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14. ABSTRACT The identification of the interaction between MKs and prostate cancer cells was the focus of this study. K562 (human megakaryocyte precursors), and primary MKs induced from mouse bone marrow hematopoietic precursor cells, potently suppressed prostate carcinoma PC-3 cells in co-culture. The inhibitory effects were specific to prostate carcinoma cells and were enhanced by direct cell-cell contact. Recombinant thrombopoietin (TPO) was used to expand MKs in the bone marrow and resulted in decreased prostate tumor skeletal lesion development after intra-cardiac tumor inoculation. Flow cytometry for propidium iodide (PI) and annexin V supported a pro-apoptotic role for K562 cells in limiting PC-3 cells. Gene expression analysis reduced mRNA levels for cyclin D1 while mRNA levels of apoptosis-associated speck-like protein containing a CARD (ASC) and death-associated protein kinase 1 (DAPK1) were increased in PC-3 cells after co-culture with K562 cells. These novel findings suggest a potent inhibitory role of MKs in prostate carcinoma cell growth <i>in vivo</i> and <i>in vitro</i> . This new interaction of metastatic tumors and hematopoietic cells, during tumor dissemination and colonization in bone, may ultimately lead to improved therapeutic interventions for prostate cancer patients.				
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Introduction

This is a revised final progress report on the project “Inhibitory Effects of Megakaryocytes in Prostate Cancer Bone Metastasis”, which has been funded by the PCRP Prostate Cancer Training Award (Postdoctoral Ph.D. Pls), Department of Defense since March 2008. In the project proposal, a potent inhibition by K562 cells (a human megakaryocyte progenitor cell line) on prostate carcinoma cells was demonstrated *in vitro* and three specific aims were proposed to investigate the inhibitory effects of K562 cells on prostate carcinoma cell growth. During the past two years, three specific aims were investigated, resulting in novel findings and a manuscript published on Journal of Bone and Mineral Research.

In the past one year since the progress report was submitted in May 2009, more *in vitro* experiments were carried out to characterize the mechanism of the interactions between PC-3 cells and K562 cells. The specificity of the inhibition of megakaryocytic cells on prostate carcinoma cells was investigated using K562 cells derived erythroids. The inhibitory potency was also compared between mature megakaryocytes versus less mature megakaryocytes. The expression of apoptosis mediators was validated at protein level. These results were added to the conditionally accepted manuscript submitted in 2009 and helped the manuscript being accepted and published in 2010.

Body

Prostate cancer cells commonly spread through the circulation, but few successfully generate metastatic foci in bone. Osteoclastic bone metabolism has been proposed as an initiating event for skeletal metastasis. Megakaryocytes (MKs) inhibit osteoclastogenesis indicating that MKs may indirectly inhibit tumor growth in bone through reduced bone resorption. Given the location of mature megakaryocytes at vascular sinusoids, they may be the first cells to physically encounter cancer cells as they enter the bone marrow acting in a direct manner. The identification of the interaction between MKs and prostate cancer cells was the focus of this study. K562 cells, and primary MKs induced from mouse bone marrow hematopoietic precursor cells, potently suppressed prostate carcinoma PC-3 cells in co-culture. The inhibitory effects were specific to prostate carcinoma cells and were enhanced by direct cell-cell contact. Using recombinant thrombopoietin (TPO) to expand MKs in athymic mice bone marrow effectively decreased prostate tumor skeletal lesion development after intra-cardiac tumor inoculation. Flow cytometry for propidium iodide (PI) and annexin V supported a pro-apoptotic role for MKs in limiting PC-3 cells. Gene expression analysis showed reduced mRNA levels for cyclin D1 while expressions of apoptosis-associated speck-like protein containing a CARD (ASC) and death-associated protein kinase 1 (DAPK1) were increased in PC-3 cells after co-culture with K562 cells. These novel findings suggest a potent inhibitory role of MKs in prostate carcinoma cell growth *in vivo* and *in vitro*. This new insight into the interaction of the metastatic tumors and hematopoietic cells during tumor dissemination and colonization in bone, may ultimately lead to improved therapeutic interventions for prostate cancer patients.

For details, please refer to the following lists: Key Research Accomplishments, Appendices (manuscript), Supporting Data and Reportable outcomes.

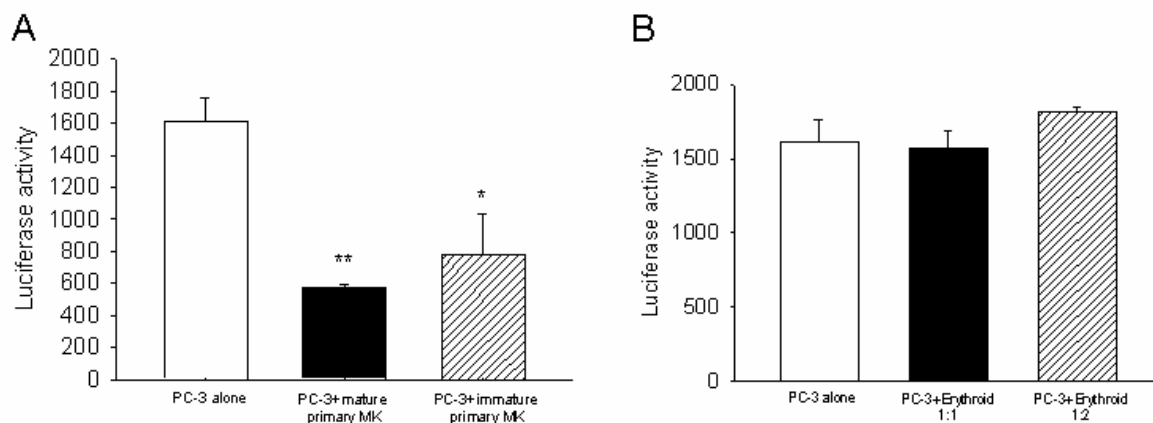
Key Research Accomplishments

Aim 1: Characterize the inhibitory effects of megakaryocytes on prostate carcinoma *in vitro*

In addition to the findings we made previously, in the past year, the following experiments were performed:

- 1) The inhibitory effects of mature and less mature primary megakaryocytes were compared where there was no significant difference (Figure 1A).
- 2) Considering that K562 cells can also be differentiated to erythroid cells, we checked the impact of erythroid cells derived from K562 cells on prostate carcinoma cell growth in vitro. And unlike megakaryocytes, erythroid cells didn't inhibit prostate carcinoma cell growth (Figure 1B).
- 3)

Figure 1.



Aim 2: Investigate the effect of MKs in prostate cancer bone metastasis *in vivo*.

The experiments proposed for this aim were fully achieved as reported in the first annual report.

Aim 3: Determine the role of TSP-1 in MK's inhibition on prostate carcinoma.

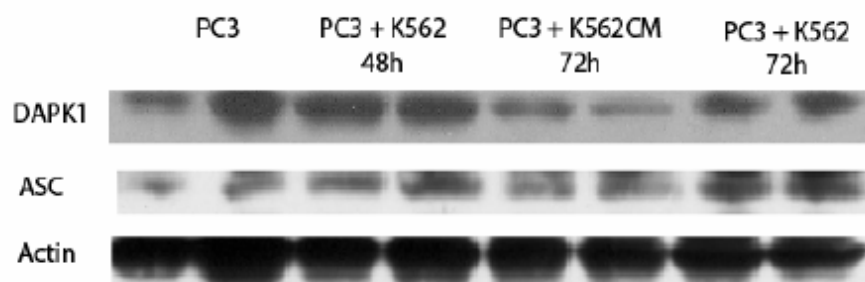
As mentioned in the previous annual report, we addressed this aim alternatively by exploring the apoptosis and cell cycle pathways after performing an experiment which found that TSP-1 mRNA levels were not highly expressed in K562 cells. And K562 cells demonstrated very potent inhibition on prostate carcinoma cell growth, it is unlikely that TSP-1 mediated the inhibitory effects as we speculated at first. Based on what we found in the apoptosis pathway analysis that ASC and DAPK1 were regulated during co-culture. We validated ASC and DAPK1 expressions at mRNA level as demonstrated in the report we submitted in 2009. This year, we performed experiments to check ASC, DAPK1 and caspase-3 expressions in prostate tumors at protein level by western and immunohistochemistry. The findings are:

- 1) The remarkable induction of both ASC and DAPK1 levels were confirmed with Western blots in vitro (Figure 2 A-C).
- 2) The detection of ASC and DAPK1 in prostate tumor lesions in skeletal sites was not successful. We are working on this issue by trying different fixation of the tumors and harvesting fresh tumors from both subcutaneous implants and bone lesions.

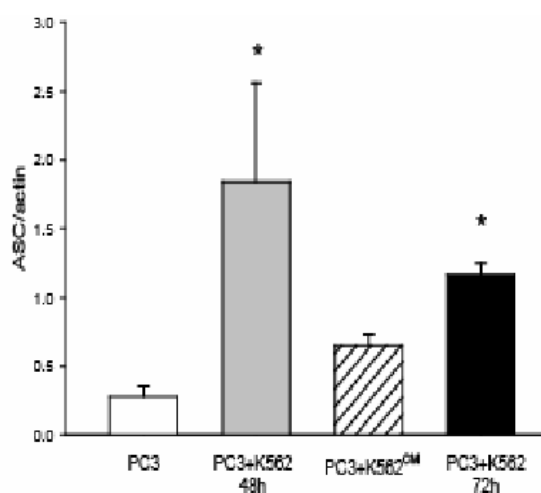
- 3) The expression of caspase-3 was not significantly increased in PC-3 cells after co-culture with K562 cells. This indicated that the apoptosis induced by K562 cells was partially mediated through caspase-3 (Figure 2D).

Figure 2.

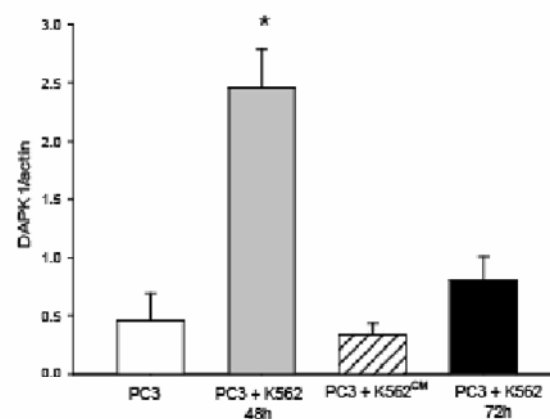
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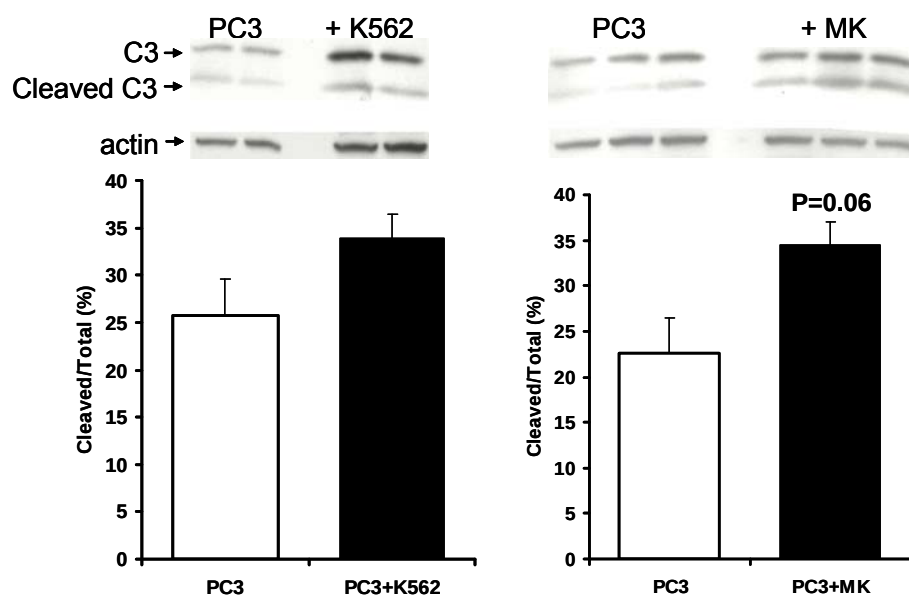
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C



D



Detailed methodology used in this study can be found in the attached manuscript (Materials and Methods), page 11-15.

Reportable outcomes

1. Inhibitory effects of megakaryocytic cells in prostate cancer skeletal metastasis. Xin Li, Amy J Koh, Zhengyan Wang, Fabiana N Soki, Serk I Park, Kenneth J Pienta, Laurie K McCauley (J Bone Miner Res. Aug 3. 2010. [Epub ahead of print])
2. Inhibitory effects of megakaryocytes in prostate cancer skeletal metastasis. (presentation at PO1 combined meeting, Feb. 2010, Seattle, WA)
3. Inhibitory effects of megakaryocytes in prostate cancer bone metastasis. (Oral presentation and Young Investigator Award, IX International Meeting on Cancer Induced Bone Disease, Nov. 2009, Arlington, VA)
4. Inhibitory effects of megakaryocytes in prostate cancer bone metastasis. (Oral presentation and ASBMR-Harold M. Frost Young Investigator Award, Aug. 2009, Sun Valley, ID)
5. Career development: The PI was offered a tenure-track assistant professor position in New York University. This study was included in her job interview presentation.

Conclusion

This project provided important and novel results which reveal a new role of bone marrow cells in the defence against prostate cancer metastases in the bone marrow and contribute to an unexplored osteoimmunologic avenue in skeletal metastasis. The present results demonstrated an inhibitory effect of megakaryocytes on prostate carcinoma cell growth *in vitro* and *in vivo*. Further investigation on the ASC and DAPK1 actions in prostate carcinoma cell growth can be addressed by establishing knock-down and/or over-expression cell lines. A clearer understanding of the microenvironment of skeletal lesions of prostate cancer has been accomplished with this study. In summary, valuable data were generated that can be used as a foundation for further research submissions. A high quality publication resulted from the work, along with several presentations including two young investigator awards from prestigious societies. This fellowship provided resources for the accomplishment of valuable new scientific information and also provided critical career development support for an aspiring young scientist.

References

Please refer to the attached manuscript, page 23-25. (JBMR Aug.3 2010 Epub ahead of print).

Appendices

1. Manuscript:

Inhibitory Effects of Megakaryocytic cells in Prostate Cancer Skeletal Metastasis

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ABSTRACT:

Prostate cancer cells commonly spread through the circulation, but few successfully generate metastatic foci in bone. Osteoclastic cellular activity has been proposed as an initiating event for skeletal metastasis. Megakaryocytes (MKs) inhibit osteoclastogenesis, which could impact tumor establishment in bone and given the location of mature megakaryocytes at vascular sinusoids, they may be the first cells to physically encounter cancer cells as they enter the bone marrow. Identification of the interaction between megakaryocytes and prostate cancer cells was the focus of this study. K562 (human megakaryocyte precursors), and primary megakaryocytes derived from mouse bone marrow hematopoietic precursor cells, potently suppressed prostate carcinoma PC-3 cells in co-culture. The inhibitory effects were specific to prostate carcinoma cells and were enhanced by direct cell-cell contact. Flow cytometry for propidium iodide (PI) and annexin V supported a proapoptotic role for K562 cells in limiting PC-3 cells. Gene expression analysis revealed reduced mRNA levels for cyclin D1 while mRNA levels of apoptosis-associated speck-like protein containing a CARD (ASC) and death-associated protein kinase 1 (DAPK1) were increased in PC-3 cells after co-culture with K562 cells. Recombinant thrombopoietin (TPO) used to expand megakaryocytes in the bone marrow resulted in decreased prostate tumor skeletal lesion development after intra-cardiac tumor inoculation. These novel findings suggest a potent inhibitory role of megakaryocytes in prostate carcinoma cell growth *in vitro* and *in vivo*. This new finding, of an interaction of metastatic tumors and hematopoietic cells during tumor colonization in bone, will ultimately lead to improved therapeutic interventions for prostate cancer patients.

Key words: megakaryocytes, prostate carcinoma, skeletal metastasis

INTRODUCTION

Prostate cancer is the most common malignancy in Western men, it alone accounts for about 25% of cancer cases in men (1). Bone metastasis is the major cause of morbidity associated with

prostate cancer and affects up to 90% of patients dying with advanced disease (2). The classic concept that circulating tumor cells need “congenial soil” to “seed”, provoked extensive attention focused on explaining the dynamic migratory abilities of tumor cells (3). Meanwhile, understanding the characteristics and early changes occurring in the bone marrow microenvironment that welcome incoming cancer cells is still lacking. Within the skeleton, prostate cancer often spreads to the axial skeleton and long bone metaphyses, sites under active remodeling that contain increased marrow cellularity (4). Bone, as a dynamic and complicated system, is filled with rich bone marrow and blood vessels. There are two kinds of stem cells that reside in the bone marrow: hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs). HSCs and their progeny are surrounded by stromal cells in the marrow. MSCs reside in the bone and give rise to the majority of marrow stromal cell lineages, including chondrocytes, osteoblasts, fibroblasts, adipocytes, endothelial cells and myocytes (5,6). Developing hematopoietic cells in the bone marrow are retained until they mature and are released into the circulation. That hematopoietic cells have been observed in close juxtaposition to osteoblasts (OBs), and that cytokines known to be important for hematopoiesis are expressed on the cell surface or secreted by osteoblasts, has lead scientists to explore the interaction between hematopoietic cells and cells of the marrow stromal cell lineage. Increased observations show that the osteoblastic niche is important in supporting and maintaining HSCs (7). Megakaryocytopoiesis occurs in the hematopoietic (extravascular) compartment of the marrow, megakaryocytes then migrate and locate parasinusoidally, the cytoplasm invaginates, penetrates the endothelial cell lining and platelets are released into the blood circulation (8). This process is regulated by the action of numerous factors including cytokines, growth factors, chemokines, and extracellular matrix molecules (ECMs), many of which are produced by stromal cells within the marrow microenvironment. The development and proliferation of osteoblasts directly

influence hematopoiesis (7,9-11) and megakaryopoiesis (12,13), further illustrating the significance of these interactions.

Recently, megakaryocytes have been demonstrated to have inhibitory effects on osteoclast formation and activity (14-17). These studies demonstrated that a factor or factors secreted by megakaryocytes inhibit osteoclast development. The emerging paradigm is that megakaryocytes play a dual role in regulating skeletal homeostasis by simultaneously stimulating OB proliferation and differentiation, and inhibiting OC development (14-17). As a result, alterations in megakaryocyte proliferation or differentiation could also affect the skeleton changes. Considering that bone resorption is favorable to tumor growth in bone (18-20) and there is a high tendency of prostate cancer to localize in bone, megakaryocytes may indirectly inhibit tumor growth via decreased osteoclastic resorption and reduced cytokine and growth factor release from the bone matrix (15-17). Another possibility is that megakaryocytes directly impact prostate cancer cells in their trajectory through the bone marrow microenvironment. However, investigations on the interaction between megakaryocytes and solid tumors are minimal. This study is based on the hypothesis that megakaryocytes inhibit prostate cancer cell growth in bone through direct inhibition of cancer cells and indirectly by their influence in the bone microenvironment. Meanwhile, in association with their location, mature megakaryocytes may be able to act like “gate-keepers” to block the entry of cancer cells into the marrow.

MATERIALS AND METHODS

Cell culture

Human hematopoietic precursor cells K562 (patient-derived leukemia cell line expressing both erythroid and megakaryocyte markers and can be induced to differentiate along each of these pathways) and Meg01 (patient derived megakaryoblastic cells) were used as precursors for megakaryocytes. HL60 cells (patient-derived promyelocytic leukemia cells which can be differentiated to neutrophilic promyelocyte) were cultured in suspension under appropriate growth conditions. K562 cells were maintained in IMDM + 10% fetal bovine serum (FBS)

(Invitrogen Corp., Carlsbad, CA) and induced with 50 nM phorbol myristate acetate (PMA) (Sigma, St. Louis, MO) to differentiate into mature megakaryocytes. To differentiate K562 cells to erythroid cells, 100 nM doxorubicin hydrochloride (DOX) (Sigma) was added to the culture media of K562 cells for 3 days. Meg01 cells and HL60 cells were maintained in RPMI 1640 + FBS. Both cells were passaged at a density of about 2×10^5 /ml when seeding.

Primary megakaryocytes were isolated and cultured from mouse bone marrow cells. Briefly, after flushing and centrifugation with Ficoll-Paque (GE Healthcare, Piscataway, NJ), bone marrow cells were harvested, counted and seeded at 10^6 /ml with addition of nucleotide mixtures (Invitrogen), TPO, IL6 and IL11 (PeproTech, Rocky Hill, NJ). Five or six days later, megakaryocytes were enriched by gravity sedimentation twice before used for co-culture assay. The upper layer cells were collected as less mature megakaryocytes and the bottom layer cells were collected as mature megakaryocytes. The primary megakaryocyte culture method was the same as described previously where it was demonstrated that after 6 days of culture, the purity of megakaryocytes (both medium and large in size population) is 98% (21).

PC-3 prostate carcinoma cells stably expressing luciferase (PC-3^{Luc+}, referred to as PC-3) (22) and C4-2b cells were maintained in RPMI 1640 + FBS. The VCaP prostate carcinoma cell line was maintained in DMEM + 10% FBS. Cells were passaged using trypsin with EDTA (Invitrogen) and resuspended in appropriate growth media.

Human osteoblastic SaOS2 cells were maintained and passaged every 4-5 days in α MEM (Invitrogen) containing 100 units/ml penicillin and streptomycin and 10% FBS. SaOS2 cells were plated at 50,000 cells/cm² and cultured to 80~90% confluence.

Viable Cell enumeration

Five thousand (5×10^3) cells (adherent prostate carcinoma cells or osteoblastic cells) were plated per well in 24-well plates in quadruplicate in the presence of serum at indicated concentrations. K562 cells, HL60 cells or megakaryocytes induced from K562 cells were seeded in the

suspension media above the adherent cells. On the indicated days, suspension cells were removed before PC-3 cells were washed and trypsinized. The luciferase activity of cell lysates as a reflection of cell number was determined with the dual luciferase reporter assay system (Promega, Madison, WI) and a Monolight 2010 luminometer (BD-Pharmingen, San Diego, California). The non luciferase expressing adherent cells were washed with PBS and fixed with 40% methanol followed by staining with crystal violet. DNA binding dye was solubilized with 10% acetic acid. Relative cell numbers were measured by optical density at 595 nm.

Protein isolation and analysis

Protein levels of ASC and DAPK1 were determined by Western blot using total cell lysates. Briefly, cells were washed with cold PBS and collected with Invitrogen SDS buffer. Samples were stored at -20°C until assays were performed. Western blots were performed using actin as a loading control in each sample (all antibodies from Cell Signaling Technology, Danvers, MA). Western films were scanned, and relative protein expression was quantified using ImageJ analysis program.

In vivo prostate cancer models

Four-week-old male athymic mice were obtained from Harlan (Indianapolis, IN). Mice (20/group) were pre-treated with TPO or vehicle for 5 days before inoculation of tumor cells. Five mice from each group were sacrificed for histology and flow cytometry analysis using FITC labeled CD41 antibody (BD-Pharmingen) to determine CD41 positive cell numbers in the bone marrow to confirm the effect of TPO. The remaining mice were sedated with 1.7% isoflurane mixed with air and 100,000 PC-3 cells were inoculated via intra-cardiac injection into the left ventricle as previously described (23). Mice were sacrificed after 4 weeks and the skeleton exposed to X-ray film (Wolverine X-Ray, Dearborn, MI) at $3\times$, 32 kV, 45 sec in a microradiography X-ray machine (Faxitron, Madison, WI) to identify gross skeletal changes. Tibiae were fixed in 10% formalin, decalcified, and processed for histological analysis. All

animal studies were approved by the University of Michigan Committee on the Use and Care of Animals (UCUCA).

In vivo localization and growth of PC-3 prostate cancer cells

In vivo bioluminescent imaging was carried out at the University of Michigan Small Animal Imaging Resource facility. Before imaging, mice were injected i.p. with 100 μ L of 40 mg/mL luciferin dissolved in PBS. Imaging was performed under 1.75% isoflurane/air anesthesia on a cryogenically cooled IVIS system equipped with a 50-mm lens and coupled to a data acquisition PC running Living Image software 2.6 (Xenogen Corp., Alameda, CA). Ventral images were acquired 12 minutes after injection. Pseudocolor images of photon emissions were overlaid on grayscale images of mice to aid in determining signal spatial distribution. Photon quantifications were calculated within regions of interest (ROI).

Histology and immunohistochemistry

Xenograft tumors were harvested and placed in fresh 10% formalin. Tibiae were decalcified in 10% EDTA for 21 days prior to paraffin embedding. Paraffin embedded specimens were sectioned (5 μ m) and stained with either hematoxylin and eosin (H&E), Trichrome (bone), tartate resistant acid phosphatase (TRAP) (osteoclasts) (Acid Phosphatase, Leukocyte Kit, Sigma), or immunohistochemistry for von Willebrand factor (vWF). Standard indirect immunoperoxidase procedures were used for immunohistochemistry using the AEC Cell and Tissue staining system (R&D systems, Minneapolis, MN). Mayer's hematoxylin (Sigma-Aldrich, Sigma) was used for counterstaining.

Flow cytometric analysis for cell cycle and apoptosis

Cells were washed twice with cold PBS and lifted with trypsin for 3 min at 37°C before being stained with propidium iodide (PI) or the Annexin V-FITC Apoptosis detection system (BD Pharmingen™). Cells were also treated with ribonuclease A before staining with PI for cell cycle

analysis. FITC-conjugated annexin V and PI staining were evaluated with a FACs Calibur (BD Bioscience).

RNA isolation and mRNA quantification

RNA was isolated using Tri reagent (Sigma) following manufacturer's protocols. One μ g total RNA was reverse transcribed in a 20- μ L reaction volume containing random hexamers using a reverse transcription assay system (Applied Biosystems, Foster City, CA). Semiquantitative RT-PCR was performed on a GeneAmp 7700 thermocycler (Applied Biosystems) and fold changes were determined via the delta-delta CT method with normalization to GAPDH mRNA levels.

Statistical Analysis

One-way ANOVA or Student's t test for independent analysis was applied to evaluate differences and Fisher's exact test was applied to compare the incidence rate of lesion development using the GraphPad InStat software program (GraphPad Software, Inc., San Diego, CA). The value $p < 0.05$ was considered statistically significant. All assays were repeated a minimum of 2 times with similar results.

RESULTS

K562/megakaryocyte cells inhibited prostate carcinoma cell growth in vitro

The K562 cell line is an established precursor cell line of megakaryocytes and erythroid cells upon differential stimulation. When induced by PMA, K562 cells differentiate into megakaryocytes accompanied by a net increase in megakaryocytic markers and a reduction of erythroid markers (24). K562 cells, with or without PMA pretreatment, were co-cultured with the prostate carcinoma cell line, PC-3 labeled with a luciferase tag. Strong inhibition of PC-3 growth was found in both conditions (Fig. 1A). This inhibitory effect of K562 on prostate carcinoma growth was further confirmed using two other prostate carcinoma cell lines, VCaP and C4-2B cells (Figs. 1B and C). In addition, primary megakaryocytes derived from mouse bone marrow cells also demonstrated significant inhibition of PC-3 cell growth in co-culture (Fig. 1D). The primary megakaryocytes used in co-culture were fairly pure (>90%) after gravity sedimentation

as measured by flow cytometry using CD41 as the marker for megakaryocytes (Supplementary Fig. 1).

K562/megakaryocytes inhibition of cell growth is specific to prostate carcinoma and enhanced by close proximity

To examine the specificity of megakaryocyte inhibition of prostate carcinoma cell growth, co-cultures with non-epithelial SaOS2 cells, a human osteosarcoma cell line and HL60, a human promyelocytic leukemia cell line were performed. Unlike the inhibitory effects seen with prostate carcinoma cells, a significant stimulation of SaOS2 cell growth was observed (Fig. 2A). Furthermore, HL60 failed to regulate the growth of prostate carcinoma (Fig. 2B). To evaluate the dependence of direct cell-cell contact on the inhibitory effects of pre- and megakaryocytes, a transwell culture system was utilized, in which PC-3 cells and K562 or megakaryocytes were cultured separately in the upper and lower wells, respectively. The inhibitory effect of megakaryocytes on PC-3 cells was still present but significantly blunted when K562 or megakaryocytes differentiated from K562 (MK-K562) were cultured with PC-3 cells in the transwell system without direct contact (Fig. 2C). Without very close proximity or cell-cell contact, K562 cells still blocked the growth of PC-3 by 35% (trans-well plate) while mature megakaryocytes only inhibited PC-3 cell growth by 15% in trans-well plates versus 55% in regular-well co-culture. This suggests that a very close proximity or direct cell-cell contact is important for the inhibitory effects of mature megakaryocytes. Thus, either a very steep inhibitory cytokine/growth factor gradient that requires very close proximity or a membrane-bound factor which requires direct cell-cell contact to exert the inhibitory effects exists.

Primary megakaryocytes inhibited PC-3 cell growth regardless of maturation phases

Since the inhibitory effect of K562 cells were more potent than mature megakaryocytes derived from K562 cells, primary megakaryocytes cultured from mouse bone marrow were used to determine whether maturation of megakaryocytes affect their inhibitory ability. Primary

megakaryocytes at different mature phases were cultured with PC-3 cells for 3-days, both population suppressed PC-3 cell growth without significant difference (Fig. 3A). Erythroid cells derived from K562 cells were also tested for the effects on prostate carcinoma cell growth in co-culture system (Fig. 3B). Unlike megakaryocytes derived from K562 cells, erythroid cells differentiated from K562 cells lost the inhibitory ability on PC-3 cell growth.

K562/megakaryocytes induced apoptosis in PC-3 cell

To understand the mechanism of the inhibitory effects of megakaryocytes on prostate carcinoma cell growth, the megakaryocytic and PC-3 co-culture system was evaluated with cell cycle and apoptosis assays. Flow cytometry for cell cycle analyses demonstrated that K562 and MK-K562 cells altered the ratios of PC-3 cells in cell cycle phases (Supplementary Fig. 2A). The percentage of cells in G0/G1 phase increased while cells in S+G2/M phases decreased by co-culture with K562 and MK-K562. It was further demonstrated by QPCR that there was a 50% decrease in PC-3 cyclin D1 mRNA levels after 48h co-culture with K562 cells (Supplementary Fig. 2B); however, these changes were not enough to decrease PC-3 cell growth to the extent we observed. K562 cells and another megakaryocyte precursor cell, Meg01 cells, also significantly induced apoptosis of PC-3 cells. A dramatic increase in annexin V⁺ cell (early apoptotic cells) number was observed in PC-3 cells after 24 and 36h co-culture with K562 cells and significantly at 48h with Meg01 cells (Fig. 4A) followed by a significant increase in both annexin V and PI positive cell populations (late apoptotic cells) in PC-3 cells after 48h co-culture with K562 cells and Meg01 cells (Fig. 4B). An apoptosis pathway analysis (Supplementary table 1) revealed that apoptosis-associated speck-like protein containing a CARD (ASC) and death-associated protein kinase (DAPK1) were two highly stimulated genes in PC-3 cells after co-culture with K562 cells. The significant induction of both ASC and DAPK1 mRNA levels were confirmed with QPCR (Fig. 4D and E). The protein levels of both ASC and DAPK1 were increased in PC-3 cells after

48h incubation with K562 cells. Up until 72h, ASC protein levels were still significantly higher than control (Fig. 4F-H).

Increased megakaryocytes in vivo were associated with reduced prostate tumor skeletal metastatic lesions

Thrombopoietin (TPO), the major megakaryocyte growth factor is required for megakaryocyte proliferation and maturation and was used to expand megakaryocytes to determine their impact on tumor metastasis *in vivo* (Fig. 5A). An increase in megakaryocytes after TPO priming was confirmed by histologic staining for the megakaryocyte marker Von Willebrand factor (vWF) and flow cytometric analysis of CD41⁺ cells in the bone marrow of a subset of athymic mice from each group 1 day after the last injection of TPO or Vehicle (veh) and before cardiac inoculations. Daily treatment of mice with TPO efficiently expanded megakaryocytes in the bone marrow as indicated by increased vWF positive cell number and percent of CD41 positive cells (Figs. 5B-D). The incidence of lesion development in the legs after intra-cardiac inoculation of PC-3 cells was significantly reduced in mice pretreated with TPO (Fig. 5E). Meanwhile, the BLI activity of bone lesions in the legs of TPO treated mice was less compared to the vehicle group at 2-weeks after tumor cell inoculation (Fig. 5F). Consistent with these live image results, histomorphometric analysis indicated that the tumor area in the tibia of mice pretreated with TPO at sacrifice was significantly less than the controls (Figs. 5G-I).

Based on these results, a schematic model to summarize our findings was proposed (Fig. 6). Megakaryocytes inhibit prostate cancer cell growth in bone through direct inhibition of cancer cell proliferation and induction of apoptosis, and indirectly by decreasing bone remodeling (16,17).

DISCUSSION

In this study, K562 cells and megakaryocytes derived from K562 cells or mouse bone marrow cells specifically suppressed prostate carcinoma cell growth while erythroid cells derived from

K562 cells and another leukemia cell line HL60 cells which differentiate along the macrophage or neutrophil lineage did not. These results revealed a novel and specific inhibitory effect of megakaryocytes, originated from bone marrow hematopoietic progenitor cells, on prostate carcinoma cell growth *in vitro* and *in vivo*. To our knowledge, this is the first report of a specific megakaryocyte mediated inhibition of prostate cancer cell growth and may contribute to the understanding of the interaction among cancer cells and native cells in the bone microenvironment. In addition, the location of mature megakaryocytes at the parasinusoids may make them the first defensive cells in the bone marrow facing tumor cells in their extravascular process. The confirmation of the inhibitory effects on PC-3 cell growth with primary megakaryocytes derived from mouse bone marrow strengthened this observation.

Bone turnover contributes to tumor localization, and bisphosphonates which suppress bone turnover via inhibition of osteoclastic bone resorption have been effectively used for the management of cancer-induced bone metastasis (4,25-27). Since megakaryocytes potently inhibit osteoclastogenesis *in vitro* (16), expansion of megakaryocytes with TPO may result in decreased osteoclast activity *in vivo*, which also inhibits prostate carcinoma cell growth in the bone marrow. Hence, the decrease in prostate tumor bone lesion development in mice pretreated with TPO could be the combined effect of altered osteoclasts (indirect) and megakaryocytic cells (direct). However, in the present study the direct effects may be the major inhibitory effects since the decrease in osteoclast activity was mild but not significant after 5 days of TPO treatment (Supplementary Fig. 3).

The inhibitory effects of megakaryocytes on prostate carcinoma were not sustained over time *in vivo* as the luciferase activity of the tumor cells in the hind limbs of TPO treated group caught up with the vehicle group at 3 weeks after tumor inoculation. These data may imply that the inhibitory effects from megakaryocytes and the precursors are early events taking place during tumor cell seeding in the marrow. During this period, megakaryocytes have advantages over the

tumor cells in regards to cell numbers and/or density. Once the tumor cells exit the blood vessels and enter into the marrow, they may be under limited influence of megakaryocytes considering a more restricted megakaryocyte spreading in the extravascular space versus megakaryocyte clustering adjacent to the vessels. It has been reported that Granulocyte colony-stimulating factor (G-CSF) enhanced bone tumor growth in mice (28). Since both G-CSF and TPO may increase the bone marrow cellularity, but they demonstrated different effects on tumor growth in bone, suggesting decrease in bone tumor burden in TPO treated mice was less likely due to the changes in bone marrow cellularity.

Megakaryocytes produce platelets, which have been shown to support the metastatic potential of tumor cells, and inhibiting platelet aggregation decreased tumor metastasis (29,30). It is possible that following the expansion of megakaryocytes in the bone marrow, the number of platelets increased which favored tumor growth later. Interestingly, it has been shown that the size of platelets decreased in patients with solid tumors and skeletal metastasis (31). Though the mechanism underlying this observation is obscure, it is known that platelet size is determined at the megakaryocyte stage, and is inversely proportional with megakaryocyte ploidy (32). Megakaryocyte ploidy is regulated by cytokines such as interleukin-3 and 6, which contribute to more reactive and larger platelets (32,33). Identifying the interaction between tumor cells and megakaryocytes could result in a better understanding of the decrease in platelet size in patients with skeletal metastasis.

In mice, PC-3 cells have a tendency to metastasize to jaw bones when they are injected into circulation. Interestingly, in contrast to the long bones, the growth of tumor development in the jaws was similar between TPO and vehicle groups with intracardiac injection of PC-3 cells (supplementary Fig. 4). Comparing the jaw to the long bones, there is a smaller marrow cavity, thus the structure of the jaw sets a limitation of the marrow cavity as well as reduced numbers of megakaryocytes. The continuous tooth eruption of rodents and high bone turnover rates may also

contribute to tumor development in the jaw areas. Hence, the inhibitory effects of megakaryocytes in this location may be overruled by other factors in this model system.

As demonstrated in the present study, precursor cells of megakaryocytes stimulated the osteoblastic cell line, SaOS2. This is consistent with recent reports that megakaryocytes stimulate osteoblast proliferation (14,17). In addition, megakaryocytes express several receptors that are known to be involved in the regulation of bone remodeling (34-36). It was demonstrated that the tumor areas in the metaphyseal areas of the tibiae in the TPO group were significantly less than that of vehicle group at 4 weeks after tumor inoculation. Considering the positive effects of megakaryocytes on osteoblasts and bone formation, the bone areas in the TPO treated group could be protected and hence result in less tumor occupation in the metaphyseal areas.

Our data indicated that K562 cells suppressed the growth of prostate carcinoma cells mainly through enhancing apoptosis. Further experiments revealed that two apoptotic genes, ASC and DAPK1 were highly regulated genes associated with the inhibitory effects during co-culture. ASC, as indicated by its name, apoptosis-associated speck-like protein containing a CARD, contains a caspase recruitment domain (CARD), suggesting a role in caspase-mediated cell death. It has been shown that in breast cancers, ASC induced apoptosis proceeds through a CARD-dependent aggregation step followed by activation of a caspase-9-mediated pathway (37). Interestingly, both ASC and DAPK1 gene promoters are reported to be methylated in prostate carcinoma including the PC-3 cell line (38-41). Methylation in the promoter often involves loss of function of a gene and thus plays an essential role in maintaining normal cell function. Aberrant DNA methylation patterns may be the earliest somatic genome changes in prostate cancer. Changes in methylation contribute to multiple cancer development (42,43). Our study revealed a significant increase in ASC and DAPK1 expression in PC-3 cells after co-culture with K562 cells suggesting K562 may induce PC-3 cell apoptosis through de-methylation in the promoters of target genes.

In summary, the original finding of megakaryocytic cell inhibitory effects on prostate carcinoma cells unmasked a novel osteoimmunology response to the invasion of prostate carcinoma cells during the skeletal metastasis. Moreover, the discovery of the downstream mediators, ASC and DAPK1, provided new potential therapeutic targets for prostate cancer.

ACKNOWLEDGEMENTS

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FIGURE LEGENDS

FIG. 1. Prostate carcinoma cell growth is inhibited by K562/MK cells *in vitro*

Five thousand luciferase tagged prostate carcinoma PC-3 cells (PC-3^{luc+}, adherent, referred to as PC-3) were co-cultured with 20,000 K562 cells (non adherent) or mature megakaryocytes derived from K562 cells or mouse bone marrow cells (non adherent, referred to as MK-K562) for up to 6 days. Megakaryocytic cells were removed with the supernatant, and only adherent cells were analyzed. (A) PC-3 cell lysates were analyzed for luciferase activity using the luciferase assay reagent (Promega). VCaP (B) and C4-2B (C) cells were fixed and stained following a protocol using crystal violet. (D) Ten thousand PC-3 cells were co-cultured with

10,000 mature primary megakaryocytes (MKs) for up to 2 days. PC-3 cell lysates were analyzed for luciferase activity using the luciferase assay reagent. All assays performed in triplicates. * $p < 0.05$, ** $p < 0.005$ versus PC-3 alone.

FIG. 2. Megakaryocyte inhibition of cell growth is specific to prostate carcinoma and enhanced by close proximity.

(A) Growth of PC-3 cells and human osteoblastic cells (SaOS2) was up-regulated by K562 cells after 3-day co-culture. (B) K562 cells but not HL60 inhibited the growth of PC-3 cells with 3-day co-culture. K562 and HL60 cells were removed with supernatant, only adherent cells were left for the analysis. (C) Five thousand PC-3 cells were cultured in the upper chamber with 20,000 K562 cells or megakaryocytes differentiated from K562 in the bottom well for 3 days. After 3 washes with PBS, PC-3 cell lysates were analyzed immediately for luciferase activity, data shown as fold change over luciferase activity of PC-3 cells alone. All assays performed in triplicates. * $p < 0.05$, ** $p < 0.005$ versus no addition of K562 or HL60 cells.

FIG. 3. Megakaryocytes but not erythroid cells inhibited PC-3 cell growth.

(A) Ten thousand PC-3 cells were co-cultured with 10,000 primary megakaryocytes at mature (larger size CD41+) or less mature (immature, smaller size CD41+) for 3 days. (B) Ten thousand PC-3 cells were co-cultured with 10,000 erythroid cells derived from K562 cells by DOX. In both experiments, PC-3 cells were washed twice with PBS and the cell lysates were collected with passive lysis buffer analyzed for luciferase activity. Assays performed in triplicates. * $p < 0.05$, ** $p < 0.001$ versus PC3 cells alone.

FIG. 4. Megakaryocytic cells increased apoptosis and ASC and DAPK1 expression in PC-3 cells.

PC-3 cells were co-cultured with megakaryocyte progenitor cell line K562 or Meg01 cells for 24, 36 and 48 hours before Annexin V and propidium iodide staining. (A) annexin V+ and (B) both annexinV+ and PI+ cell populations in PC-3 cells after co-culture with K562 or Meg01 cells. (C)

Representative FACS images of PC3 cell cultured with or without K562 cells at 24h and 48h. QPCR using FAM labeled primers for ASC (D) and DAPK1 (E) mRNA levels. (F) Representative western blot of ASC and DAPK1 protein detection in PC-3 cells cultured with K562 cells for 48h and 72 h. Quantitative protein levels of ASC (G) and DAPK1 (H). All assays performed in triplicates. N.D.= non detectable; * $p < 0.05$, ** $P < 0.001$

FIG. 5. Expansion of megakaryocytes *in vivo* decreased prostate tumor metastatic lesion development

(A) Schematic model of experimental design: mice (20/group) were treated with recombinant TPO intraperitoneally to increase megakaryocytes. One-day after the last injection, 5 mice from each group were sacrificed and one tibia collected for (B) vWF IHC and (C) representative images of vWF staining of the tibia sections from Veh and TPO treated group before tumor injection. (C) The other hind limb was flushed with PBS for flow cytometric staining of CD41+ megakaryocytic cells. (E-F) The remaining mice were injected via the intracardiac route with 200,000 PC-3 cells and tumor cell location and activity recorded with weekly bioluminescence (BLI). The location and progress (E) of inoculated prostate carcinoma cells in mice were recorded with BLI imaging weekly and the incidence rate of tumor in the legs (F) were enumerated weekly and analyzed by Fisher's Exact test (* $p < 0.05$). (G) Representative images of BLI of tumor development in hind limbs of vehicle (Veh) and TPO treated groups. (H) Histomorphometric analysis of tumor area in tibia was decreased in mice pretreated with TPO. (I) Representative radiographs of hind limb and H&E staining of the tibia sections from Veh and TPO treated groups 4 weeks after tumor injection. T: tumor. * $p < 0.05$ versus Veh.

FIG 6. A schematic model to summarize the findings.

Megakaryocytes inhibit prostate cancer cell growth in bone through direct inhibition of cancer cells and indirectly by decreasing bone remodeling and angiogenesis. The direct inhibition involves a marginal cell cycle arrest via reduction in cyclin D1 and dramatic stimulation of

apoptosis via induction of ASC and DAPK1. Meanwhile, megakaryocytes exert an anabolic effect in bone by stimulating osteoblasts and inhibiting osteoclasts.

2. curriculum vitae

BIOGRAPHICAL SKETCH

NAME		POSITION TITLE	
Xin Li, Ph. D.		Research Fellow	
EDUCATION/TRAINING <i>(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)</i>			
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Institute of Zoology, Chinese Academy of Sciences, Beijing, China	M.S. 9/19	98-6/2001	Insect physiology
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IX International Cancer and Bone Meeting Young Investigator Award, 2009

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Plenary Poster Award, ASBMR 30th Annual Meeting. 2008, Montreal, Canada

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PI: Department of Defense Prostate Cancer Postdoctoral Training Grant, 2008-2010

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Outstanding Graduate Fellowship, Graduate School of Chinese Academy of Sciences, 1999/2000

Outstanding Academic Performance Award, Shandong University, 1994/1995, 1995/1996, 1996/1997

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Support Data

FIG. 1

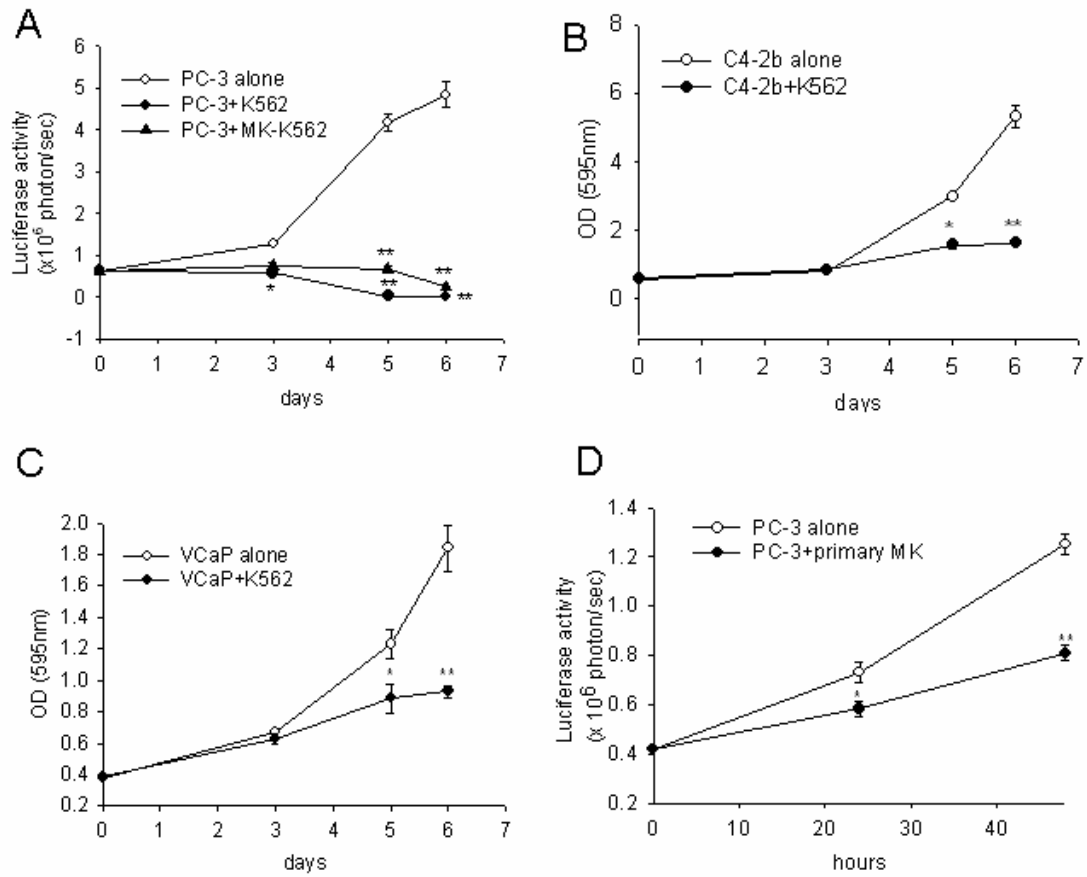


FIG. 2

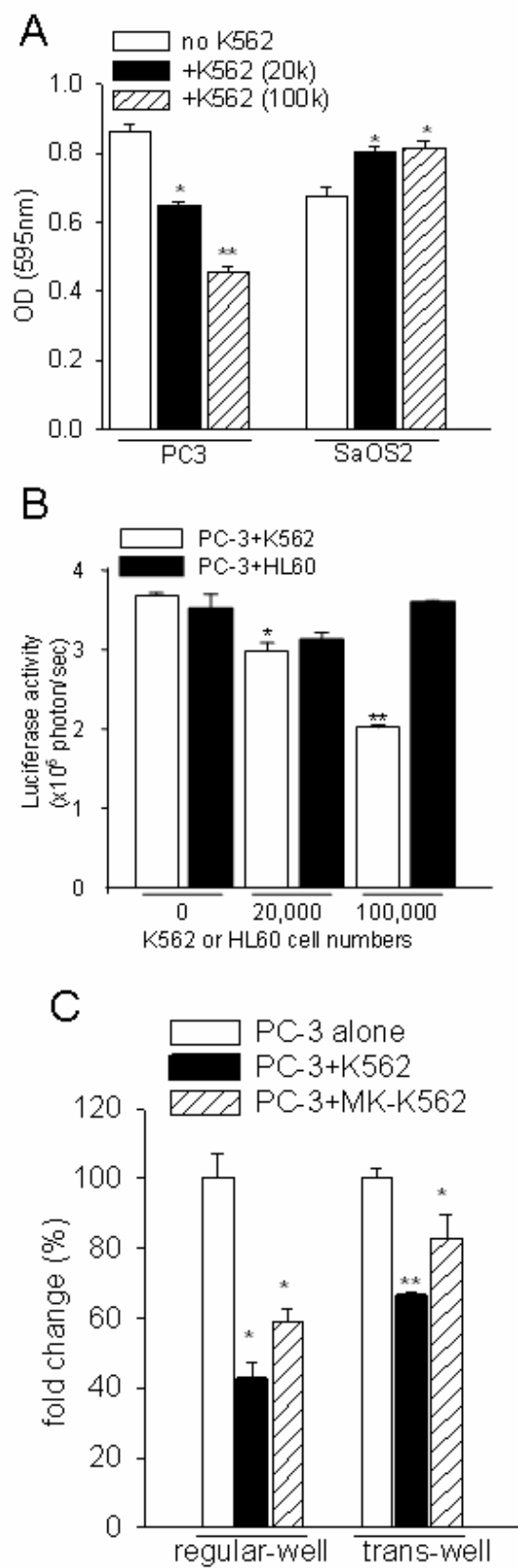


FIG. 3

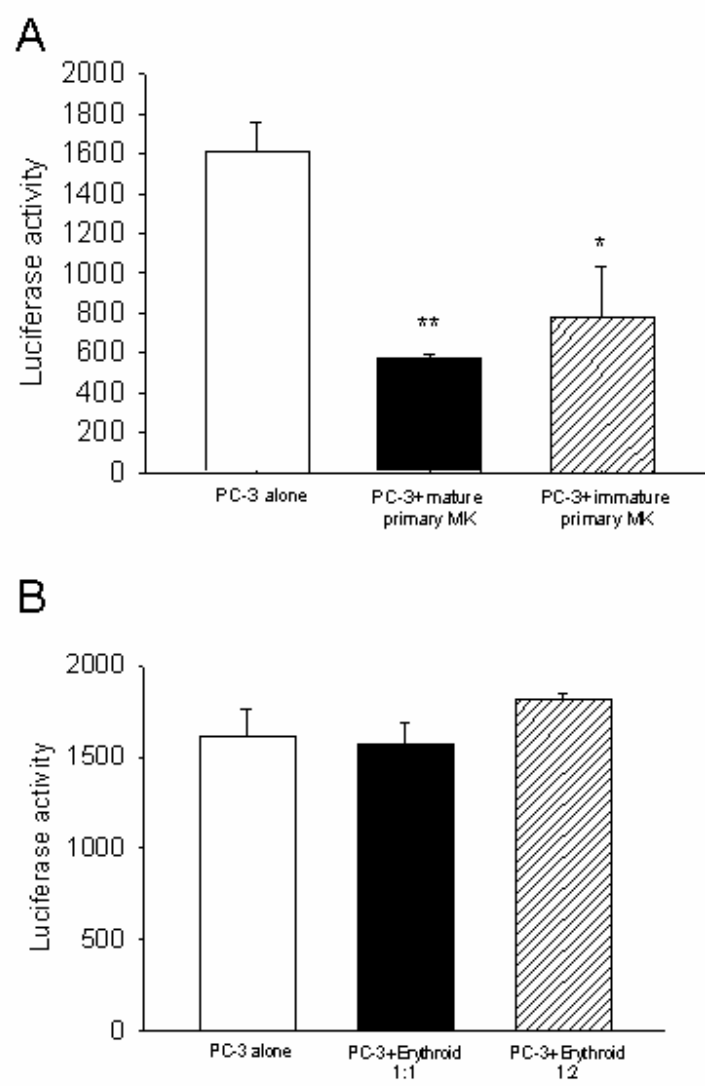


FIG. 4

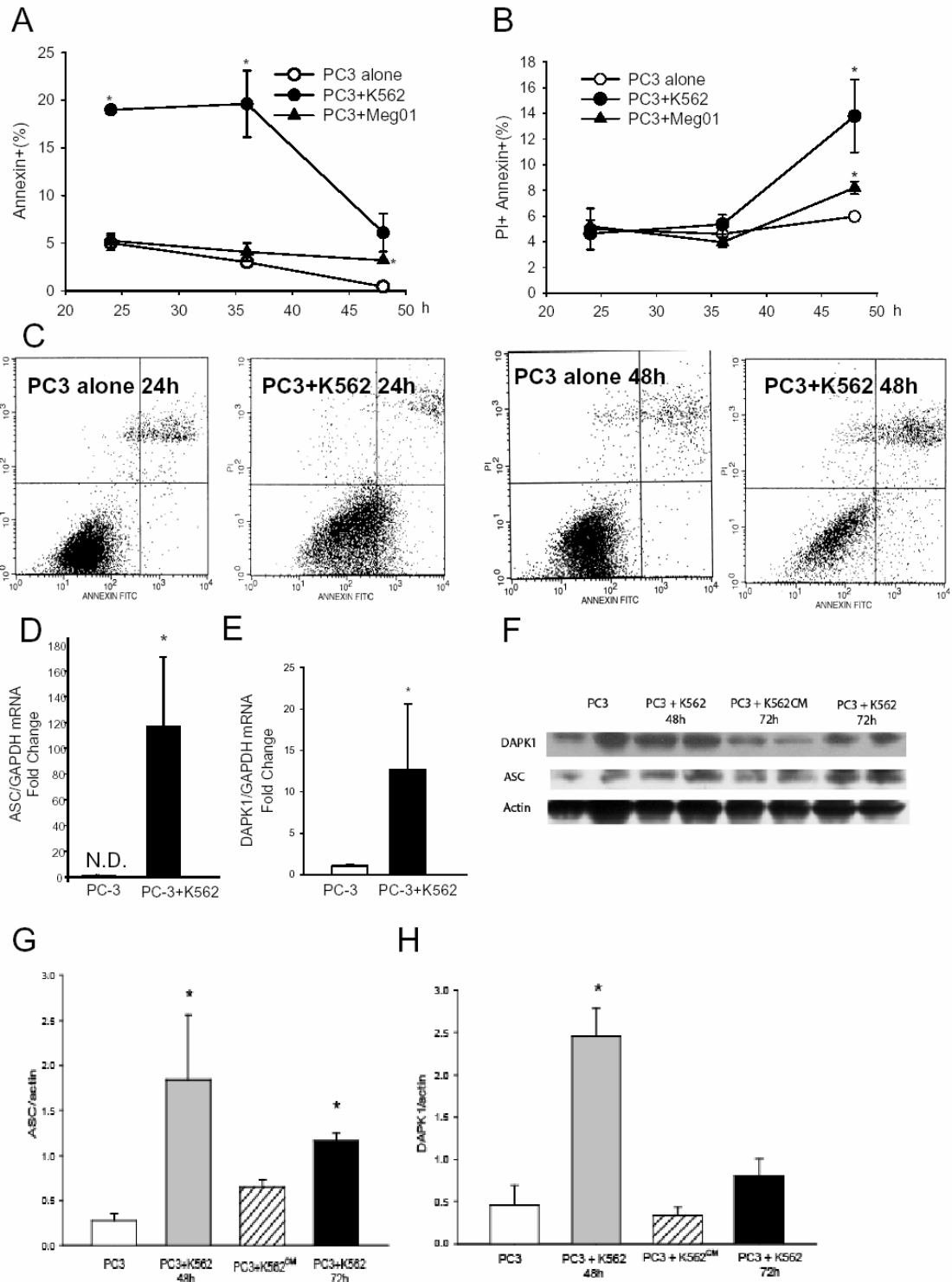
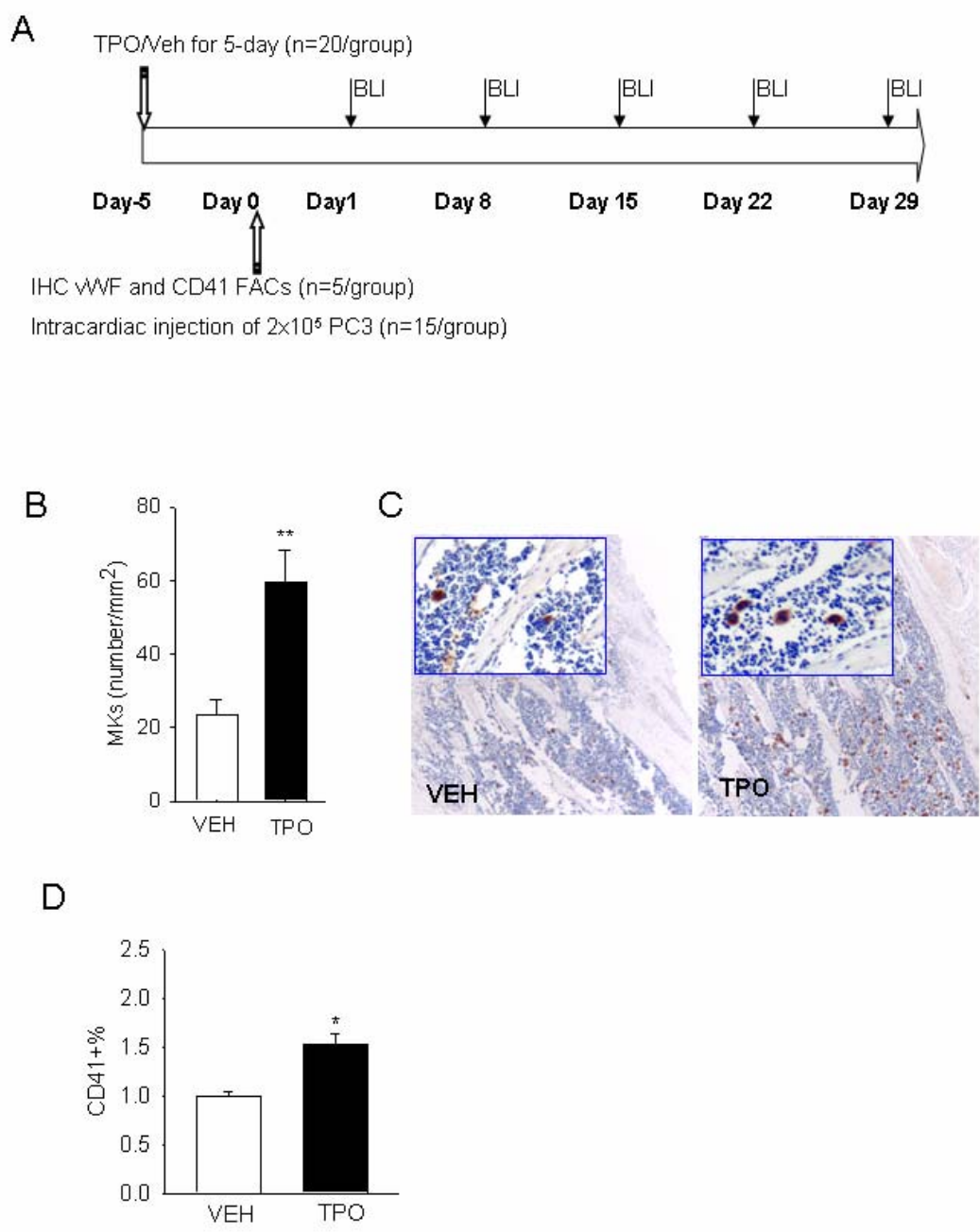
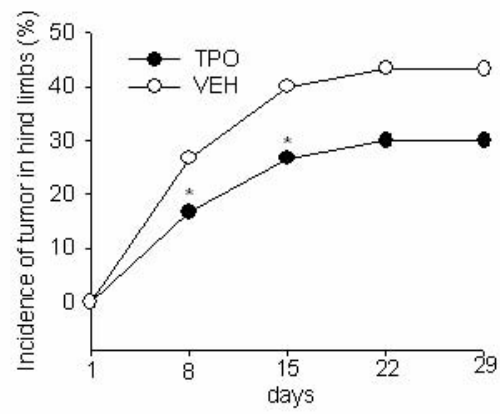


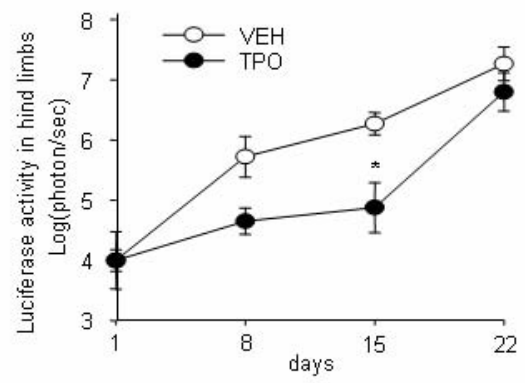
FIG. 5



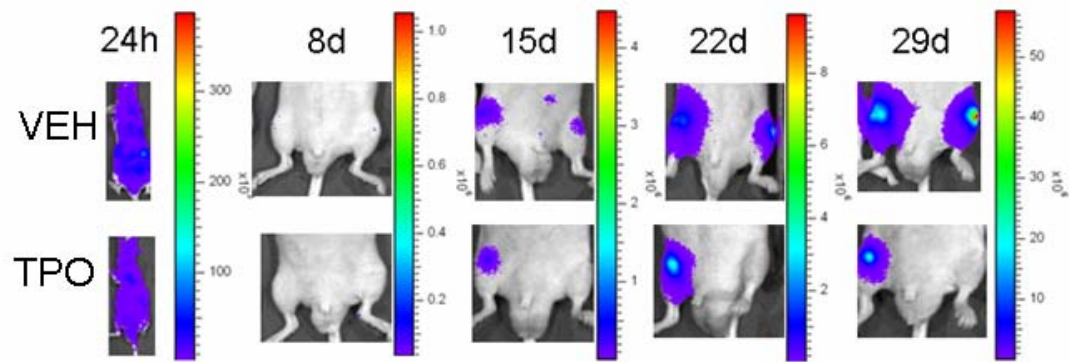
E



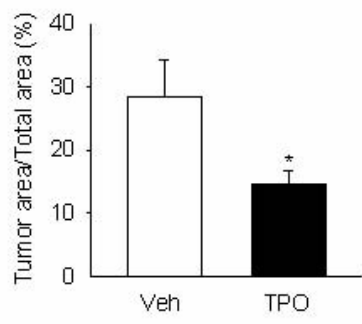
F



G



H



I

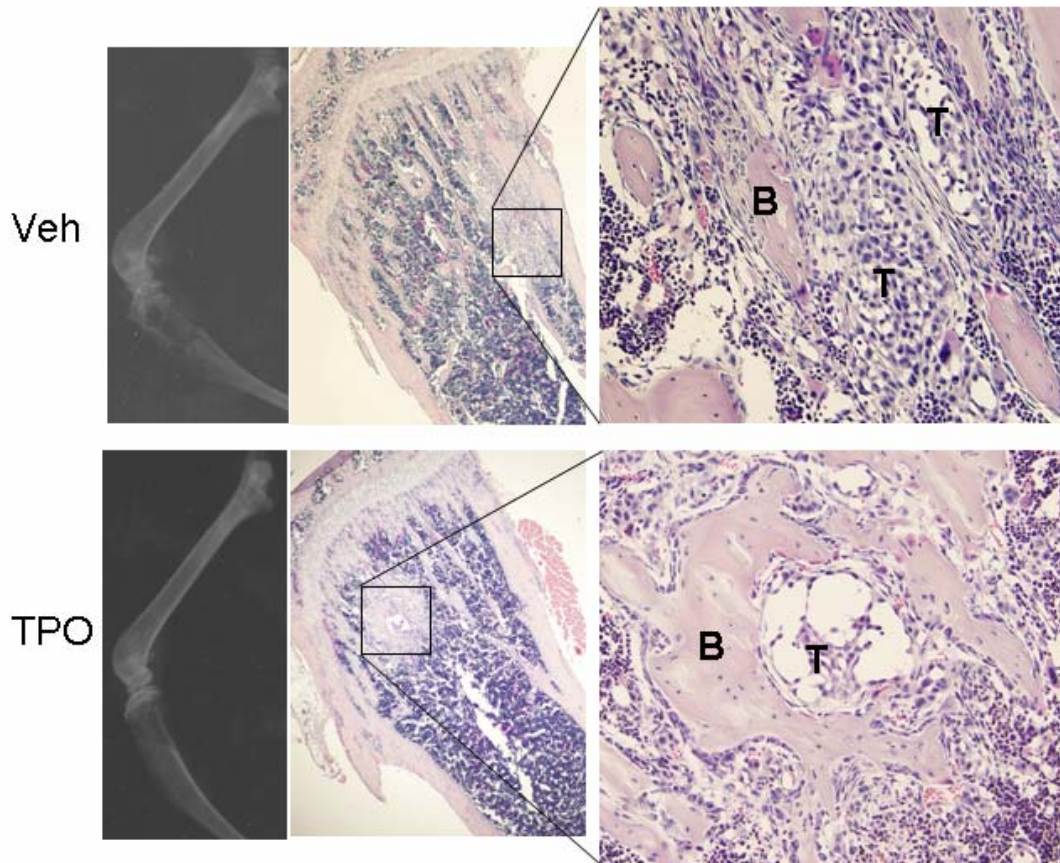
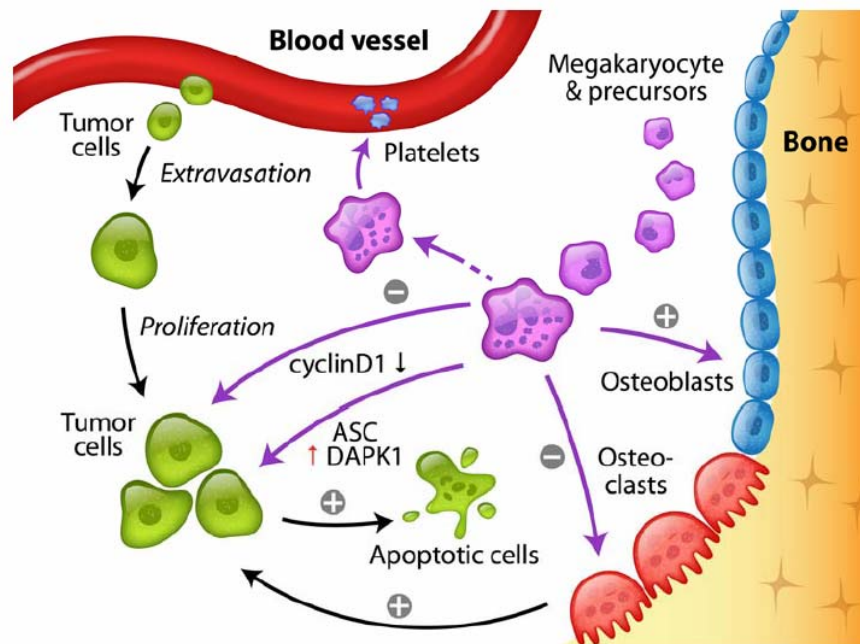
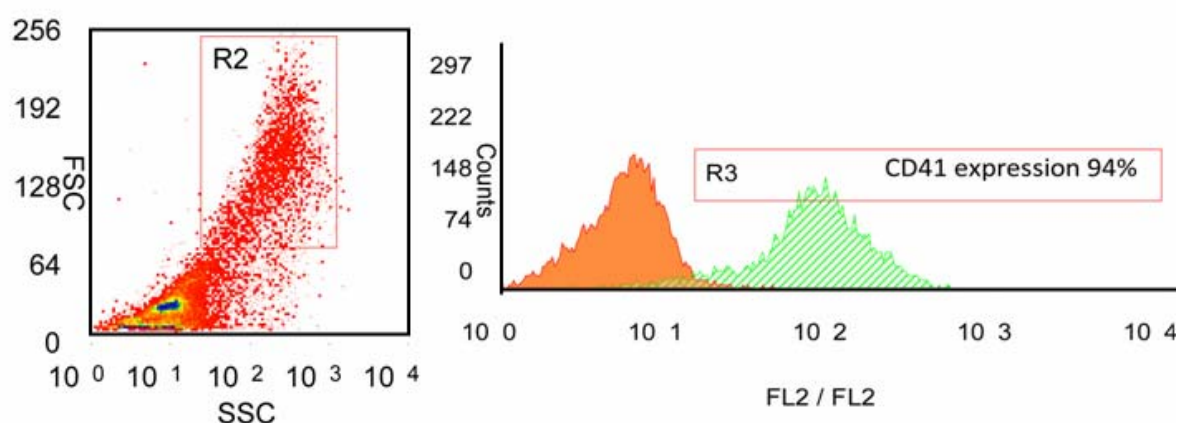


FIG. 6

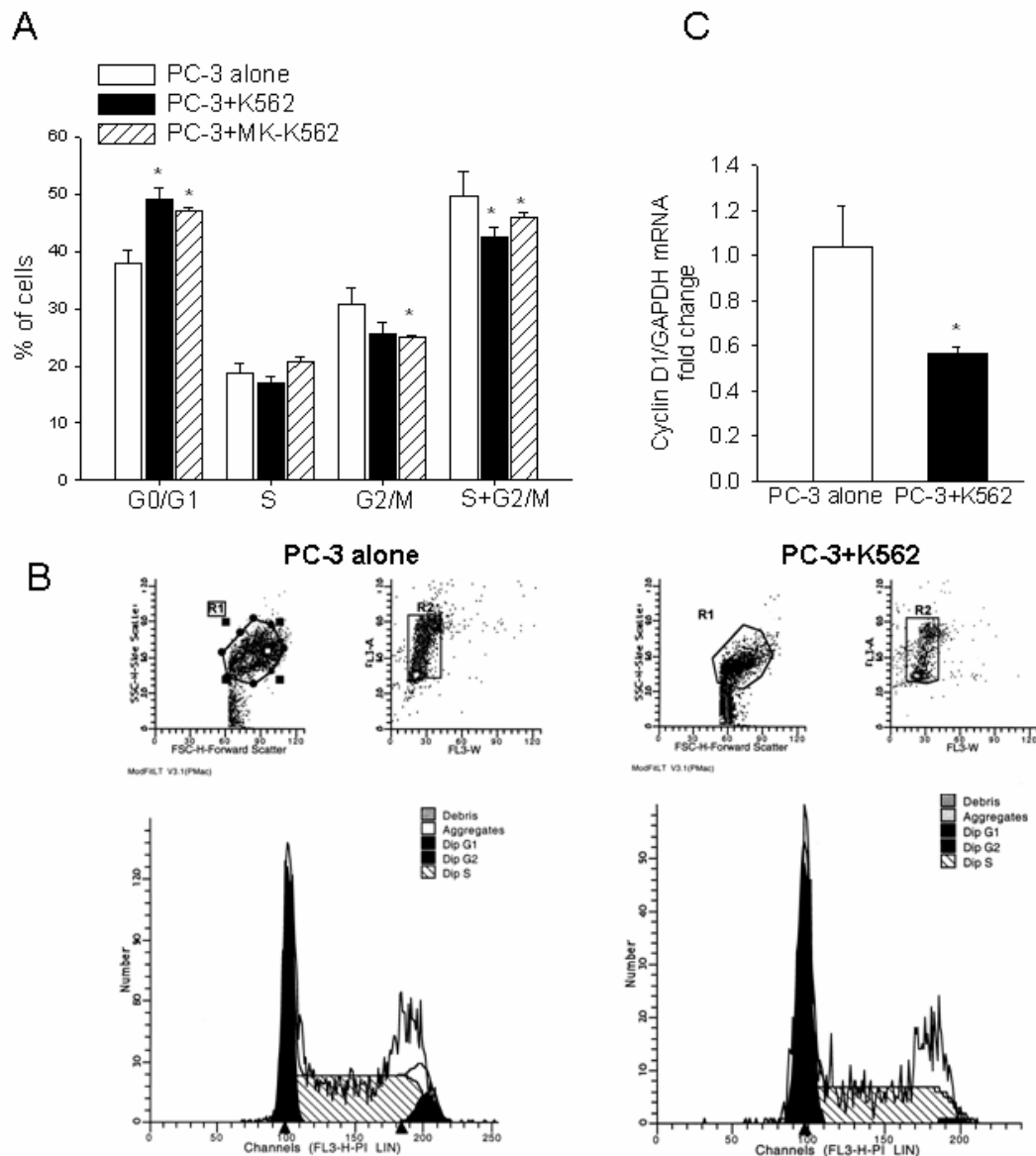


Supplementary FIG 1. Purity of mature primary megakaryocytes



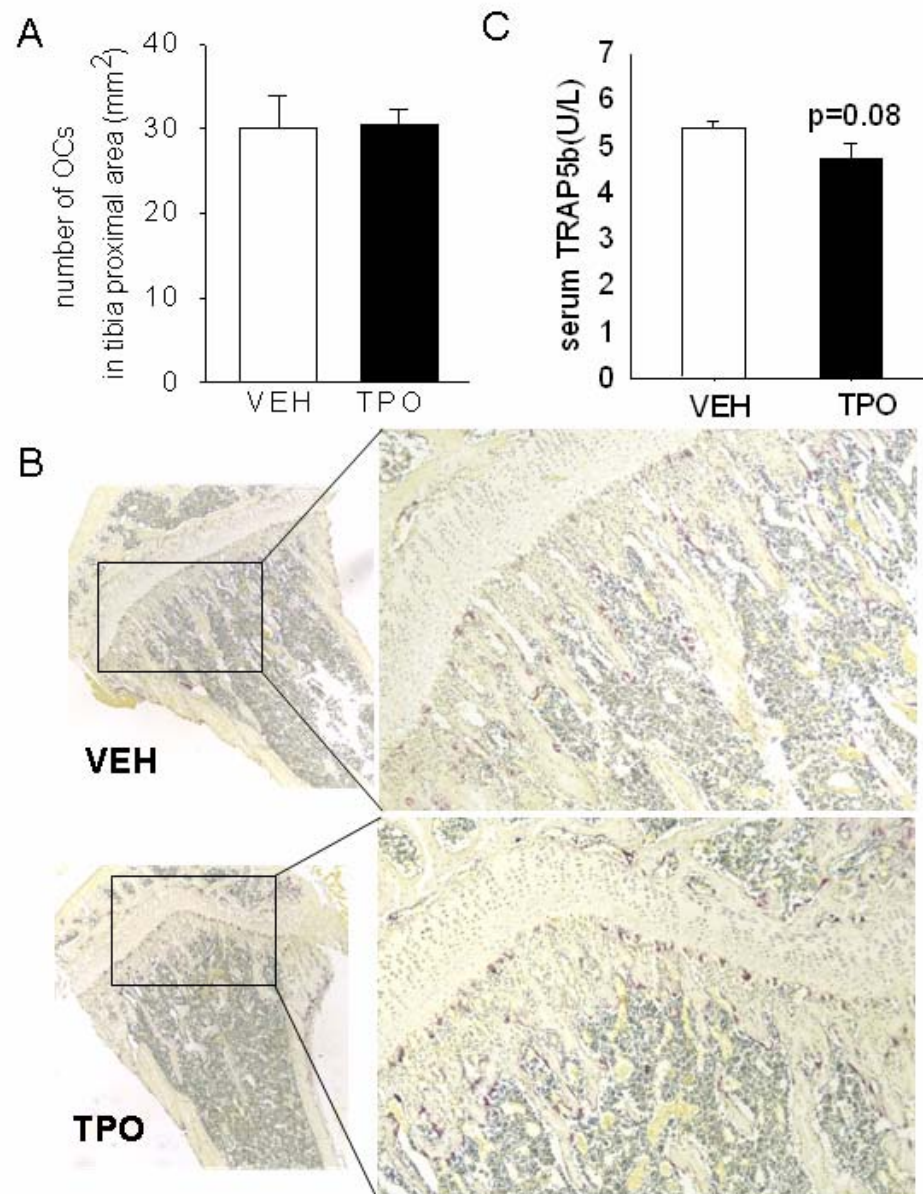
Primary megakaryocytes cultured from mouse bone marrow as described in Materials and Methods were incubated with primary antibody for integrin and secondary fluorescence labeled secondary antibody and integrin expression was evaluated by Flow cytometry. Mature megakaryocyte (large) enriched population, which was used for co-culture with PC-3 cells, was gated in large FS and SS (R2). Over 90% of these cells expressed CD41 (R3), marker for megakaryocyte lineage cells.

Supplementary FIG 2. K562/MKs induced PC-3 cell cycle arrest via cyclin D1



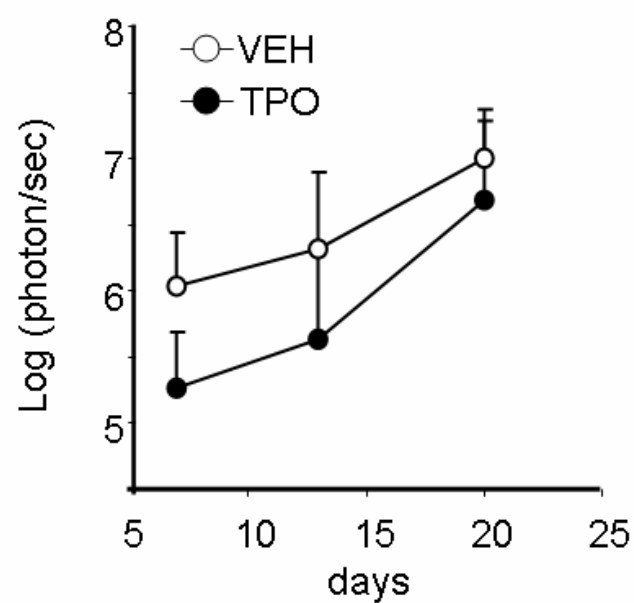
(A) PC-3 cells were serum starved overnight, co-cultured with megakaryocytic cells for 48h, then incubated with propidium iodide solution containing ribonuclease A. Flow cytometry analysis showed PC-3 cells were arrested by K562 and MK-K562 at G0/G1 phase with less cell entry to S, G2/M phases of cell cycle. (B) Representative FACS images analyzed with ModFit. (C) After co-culture with K562 cells for 48h, PC-3 cells were washed, RNA isolated and QPCR assay performed. Co-culture caused a 50% decrease in cyclin D1 mRNA in PC-3 cells. n=3/group, * p < 0.05.

Supplementary FIG 3. Osteoclast number and activity after TPO treatment before tumor inoculation.



Mice (20/group) were treated with recombinant TPO intraperitoneally to increase megakaryocytes. Five mice from each group were sacrificed. Serum and one tibia were collected for TRAP5b assay and TRAP staining on osteoclasts. (A) Osteoclast number in tibia proximal area; (B) representative images of TRAP staining on tibia sections; (C) Serum TRAP5b levels in TPO and Vehicle (VEH) treated groups.

Supplementary FIG 4. TPO administration did not affect tumor development in jaws.



Mice (20/group) were treated with recombinant TPO intraperitoneally to increase megakaryocytes. The development of inoculated prostate carcinoma cells in the jaws of mice were recorded with BLI imaging weekly. Although there was an early trend for TPO to result in less bioluminescence there was no significant difference between groups.

Supplementary Table 1. Other apoptosis pathway analysis revealed genes regulated by co-culture with K562 cells

Gene Symbol	Fold Change
APAF 1	2.385
BAG3	4.039
BAK1	2.1495
BAX	2.7207
BCL2A1	3.2807
CARD6	2.5919
CASP5	2.1053
CASP9	2.2099
CD27	2.1199
CD40	2.1053
CD40LG	2.0195
DFFA	2.7587
FADD	2.0619
FAS	2.0195
LTBR	2.6833
MCL1	2.2564
NOL3	2.0195
RIPK2	2.0907
TNFRSF10A	2.7397
TNFRSF10B	2.7207
TNFRSF1A	2.0907
TNFRSF9	3.9559
TNFSF10	2.856
TNFSF8	2.1199
TP53BP2	2.2099
BCL2	-57.123
BCLAF1	-47.7027
HGDC	-8.6099
NAIP	-2.6317

PC-3 cells were co-cultured with or without K562 cells for 48 hours before RNA isolation. Apoptosis pathway PCR array was performed following the instruction of the manufacture (SABiosciences, MA). Raw data were uploaded to the online data analysis program provided by SABiosciences and genes with at least 2-fold change with K562 cells were listed.