al.* 2011). The presence of tumour cells in the bone, however, is not predictive of future development of metastases, and many patients with detectable tumour cells in their bone marrow never go on to develop overt metastases (Townson & Chambers 2006, Aguirre-Ghiso 2007, Weilbaecher *et al.* 2011). Therefore, for bone metastases to develop one of two things must happen; either disseminated tumour cells acquire mutations rendering them capable of proliferating in their new environment, or the local environment undergoes changes that initiate proliferation of disseminated tumour cells.

Evidence from mouse models has shown that the microenvironment significantly influences tumour progression in bone, and that increasing bone turnover can induce tumour growth (Ottewell *et al.* 2014). Osteoblasts and osteoclasts may both play important roles in this process. In models of prostate cancer, expansion of the osteoblast niche by administration of parathyroid hormone increased subsequent colonisation of bone by prostate cancer cells (Shiosawa *et al.* 2011). Whereas in breast cancer models, inhibition of osteoclast activity by pre-treatment with anti-resorptive agents such as zoledronic acid (ZOL) prevents subsequent tumour growth in bone and delays growth of established tumours (van der Pluijm *et al.* 2005). To maximise tumour take, the majority

of experimental models of bone metastasis use 5- to 6-week-old mice that have high bone remodelling rates, providing additional evidence that increased bone turnover promotes skeletal tumour growth. In keeping with this, administration of ZOL in young mice has been shown to reduce the development of bone metastasis (Brown & Holen 2009).

Increased bone turnover and subsequent bone loss are well-described side effects of castration (Verhas *et al.* 1986, Reim *et al.* 2008), but the precise cellular and molecular consequences of this for the bone microenvironment, including on effects on disseminated tumour cells, have not been characterised. In the current study, we have used mouse models of prostate cancer bone colonisation to investigate the effects of castration-induced bone loss on growth of disseminated tumour cells. We deliberately chose a tumour model that is androgen insensitive, as this allowed us to investigate the effects of androgen deprivation on the bone microenvironment without directly affecting growth of the tumour cells. We hypothesised that if castration can stimulate growth of disseminated tumour cells in the bone microenvironment to produce overt metastases in mice, this may also cause the development of bone metastases from disseminated tumour cells in prostate cancer patients.

ZOL is licenced to treat skeletal complications in metastatic prostate cancer (reviewed by Morgans & Smith (2012) and El-Amm *et al.* (2013)). However, this drug is not routinely administered until bone metastases are confirmed or when the first skeletal-related event (SRE) occurs. A number of *in vivo* studies have supported that ZOL inhibits prostate cancer-induced bone disease, but the majority of these have focussed on treatment effects of advanced disease with extensive bone destruction, and hence shown limited effects on disease progression (Corey *et al.* 2003, Thudi *et al.* 2008, Hung *et al.* 2011). A recent clinical trial assessing the effects of starting ZOL treatment within 6 months of androgen-deprivation therapy (ADT) in patients with castration-sensitive, metastatic, prostate cancer found no delay in time to first SRE in the ZOL group (Smith *et al.* 2014). These data demonstrate that ZOL does not prevent the progression of established bone metastases. We have, therefore, investigated the potential benefits of giving ZOL before castration, in order to inhibit resorption-mediated growth of disseminated tumour cells and thus prevent prostate cancer relapse in bone. This is the first *in vivo* study to demonstrate that castration results in changes to the bone microenvironment, triggering growth of disseminated tumour cells and development of bone metastases.

## Materials and methods

### Cell culture

Low passage (<P10) human androgen-insensitive (PC3) and androgen-sensitive (VCAP, DUCAP, CWR22 and LNCAP) prostate cancer cells (European Collection of Cell Cultures, Wiltshire, UK), and an androgen-insensitive clone of LNCAP, C4-2B4 (made in house) were cultured in DMEM + 10% FCS (Gibco, Invitrogen).

For real-time analysis of tumour growth *in vivo*, PC3 cells were transfected with the red fluorescent protein, mCherry (RFP) or second-generation luciferase (Luc2). Before *in vivo* inoculation, eRFP-expressing cells were incubated for 15 min with 25  $\mu$ M of 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine and 4-chlorobenzenesulfonate (DiD; Life Technologies). Tumour growth was monitored using an IVIS (luminol) system (resolution 20  $\mu$ m/3.1 cm field of view) (Caliper Life Sciences, Waltham, MA, USA) (Luc2) or an Illumatool Lighting System (LightTools Research, Encintas, CA, USA) (RFP).

### *In vivo* studies

We used 6- and 12-week-old male BALB/c nude mice (Charles River, Kent, UK). Experiments were carried out in accordance with local guidelines and with Home Office approval under project licence 40/3462, University of Sheffield, UK.

Identification of a prostate cancer cell line that mimics tumour cell dissemination and subsequent growth in bone was done by injecting  $1 \times 10^5$  PC3, VCAP, DUCAP, CWR22, LNCAP or C4-2B4 cells into the left cardiac ventricle (intracardiac (i.c.)) or directly into the left tibia (i.t.) of 6-week-old mice as described previously (Ottewell *et al.* 2009).

For analyses of bone turnover markers in young mice, serum was isolated from 6-week-old animals ( $n=6$ ) weekly up to 12 weeks via tail vein bleed. For mice with a mature skeleton, 12-week-old mice were castrated or sham operated and killed 1–8 weeks later ( $n=5$ /group) and bone effects assessed. Effects of castration on disseminated tumour cells were determined following injection of prostate cancer cells into 12-week-old mice 7 days before castration, sham or no operation ( $n=10$ /group). Six-week-old mice were used as tumour growth controls. A total of  $1 \times 10^5$  DiD-labelled PC3-RFP or PC3-Luc2 cells were injected into the left cardiac ventricle (i.c.), tumour growth was monitored for 5 weeks.

Effects of ZOL on castration-induced tumour growth were investigated in mice injected with  $1 \times 10^5$  DiD-labelled PC3-Luc2 cells i.c., and given weekly ZOL (100  $\mu$ g/kg) or saline ( $n=20$ /group) from day 5. Seven days following tumour cell injection, animals from both groups underwent either sham or castration ( $n=10$ ). This experiment was carried out twice ( $n=19$ – $20$  mice/group). We refer to the treatment groups in the manuscript as follows: control (no operation), sham control (sham operation, PBS treatment), sham ZOL (sham operation, ZOL treatment), castration control (castration, PBS treatment) and castration ZOL (castration, ZOL treatment).

Serum was stored at  $-80^\circ\text{C}$  for ELISA, tibiae and femurs were fixed in 4% PFA for microcomputed tomography ( $\mu$ CT) analysis before decalcification in 1%PFA/0.5% EDTA and processing for histology. For two-photon analysis, bones were stored in optimum cutting temperature (OCT) embedding reagent at  $-80^\circ\text{C}$ .

### $\mu$ CT imaging

$\mu$ CT analysis was carried out using a Skyscan 1172 X-ray-computed microtomography scanner (Skyscan, Aartselaar, Belgium) equipped with an X-ray tube (voltage, 49 kV; current, 200  $\mu$ A) and a 0.5-mm aluminium filter. Pixel size was set to 5.86  $\mu$ m and scanning initiated from the top of the proximal tibia as described previously (Ottewell *et al.* 2008).

### Bone histology and measurement of tumour volume

Osteoclasts were detected using toluidine blue and tartrate-resistant acid phosphatase (TRAP) staining as described previously (Cole & Walters 1987). The osteoblasts were identified as mononuclear, cuboidal cells residing in chains along the bone surface. The number of osteoclasts/osteoblasts per millimetre of cortical-endosteal bone surface and trabecular bone surfaces and the proportion of bone surface occupied by osteoclasts/osteoblasts was determined using a Leica RMRB upright microscope and OsteoMeasure Software (Osteometrics, Inc., Decatur, GA, USA) as described previously (Parfitt *et al.* 1987).

### Two-photon microscopy

Tibiae were imaged using a multiphoton confocal microscope (LSM510 NLO upright; Zeiss, Cambridge, UK). DiD-labelled cells were visualised using a 633 nm Chameleon laser, bone was detected using the 900 nm

multiphoton laser (Coherent, Santa Clara, CA, USA) and images were reconstructed in LSM Software version 4.2 (Zeiss). Velocity 3D Image Analysis Software (Zeiss) was used to count the number of disseminated tumour cells in  $2104\ \mu\text{m}$  ( $X$ -axis)  $\times$   $2525\ \mu\text{m}$  ( $Y$ -axis)  $\times$   $100\ \mu\text{m}$  ( $Z$ -axis) of the proximal tibiae just below the growth plate.

### Biochemical analysis

Serum concentrations of TRAP 5b, P1NP and osteocalcin were measured using commercially available ELISA Kits: MouseTRAP Assay (Immunodiagnostic Systems, Boldon, Tyne and Wear, UK), Rat/Mouse P1NP Competitive Immunoassay Kit (Immunodiagnostic Systems) and Mouse Osteocalcin Kit (Biomedical Technologies, Inc., Stoughton, MA, USA) respectively.

### Statistical analyses

Statistical analyses were one-way ANOVA followed by Newman–Keuls multiple comparison test. Statistical significance was defined as  $P$  value  $\leq 0.01$ . All  $P$  values are two sided.

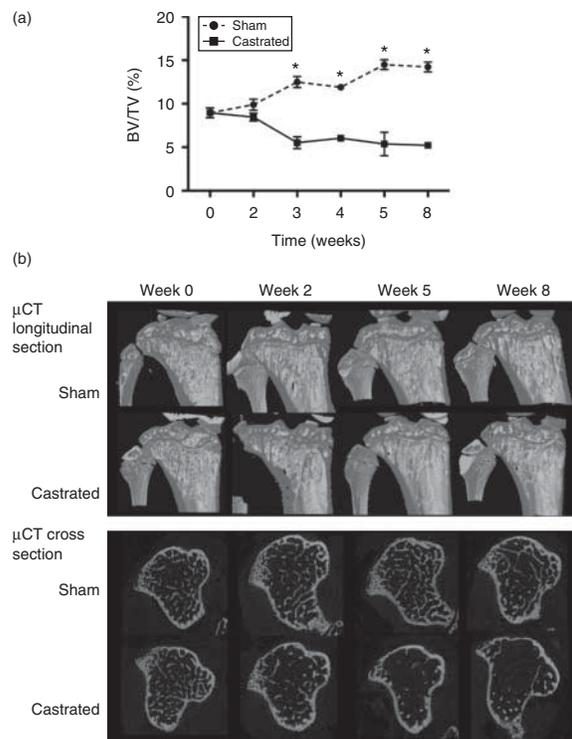
## Results

### Characterisation of castration-induced changes in the bone microenvironment

We carried out a longitudinal study to establish how castration modified the bone microenvironment in nude mice. Castrated animals had significantly reduced trabecular bone volume by week 3 compared with sham-operated animals ( $P < 0.01$ ; Fig. 1). Decreased bone volume was preceded by an increase in the serum levels of the osteoclast marker TRACP (Fig. 2a), accompanied by increased numbers of osteoclasts, detected 2 weeks following castration (Fig. 2b). In addition, at 2 weeks, there was a decrease in the osteoblast activity marker P1NP (Fig. 2c) as well as in the number of osteoblasts per millimetre of bone (Fig. 2d). In subsequent experiments, tumour cells were injected for 7 days before castration to allow sufficient time for tumour cell colonisation to occur before the initiation of bone loss.

### Effects of castration on growth of disseminated prostate cancer cells in bone

Tumour growth in bone was investigated following *i.c.* injection of PC3–RFP prostate cancer cells in male mice.

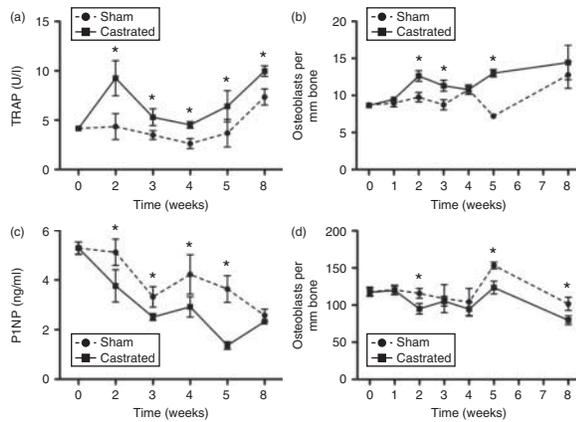


**Figure 1**

Effects of castration on bone volume and structure over time.  $\mu$ CT analysis of tibiae up to 8 weeks following castration in 12-week-old male BALB/c nude mice ( $n = 5/\text{group}$ ). (a) Graph showing % total bone volume compared with tissue volume (BV/TV %)  $\pm$  S.E.M.; \* $P < 0.01$  by ANOVA. (b) X-ray images show representative longitudinal and cross-sectional  $\mu$ CT images of left proximal tibiae containing trabecular bone.

In pilot studies, 90% of 6-week-old animals had detectable skeletal tumour growth following this protocol, compared with only 10% of 12-week-old animals. This difference was attributed to the higher rate of bone turnover in young animals as demonstrated by a gradual drop in serum levels of bone turnover markers with age (Fig. 3). Throughout the study, a control group of 6-week-old mice was included to confirm tumour cell growth *in vivo*.

Following *i.c.* injection of PC3–RFP cells in 12-week-old animals, the majority of cancer cells that reach bone remain quiescent and do not form overt tumours as shown in Figs 4 and 5. We investigated whether modification of the bone microenvironment through castration affects the ability of these disseminated tumour cells to form colonies. Castration carried out 7 days after tumour cell injection resulted in a substantial increase in tumour growth in bone (Fig. 4), accompanied by a 42 and 43% loss in trabecular bone, respectively, compared with the sham or control group ( $P < 0.001$ , Fig. 4c and d). Four weeks following castration, there was a trend towards increased



**Figure 2**

Effects of castration on bone turnover over time. ELISA analysis showing (a) serum TRAP levels up to 8 weeks following castration or sham operation and (b) numbers of osteoclasts per millimetre of bone. (c) P1NP ELISA showing serum concentrations up to 8 weeks following castration or sham operation and (d) numbers of osteoblasts lining bone. Graphs are shown as mean  $\pm$  S.E.M.; \* $P < 0.01$  compared with sham operated mice ( $n = 5$  mice/group).

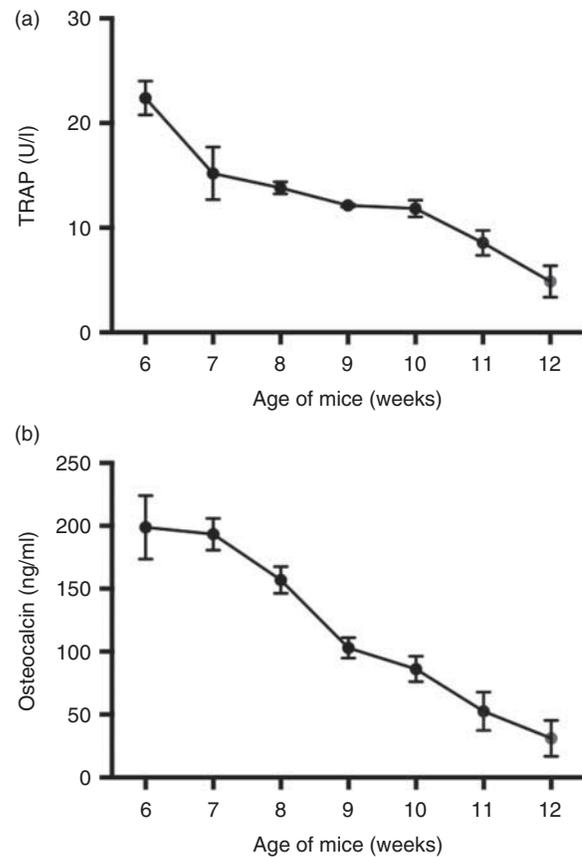
serum TRAP and P1NP levels in 6-week-old control and 12-week-old castrated mice compared with 12-week control and sham-operated animals, but, this did not reach significance (Fig. 4e and f). In all other experiments carried out in this study, castration caused significant increases in concentrations of circulating TRAP: 70% of animals had undergone castration-developed tumours in bone, a comparable frequency to that detected in 6-week-old tumour control animals (80%). In mice with lower bone turnover, or in which bone turnover had not been stimulated, a much lower percentage of animals developed skeletal tumours; 10% of the animals in the 12-week-old control and 10% in the sham group ( $P < 0.001$  for castration vs control or sham; Fig. 4a). Histological analysis revealed that tumour growth in long bone was primarily in the tibiae, with significantly increased mean tumour volume per tibiae of castrated mice compared with control ( $632.5 \pm 187.3$  vs  $107.5 \pm 107.5$  mm<sup>3</sup>; Fig. 4b). However, once tumour growth was established, castration had no effect on the rate of tumour growth. A number of mice with tumours and tumour volume were similar in castrated mice and 6-week-old control mice, both of which had decreased bone volume compared with 12-week control (Fig. 4).

To ensure that our findings were not a result of particular properties of the strain of PC3 cells used, we tested a second, independent, PC3 strain that has been stably transfected to express luciferase (PC3-Luc2). Using this, tumour growth in bone was detected in 70% of

castrated and 30% of sham-operated animals (data not shown). Subsequent experiments were carried out using PC3-Luc2 cells to enable visualisation of overall tumour growth *in vivo* (see examples in Fig. 5).

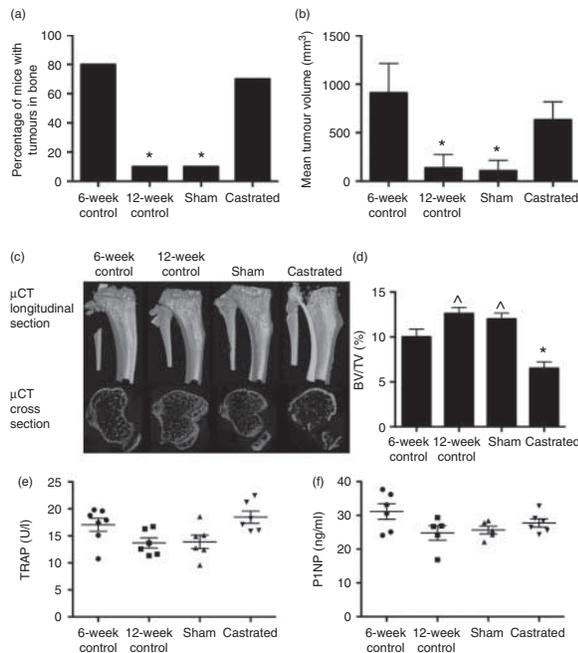
### Differential effects of osteoclast inhibition on tumour growth in bone in control and androgen-deprived animals

We investigated whether tumour growth in bone was driven by increased bone resorption by treating sham and castrated animals with a clinically relevant dose of the potent osteoclast inhibitor ZOL. PC3-Luc2 cells were injected *i.c.* in two groups of animals that received weekly injections of saline (control,  $n = 20$ ) or ZOL (100  $\mu$ g/kg, equivalent to 4 mg dose given to patients with prostate cancer-induced bone disease,  $n = 20$ ) for 5 weeks, starting 4 days after tumour cell injection. Half of the animals from each group underwent either a sham operation or castration on day 7 ( $n = 10$ /group), and tumour growth



**Figure 3**

Association between age and bone turnover markers in BALB/c nude mice. ELISA analysis showing serum levels of (a) the osteoclast marker TRAP and (b) osteocalcin in mice age 6–12 weeks.

**Figure 4**

Effect of castration on tumour growth in long bones of 12-week-old mice. Histograms show percentage of mice with detectable tumours in bone (a) and mean tumour volume per mouse  $\pm$  S.E.M. in 6-week control and 12-week castrated and sham-operated mice (b). X-ray images show representative longitudinal and cross-sectional  $\mu$ CT images of left proximal tibiae containing trabecular bone from 6-week and 12-week-old control animals, as well as sham-operated and castrated animals (c). Graphs show % total bone volume compared with trabecular volume  $\pm$  S.E.M. (d), total serum TRAP (U/l) (e) and total serum P1NP (ng/ml) (f).  $^{\wedge}P < 0.01$  increased and  $*P < 0.01$  decreased compared with sham saline (control) by ANOVA ( $n = 10$  mice/group).

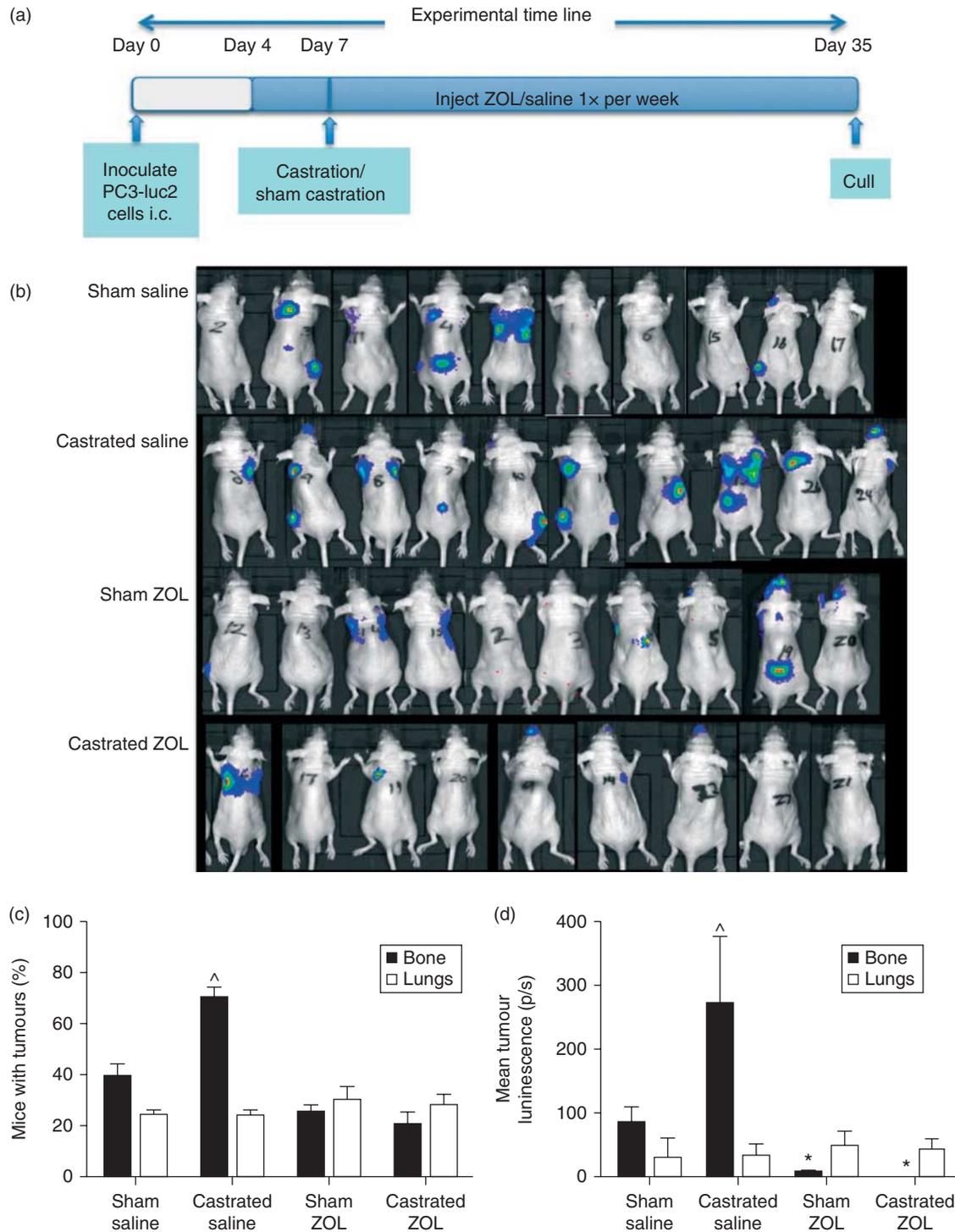
was monitored until day 35 (Fig. 5a). 70% animals that underwent castration had detectable tumours in bone compared with 39% of sham ( $P < 0.001$ ), supporting that increased bone resorption stimulated tumour growth (Fig. 5c). There was a significant reduction in tumour growth in bone in castrated animals treated with ZOL compared with castrated control (bone tumours were detected in 20% of castrated mice treated with ZOL and 70% of castrated control mice;  $P < 0.001$ ). In contrast, ZOL treatment did not alter the number of animals in which skeletal tumours in the sham group, with 22% of control and 20% ZOL treated sham-operated animals having detectable tumours in bone. However, ZOL significantly reduced the size of skeletal tumours in both sham and castrated mice compared with saline-treated animals ( $P < 0.01$ ; Fig. 5d). Despite the differential effects on tumour growth, ZOL caused significant alterations in bone in both castrated and sham-operated animals compared with the respective controls (Fig. 6). Bone

volume/trabecular volume increased from  $10.47 \pm 1.06\%$  in sham castrated to  $13.89 \pm 0.55\%$  in sham ZOL ( $P < 0.01$ ) and from  $4.12 \pm 1.11\%$  in castrated control compared with  $13.80 \pm 1.68\%$  in castrated ZOL ( $P < 0.01$ ) (Fig. 6b). In agreement with this finding, measurement of osteoclastic bone resorption demonstrated significant reductions in circulating levels of TRACP from  $13.73 \pm 0.89$  U/l in sham castrated to  $9.71 \pm 0.47$  U/l in sham ZOL ( $P < 0.01$ ) and from  $18.63 \pm 0.01$  U/l in castrated control compared with  $9.22 \pm 0.45$  U/l ( $P < 0.01$ ) in castrated ZOL. In addition, serum concentrations of the osteoblast activity marker P1NP were reduced in sham castrated compared with sham ZOL ( $P < 0.01$ ) and in castrated control compared with castrated ZOL ( $P < 0.01$ ). No significant differences in bone volume, serum TRACP or serum P1NP were detected between sham or castrated animals treated with ZOL, demonstrating that bone resorption is reduced to the same level in control and androgen-deprived conditions (Fig. 6d and e). Decreased bone resorption following administration of ZOL was associated with decreased lytic lesion formation from  $3.26 \pm 1.13$  mm<sup>3</sup> in sham castrated control to  $1.55 \pm 0.47$  mm<sup>3</sup> in sham ZOL ( $P < 0.01$ ) and from  $8.72 \pm 1.98$  in castrated compared with  $1.47 \pm 0.33$  in castrated ZOL ( $P < 0.01$ ) (Fig. 6c). These data demonstrate that lytic lesion formation is reduced to similar low levels in sham and castrated mice following administration of ZOL.

Multiphoton microscopy and Velocity 3D Image Analysis Software confirmed the presence of individual disseminated PC3 cells in the long bones of animals 7 days after injection (Fig. 7). Analysis of a three dimensional area comprising  $2104 \mu\text{m}$  (X-axis)  $\times$   $2525 \mu\text{m}$  (Y-axis)  $\times$   $100 \mu\text{m}$  (Z-axis) of the proximal tibiae just below the growth plate identified  $16.39 \pm 4.66$  DiD-labelled PC3 cells ( $n = 6$ ). Individual PC3 cells remained in the long bones of animals did not develop overt bone tumours by day 35, regardless of group (Fig. 5). Similar numbers of single tumour cells were seen disseminated in bone in all experimental groups ( $7.24 \pm 0.44$  in sham saline ( $n = 6$ ),  $7.76 \pm 0.24$  in castrated saline ( $n = 2$ ),  $7.18 \pm 0.21$  in sham ZOL ( $n = 6$ ) and  $7.92 \pm 0.42$  ( $n = 6$ )), demonstrating that the tumour cells were successfully engrafted in bone but remained non-proliferative for extensive periods in all settings.

## Discussion

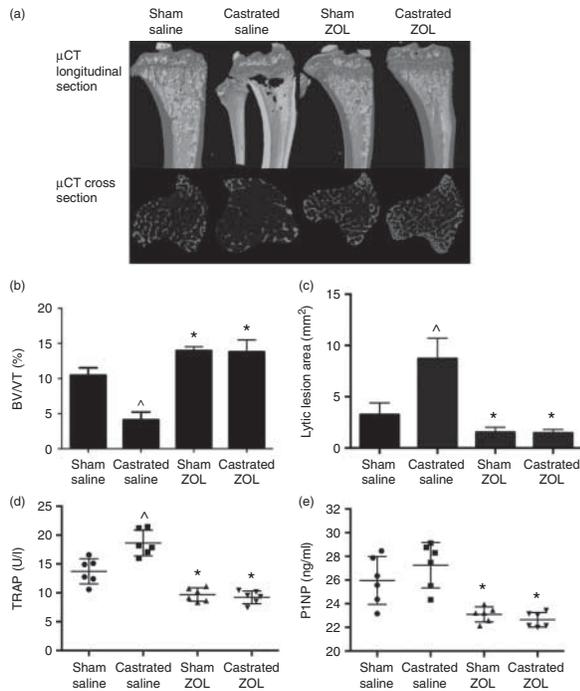
Using experimental systems that separately model the normal and the castrated bone microenvironment, combined with advanced imaging of disseminated



**Figure 5**

Zoledronic acid (ZOL) inhibits castration-induced tumour growth in bone. 12-week-old BALB/c mice were administered 100 µg/kg ZOL for 4 days after intracardiac injection of PC3-Luc2 cells followed by castration/sham operation 3 days later (a). Photographs show images for expression of luciferase in PC3-Luc2 cells growing in BALB/c nude mice 5 weeks following

tumour cell injection (b). Graphs show mean percentage of mice with tumours in bone or lung (c) and mean tumour fluorescence (photons/s) (d) 5 weeks following injection of PC3-Luc2 cells. <sup>^</sup>*P*<0.01 increased and <sup>\*</sup>*P*<0.01 decreased compared with sham saline (control), by ANOVA.

**Figure 6**

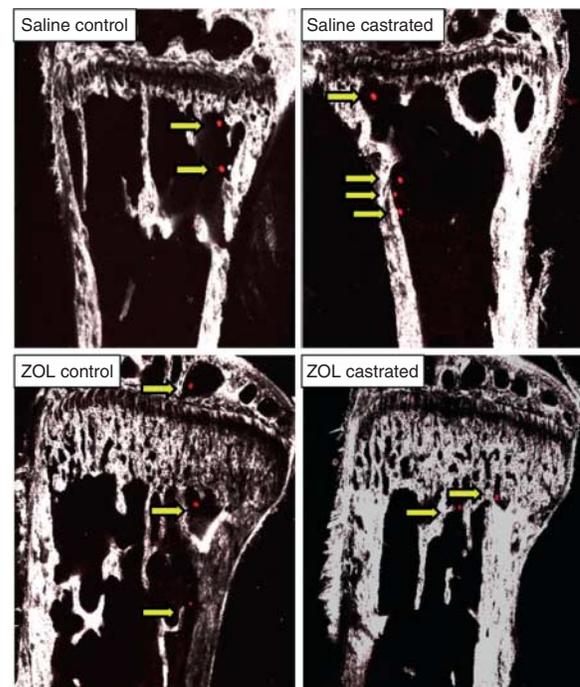
Zoledronic acid inhibits castration-induced bone loss. X-ray images show representative longitudinal and cross-sectional  $\mu$ CT images of left proximal tibiae containing trabecular bone (a). Graphs show % total bone volume compared with trabecular volume  $\pm$  s.e.m. (b), lytic lesion area ( $\text{mm}^2$ ) (c), serum concentrations of TRAP (U/l) (d) and serum concentrations of P1NP (ng/ml) (e) in sham and castrated animals treated with saline or 100  $\mu\text{g}/\text{kg}$  ZOL weekly for 5 weeks;  $^{\wedge}P < 0.01$  increased and  $^*P < 0.01$  decreased compared with sham saline (control) by ANOVA ( $n = 10$  mice/group).

tumour cells in bone, we have identified major differences in the growth of disseminated androgen-insensitive PC3 tumour cells and response to anti-resorptive therapy in these two settings. Prostate cancer cells homed to and grew in the long bones of young (6-week-old) BALB/c mice that had rapid bone turnover. Prostate cancer cells also homed to the long bones in 12-week-old mice, but failed to progress to form overt tumours in the majority of animals unless bone turnover was increased by subsequent castration (Fig. 4). This is the first study showing that castration-induced changes to the bone microenvironment can trigger proliferation of disseminated androgen-insensitive tumour cells and hence initiate bone metastasis.

For the current study, we have used mouse systems to model early stages of prostate cancer cell colonisation of bone. It should be noted, however, that there are major differences in bone turnover between mice and humans. In mice, castration-induced modification of the bone microenvironment is evident within a few days.

These rapid changes may have a greater influence on growth of disseminated tumour cells than would be the case in humans. To inhibit the high bone resorption rate in mice, a dose of ZOL equivalent to the standard 4 mg infusion was administered weekly, rather than the 3–4 weekly interval used in clinical treatment of cancer-induced bone disease. It is impossible to study tumour cell colonisation of bone in patients and we therefore rely on model systems to help decipher the key early events that drive prostate cancer bone metastasis.

Although PC3 cells are commonly used as a model for prostate cancer bone metastasis (Thudi *et al.* 2008, Das *et al.* 2010, Kim *et al.* 2013, Lee *et al.* 2013, Hansen *et al.* 2014), these cells do not mimic the majority of prostate cancers. PC3 cells do not express androgen receptors and hence are androgen insensitive (Veldscholte *et al.* 1990). In contrast, the majority of prostate cancers are initially androgen dependent but develop resistance to castration as the disease progresses. This is not mediated by the loss of androgen receptors; instead, the tumours acquire additional mechanisms that enable their survival in an androgen-deprived environment (Veldscholte *et al.* 1990).

**Figure 7**

Non-proliferating tumour cells are present in trabecular bone of mice without detectable metastases. Confocal images of disseminated DiD-labelled tumour cells (red cells highlighted with yellow block arrows) that have homed to bone but not formed tumours in tibiae of sham and castrated mice treated with either saline or 100  $\mu\text{g}/\text{kg}$  ZOL.

The completely androgen-dependent PC3 cells were deliberately used in this study to allow us to independently assess the effects of androgen on the bone microenvironment and the influence of these changes on tumour growth. In addition, bone lesions generated by PC3 cells are strongly osteolytic. Studies of postmortem samples from prostate cancer patients have shown that the majority of bone metastases result in either predominantly osteoblastic (30%), or mixed osteoblastic, and osteolytic diseases (44%), with only 14% of patients having predominantly osteolytic disease (Morrissey *et al.* 2013). However, bone resorption markers are significantly elevated in patients with metastatic prostate cancer regardless of lesion type (Garnero *et al.* 2000, Coleman *et al.* 2013), and the use of anti-resorptive agents such as ZOL is therefore common in this setting (Coleman *et al.* 2010). Despite these limitations, we found that the PC3 model was the only prostate cancer model to reflect tumour cell colonisation, quiescence and subsequent growth in the bone microenvironment following injection into the circulation (Supplementary Table 1, see section on supplementary data given at the end of this article). The androgen-dependent VCAP and DUCAP lines form osteoblastic lesions in bone following intratibial injection and may provide a more clinically relevant model for prostate cancer-induced bone disease. We and others have shown that implanting prostate and breast cancer cells directly into the tibia enables tumours to grow in this environment both in 6-week-old mice with high bone turnover and in 12-week-old mice with low bone turnover (Ottewell *et al.* 2009, Herroon *et al.* 2013, Graham *et al.* 2014). This method of tumour cell injection is not appropriate for studies of disseminated tumour cells in bone and effects of therapies on early stage disease, as it involves introduction of a large number of tumour cells directly into the bone marrow. We were therefore limited in our choice of model for this study as PC3 cells are the only prostate cancer cell line that could be used to investigate seeding and dormancy in the bone environment.

This study identifies a clear link between osteoclast activity and growth of disseminated prostate cancer cells. Our data show that castration results in increased osteoclast activity and increased bone resorption leading to significant bone loss (Figs 1 and 2). We provide the first demonstration that castration-induced changes to bone turnover can initiate growth of disseminated prostate tumour cells, and that these changes are abolished following administration of the anti-resorptive agent ZOL. In our model, disseminated tumour cells remain

quiescent in the mature skeleton unless the bone microenvironment is modified to increase bone turnover. This is in keeping with the hypothesis that changes in the bone microenvironment are a key component that stimulates metastatic tumour growth from prostate cancer cells already seeded in bone. It should be noted that this situation does not mimic the majority of prostate cancers. ADT is most commonly administered as a treatment for patients following detection of tumour recurrence in bone as assessed by increases in serum PSA (Harris *et al.* 2009). In these patients, disseminated tumour cells in the bone environment have already been stimulated to proliferate and tumour growth is established. Furthermore, androgen deprivation still remains as a successful treatment for androgen-naïve tumours that express androgen receptors, and this treatment can prolong the life of a patient for 12–30 months (Seruga *et al.* 2011). Taking this into account, it is possible that castration-induced stimulation of prostate cancer growth in bone may only be clinically relevant to patients with disseminated castration-resistant prostate cancer cells in bone. For patients with androgen-sensitive prostate cancer, the benefits of androgen deprivation may outweigh the tumour growth, stimulating effects on increased bone turnover seen following castration. However, it is generally accepted that increasing bone turnover results in the release of growth factors from bone that in turn stimulate growth of tumour cells (reviewed by Sturge *et al.* (2011) and Cook *et al.* (2014)). It is, therefore, likely that increased bone turnover following castration may drive tumour growth in both androgen-sensitive and castration-resistant prostate cancer via the release of tumour-stimulating growth factors. This may be particularly important when metastatic cell numbers are low and potentially more dependent on the environment, rather than in large more autonomous lesions. The majority of androgen-sensitive prostate cancers eventually relapse following ADT and these tumours become castration resistant (Debes & Tindall 2002). It is possible that these tumours may develop the ability to grow in an androgen-reduced environment by utilising bone-derived growth factors, such as TGF $\beta$ , IGF1, FGF, PDGF, BMPs and chemokines to stimulate tumourigenesis (Guise 2010). Therefore, blocking castration-induced bone turnover may inhibit prostate cancer relapse in bone. The efficacy of decreasing bone turnover by ZOL treatment at the same time as administering androgen deprivation in androgen-sensitive tumours warrants further investigation in clinical studies.

Following homing to bone, it is suggested that tumour cells occupy specific niches identical to (or overlapping

with) the hematopoietic stem cell (HSC) niche, where they remain dormant until triggered to proliferate (Shiosawa *et al.* 2011). Tumour cell dormancy and proliferation in bone and HSC mobilisation and quiescence may therefore be regulated by many of the same processes, including osteoblast and osteoclast activities (Kollet *et al.* 2007, Renstrom *et al.* 2010, Ellis *et al.* 2011). In models of prostate cancer, there is evidence suggesting that the 'bone metastatic niche' may be located either in the perivascular niches of the bone marrow sinusoids (Aguirre-Ghiso 2007), or at endosteal bone surfaces in the long bones. Our model supports the idea that the bone metastatic niche is located on the endosteal surfaces as this is where tumour cells were most commonly detected (Fig. 7). However, we cannot rule out the involvement of the perivascular niche as this is also a highly vascularised region of bone.

In keeping with our finding that prostate tumour cells home to the areas of bone comprising the HSC niche, and that stimulation of these tumour cells to proliferate is driven by osteoclast-mediated bone loss, osteoclasts have also been shown to be central to mobilisation of HSCs (Kollet *et al.* 2007). Experimental evidence has come from mouse models showing that RANKL-induced osteoclastic bone resorption stimulates HSCs to leave bone marrow niches and enter the circulation. In addition, bone resorption disrupts adhesion of HSCs to niche components that maintain cell quiescence resulting in proliferation (Kollet *et al.* 2006). Furthermore, RANKL-mediated signalling from prostate cancer cells is shown to establish the pre-metastatic niche and induce colonisation and metastasis to bone (Chu *et al.* 2014). In addition, bone resorption disrupts adhesion of HSCs to niche components that maintain cell quiescence resulting in proliferation (Shiozawa *et al.* 2013). It is therefore likely that other mechanisms that disrupt the endosteal niche, including castration-induced bone loss, may have similar effects altering integrin interactions that maintain tumour cells in a quiescent state (Barkan *et al.* 2010).

We found significantly decreased bone turnover in mice treated with ZOL, and this was associated with inhibition of castration-induced proliferation of disseminated prostate cancer cells. Emerging data strongly suggest that administration of ZOL, before detection of bone metastases, may have significant anti-tumour benefit. We and others have shown that administration of ZOL, in the absence of anti-cancer therapies, does not reduce existing bone metastases from solid tumours, including prostate and breast (Ottewell *et al.* 2009, 2012, Hung *et al.* 2011). However, when given in a preventive setting, i.e. ZOL is administered before tumour cells are introduced,

this results in a significant reduction in metastatic tumour growth in bone from both osteoblastic LnCAP and osteolytic PC3 prostate cancer cells, as well as from MDA-MB-231 breast cancer cells (Daubin  *et al.* 2007, Hung *et al.* 2011). Our data suggest that this is due to inhibition of processes in the metastatic niche, causing disseminated tumour cells to be held in a quiescent state. Recent evidence has suggested that inhibiting bone turnover with ZOL may have limited usefulness for specific tumour types. Studies in which dog (Ace-1) and mouse (RM1) prostate cancer cells have been injected i.c. into mice showed no difference in tumour growth between mice that had received ZOL before or after tumour cell injection, or in untreated control mice (Thudi *et al.* 2008, Hung *et al.* 2011). These findings warrant further investigation involving clinical trials of prostate cancer metastasis to bone.

The use of ADT in prostate cancer causes marked changes in hormone levels, most notably a drop in circulating androgens. As male oestrogen production is mediated by the aromatisation of testosterone, ADT also reduces circulating oestrogen levels. This leads to loss of bone mineral density (BMD; Smith *et al.* 2001) and is associated with increased risk of fractures (Shahinian *et al.* 2005). Clinical trials have reproducibly shown that treatment with a bisphosphonate, including ZOL, improves BMD in prostate cancer patients undergoing ADT (Smith 2003, Michaelson *et al.* 2007). However, these trials did not record data on effects on future development of bone metastasis. Only two phase III clinical trials have aimed to investigate whether ZOL treatment can prevent development of bone metastasis in men with castrate-resistant and castrate-sensitive prostate cancer. However, both studies were stopped early: the castrate-resistant prostate cancer trial due to poor accrual and lower than expected rate of bone metastasis (Smith *et al.* 2005), and the trial of castrate-sensitive prostate cancer due to withdrawal of drug supply by the corporate sponsor (Smith *et al.* 2003). A recently published clinical trial has shown that ZOL treatment initiated within 6 months of ADT did not reduce the time to first SRE in patients with established bone metastasis (Smith *et al.* 2014). This supports our hypothesis that ADT induces rapid changes to the bone microenvironment and that ZOL therefore must be given at the time of ADT in order to modify disease progression. For now the benefits of early intervention with ZOL remain inconclusive. However, ZOL is currently under study for use in men with castrate-sensitive prostate cancer without evidence of metastasis in a phase III trial that has not yet reported its results (NCT00242567). The primary endpoint

of this study is skeletal event free survival at 18 months and 3 years (Saylor 2014). The findings from this small study involving 522 men are currently being analysed and should give an indication into the effectiveness of ZOL as a preventative treatment for prostate cancer-induced bone metastasis.

While our studies have implications for castration-resistant prostate cancer growth in patients receiving ADT, the studies may have a more general implication for the treatment of apparently localised T1/T2, prostate cancers. Currently a significant proportion of patients deemed to be metastases free at diagnosis and who receive radical prostatectomy, return to clinics within 5–10 years with clear evidence of disease progression (Han et al. 2003). This suggests that in these patients, prostate cancer cells have already taken up residency in metastatic sites in such low numbers and activity as to be undetected at the time of surgery. While we currently do not know exactly how these cells are triggered to form growing lesions in the skeleton, our studies would suggest that alterations in bone turnover are pivotal to these events and therefore this may be worth targeting in these patients to prevent relapse.

ZOL (4 mg infusion over 15 min) is currently licenced to treat bone complications that are associated with androgen deprivation and bone metastases in prostate cancer (reviewed by Morgans & Smith (2012) and El-Amm et al. (2013)). Administration of ZOL is usually reserved until patients present with SREs or are deemed likely to develop skeletal complications. Our data suggest that earlier intervention, with ZOL administered before, or simultaneously to, the initiation of ADT, may not only prevent androgen deprivation-induced bone loss but could also inhibit prostate cancer relapse in bone. In conclusion, our study presents a potential treatment strategy that warrants further investigation in clinically relevant models of androgen-sensitive prostate cancer.

#### Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/ERC-14-0199>.

#### Declaration of interest

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

#### Funding

This study was supported by a programme grant from Cancer Research UK (to C L Eaton, P I Croucher and I Holen). P I Croucher is supported by

Mrs Janice Gibson and the Ernest Heine Family Foundation. IVIS lumino imaging system was funded by an equipment grant from Yorkshire Cancer Research.

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Received in final form 11 July 2014

Accepted 21 July 2014

Made available online as an Accepted Preprint

22 July 2014