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(54) Title: METHODS FOR THE TREATMENT OF CANCER-ASSOCIATED BONE DISEASE

(57) Abstract: Provided herein are methods and compositions for the treatment of bone disease, particularly cancer-induced bone disease, comprising administering an inhibitor of thymidine phosphorylase or an inhibitor of a product of thymidine metabolism, such as 2DDR, to the subject.



DESCRIPTION

METHODS FOR THE TREATMENT OF CANCER-ASSOCIATED BONE DISEASE

[0001] This application claims the benefit of United States Provisional Patent Application No. 62/378,517, filed August 23, 2016, the entirety of which is incorporated
5 herein by reference.

[0002] The sequence listing that is contained in the file named “UTFCP1303WO_ST25.txt”, which is 13 KB (as measured in Microsoft Windows) and was created on August 18, 2017, is filed herewith by electronic submission and is incorporated by reference herein.

10 BACKGROUND OF THE INVENTION

[0003] The invention was made with government support under Grant Nos. R01 CA190863 and R01 CA193362 awarded by the National Institutes of Health. The government has certain rights in the invention.

1. Field of the Invention

15 [0004] The present invention relates generally to the fields of medicine and molecular biology. More particularly, it concerns compositions and methods for the treatment of bone disease.

2. Description of Related Art

[0005] Bone is constantly being remodeled in a process where osteoclasts resorb
20 bone, then osteoblasts deposit type I collagen and other proteins in the resorbed lacunae, and lastly the collagen mineralizes to form bone (Rucci, 2008; Karsenty *et al.*, 2009). The first partner in this *pas de deux* is the osteoclast, which arises from hematopoietic monocytic precursors and resorbs bone. The formation of osteoclasts requires the cytokine receptor activator of nuclear factor- κ B ligand (RANKL) and macrophage colony-stimulating factor
25 (M-CSF) (Bruzaniti and Baron, 2006). RANKL enhances the expression of nuclear factor of activated T-cells, cytoplasmic 1 protein (NFATc1), a transcriptional factor that upregulates the expression of osteoclast differentiation-associated genes, such as tartrate-resistant acid phosphatase (*TRAP*), calcitonin receptor (*CALCR*), and cathepsin K (*CTSK*); whereas the transcriptional factor interferon regulatory factor 8 (*IRF8*) can suppress RANKL-induced

NFATc1 expression (Nishikawa *et al.*, 2015). The second player in the remodeling cycle is the osteoblast, which is differentiated from mesenchymal stem cells (MSC). This process requires the activation of core-binding factor α -1/runt-related transcription factor 2 (*RUNX2*) and osterix, which stimulate the expression of osteoblast differentiation-associated genes, such as bone gamma-carboxyglutamic acid-containing protein (*BGLAP*), alkaline phosphatase (*ALP*), and collagen type I α 1 (*COL1A1*).

[0006] This delicate balance between osteoclast-mediated bone resorption and osteoblast deposition of matrix is disrupted in certain types of malignancies, including multiple myeloma and solid tumors, such as breast and lung cancer, among others (Roodman, 2001; Roodman, 2009). In multiple myeloma, tumor cells secrete RANKL or stimulate the release of RANKL by surrounding stromal cells, leading to enhanced osteoclast differentiation. Myeloma cells can also secrete dickkopf-related protein 1 (*DKK1*), which inhibits the Wnt/ β -catenin signaling pathway and suppresses osteoblast differentiation. Attempts to target RANKL and *DKK1* in myeloma therapeutically have achieved only modest success. For example, the anti-resorptive agent denosumab (*i.e.*, a monoclonal antibody against RANKL) was examined in a Phase III trial, but only moderately affected myeloma-induced lytic lesions (Terpos *et al.*, 2015). *BHQ880* (*i.e.*, a monoclonal antibody against *DKK-1*) is in a Phase I/II study, but its application in myeloma fails to restore new bone formation (Fulciniti *et al.*, 2009). Thus bisphosphonates, which suppress osteoclast function, remain the mainstay in treatment of myeloma-induced bone disease. Unfortunately, bisphosphonates are less than fully effective and cause osteonecrosis of the jaw in 2-5% of treated patients (Terpos *et al.*, 2015). Thus, there is an unmet need to identify other factors produced by myeloma cells which regulate both resorption and formation, and to determine whether this information can be used to prevent myeloma-induced bone disease.

SUMMARY OF THE INVENTION

[0007] Certain embodiments of the present disclosure provides methods and compositions for the treatment of bone disease, such as cancer-induced bone disease. In a first embodiment, there is provided a method of treating a bone disease in a subject comprising administering an effective amount of an inhibitor of thymidine phosphorylase (TP) to the subject. In another embodiment, there is provided a method of treating a bone disease in a subject comprising administering an effective amount of an inhibitor of a product of thymidine metabolism (*e.g.*, 2DDR) to the subject. In some embodiments, there is

provided a method of treating a bone disease in a subject comprising administering an effective amount of an inhibitor of TP and an inhibitor of a product of thymidine metabolism, such as 2DDR. In some aspects, the subject is a human. In certain aspects, the inhibitor of thymidine phosphorylase is tipiracil hydrochloride (TPI), 7-deazaxanthine (7DX), 5'-O-tritylinosine (KIN-59), or 9-(8-phosphonooctyl)-7-deazaxanthine (TP65).

[0008] In some aspects, the bone disease is cancer-induced bone disease. In particular aspects, the subject is diagnosed as having cancer, such as multiple myeloma, breast cancer, colorectal cancer, or lung cancer. In some aspects, the multiple myeloma is further defined as CD138-positive multiple myeloma. In specific aspects, the bone disease is multiple myeloma-induced bone disease. In certain aspects, the bone disease is further defined as osteolytic bone lesions.

[0009] In some aspects, the subject has an increased serum level of 2DDR as compared to the serum level of 2DDR in a healthy subject. In certain aspects, the subject has an increased serum level of TP as compared to the serum level of TP in a control, such as a healthy subject or a subject with no bone disease. In specific aspects, the subject has an increased level of RANKL and/or sclerostin as compared to a control level.

[0010] In further aspects, the treatment with the TP inhibitor or the inhibitor of a product of thymidine metabolism, such as 2DDR, results in a decreased serum level of 2DDR as compared to the serum level prior to the treatment. In some aspects, the treatment with the TP inhibitor or the inhibitor of a product of thymidine metabolism results in the subject having a decreased expression of *DNMT3A*, *TRAP*, *CALCR*, and/or *CTSK* as compared to before the treatment was administered. In some aspects, the subject has increased expression of *RUNX2*, *osterix*, and/or *IRF8* after administration of the inhibitor of TP and/or product of thymidine metabolism. In certain aspects, the subject has reduced osteoclast differentiation, decreased osteolytic bone lesions, and/or increased osteoblast-mediated bone formation (*i.e.*, bone volume) after administration of the inhibitor of TP and/or product of thymidine metabolism. In particular aspects, the subject has decreased levels of TP, 2DDR, RANKL, and/or sclerostin after administration of the inhibitor of TP and/or the inhibitor of a product of thymidine metabolism, such as 2DDR.

[0011] In some aspects, the inhibitor of TP and/or product of thymidine metabolism is administered intravenously, intraperitoneally, intratracheally, intratumorally, intramuscularly,

endoscopically, intralesionally, percutaneously, subcutaneously, regionally, or by direct injection or perfusion. In some aspects, the inhibitor of TP and/or product of thymidine metabolism is injected directly into a bone, such as the bone marrow, of the subject.

[0012] In further aspects, the method further comprises administering at least a second therapeutic agent. In some aspects, the at least a second therapeutic agent is an anti-resorptive agent and/or an anti-cancer agent. In certain aspects, the at least a second therapeutic agent is a DNMT inhibitor. In some aspects, the anti-resorptive agent is an anti-RANKL antibody, anti-DKK-1 antibody, bisphosphonate, or hormone therapy. In some aspects, the bisphosphonate is alendronate, risedronate, ibandronate, or etidronate. In particular aspects, the hormone therapy is a selective estrogen receptor modulator (SERM) or calcitonin. In certain aspects, the anti-cancer therapy is chemotherapy, immunotherapy, surgery, radiotherapy, or biotherapy. In some aspects, the anti-cancer therapy is a nucleoside analog. In one particular aspect, the nucleoside analog is trifluridine. In some aspects, the anti-cancer therapy is a proteasome inhibitor. In particular aspects, the proteasome inhibitor is bortezomib or carfilzomib. In some aspects, the anti-cancer therapy is anti-integrin alpha-v antibody or anti-integrin alpha-5 antibody.

[0013] A further embodiment provides a pharmaceutical composition comprising an inhibitor of TP and/or 2DDR and a pharmaceutically acceptable carrier for use in the treatment of a bone disease, such as a cancer-induced bone disease. Also provided herein is a composition comprising an effective amount of an inhibitor of TP or 2DDR for the treatment of a bone disease in a subject.

[0014] In another embodiment, there is provided a method of treating bone disease in a subject comprising administering an effective amount of an inhibitor of TP to the subject, wherein the subject is identified to have an increased level of TP as compared to a control. In particular aspects, the increased level of TP identifies the presence of bone lesions in said subject. In some aspects, the level of TP is measured in the serum of said subject. In particular aspects, the inhibitor of thymidine phosphorylase is tipiracil hydrochloride (TPI), 7-deazaxanthine (7DX), 5'-O-tritylinosine (KIN-59), or 9-(8-phosphonooctyl)-7-deazaxanthine (TP65). In specific aspects, the subject is human.

[0015] In some aspects, the subject has increased levels of RANKL and/or sclerostin as compared to a control. In specific aspects, the administration of an inhibitor of TP results in decreased levels of RANKL and/or sclerostin as compared to levels prior treatment.

5 [0016] In some aspects, the bone disease is cancer-induced bone disease. In certain aspects, the subject is diagnosed as having cancer. In some aspects, the cancer is multiple myeloma, breast cancer, or lung cancer. In some aspects, the bone disease is further defined as osteolytic bone lesions.

10 [0017] In some aspects, the inhibitor of TP and/or product of thymidine metabolism is administered intravenously, intraperitoneally, intratracheally, intratumorally, intramuscularly, endoscopically, intralesionally, percutaneously, subcutaneously, regionally, or by direct injection or perfusion. In some aspects, the inhibitor of TP is injected directly into a bone of the subject.

15 [0018] In additional aspects, the method further comprises administering at least a second therapeutic agent. In some aspects, the at least a second therapeutic agent is an anti-resorptive agent and/or an anti-cancer agent. In certain aspects, the at least a second therapeutic agent is a DNMT inhibitor. In specific aspects, the anti-resorptive agent is an anti-RANKL antibody, anti-DKK-1 antibody, bisphosphonate, or hormone therapy. In particular aspects, the bisphosphonate is alendronate, risedronate, ibandronate, or etidronate. In certain aspects, the hormone therapy is a selective estrogen receptor modulator (SERM) or calcitonin. In some aspects, the anti-cancer therapy is chemotherapy, immunotherapy, surgery, radiotherapy, or biotherapy. In particular aspects, the anti-cancer therapy is a nucleoside analog, such as trifluridine. In some aspects, the anti-cancer therapy is a proteasome inhibitor. In particular aspects, the proteasome inhibitor is bortezomib or carfilzomib. In some aspects, the anti-cancer therapy is anti-integrin alpha-v antibody or anti-integrin alpha-5 antibody.

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[0019] As used herein, “essentially free,” in terms of a specified component, is used herein to mean that none of the specified component has been purposefully formulated into a composition and/or is present only as a contaminant or in trace amounts. The total amount of the specified component resulting from any unintended contamination of a composition is therefore well below 0.05%, preferably below 0.01%. Most preferred is a composition in

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which no amount of the specified component can be detected with standard analytical methods.

[0020] As used herein the specification, “a” or “an” may mean one or more. As used herein in the claim(s), when used in conjunction with the word “comprising,” the words “a” or “an” may mean one or more than one.

[0021] The use of the term “or” in the claims is used to mean “and/or” unless explicitly indicated to refer to alternatives only or the alternatives are mutually exclusive, although the disclosure supports a definition that refers to only alternatives and “and/or.” As used herein “another” may mean at least a second or more.

[0022] Throughout this application, the term “about” is used to indicate that a value includes the inherent variation of error for the device, the method being employed to determine the value, or the variation that exists among the study subjects.

[0023] Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

[0024] The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

[0025] **FIGS. 1A-C: TP is highly expressed in myeloma.** (A) Representative immunohistochemical images of bone marrow biopsies from tissue arrays from 14 healthy and 14 myeloma patients stained for CD138 and TP. High expression of TP and CD138 was seen in the multiple myeloma samples and almost no expression in the healthy samples. (B) Densitometry analysis of CD138⁺ cells or TP⁺ cells in (A). Data are box plots showing the distribution and median value of quantitative staining ($n = 14$). Scale bars, 50 μm . P values

were determined by Student's *t* test. (C) Western blot analysis of TP expression in normal plasma cells from 4 healthy donors, malignant plasma cells of 6 myeloma patients, and 6 established human myeloma cell lines. Primary plasma cells were isolated from the bone marrow aspirates of healthy donors or myeloma patients. β -Actin served as loading control.

5 Data are representative of triplicate blots.

[0026] **FIGS. 2A-H: Association of TP expression and lytic bone lesion in myeloma.** (A) Shown is the correlation coefficient between the mRNA levels of *TP* and numbers of bone lesion in myeloma patients ($n = 52$). *P* values were determined by Pearson Coefficient. (B and C) Bone marrow biopsy samples from $n = 13$ patients in (A) were labeled with an anti-TP antibody. TP staining was analyzed using the Image Pro Plus. (B) The correlation between TP staining in bone marrow biopsies and the numbers of bone lesions in myeloma patients. *P* values were determined by Pearson Coefficient. (C) Representative images of immunohistochemical staining show TP expression in myeloma cells and CD138⁺ infiltrated myeloma cells within bone marrow of the patient samples from (B) highlighted with circles. Scale bar, 10 μ m. (D to H) Based on the levels of TP expression in myeloma cells, patients' myeloma cells were separated into high and low TP expression groups (TP_{high} and TP_{low}; $n = 5$ patients' bone marrow aspirates/group). In addition, myeloma cells were injected into the bone chips of SCID-hu mice or SCID mouse femurs. Shown are representative X-ray images (D-G) and summarized data of the percentage of bone volumes vs total volumes (BV/TV) (H) of lytic lesion in the implanted human bone chips of SCID-hu mice injected with TP_{high} and TP_{low} cells or in the femurs of SCID mice injected with myeloma cell lines ARP-1 [wild-type (WT), non-targeted shRNA (shCtrl), and *TP* shRNA (shTP)] and MM.1S [WT, control vector (*Vec*), and *TP* cDNA (*TP*)]. Data are averages $\pm$ SD ($n = 5$ mice/group, 3 replicate studies). *P* values were determined by Student's *t* test.

25 [0027] **FIGS. 3A-F Myeloma-expressed TP enhances osteoclast-mediated bone resorption and inhibits osteoblast-mediated bone formation *in vivo*.** The implanted human bone chips from SCID-hu mice injected with TP_{high} and TP_{low} cells ($n = 5$ patients' bone marrow aspirates/group) or the femurs from SCID mice injected with myeloma cell lines ARP-1 (wild-type [WT], non-targeted shRNA [shCtrl], and *TP* shRNA [shTP]) and MM.1S (WT, control vector [*Vec*], and *TP* cDNA [*TP*]) were fixed, TRAP- or toluidine blue-stained, and analyzed by BIOQUANT OSTEO software. (A to D) The percentage of bone surface eroded by osteoclasts (ES/BS) (A), the percentage of bone surface covered with

osteoclasts (Oc.S/BS) (**B**), the percentage of osteoid surface (OS/BS) (**C**), and the percentage of total bone surface lined with osteoblasts (Ob.S/BS) (**D**) in myeloma-bearing human bone chips or mouse femurs. (**E and F**) Bone formation rate (BFR/BS) was measured by calcein injection, and the undecalcified bone sections were imaged and analyzed. Shown are representative images or summarized data of bone formation in the femurs from SCID mice injected with myeloma cell lines ARP-1 (sh*Ctrl* and sh*TP*) and MM.1S (*Vec* and *TP*). Scale bar, 20 μ m. All data are averages $\pm$ SD ($n = 5$ mice/group, 3 replicate studies). All *P* values were determined by Student's *t* test.

[0028] FIGS. 4A-F: TP inhibits the expression of *RUNX2*, *osterix*, and *IRF8* through hypermethylation of their CpG islands. (**A**) Schematic diagrams of CpG-rich test regions on the promoter of *RUNX2* or *osterix* in human MSCs, and on the promoter of *IRF8* in human preOCs. TSS represents the transcription start site. The arrow indicates the translation-initiating ATG site. The CpG-rich test region is marked with a horizontal bar. (**B to E**) MSCs or preOCs were co-cultured with myeloma cells ARP-1 (wild-type [WT], non-targeted shRNA [sh*Ctrl*], and *TP* shRNA [sh*TP*]) and MM.1S (WT, control vector [*Vec*], and *TP* cDNA [*TP*]) in their respective medium for 7 days. After cultures, bisulfite-treated genomic DNA was subjected to methylation-specific PCR (MSP) or bisulfite sequencing PCR (BSP) analysis. (**B**) DNA gel electrophoresis shows the unmethylated (U) and methylated (M) PCR products from MSP analysis. Sequencing results from BSP analysis shows percentage of methylation in the promoter of *RUNX2* or *osterix* in MSCs, and in the promoter of *IRF8* in preOCs co-cultured with myeloma cell lines ARP-1 and MM.1S (**C**), sh*Ctrl* or sh*TP* ARP-1 cells (**D**), or *Vec* or *TP* MM.1S cells (**E**). Cultured MSCs or preOCs without myeloma (No MM) served as a control. Data are individual samples with averages $\pm$ SD ($n = 5$) of 3 experiments. *P* values were determined by Student's *t* test. (**F**) Summary of BSP analysis shows percentage of methylation in the promoter of *RUNX2* or *osterix* in MSCs, and in the promoter of *IRF8* in preOCs, of healthy donors and TP_{high} or TP_{low} patients. Data are individual samples with averages $\pm$ SD ($n = 5$) of 3 experiments. *P* values were determined by Student's *t* test.

[0029] FIGS. 5A-H: 2DDR inhibits osteoblast differentiation and activates osteoclast differentiation by upregulating *DNMT3A* expression. Human MSCs or preOCs were cultured in medium without (0) or with 0.5, 1, or 2 mM of 2DDR for 48 hours. In some studies, MSCs or preOCs carried with non-targeted shRNAs (sh*Ctrl*) or *DNMT3A* shRNAs

(sh*DNMT3A*) were cultured with PBS or 1 mM 2DDR. (A to D) Expression of RUNX2 and osterix (A), *DNMT3A* mRNA expression and activity (B), methylation of CGIs in the promoter regions of *RUNX2* and *osterix* (C), and ALP activity and Alizarin red S staining (D) in MSC-derived cells after 2DDR treatment. (E to H) Expression of IRF8 (E), *DNMT3A* mRNA expression and activity (F), methylation of CGIs in the promoter region of *IRF8* (G), and the number of multiple nuclear (≥ 3) TRAP⁺ cells and secretion of TRAP5b (H) in preOC-derived cells after 2DDR treatment. mRNA expression was normalized to cells without 2DDR (set at 1). The levels of β -Actin served as loading controls. Data are averages $\pm$ SD ($n = 3$). *P* values were determined by Student's *t* test. Each experiment was repeated three times.

[0030] FIGS. 6A-G: Administration of TP inhibitor in myeloma-bearing mice reduces bone lesions and osteoclastogenesis and enhances osteoblastogenesis. ARP-1 cells were injected into the femurs of SCID mice. Mice without myeloma cells served as controls (No MM). After 3 weeks, mice were treated with PBS as vehicle controls or TP inhibitor 7DX (200 μ g/kg) or TPI (300 μ g/kg). After treatment, mice were scanned for radiography, and mouse femurs were subjected to toluidine blue staining or TRAP staining. (A) Representative X-ray images of mouse femurs. (B to D) The percentage of bone volume to total volume (BV/TV) (B), bone surface eroded by osteoclasts (ES/BS) and bone surface covered with osteoclasts (Oc. S/BS) (C), and percentage of osteoid surface (OS/BS) and of bone surface lined with osteoblasts (Ob.S/BS) (D). Data are averages $\pm$ SD ($n = 5$ mice/group, 3 replicate studies). (E) *Dnmt3a* mRNA expression in murine MSCs and preOCs isolated from bone marrow aspirates of ARP-1 bearing mice. Data are averages relative to no MM bearing mice (No MM) treated with vehicle (set at 1) $\pm$ SD ($n = 5$ mice/group, 3 replicate studies). (F) 2DDR levels in the serum of ARP-1 bearing mice. Data are averages relative to that in no MM bearing mice (No MM) treated with vehicle (set at 1) $\pm$ SD ($n = 5$ mice/group, 3 replicate studies). All *P* values were determined by Student's *t* test. (G) A depiction of signaling pathways involved in the myeloma TP-mediated suppression of osteoblastogenesis and activation of osteoclastogenesis.

[0031] FIGS. 7A-F: TP expressed by myeloma cells regulates osteoblast and osteoclast differentiation *in vitro* and *in vivo*. (A) Relative levels of 2DDR in human myeloma cell lines RPMI8226 or U266 were measured after 48 hours of culture. Data are relative to RPMI8226. Data are averages $\pm$ SD ($n = 3$) of 3 experiments. (B) PreOCs were

cultured alone or co-cultured with RPMI8226 or U266 cells in medium without or with RANKL (10 ng/ml) for 1 week. preOCs alone without or with 10 ng/ml of RANKL served as controls. Numbers of multinuclear (≥ 3) TRAP⁺ cells and the relative expression of osteoclast differentiation-associated genes *TRAP*, *CALCR*, and *CTSK* were measured. mRNA expression was normalized to cells without myeloma (No MM, set to 1). Data are averages $\pm$ SD ($n = 3$) of 3 experiments. (C) MSCs were co-cultured with RPMI8226 or U266 in osteoblast medium for 2 weeks and then stained for Alizarin red S. The relative expression of osteoblast differentiation-associated genes *BGLAP*, *ALP*, and *COL1A1* were determined in attached cells. mRNA expression was normalized to cells without myeloma (No MM, set to 1). Data are averages $\pm$ SD ($n = 3$) of 5 experiments. (D to F) RPMI8226 cells (5×10^5 cells/mouse) were injected into the femurs of SCID mice. Mice without myeloma cell injection served as controls (No MM). After 3 weeks, mice were intraperitoneally injected with PBS as vehicle control or the TP inhibitors 7DX (200 μ g/kg) or TPI (300 μ g/kg) three times per week for 2 weeks. After treatment, mice were scanned for radiography, and mouse femurs were subjected to toluidine blue staining or TRAP staining. The percentages of bone volume to total volume (BV/TV) (D), bone surface eroded by osteoclasts (ES/BS) and bone surface covered with osteoclasts (Oc. S/BS) (E), as well as percentage of osteoid surface (OS/BS) and bone surface lined with osteoblasts (Ob.S/BS) were calculated (F). Data are averages $\pm$ SD ($n = 5$ mice/group, 3 replicate studies). All *P* values were determined by Student's *t* test.

[0032] FIGS. 8A-G: Modulation of TP expression does not affect the growth and survival of myeloma cells. (A to D) TP expression in myeloma cells by representative western blots. β -Actin served as loading control. (A) TP_{high} and TP_{low} cells isolated from the bone marrow aspirates of newly diagnosed patients ($n = 5$ per group). (B) Wild-type (WT) ARP-1 and WT MM.1S cell lines. (C) ARP-1 cells transfected with non-targeted shRNA (shCtrl) or TP shRNA (shTP). (D) MM.1S transfected with TP cDNA (TP) or control vector (Vec). Each blot was repeated three times. (E) Myeloma cells (5×10^5 per mouse) (shCtrl or shTP ARP-1, or Vec or TP MM.1S) were injected into SCID mouse femurs. Four weeks after cell injection, mouse sera were collected and M-protein levels were measured by ELISA. Data are averages $\pm$ SD ($n = 5$ mice/group). Each experiment was repeated three times. (F and G) Proliferation of shCtrl or shTP ARP-1 cells or Vec or TP MM.1S cells in culture for 5 days (F). Percentages of apoptotic cells in culture examined at 0 and 48 hours (G). Data are averages $\pm$ SD ($n = 3$). Each experiment was repeated three times.

[0033] FIGS. 9A-F: Myeloma-expressed TP enhances RANKL-mediated osteoclast differentiation and activity *in vitro*. PreOCs were cultured alone or co-cultured with myeloma cells in medium without or with RANKL (10 ng/ml) for 1 week, and then TRAP5b was measured in the supernatant. Multinuclear osteoclast-like cells were stained for TRAP and enumerated. mRNA from the attached cells were collected for real-time RT-PCR. Culturing preOCs alone without or with 10 ng/ml of RANKL served as negative or positive controls, respectively, for osteoclast differentiation. (A) Multinuclear (≥ 3) TRAP⁺ cells. (B) The levels of TRAP5b. (C to F) Relative expression of osteoclast differentiation-associated genes *TRAP*, *CALCR*, and *CTSK* in co-culture with TP_{high} ($n = 5$ patients' bone marrow aspirates) or TP_{low} ($n = 5$ patients' bone marrow aspirates) cells (C), wild-type (WT) myeloma cell lines ARP-1 and MM.1S (D), non-targeted shRNA (*shCtrl*) or *TP* shRNA (*shTP*) ARP-1 cells (E), or control vector (*Vec*) or *TP* cDNA (*TP*) MM.1S cells (F). Culturing preOCs alone (No MM) served as controls. Each experiment was repeated three times. Data are averages $\pm$ SD ($n = 3$). All *P* values were determined by Student's *t* test.

[0034] FIGS. 10A-C: Myeloma expressed-TP enhances NFATc1 expression and activity via inhibition of IRF8. (A) PreOCs were co-cultured without or with myeloma cells ARP-1, MM.1S, non-targeted shRNA (*shCtrl*) or *TP* shRNA (*shTP*) ARP-1 cells, or control vector (*Vec*) or *TP* cDNA (*TP*) MM.1S cells. Western blotting shows the expression of NFATc1 and IRF8 in preOCs co-cultured with myeloma cells. (B) IRF8 was knocked down in preOCs by a lentivirus carrying shRNA targeting *IRF8* (*shIRF8*). The expression of IRF8 and NFATc1 were measured by western blot in preOCs co-cultured with or without ARP-1 cells. The expression of β -Actin served as loading control. (C) PreOCs were co-cultured with or without ARP-1 myeloma cells with 10 ng/ml of RANKL for 7 days. After culture, TRAP5b secretion was measured in supernatant by ELISA. Data are averages $\pm$ SD ($n = 3$) normalized to control (set as 1). *P* value was determined by Student's *t* test. Each experiment was repeated three times.

[0035] FIGS. 11A-F: Myeloma-expressed TP inhibits osteoblast differentiation and activity *in vitro*. MSCs were co-cultured with patient myeloma cells or myeloma cell lines in osteoblast medium for 14 days. (A and B) After culture, the cells were stained for soluble ALP (A) and Alizarin red S (B). Data are averages $\pm$ SD ($n = 3$). All *P* values were determined by Student's *t* test. (C to F) The mRNAs of the attached cells were collected for real-time RT-PCR. The relative expression of osteoblast differentiation-associated genes

BGLAP, *ALP*, and *COL1A1* were determined in co-culture with TP_{high} ($n = 5$ patients' bone marrow aspirates) or TP_{low} ($n = 5$ patients' bone marrow aspirates) cells (C), wild-type (WT) myeloma cell lines ARP-1 and MM.1S (D), non-targeted shRNA (shCtrl) or *TP* shRNA (sh*TP*) ARP-1 cells (E), or control vector (*Vec*) or *TP* cDNA (*TP*) MM.1S cells (F).
 5 Culturing MSCs alone (No MM) served as a control. Each experiment was repeated three times. Data are averages $\pm$ SD ($n = 3$) normalized to control (set as 1). All *P* values were determined by Student's *t* test.

[0036] FIGS. 12A-C: TP inhibits the expression and activities of RUNX2 and osterix *in vitro*. MSCs were co-cultured with high-TP ARP-1 cells or low-TP MM.1S cells,
 10 non-targeted shRNA (shCtrl) or *TP* shRNA ARP-1 cells (sh*TP*), or control vector (*Vec*) or *TP* cDNA MM.1S cells (*TP*). (A) Western blot analysis shows the expression of RUNX2 and osterix. β -Actin served as a loading control. (B and C) ChIP assay was used to detect the binding of RUNX2 (B) or osterix (C) onto the *BGLAP* and *COL1A1* promoters in MSCs. Left panels show the ChIP-PCR analysis and right panels show the summarized data of
 15 transcription factor binding activities in MSCs. Data are fold enrichment relative to ARP-1 (upper panels), shCtrl ARP-1 (middle panels), and *Vec* MM.1S (lower panels) (all set as 1). Data are average $\pm$ SD ($n = 3$). *P* values were determined by Student's *t* test. Each experiment was repeated three times.

[0037] FIGS. 13A-C: Myeloma-expressed TP enhances DNMT3A levels in MSCs
 20 and preOCs. (A and B) MSCs (A) or preOCs (B) were co-cultured with myeloma cell lines, and the expression of *DNMT* mRNAs and the enzymatic activity of DNMT3A were quantified after 2 weeks. *GAPDH* served as a control. Gels are representative of $n = 3$. Enzymatic data are averages $\pm$ SD ($n = 3$). *P* values determined with Student's *t* test. (C) The expression of *DNMT3A* in myeloma patient-derived MSCs and preOCs was correlated with
 25 *TP* expression in patient myeloma cells and with bone lesion numbers. *P* values were determined by Pearson Coefficient. Each point represents the aggregate MSCs or preOCs from one patient sample ($n = 20$ patients' bone marrow aspirates).

[0038] FIGS. 14A-D: Myeloma cells with high TP expression secrete more 2DDR, which affects osteoclast and osteoblast differentiation *in vitro*. Myeloma cells
 30 were cultured for 48 hours, and then 2DDR levels in the supernatants were measured. (A) The relative levels of 2DDR in cultures of myeloma cell lines ARP-1 and MM.1S (relative to ARP-1 [set at 1]); control vector (*Vec*) or *TP* cDNA (*TP*) MM.1S cells; and non-targeted

shRNA (shCtrl) or TP shRNA (shTP) ARP-1 cells. 2DDR levels in medium without any cell culture (No MM) served as controls (set at 1). (B) 2DDR levels in the serum of mice bearing Vec or TP MM.1S cells, or shCtrl or shTP ARP-1 cells. 2DDR levels in serum of mice that did not receive myeloma cells (No MM) served as controls (set at 1). (C and D) MSCs or preOCs were treated without or with various concentrations of 2DDR. After culture, the cells or supernatants were assayed for soluble ALP and Alizarin red S staining (for MSC-derived osteoblasts) (C) or TRAP staining and soluble TRAP5b (for preOC-derived osteoclasts) (D). The mRNAs of the attached cells were collected for real-time RT-PCR. The relative expression of osteoblast differentiation-associated genes *BGLAP*, *ALP*, and *COL1A1* was determined in MSCs with or without 2DDR treatment (C), and the relative expression of osteoclast differentiation-associated genes *TRAP*, *CALCR*, and *CTSK* was determined in preOCs with or without 2DDR treatment. The mRNA expression of genes in cells without 2DDR was set to 1. Each experiment was repeated three times. Data are averages $\pm$ SD ($n = 3$). *P* values versus respective controls were determined by Student's *t* test.

[0039] FIGS. 15A-J: 2DDR upregulates DNMT3A via $\alpha_5\beta_1/\alpha_v\beta_3$ -PI3K/Akt signaling pathways. (A and B) Alizarin red S staining (A) and expression of *DNMT3A* (B) in human MSCs cultured without or with 1 mM of 2DDR in the presence of control IgG or 10 μ g/ml antibody against either α_5 subunit of $\alpha_5\beta_1$ (α_5 Ab) or α_v subunit of $\alpha_v\beta_3$ (α_v Ab), or both. Data are averages $\pm$ SD ($n = 3$), with mRNA normalized to negative control. (C) Phosphorylated and non-phosphorylated Akt, ERK1/2, FAK, Paxillin, and p130Cas in MSCs cultured without or with 0.5, 1, or 2 mM of 2DDR. β -Actin served as loading control. (D) Phosphorylated Akt in MSCs cultured without or with 1 mM of 2DDR in the presence of IgG or a combination of 10 μ g/ml of α_5 Ab and α_v Ab. β -Actin levels served as control. (E) Expression of *DNMT3A* in MSCs cultured without or with 1 mM of 2DDR in the presence or absence of an inhibitor against PI3K/Akt (10 μ M; LY294002) for 24 hours. Data are averages $\pm$ SD normalized to negative control ($n = 3$). (F and G) PreOCs were treated with or without 1 mM of 2DDR in the presence of IgG or 10 μ g/ml α_v Ab. The secretion of TRAP5b was determined at day 7 (F) and *DNMT3A* expression was determined after 24 hours of incubation (G). Data are averages $\pm$ SD ($n = 3$), with mRNA normalized to negative control. (H) Phosphorylated and non-phosphorylated Akt, ERK1/2, FAK, Paxillin, and p130Cas in the preOCs cultured without or with 0.5, 1, or 2 mM of 2DDR. β -Actin served as loading control. (I) The levels of phosphorylated Akt in preOCs cultured without or with 1 mM of 2DDR in the presence of IgG or 10 μ g/ml of α_v Ab. β -Actin served as loading control. (J)

DNMT3A expression in preOCs cultured without or with 1 mM of 2DDR in the presence or absence of an inhibitor against PI3K/Akt (10 μ M; LY294002) for 24 hours. Data are averages $\pm$ SD ($n = 3$). Each experiment was repeated three times. All *P* values were determined by Student's *t* test.

5 **[0040] FIGS. 16A-L: Knockdown of integrins or Akt1/2 abrogates the effects of 2DDR on DNMT3A expression.** (A) Levels of α_5 or α_v proteins in MSCs transfected with siRNAs against α_5 or α_v , respectively. (B and C) Alizarin red S staining (B) and expression of *DNMT3A* (C) in human MSCs receiving non-targeted siRNA (siCtrl), α_5 siRNA (si α_5), and/or α_v siRNA (si α_v), and cultured without or with 1 mM of 2DDR. (D) Western blot analysis shows the levels of phosphorylated Akt in mouse MSCs with Ctrl or si α_5/α_v in the absence or presence of 2DDR (1 mM). (E) Western blot analysis shows the reduced levels of Akt1 and Akt2 proteins in MSCs transfected with pooled siRNAs against *Akt1* and *Akt2* (si*Akt1/2*). (F) Expression of *DNMT3A* in MSCs with siCtrl or si*Akt1/2* in the absence or presence of 2DDR (1 mM). (G) Levels of α_v in preOCs transfected with si α_v . (H and I) TRAP5b levels (H) and expression of *DNMT3A* (I) in siCtrl or si α_v preOCs cultured without or with 1 mM of 2DDR. (J) The levels of p-Akt in the preOCs with siCtrl or si α_v in the absence or presence of 2DDR (1 mM). (K) Western blot analysis shows the reduced levels of Akt1 and Akt2 proteins in preOCs transfected with si*Akt1/2*. (L) *DNMT3A* mRNA expression in preOCs with siCtrl or si*Akt1/2* in the absence or presence of 2DDR (1 mM). In all western blot analysis, β -actin levels served as control. In all real-time RT-PCR analysis, *DNMT3A* mRNA expression was relative to that in cells without any reagent (set at 1). Data are averages $\pm$ SD ($n = 3$) representative of 3 experiments. All *P* values were determined by Student's *t* test.

25 **[0041] FIGS. 17A-B: The effects of the TP inhibitor on osteoblast (OB) and enhances osteoclast (OC) differentiation in myeloma (MM).** (A) The OB progenitors, mesenchymal stem cells, were co-cultured with the MM cell lines ARP-1 or RPMI8226 in OB medium for 14 days. In some experiments, 100 μ M of the TP inhibitor (TPI) was added to the cultures. The cultured cells were stained with Alizarin red solution. The positive cells are characterized as mature OBs. The dye was further resolved and measured at OD 490nm. Shown is the staining level in cultures. (B) The precursors of OCs were co-cultured with the MM cell lines ARP-1 or RPMI8226 in medium with RANKL (10 ng/ml) for 1 week. In some experiments, 100 μ M of the TP inhibitor, TPI, was added to the cultures. The cultured cells

were stained with tartrate resistant acid phosphatase (TRAP) staining. The multinuclear TRAP-positive (OC-like) cells were enumerated. Shown is the number of multinuclear TRAP-positive cells in cultures. Culture of the precursors alone (No MM) served as controls. Data are averages $\pm$ SD ($n = 3$). $*P < 0.05$. All P values were determined by Student's t test.

5 **[0042] FIGS. 18A-B: The efficacy the TP inhibitor to treat MM-induced bone lesions in mouse models.** MM ARP-1 cells were injected into the femurs of SCID mice. Mice without injection of MM cells served as controls (No MM). After 3 weeks, mice were treated with PBS as vehicle controls or TP inhibitor 7DX (200 μ g/kg) or TPI (300 μ g/kg). After treatment, mice were scanned for micro-computed tomography and analyzed using
10 BIOQUANT OSTEO software (BIOQUANT Image Analysis Co.). Shown are the trabecular number (Tb. N) (A) and trabecular thickness (Tb. Th) (B) in mouse femurs. Data are averages $\pm$ SD ($n = 5$ mice per group, three replicate studies). $*P < 0.05$. All P values were determined by Student's t test.

15 **[0043] FIGS. 19A-B: The mechanism of TP-induced upregulation of cytokine production.** The murine osteocytes cell lines (MLO-Y4 and MLO-A5) were cultured in medium without (0) or with 0.5, 1, or 2mM of 2DDR for 48 hours. The mRNA expression of osteolytic cytokine genes *Tnfsf11* (A) and *Sost* (B) in osteocytes were determined by real time PCR analysis and the expression was normalized to cells without 2DDR (set at 1). Data are averages $\pm$ SD ($n = 3$). P values were determined by Student's t test. Each experiment was
20 repeated three times. $*P < 0.05$, $**P < 0.01$.

25 **[0044] FIGS. 20A-B: The effects of the TP inhibitor in the production of osteolytic cytokines RANKL and sclerostin *in vivo*.** MM cells ARP-1 or RPMI8226 (5×10^5 cell/mouse) were injected into the femurs of 6- to 8-week-old SCID mice. In some experiments, PBS (served as vehicle controls) or 7DX (200 μ g/kg) or TPI (300 μ g/kg) were
30 injected into the peritoneum of mice three times a week for two weeks beginning 3 weeks after myeloma cell injection. Mice without MM cell injection served as controls (No MM). After treatment, mouse sera were collected and the serum levels of cytokines levels were measured by ELISA. Shown are the levels of RANKL (A) and sclerostin (B). Data are averages $\pm$ SD ($n = 5$ mice/group). Each experiment was repeated three times. $*P < 0.05$. $**P < 0.01$. All P values were determined by Student's t test.

[0045] FIGS. 21A-B: Correlation between the expression of TP in MM cells and the serum levels of the osteolytic cytokines RANKL and SOST in MM patients. Primary MM cells were isolated from the bone marrow aspirates of newly diagnosed MM patients (n = 20) using anti-CD138 antibody-coated magnetic beads (Miltenyi Biotec, Inc). The matched sera were obtained from Myeloma Tissue Bank in MD Anderson Cancer Center. ELISA was performed to determine the levels of cytokines in serum and quantitative PCR was performed to assess the relative expression levels of TP in primary MM cells. Shown is correlation coefficient between the mRNA expression of TP in MM cells and the serum levels of RANKL (A) and sclerostin (B) in MM patients. *P* values were determined by Pearson coefficient.

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

[0046] Thymidine phosphorylase (TP), also called platelet-derived endothelial cell growth factor, is an enzyme that can reversibly catalyze the conversion of thymidine to thymine and 2-deoxy-D-ribose-1-phosphate (2DDR1P), which is further dephosphorylated into a smaller, more stable molecule, 2-deoxy-D-ribose (2DDR) (Toi *et al.*, 2005). TP has been found in a wide range of normal tissues (Fox *et al.*, 1995), and participates in wound healing and a variety of chronic inflammatory diseases (Bronckaers *et al.*, 2009). TP is highly expressed in many types of cancers, including lung and breast, and plays a role in angiogenesis and anti-apoptosis (Moghaddam *et al.*, 1995; Takebayashi *et al.*, 1996). Clinically, elevated levels of TP are associated with cancer aggressiveness and poor prognosis, but TP has not implicated in the regulation of bone resorption or formation. Accordingly, in certain embodiments, the present disclosure provides methods of treating cancer-induced bone disease by targeting TP.

[0047] Specifically, the present studies discovered an association of cancer-expressed TP with osteolytic bone lesions, as well as the ability of TP to orchestrate osteoclast-mediated resorption and decreased bone formation. Specifically, TP downregulated the expression of IRF8 and thereby activated RANKL-induced NFATc1 expression, leading to an increase in osteoclastogenesis and bone resorption. It was further observed that myeloma-expressed TP suppressed osteoblastogenesis and bone formation by downregulating the expression of RUNX2 and osterix in human MSCs. These findings not only elucidate a mechanism of cancer-induced suppression of osteoblast differentiation and activation of osteoclast differentiation and activity, but also implicate a therapeutic approach for cancer patients with

osteolytic bone lesions by targeting TP. Thus, in some embodiments, the present disclosure provides a therapeutic strategy that targets thymidine phosphorylase (TP) and/or products of thymidine metabolism (*e.g.*, 2DDR) for the treatment of bone disease particularly, cancer-associated bone disease. In particular, inhibition of TP/2DDR suppresses osteoclastogenesis and also helps regeneration of new bone from osteoblasts, resulting in an improved bone formation. Patients with cancer-related bone disease, including those with multiple myeloma and bone-metastatic solid tumors such as breast, lung, and colorectal cancers can be treated by the present methods.

I. Methods of Treatment of Bone Disease

[0048] Provided are methods for treating, preventing or delaying the progression of bone disease in a subject comprising administering an effective amount an inhibitor of thymidine phosphorylase and/or an inhibitor of a product of thymidine metabolism to the subject. In some aspects, the inhibitor of thymidine phosphorylase is tipiracil hydrochloride (TPI), 7-deazaxanthine (7DX), 5'-O-tritylinosine (KIN-59), or 9-(8-phosphonooctyl)-7-deazaxanthine (TP65). Other exemplary TP inhibitors that may be used in the present methods include, but are not limited to, 5-chloro-6-(2-iminopyrrolidin-1-yl)methyl-2,4(1H,3H)-pyrimidinedione (U.S. Patent Publication No. 20100240682), TUPI (5-chloro-6-aminouracil), 7-deazaxanthine, KIN-59 (5'-O-tritylinosine), SHetA2 ([{(4-nitrophenyl)amino}[(2,2,4,4-tetramethyl thiochroman-6-yl)amino]methane-1-thione)), 5-chloro-6-(1-[2-iminopyrrolidinyl]methyl)uracil hydrochloride, and salts thereof. The TP inhibitor may be selected from the group comprising 5-chloro-6-(1-[2-iminopyrrolidinyl]methyl)uracil hydrochloride, 6-imidazolylmethyl-5-fluorouracil, 5-chloro-6-(1-pyrrolidinylmethyl)uracil, 5-bromo-6-(1-pyrrolidinylmethyl)uracil, 5-chloro-6-(1-azetidinylmethyl)-uracil, 5-bromo-6-(1-(2-iminopyrrolidinyl)methyl)uracil hydrochloride, 5-cyano-6-(1-(2-iminopyrrolidinyl)methyl)uracil, 5-chloro-6-(1-(2-iminoimidazolidinyl)methyl) uracil, 5-bromo-6-(1-(2-iminoimidazolidinyl)-methyl) uracil, 5-chloro-6-(1-imidazolylmethyl)uracil hydrochloride, 2-(5-chlorouracil-6-ylmethyl)isothiurea hydrochloride, 2-(5-cyanouracil-6-ylmethyl)isothiurea hydrochloride and 5-chloro-6-(1-guanidino)methyl-uracil hydrochloride. In some aspects, the product of thymidine metabolism is 2DDR. An "effective amount" of the pharmaceutical composition, generally, is defined as that amount sufficient to detectably and repeatedly to achieve the stated desired result, for example, to ameliorate, reduce, minimize or limit the extent of the osteolytic

condition or disease, or its symptoms. In certain aspects, more rigorous definitions may apply, including prevention, elimination, eradication or cure of disease.

[0049] The compounds and methods of the present disclosure may be used for treating, preventing, or inhibiting bone resorption, pathological bone resorption, osteoclast activity, osteoclastogenesis, osteolytic lesions, or other pathologic conditions with associated bone loss or destruction. In certain embodiments, the compounds, such as a TP inhibitor, are used as part of a pharmaceutical composition.

[0050] Examples of such bone diseases include, but are not limited to osteoporosis, including type I osteoporosis, type II osteoporosis, age-related osteoporosis, disuse osteoporosis, diabetes-related osteoporosis, and steroid-related osteoporosis, periodontal disease, osteopenia, osteomalacia, osteolytic bone disease, primary and secondary hyperparathyroidism, multiple myeloma, metastatic cancers of the bone, for example, of the spine, pelvis, limbs, hip, and skull, osteomyelitis, osteoclerotic lesions, osteoblastic lesions, fractures, osteoarthritis, infective arthritis, ankylosing spondylitis, gout, fibrous dysplasia, and Paget's disease of the bone. In some embodiments, the bone disease is selected from the group consisting of bone resorption, osteoarthritis, osteoporosis, osteomalacia, osteitis fibrosa cystica, osteoblastogenesis, osteochondritis dissecans, osteomalacia, osteomyelitis, osteopenia, osteonecrosis, and porotic hyperostosis.

[0051] Examples of cancers that may be associated with bone disease include bladder cancer, breast cancer, clear cell kidney cancer, head/neck squamous cell carcinoma, lung squamous cell carcinoma, melanoma, non-small-cell lung cancer (NSCLC), ovarian cancer, pancreatic cancer, prostate cancer, renal cell cancer, small-cell lung cancer (SCLC), triple negative breast cancer, acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), chronic lymphocytic leukemia (CLL), chronic myeloid leukemia (CML), diffuse large B-cell lymphoma (DLBCL), follicular lymphoma, Hodgkin's lymphoma (HL), mantle cell lymphoma (MCL), multiple myeloma (MM), myeloid cell leukemia-1 protein (Mcl-1), myelodysplastic syndrome (MDS), non-Hodgkin's lymphoma (NHL), or small lymphocytic lymphoma (SLL). In particular aspects, the patient has myeloma-induced bone disease.

[0052] Arthritic conditions include, but are not limited to adjuvant-, collagen-, bacterial- and antigen-induced arthritis, particularly rheumatoid arthritis. Osteolytic lesions include, but are not limited to adamantinoma, aneurysmal bone cyst (lesion), angiosarcoma—

high grade, angiosarcoma—low grade, bone lesions of gaucher's disease, brown tumor of hyperparathyroidism, chondroblastoma, chondromyxoid fibroma, chondrosarcoma, chordoma, clear cell chondrosarcoma, conventional intramedullary osteosarcoma, degenerative joint disease, desmoplastic fibroma, diaphyseal medullary stenosis with malignant fibrous histiocytoma, enchondroma, eosinophilic granuloma, epithelioid hemangioendothelioma, ewing's sarcoma of bone, extraosseous osteosarcoma, fibrosarcoma, fibrous dysplasia, florid reactive periostitis, giant cell tumor, glomus tumor, granulocytic sarcoma in bone, hardcastle's syndrome, hemangioma, hemangiopericytoma, high-grade surface osteosarcoma, hodgkin lymphoma of bone, intracortical osteosarcoma, intraosseous well-differentiated osteosarcoma, juxtacortical chondroma, leukemia, malignant fibrous histiocytoma, melorheostosis, metastatic breast cancer, metastatic kidney cancer, metastatic lung cancer, metastatic prostate cancer, multifocal osteosarcoma, multiple myeloma, myositis ossificans, neurofibroma of bone, non hodgkin lymphoma, nonossifying fibroma (fibrous cortical defect), nora's lesion, osteoblastoma, osteochondroma, osteochondromatosis (hmoce), osteofibrous dysplasia, osteoid osteoma, osteoma, osteomyelitis, osteopathia striata, osteopoikilosis, osteosarcoma, paget's disease, parosteal osteosarcoma, periosteal chondroma, periosteal osteosarcoma, pigmented villonodular synovitis, post-paget's sarcoma, schwannoma of bone, small cell osteosarcoma, solitary bone cyst, solitary fibrous tumor, solitary myeloma (plasmacytoma), subchondral cyst, synovial chondromatosis, telangeatic osteosarcoma, “Tug” lesions—metaphyseal fibrous defect, or unicameral bone cyst

[0053] In some embodiments, the individual has cancer that is resistant (has been demonstrated to be resistant) to one or more anti-cancer therapies. In some embodiments, resistance to anti-cancer therapy includes recurrence of cancer or refractory cancer. Recurrence may refer to the reappearance of cancer, in the original site or a new site, after treatment. In some embodiments, resistance to anti-cancer therapy includes progression of the cancer during treatment with the anti-cancer therapy. In some embodiments, the cancer is at early stage or at late stage.

[0054] In some embodiments, the inhibitor is administered intravenously, intramuscularly, subcutaneously, topically, orally, transdermally, intraperitoneally, intraorbitally, by implantation, by inhalation, intrathecally, intraventricularly, or intranasally. An effective amount of the inhibitor may be administered for prevention or treatment of disease. The appropriate dosage of the agent be determined based on the type of disease to be

treated, severity and course of the disease, the clinical condition of the individual, the individual's clinical history and response to the treatment, and the discretion of the attending physician.

[0055] In certain specific embodiments, it is desired to inhibit osteoclastogenesis or otherwise reverse, hinder or reduce the resorption of bone using the methods and compositions of the present disclosure. The routes of administration will vary, naturally, with the location and nature of the lesion and may include, for example, intradermal, subcutaneous, regional, parenteral, intravenous, intramuscular, intranasal, systemic, and oral administration and formulation.

[0056] Continuous administration also may be applied where appropriate. Delivery via syringe or catheterization is contemplated. Such continuous perfusion may take place for a period from about 1-2 hours, to about 2-6 hours, to about 6-12 hours, to about 12-24 hours, to about 1-2 days, to about 1-2 weeks or longer following the initiation of treatment. Generally, the dose of the therapeutic composition via continuous perfusion will be equivalent to that given by a single or multiple injections, adjusted over a period of time during which the perfusion occurs.

[0057] The treatments may include various "unit doses." Unit dose is defined as containing a predetermined-quantity of the therapeutic composition. The quantity to be administered, and the particular route and formulation, are within the skill of those in the clinical arts. A unit dose need not be administered as a single injection but may comprise continuous infusion over a set period of time. Unit dose of the present disclosure may conveniently be described in terms of mg per volume of formulation or weight (*e.g.*, milligrams or mg) of therapeutic composition.

[0058] In some embodiments, the method for the delivery of a composition comprising one or more compositions of the present disclosure is via systemic administration. However, the pharmaceutical compositions disclosed herein may alternatively be administered parenterally, subcutaneously, intratracheally, intravenously, intradermally, intramuscularly, or even intraperitoneally. Injection may be by syringe or any other method used for injection of a solution.

A. Pharmaceutical Compositions

[0059] Also provided herein are pharmaceutical compositions and formulations comprising the inhibitor of thymidine phosphorylase or product of thymidine metabolism, optionally an anti-cancer agent, and a pharmaceutically acceptable carrier.

5 [0060] Pharmaceutical compositions and formulations as described herein can be prepared by mixing the active ingredients (such as an antibody or a polypeptide) having the desired degree of purity with one or more optional pharmaceutically acceptable carriers (Remington's Pharmaceutical Sciences 22nd edition, 2012), in the form of lyophilized formulations or aqueous solutions. Pharmaceutically acceptable carriers are generally
10 nontoxic to recipients at the dosages and concentrations employed, and include, but are not limited to: buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride; benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about
15 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrans; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (*e.g.* Zn- protein complexes); and/or non-ionic surfactants such as polyethylene glycol (PEG). Exemplary pharmaceutically acceptable carriers herein further include interstitial drug dispersion agents such as soluble neutral-active hyaluronidase glycoproteins (sHASEGP), for example, human soluble PH-20 hyaluronidase glycoproteins,
20 such as rHuPH20 (HYLENEX®, Baxter International, Inc.). Certain exemplary sHASEGPs and methods of use, including rHuPH20, are described in U.S. Patent Publication Nos. 2005/0260186 and 2006/0104968. In one aspect, a sHASEGP is combined with one or more additional glycosaminoglycanases such as chondroitinases.

 [0061] As used herein, "carrier" includes any and all solvents, dispersion media,
30 vehicles, coatings, diluents, antibacterial and antifungal agents, isotonic and absorption delaying agents, buffers, carrier solutions, suspensions, colloids, and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except

insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

[0062] The phrase “pharmaceutically acceptable” refers to molecular entities and compositions that do not produce an allergic or similar untoward reaction when administered to a human. The preparation of an aqueous composition that contains a protein as an active ingredient is well understood in the art. Typically, such compositions are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection can also be prepared.

B. Combination Therapies

[0063] In certain embodiments, the compositions and methods of the present embodiments involve an inhibitor of thymidine phosphorylase or product of thymidine metabolism in combination with at least one additional anti-resorptive agent and/or anti-cancer agent. The anti-cancer agent may be radiation therapy, surgery (*e.g.*, lumpectomy and a mastectomy), chemotherapy, gene therapy, DNA therapy, viral therapy, RNA therapy, immunotherapy, bone marrow transplantation, nanotherapy, monoclonal antibody therapy, or a combination of the foregoing. The anti-resorptive agent may be an anti-RANKL antibody, anti-DKK-1 antibody, bisphosphonate (*e.g.*, alendronate, risedronate, ibandronate, or etidronate), or hormone therapy (*e.g.*, selective estrogen receptor modulator (SERM) or calcitonin). Examples of bisphosphonates include Etidronate, Elodronate, Tiladronate, Pamidronate, Neridronate, Olpadronate, Alendronate, Ibandronate, Residronate, and/or Zoledronate. In some embodiments, the inhibitor is administered with calcium and/or vitamin D. In some embodiments, the inhibitor is administered with compounds for the treatment of osteoporosis. In some embodiments, the inhibitor is administered with Teriparatide, strontium ranelate, raloxifene, and/or Denosumab. The additional therapy may be in the form of adjuvant or neoadjuvant therapy.

[0064] In certain embodiments, the compositions and methods of the present disclosure involve a compound inhibiting or regulating bone resorption, osteoclast activity, and/or osteoclastogenesis, which in turn may be used in combination with other agents or compositions to enhance the effect of other treatments, such as anti-neoplastic treatments, to better the quality of life of a subject being treated. These compositions would be provided in a combined amount effective to achieve the desired effect, for example, the killing or growth

inhibition of a cancer cell and the inhibition of osteoclastogenesis, the activity of osteoclasts, or the resorption of bone. This process may involve contacting the cells with a therapeutic agent, and a second therapeutic agent(s) or multiple factor(s) at the same time. This may be achieved by contacting the cell with a single composition or pharmacological formulation that includes two or more agents, or by contacting the cell with two or more distinct compositions or formulations wherein at least one composition includes a therapeutic agent described herein and one or more other compositions includes at least a second therapeutic agent.

[0065] In some embodiments, the anti-cancer agent is the administration of small molecule enzymatic inhibitor or anti-metastatic agent. In some embodiments, the anti-cancer is the administration of side-effect limiting agents (*e.g.*, agents intended to lessen the occurrence and/or severity of side effects of treatment, such as anti-nausea agents, *etc.*). In some embodiments, the anti-cancer agent is radiation therapy. In some embodiments, the anti-cancer therapy is surgery. In some embodiments, the anti-cancer therapy is a combination of radiation therapy and surgery. In some embodiments, the anti-cancer therapy is gamma irradiation. In some embodiments, the anti-cancer therapy is therapy targeting PBK/AKT/mTOR pathway, HSP90 inhibitor, tubulin inhibitor, apoptosis inhibitor, and/or chemopreventative agent. The anti-cancer therapy may be one or more of the chemotherapeutic agents known in the art.

[0066] Various combinations may be employed. For the example below a TP inhibitor therapy and/or inhibitor of thymidine metabolism is “A” and an anti-resorptive or anti-cancer therapy is “B”:

A/B/A B/A/B B/B/A A/A/B A/B/B B/A/A A/B/B/B B/A/B/B
 B/B/B/A B/B/A/B A/A/B/B A/B/A/B A/B/B/A B/B/A/A
 B/A/B/A B/A/A/B A/A/A/B B/A/A/A A/B/A/A A/A/B/A

[0067] Administration of any compound or therapy of the present embodiments to a patient will follow general protocols for the administration of such compounds, taking into account the toxicity, if any, of the agents. Therefore, in some embodiments there is a step of monitoring toxicity that is attributable to combination therapy.

1. Chemotherapy

[0068] A wide variety of chemotherapeutic agents may be used in accordance with the present embodiments. The term “chemotherapy” refers to the use of drugs to treat cancer. A “chemotherapeutic agent” is used to connote a compound or composition that is administered in the treatment of cancer. These agents or drugs are categorized by their mode of activity within a cell, for example, whether and at what stage they affect the cell cycle. Alternatively, an agent may be characterized based on its ability to directly cross-link DNA, to intercalate into DNA, or to induce chromosomal and mitotic aberrations by affecting nucleic acid synthesis.

[0069] Examples of chemotherapeutic agents include alkylating agents, such as thiotepa and cyclophosphamide; alkyl sulfonates, such as busulfan, improsulfan, and piposulfan; aziridines, such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines, including altretamine, triethylenemelamine, trietylenephosphoramide, triethylenethiophosphoramide, and trimethylolomelamine; acetogenins (especially bullatacin and bullatacinone); a camptothecin (including the synthetic analogue topotecan); bryostatin; callystatin; CC-1065 (including its adozelesin, carzelesin and bizelesin synthetic analogues); cryptophycins (particularly cryptophycin 1 and cryptophycin 8); dolastatin; duocarmycin (including the synthetic analogues, KW-2189 and CB1-TM1); eleutherobin; pancratistatin; a sarcodictyin; spongistatin; nitrogen mustards, such as chlorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, and uracil mustard; nitrosureas, such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, and ranimustine; antibiotics, such as the enediyne antibiotics (e.g., calicheamicin, especially calicheamicin gammaII and calicheamicin omegaII); dynemicin, including dynemicin A; bisphosphonates, such as clodronate; an esperamicin; as well as neocarzinostatin chromophore and related chromoprotein enediyne antibiotic chromophores, aclacinomysins, actinomycin, authrarnycin, azaserine, bleomycins, cactinomycin, carabycin, carminomycin, carzinophilin, chromomycinis, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, doxorubicin (including morpholino-doxorubicin, cyanomorpholino-doxorubicin, 2-pyrrolino-doxorubicin and deoxydoxorubicin), epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins, such as mitomycin C, mycophenolic acid, nogalarnycin, olivomycins, peplomycin, potfiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozocin,

tubercidin, ubenimex, zinostatin, and zorubicin; anti-metabolites, such as methotrexate and 5-fluorouracil (5-FU); folic acid analogues, such as denopterin, pteropterin, and trimetrexate; purine analogs, such as fludarabine, 6-mercaptopurine, thiamiprine, and thioguanine; pyrimidine analogs, such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, and floxuridine; androgens, such as calusterone, dromostanolone propionate, epitio stanol, mepitio stanane, and testolactone; anti-adrenals, such as mitotane and trilostane; folic acid replenisher, such as frolinic acid; aceglatone; aldophosphamide glycoside; aminolevulinic acid; eniluracil; amsacrine; bestrabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elformithine; elliptinium acetate; an epothilone; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidainine; maytansinoids, such as maytansine and ansamitocins; mitoguazone; mitoxantrone; mopidanmol; nitraerine; pentostatin; phenamet; pirarubicin; losoxantrone; podophyllinic acid; 2-ethylhydrazide; procarbazine; PSK polysaccharide complex; razoxane; rhizoxin; sizofiran; spirogermanium; tenuazonic acid; triaziquone; 2,2',2''-trichlorotriethylamine; trichothecenes (especially T-2 toxin, verracurin A, roridin A and anguidine); urethan; vindesine; dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); cyclophosphamide; taxoids, e.g., paclitaxel and docetaxel; gemcitabine; 6-thioguanine; mercaptopurine; platinum coordination complexes, such as cisplatin, oxaliplatin, and carboplatin; vinblastine; platinum; etoposide (VP-16); ifosfamide; mitoxantrone; vincristine; vinorelbine; novantrone; teniposide; edatrexate; daunomycin; aminopterin; xeloda; ibandronate; irinotecan (e.g., CPT-11); topoisomerase inhibitor RFS 2000; difluoromethylornithine (DMFO); retinoids, such as retinoic acid; capecitabine; carboplatin, procarbazine, plicomycin, gemcitabine, navelbine, farnesyl-protein transferase inhibitors, transplatinum, and pharmaceutically acceptable salts, acids, or derivatives of any of the above.

2. Radiotherapy

[0070] Other factors that cause DNA damage and have been used extensively include what are commonly known as γ -rays, X-rays, and/or the directed delivery of radioisotopes to tumor cells. Other forms of DNA damaging factors are also contemplated, such as microwaves, proton beam irradiation, and UV-irradiation. It is most likely that all of these factors affect a broad range of damage on DNA, on the precursors of DNA, on the replication and repair of DNA, and on the assembly and maintenance of chromosomes. Dosage ranges for X-rays range from daily doses of 50 to 200 roentgens for prolonged periods of time (3 to

4 wk), to single doses of 2000 to 6000 roentgens. Dosage ranges for radioisotopes vary widely, and depend on the half-life of the isotope, the strength and type of radiation emitted, and the uptake by the neoplastic cells.

3. Immunotherapy

5 [0071] The skilled artisan will understand that additional immunotherapies may be used in combination or in conjunction with methods of the embodiments. In the context of cancer treatment, immunotherapeutics, generally, rely on the use of immune effector cells and molecules to target and destroy cancer cells. Rituximab (RITUXAN®) is such an example. The immune effector may be, for example, an antibody specific for some marker
10 on the surface of a tumor cell. The antibody alone may serve as an effector of therapy or it may recruit other cells to actually affect cell killing. The antibody also may be conjugated to a drug or toxin (chemotherapeutic, radionuclide, ricin A chain, cholera toxin, pertussis toxin, etc.) and serve as a targeting agent. Alternatively, the effector may be a lymphocyte carrying a surface molecule that interacts, either directly or indirectly, with a tumor cell target.
15 Various effector cells include cytotoxic T cells and NK cells

 [0072] Antibody-drug conjugates have emerged as a breakthrough approach to the development of cancer therapeutics. Cancer is one of the leading causes of deaths in the world. Antibody–drug conjugates (ADCs) comprise monoclonal antibodies (MAbs) that are covalently linked to cell-killing drugs. This approach combines the high specificity of MAbs
20 against their antigen targets with highly potent cytotoxic drugs, resulting in “armed” MAbs that deliver the payload (drug) to tumor cells with enriched levels of the antigen. Targeted delivery of the drug also minimizes its exposure in normal tissues, resulting in decreased toxicity and improved therapeutic index. The approval of two ADC drugs, ADCETRIS® (brentuximab vedotin) in 2011 and KADCYLA® (trastuzumab emtansine or T-DM1) in
25 2013 by FDA validated the approach. There are currently more than 30 ADC drug candidates in various stages of clinical trials for cancer treatment (Leal *et al.*, 2014). As antibody engineering and linker-payload optimization are becoming more and more mature, the discovery and development of new ADCs are increasingly dependent on the identification and validation of new targets that are suitable to this approach and the generation of targeting
30 MAbs. Two criteria for ADC targets are upregulated/high levels of expression in tumor cells and robust internalization.

[0073] In one aspect of immunotherapy, the tumor cell must bear some marker that is amenable to targeting, *i.e.*, is not present on the majority of other cells. Many tumor markers exist and any of these may be suitable for targeting in the context of the present embodiments. Common tumor markers include CD20, carcinoembryonic antigen, tyrosinase (p97), gp68, TAG-72, HMFG, Sialyl Lewis Antigen, MucA, MucB, PLAP, laminin receptor, erb B, and p155. An alternative aspect of immunotherapy is to combine anticancer effects with immune stimulatory effects. Immune stimulating molecules also exist including: cytokines, such as IL-2, IL-4, IL-12, GM-CSF, gamma-IFN, chemokines, such as MIP-1, MCP-1, IL-8, and growth factors, such as FLT3 ligand.

[0074] Examples of immunotherapies are immune adjuvants, *e.g.*, *Mycobacterium bovis*, *Plasmodium falciparum*, dinitrochlorobenzene, and aromatic compounds (U.S. Patents 5,801,005 and 5,739,169); cytokine therapy, *e.g.*, interferons α , β , and γ , IL-1, GM-CSF, and; gene therapy, *e.g.*, TNF, IL-1, IL-2, and p53 (U.S. Patents 5,830,880 and 5,846,945); and monoclonal antibodies, *e.g.*, anti-CD20, anti-ganglioside GM2, and anti-p185 (U.S. Patent 5,824,311). It is contemplated that one or more anti-cancer therapies may be employed with the antibody therapies described herein.

[0075] In some embodiments, the immunotherapy may be an immune checkpoint inhibitor. Immune checkpoints are molecules in the immune system that either turn up a signal (*e.g.*, co-stimulatory molecules) or turn down a signal. Inhibitory checkpoint molecules that may be targeted by immune checkpoint blockade include adenosine A2A receptor (A2AR), B7-H3 (also known as CD276), B and T lymphocyte attenuator (BTLA), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4, also known as CD152), indoleamine 2,3-dioxygenase (IDO), killer-cell immunoglobulin (KIR), lymphocyte activation gene-3 (LAG3), programmed death 1 (PD-1), T-cell immunoglobulin domain and mucin domain 3 (TIM-3) and V-domain Ig suppressor of T cell activation (VISTA). In particular, the immune checkpoint inhibitors target the PD-1 axis and/or CTLA-4.

[0076] The immune checkpoint inhibitors may be drugs such as small molecules, recombinant forms of ligand or receptors, or, in particular, are antibodies, such as human antibodies (*e.g.*, International Patent Publication No. WO2015016718). Known inhibitors of the immune checkpoint proteins or analogs thereof may be used, in particular chimerized, humanized or human forms of antibodies may be used. As the skilled person will know, alternative and/or equivalent names may be in use for certain antibodies mentioned in the

present disclosure. Such alternative and/or equivalent names are interchangeable in the context of the present invention. For example, it is known that lambrolizumab is also known under the alternative and equivalent names MK-3475 and pembrolizumab.

[0077] In some embodiments, the PD-1 binding antagonist is a molecule that inhibits the binding of PD-1 to its ligand binding partners. In a specific aspect, the PD-1 ligand binding partners are PDL1 and/or PDL2. In another embodiment, a PDL1 binding antagonist is a molecule that inhibits the binding of PDL1 to its binding partners. In a specific aspect, PDL1 binding partners are PD-1 and/or B7-1. In another embodiment, the PDL2 binding antagonist is a molecule that inhibits the binding of PDL2 to its binding partners. In a specific aspect, a PDL2 binding partner is PD-1. The antagonist may be an antibody, an antigen binding fragment thereof, an immunoadhesin, a fusion protein, or oligopeptide. Exemplary antibodies are described in U.S. Patent Nos. 8,735,553, 8,354,509, and 8,008,449, all incorporated herein by reference. Other PD-1 axis antagonists for use in the methods provided herein are known in the art such as described in U.S. Patent Application Nos. 20140294898, 2014022021, and 20110008369, all incorporated herein by reference.

[0078] In some embodiments, the PD-1 binding antagonist is an anti-PD-1 antibody (*e.g.*, a human antibody, a humanized antibody, or a chimeric antibody). In some embodiments, the anti-PD-1 antibody is selected from the group consisting of nivolumab, pembrolizumab, and CT-011. In some embodiments, the PD-1 binding antagonist is an immunoadhesin (*e.g.*, an immunoadhesin comprising an extracellular or PD-1 binding portion of PDL1 or PDL2 fused to a constant region (*e.g.*, an Fc region of an immunoglobulin sequence)). In some embodiments, the PD-1 binding antagonist is AMP-224. Nivolumab, also known as MDX-1106-04, MDX-1106, ONO-4538, BMS-936558, and OPDIVO®, is an anti-PD-1 antibody described in WO2006/121168. Pembrolizumab, also known as MK-3475, Merck 3475, lambrolizumab, KEYTRUDA®, and SCH-900475, is an anti-PD-1 antibody described in WO2009/114335. CT-011, also known as hBAT or hBAT-1, is an anti-PD-1 antibody described in WO2009/101611. AMP-224, also known as B7-DCIg, is a PDL2-Fc fusion soluble receptor described in WO2010/027827 and WO2011/066342.

[0079] Another immune checkpoint that can be targeted in the methods provided herein is the cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), also known as CD152. The complete cDNA sequence of human CTLA-4 has the Genbank accession number L15006. CTLA-4 is found on the surface of T cells and acts as an “off” switch when bound to

CD80 or CD86 on the surface of antigen-presenting cells. CTLA4 is a member of the immunoglobulin superfamily that is expressed on the surface of Helper T cells and transmits an inhibitory signal to T cells. CTLA4 is similar to the T-cell co-stimulatory protein, CD28, and both molecules bind to CD80 and CD86, also called B7-1 and B7-2 respectively, on antigen-presenting cells. CTLA4 transmits an inhibitory signal to T cells, whereas CD28 transmits a stimulatory signal. Intracellular CTLA4 is also found in regulatory T cells and may be important to their function. T cell activation through the T cell receptor and CD28 leads to increased expression of CTLA-4, an inhibitory receptor for B7 molecules.

[0080] In some embodiments, the immune checkpoint inhibitor is an anti-CTLA-4 antibody (*e.g.*, a human antibody, a humanized antibody, or a chimeric antibody), an antigen binding fragment thereof, an immunoadhesin, a fusion protein, or oligopeptide.

[0081] Anti-human-CTLA-4 antibodies (or VH and/or VL domains derived therefrom) suitable for use in the present methods can be generated using methods well known in the art. Alternatively, art recognized anti-CTLA-4 antibodies can be used. For example, the anti-CTLA-4 antibodies disclosed in: US 8,119,129, WO 01/14424, WO 98/42752; WO 00/37504 (CP675,206, also known as tremelimumab; formerly ticilimumab), U.S. Patent No. 6,207,156 can be used in the methods disclosed herein. The teachings of each of the aforementioned publications are hereby incorporated by reference. Antibodies that compete with any of these art-recognized antibodies for binding to CTLA-4 also can be used. For example, a humanized CTLA-4 antibody is described in International Patent Publication No. WO2001014424, WO2000037504, and U.S. Patent No. 8,017,114; all incorporated herein by reference.

[0082] An exemplary anti-CTLA-4 antibody is ipilimumab (also known as 10D1, MDX- 010, MDX- 101, and Yervoy®) or antigen binding fragments and variants thereof (see, *e.g.*, WO2001014424). In other embodiments, the antibody comprises the heavy and light chain CDRs or VRs of ipilimumab. Accordingly, in one embodiment, the antibody comprises the CDR1, CDR2, and CDR3 domains of the VH region of ipilimumab, and the CDR1, CDR2 and CDR3 domains of the VL region of ipilimumab. In another embodiment, the antibody competes for binding with and/or binds to the same epitope on CTLA-4 as the above- mentioned antibodies. In another embodiment, the antibody has at least about 90% variable region amino acid sequence identity with the above-mentioned antibodies (*e.g.*, at least about 90%, 95%, or 99% variable region identity with ipilimumab).

[0083] Other molecules for modulating CTLA-4 include CTLA-4 ligands and receptors such as described in U.S. Patent Nos. 5844905, 5885796 and International Patent Application Nos. WO1995001994 and WO1998042752; all incorporated herein by reference, and immunoadhesions such as described in U.S. Patent No. 8329867, incorporated herein by
5 reference.

4. Surgery

[0084] Approximately 60% of persons with cancer will undergo surgery of some type, which includes preventative, diagnostic or staging, curative, and palliative surgery. Curative surgery includes resection in which all or part of cancerous tissue is physically
10 removed, excised, and/or destroyed and may be used in conjunction with other therapies, such as the treatment of the present embodiments, chemotherapy, radiotherapy, hormonal therapy, gene therapy, immunotherapy, and/or alternative therapies. Tumor resection refers to physical removal of at least part of a tumor. In addition to tumor resection, treatment by surgery includes laser surgery, cryosurgery, electrosurgery, and microscopically-controlled
15 surgery (Mohs' surgery).

[0085] Upon excision of part or all of cancerous cells, tissue, or tumor, a cavity may be formed in the body. Treatment may be accomplished by perfusion, direct injection, or local application of the area with an additional anti-cancer therapy. Such treatment may be repeated, for example, every 1, 2, 3, 4, 5, 6, or 7 days, or every 1, 2, 3, 4, and 5 weeks or
20 every 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 months. These treatments may be of varying dosages as well.

5. Other Agents

[0086] It is contemplated that other agents may be used in combination with certain aspects of the present embodiments to improve the therapeutic efficacy of treatment. These
25 additional agents include agents that affect the upregulation of cell surface receptors and GAP junctions, cytostatic and differentiation agents, inhibitors of cell adhesion, agents that increase the sensitivity of the hyperproliferative cells to apoptotic inducers, or other biological agents. Increases in intercellular signaling by elevating the number of GAP junctions would increase the anti-hyperproliferative effects on the neighboring
30 hyperproliferative cell population. In other embodiments, cytostatic or differentiation agents can be used in combination with certain aspects of the present embodiments to improve the

anti-hyperproliferative efficacy of the treatments. Inhibitors of cell adhesion are contemplated to improve the efficacy of the present embodiments. Examples of cell adhesion inhibitors are focal adhesion kinase (FAKs) inhibitors and Lovastatin. It is further contemplated that other agents that increase the sensitivity of a hyperproliferative cell to apoptosis, such as the antibody c225, could be used in combination with certain aspects of the present embodiments to improve the treatment efficacy.

II. Articles of Manufacture or Kits

[0087] An article of manufacture or a kit is provided comprising an inhibitor of TP or product of thymidine metabolism for the treatment of bone disease is also provided herein.

The article of manufacture or kit can further comprise a package insert comprising instructions for using the inhibitor of TP to treat or delay progression of cancer in an individual or to enhance immune function of an individual having cancer. Any of the TP inhibitors described herein may be included in the article of manufacture or kits. Suitable containers include, for example, bottles, vials, bags and syringes. The container may be formed from a variety of materials such as glass, plastic (such as polyvinyl chloride or polyolefin), or metal alloy (such as stainless steel or hastelloy). In some embodiments, the container holds the formulation and the label on, or associated with, the container may indicate directions for use. The article of manufacture or kit may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, syringes, and package inserts with instructions for use. In some embodiments, the article of manufacture further includes one or more of another agent (*e.g.*, a chemotherapeutic agent, and anti-neoplastic agent). Suitable containers for the one or more agent include, for example, bottles, vials, bags and syringes.

III. Examples

[0088] The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments

which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

Example 1 – Thymidine phosphorylase exerts complex effects on bone resorption and formation in myeloma

5 **[0089]** *Myeloma-expressed TP enhances lytic bone lesions:* The presence of myeloma (CD138⁺) cells in tissue array biopsies was confirmed, and it was found that TP⁺ cells were significantly greater in the bone marrow from 14 myeloma patients than 14 healthy donors (FIG. 1, A and B). TP was expressed in a majority of bone marrow aspirates of primary myeloma cells (4 of 6 patients) and in a majority of established human myeloma cell lines (4
10 of 6), but not in aspirates of plasma cells from normal subjects (FIG. 1C).

[0090] To determine whether TP expression correlated with the frequency of bone lesions in myeloma patients, two experiments were performed using samples from tissue banks. In the first, there was a strong positive correlation between *TP* gene expression in myeloma cells and bone lesion numbers in 52 patients (FIG. 2A); in the second, there was a
15 robust positive correlation between TP immunohistochemistry and bone lesion numbers (FIG. 2B). TP expression was higher in myeloma cells from patients with high bone lesion scores than those from patients with low lesion numbers (FIG. 2C).

[0091] Primary myeloma cells were isolated from the bone marrow aspirates of 10 newly diagnosed patients. Based on the TP expression in myeloma cells as detected by
20 western blot, the aspirates were separated into high and low TP expression groups: TP_{high} and TP_{low} (*n* = 5 patients' bone marrow aspirates/group) (FIG. 8A). To determine the functional role of myeloma-expressed TP in lytic bone lesions, TP_{high} or TP_{low} cells were injected into human bone chips that had been implanted into severe combined immunodeficient (SCID)-hu mice. Mice with no tumor cells (but with bone chips implanted) were used as controls. More
25 lytic lesions and a lower percentage of bone volume vs total volume (BV/TV) were observed in the bone chips of mice injected with TP_{high} than TP_{low} cells (FIG. 2, D and H). Moreover, injecting WT ARP-1 cells, which have high TP expression, into mouse femurs caused more lytic lesions than WT MM.1S cells, which have low TP expression (FIG. 2, E and H; FIG. 8B). ARP-1 cells with TP knocked down by shRNAs (sh*TP* ARP-1) were injected into mouse
30 femurs and caused fewer lytic lesions than ARP-1 cells with a non-targeted shRNA (sh*Ctrl* ARP-1) (FIG. 2, F and H; FIG. 8C). Conversely, MM.1S cells expressing *TP* cDNA (*TP*

MM.1S) caused more femur lesions than WT MM.1S cells expressing a control vector (*Vec* MM.1S) (FIG. 2, G and H; FIG. 8D). In summary, cells with high TP expression induced the formation of lytic lesions in mouse and human bone.

[0092] To determine whether lytic lesions result from a change in the tumor burden within the marrow milieu, the levels of myeloma-secreted M-protein, a reflection of tumor burden, were measured in the mouse sera. The modulation of TP expression in myeloma cells did not change the serum levels of M-protein (FIG. 8E). In addition, there were no differences in viability or apoptosis in control and modified ARP-1 or MM.1S cell lines (FIG. 8, F and G), indicating that changes in TP expression did not affect myeloma cell growth or survival. Together, these results reveal that myeloma cells express TP and enhance lytic bone lesions in patients and mice with myeloma.

[0093] *Myeloma-expressed TP enhances RANKL-induced osteoclastogenesis and bone resorption:* The ability of myeloma-expressed TP to regulate osteoclast differentiation was evaluated *in vitro* using a standard osteoclast differentiation protocol (Yaccoby *et al.*, 2004) in which TRAP⁺ cell number and TRAP5b secretion were assessed in the presence of RANKL (10 ng/ml) and M-CSF (25 ng/ml)—both of which are needed for osteoclast formation. Co-culture of preOCs with TP_{high} patient myeloma cells or WT ARP-1 cells induced higher numbers of multinuclear TRAP⁺ cells (FIG. 9A), more TRAP5b secretion (FIG. 9B), and higher expression of the osteoclast genes *TRAP*, *CALCR*, and *CTSK* (FIG. 9, C and D) than co-culture with cells expressing low levels of TP (TP_{low} or WT MM.1S). Knockdown of TP expression in WT ARP-1 cells reduced osteoclast differentiation and activity (FIG. 9, A, B, and E). In contrast, overexpression of TP in WT MM.1S cells enhanced the differentiation and activity (FIG. 9, A, B, and F).

[0094] To assess the role of myeloma-expressed TP in osteoclast-mediated bone resorption *in vivo*, myeloma-bearing human bone chips or mouse femurs were stained for TRAP and analyzed the percentage of bone surface eroded by osteoclasts (ES/BS) and the percentage of bone surface covered with osteoclasts (Oc. S/BS). The percentage of ES/BS (FIG. 3A) and Oc. S/BS were higher in the mice injected with myeloma cells that had high TP expression (TP_{high}, WT ARP-1, *shCtrl* ARP-1 or *TP* MM.1S) than those with low TP expression (TP_{low}, *shTP* ARP-1, WT MM.1S or *Vec* MM.1S) (FIG. 3, A and B). These results indicate that myeloma-expressed TP enhances osteoclastogenesis.

[0095] In preOCs, myeloma cells have been shown to enhance the expression of NFATc1, a transcriptional factor that plays a pivotal role in osteoclast gene expression and can be upregulated by RANKL (Takayanagi *et al.*, 2002). This effect of myeloma cells on NFATc1 expression was confirmed in preOCs (FIG. 10A), and further demonstrated that myeloma cells with high but not low TP downregulated expression of the transcription factor *IRF8* in preOCs (FIG. 10A). To determine whether *IRF8* mediates TP-induced osteoclast differentiation, its expression in preOCs was knocked down using *IRF8* shRNA (*shIRF8*). When co-cultured with WT ARP-1 cells, *shIRF8*-preOCs expressed higher levels of NFATc1 protein (FIG. 10B) and secreted more TRAP5b (FIG. 10C) than control preOCs. Thus, myeloma-expressed TP abrogated the inhibitory effect of *IRF8* on NFATc1 shown previously (Bruzzaniti and Baron, 2006), leading to the promotion of osteoclast gene expression and osteoclastogenesis.

[0096] *Myeloma-expressed TP inhibits osteoblastogenesis in vitro and bone formation in vivo*: To determine whether myeloma-expressed TP regulates osteoblast differentiation *in vitro*, the precursors of osteoblasts, MSCs, were co-cultured with patient myeloma cells and myeloma cell lines in osteoblast medium for 2 weeks. MSCs cultured alone in this medium served as a positive control. Mature osteoblasts produced soluble alkaline phosphatase, were positive for Alizarin red S staining (which is indicative of osteoblast-mediated bone formation activity), and expressed osteoblast differentiation-associated genes (FIG. 11). In line with previous studies (Yaccoby, 2010), co-culture of MSCs with myeloma cells inhibited osteoblast activity, but co-culture with TP_{low} myeloma cells and low-TP-expressing myeloma cell lines (WT MM.1S, *Vec* MM.1S, or *shTP* ARP-1) had comparatively more mature osteoblasts than cells with high levels of TP (FIG. 11).

[0097] To determine the role of myeloma-expressed TP in osteoblast-mediated bone formation *in vivo*, myeloma-bearing human bone chips or mouse femurs were collected and the osteoblasts localized on trabecular bone were enumerated and the percentage of osteoid surface (OS/BS) and of bone surface lined with osteoblasts was counted (Ob. S/BS). The percentages of OS/BS (FIG. 3C) and Ob. S/BS (FIG. 3D) were lower in the mice injected with myeloma cells expressing high level of TP (TP_{high}, WT ARP-1, *shCtrl* ARP-1 or *TP* MM.1S) than low-TP myeloma cells (TP_{low}, *shTP* ARP-1, WT MM.1S or *Vec* MM.1S). In agreement with these data, bone formation rate was increased in mice injected with *shTP* ARP-1 cells (FIG. 3E) and decreased in mice with *TP* MM.1S cells (FIG. 3F).

[0098] Transcription factors such as *RUNX2* and *osterix* promote osteoblast differentiation by upregulating the expression of osteoblast differentiation-associated genes (Komori, 2006). In line with previous studies (20), it was observed that co-culture of MSCs with myeloma cells reduced the expression of *RUNX2* and *osterix* (FIG. 12). Moreover, the expression of these transcription factors was significantly lower when co-cultured with myeloma cells with high levels of TP than with lower TP levels (FIG. 12A). Moreover, myeloma-expressed TP modulated the binding activity of *RUNX2* and *osterix* to the promoter of osteoblast genes *BGLAP* and *COL1A1* (FIG. 12, B and C), a finding consistent with the effect of TP on bone formation.

[0099] *TP downregulates the expression of RUNX2, osterix, and IRF8 via hypermethylation of CpG islands:* DNA methylation of CpG dinucleotides is a key epigenetic modification that influences tissue- and context-specific gene expression (Attwood *et al.*, 2002) and is generally associated with gene silencing (Rishi *et al.*, 2010). To determine whether myeloma-expressed TP regulates the methylation of CpG islands (CGI) in *RUNX2*, *osterix*, and *IRF8*, methylation-specific PCR and bisulfite sequencing PCR primers targeting their CpG-rich regions were designed (FIG. 4A; Table 2). Methylation of *RUNX2* and *osterix* in MSCs and *IRF8* in preOCs was higher in the co-culture of myeloma cells with high TP expression (WT ARP-1, shCtrl ARP-1, and TP MM.1S) than in the co-culture of those with low TP expression (shTP ARP-1, WT MM.1S, and Vec MM.1S) (FIG. 4B). Bisulfite sequencing PCR analysis confirmed these results (FIG. 4, C-E), and similar methylation data were obtained from healthy individual and myeloma patient samples expressing different levels of TP (FIG. 4F).

[00100] DNA methyltransferases (DNMTs) are important for the methylation of gene promoters. Higher gene expression and enzyme activity of DNMT3A—but not DNMT1 or DNMT3B—was observed in MSCs and preOCs co-cultured with high-TP myeloma cells (WT ARP-1, shCtrl ARP-1, and TP MM.1S) than with low-TP cells (shTP ARP-1, WT MM.1S, and Vec MM.1S) (FIG. 13, A and B). The clinical relevance was further studied by correlating *TP* expression in myeloma cells from patients or number of bone lesions in these patients with *DNMT3A* expression in MSCs and preOCs from the same patients. Positive correlations were found between *TP* expression and number of bone lesions with *DNMT3A* in MSCs or preOCs (FIG. 13C). Collectively these data demonstrate that myeloma-expressed TP upregulates DNMT3A expression and activity in MSCs and preOCs,

consequently enhancing methylation of CGIs in the promoters of *RUNX2*, *osterix*, and *IRF8* genes, suppressing osteoblast differentiation, and increasing osteoclast activity. These signaling changes shift the balance of bone remodeling toward a net loss of bone tissue.

[00101] *TP upregulates DNMT3A expression through 2DDR-mediated signaling:* TP degrades thymidine into thymine and 2DDR1P; the unstable 2DDR1P is further dephosphorylated to 2DDR (Bronckaers *et al.*, 2009). Not unexpectedly, *in vitro* myeloma cells with high levels of TP secreted more 2DDR than low-TP cells (FIG. 14A). Similarly, higher levels of 2DDR were observed in the serum of mice bearing high-TP myeloma cells (*TP* MM.1S, WT ARP-1) than in mice bearing low-TP cells (WT MM.1S, sh*TP* ARP-1) (FIG. 14B).

[00102] To test whether 2DDR mediates TP-induced suppression of osteoblastogenesis, MSCs were cultured in osteoblast medium with incremental doses of 2DDR for 2 weeks. The addition of 2DDR downregulated ALP production, Alizarin red S staining, and the expression of osteoblast genes in a dose-dependent manner (FIG. 14C). Furthermore, the addition of 2DDR decreased the expression of *RUNX2* and *osterix* (FIG. 5A), and upregulated the expression and activity of *DNMT3A* in MSCs (FIG. 5B). Culture of MSCs in medium with 2DDR induced the hypermethylation of CGIs on the *RUNX2* and *osterix* genes (FIG. 5C) and inhibited ALP production and Alizarin red S staining (FIG. 5D). Knockdown of *DNMT3A* in MSCs partially abrogated the effects of 2DDR (FIG. 5, C and D).

[00103] To examine the role of 2DDR in the activation of osteoclastogenesis, preOCs were cultured with RANKL (10 ng/ml) and M-CSF (25 ng/ml) without or with escalating doses of 2DDR for 1 week. 2DDR enhanced multinuclear TRAP⁺ cell numbers, TRAP5b secretion, and the expression of the osteoclast genes *TRAP*, *CALCR*, and *CTSK* in a dose-dependent manner (FIG. 14D). 2DDR also reduced *IRF8* expression (FIG. 5E), upregulated *DNMT3A* expression (FIG. 5F) and induced hypermethylation of CGIs in *IRF8* (FIG. 5G). Knocking down *DNMT3A* in preOCs reversed 2DDR-mediated *IRF8* hypermethylation (FIG. 5G) and increased osteoclast formation (FIG. 5H).

[00104] The signaling pathways by which 2DDR may regulate *DNMT3A* expression was investigated next. 2DDR is known to bind integrins $\alpha_v\beta_3$ and $\alpha_5\beta_1$ (Bronckaers *et al.*, 2009). It is known that all MSCs in this study express both integrins (Marie, 2013), and that preOCs express $\alpha_v\beta_3$ (Nakamura *et al.*, 1999). The addition of an

antibody against either the α_5 subunit of $\alpha_5\beta_1$ or the α_v subunit of $\alpha_v\beta_3$, but not control IgG, to the co-culture of MSCs with 2DDR enhanced osteoblast formation; the addition of both anti- α_5 and anti- α_v antibodies had a synergistic effect (FIG. 15A). Moreover, application of both antibodies inhibited 2DDR-stimulated *DNMT3A* expression in MSCs (FIG. 15B),
 5 indicating that 2DDR regulates osteoblast differentiation and *DNMT3A* expression via $\alpha_v\beta_3$ and/or $\alpha_5\beta_1$.

[00105] Furthermore, the ERK and PI3K/Akt signaling pathways, downstream of $\alpha_5\beta_1$ and $\alpha_v\beta_3$, were examined. The addition of 2DDR to culture of MSCs upregulated the phosphorylated levels of Akt and the integrin-mediated downstream molecules focal adhesion
 10 kinase (FAK), Paxillin, and p130Cas, but did not change phosphorylated levels of ERK or non-phosphorylated levels of these molecules (FIG. 15C). Akt phosphorylation induced by 2DDR was abrogated with antibodies against both α_5 and α_v (FIG. 15D). An Akt inhibitor LY294002 blocked 2DDR-induced *DNMT3A* expression in MSCs (FIG. 15E).

[00106] Addition of anti- α_v antibodies to the culture of preOCs reduced
 15 2DDR-induced TRAP5b secretion and *DNMT3A* expression (FIG. 15, F-G). Addition of 2DDR upregulated the levels of phosphorylated Akt, FAK, Paxillin, and p130Cas in cultured preOCs (FIG. 15H), and blocking α_v with the antibodies (FIG. 15I) reduced the phosphorylation of Akt. Similar to the results in MSCs, administration of LY294002 significantly reduced 2DDR-induced *DNMT3A* expression in preOCs (FIG. 15J).

[00107] To validate the antibody blocking study, α_v and/or α_5 expression was
 20 knocked down in MSCs using siRNAs (FIG. 16A). Adding 2DDR to cultures of si α_v or si α_5 MSCs reduced osteoblast formation, *DNMT3A* expression, and Akt phosphorylation in MSCs (FIG. 16, B-D). The siRNAs against Akt1/2 blocked 2DDR-induced *DNMT3A* expression in MSCs (FIG. 16E-F). Moreover, knockdown of α_v expression in preOCs reduced 2DDR-
 25 induced TRAP5b secretion and *DNMT3A* expression (FIG. 16, G-I) and knockdown of Akt1/2 (FIG. 16J) significantly reduced 2DDR-induced *DNMT3A* mRNA (FIG. 16, K-L). These results suggest that TP regulates 2DDR secretion from myeloma cells, and 2DDR enhances DNA methylation of *RUNX2* and *osterix* in MSCs and *IRF8* in preOCs through the $\alpha_v\beta_3/\alpha_5\beta_1$ -PI3K/Akt-*DNMT3A*. signaling pathway.

[00108] *Inhibiting TP reduces myeloma-induced osteolytic bone lesions:*

Toward a therapeutic, it was asked whether inhibiting TP can prevent myeloma-induced osteolytic bone lesions. For this purpose, ARP-1 cells, which express high levels of TP, were directly injected into the femurs of SCID mice. Mouse serum was collected to measure circulating M-protein levels for monitoring tumor burden. When myeloma was established, mice were treated with vehicle control or TP inhibitors, 7-deazaxanthine (7DX) or tipiracil hydrochloride (TPI). ARP-1 cells caused osteolytic bone lesions and increased osteoclastogenesis, reduced bone volume and osteoblastogenesis (FIG. 6, A-D). TPI or 7DX treatment significantly reduced ARP-1 induced bone lesions (FIG. 6, A-D). Treatment with 7DX or TPI significantly reduced *Dnmt3a* expression in mouse MSCs or preOCs (FIG. 6E) and 2DDR levels in the serum of ARP-1 tumor-bearing mice (FIG. 6F).

[00109] Using two additional human myeloma cell lines, RPMI8226 (high TP

expression) and U266 (low TP expression), similar effects of TP inhibitors on myeloma-induced bone lesions were observed both *in vitro* and *in vivo* (FIG. 7). Co-culture of RPMI8226 cells, secreting more 2DDR (FIG. 7A), increased osteoclast (FIG. 7B) and suppressed osteoblast (FIG. 7C) differentiation and activity, compared to those in co-culture of U266 cells (secreting less 2DDR). Treatment of RPMI8226-bearing mice with TPI or 7DX enhanced bone volume and osteoblastogenesis and reduced osteoclastogenesis (FIG. 7, D-F).

[00110] This study reveals an important biological function of TP in the

pathogenesis of myeloma-associated osteolytic bone lesions (FIG. 6G) and indicates that counteracting TP activity may be effective for prevention or treatment of osteolytic bone lesions in myeloma patients. It was found that TP reversibly catalyzes conversion of thymidine into thymine and 2DDR. Myeloma-secreted 2DDR binds to integrins $\alpha_v\beta_3/\alpha_5\beta_1$ in osteoblast progenitors, activates PI3K/Akt signaling, and increases DNMT3A expression and methylation of *RUNX2* and *osterix*, leading to decreased osteoblastogenesis. The secreted 2DDR also binds to integrin $\alpha_v\beta_3$ in osteoclast progenitors, activates PI3K/Akt signaling, and increases DNMT3A expression and methylation of *IRF8*, leading to increased NFATc1 expression and osteoclastogenesis. The net effect of TP is to suppress osteoblast-mediated bone formation and activate osteoclast-mediated bone resorption, the hallmarks of myeloma-induced bone disease.

Example 2 – Materials and Methods

[00111] *Study design:* In a myeloma setting, this study was designed to evaluate the relationship between TP expression and cancer-associated bone lesions. It encompassed three main objectives: to determine the role of TP in myeloma-induced bone lesions; to elucidate the mechanism of TP-induced bone lesions; and to validate the mechanisms *in vivo* and *in vitro* using mouse models and patient samples, respectively. In the first objective, all tested primary myeloma cells and human myeloma cell lines were separated into high- and low-TP expressing cells, injected these cells into mice, and assessed osteoclast-mediated bone resorption and osteoblast-mediated bone formation by radiography and bone histomorphometry. TP was also knocked down or overexpressed in myeloma cells to assess whether modulating TP expression affects bone formation/resorption. TRAP and Alizarin red S staining in co-culture of myeloma cells with MSCs or preOCs determined the importance of TP to bone cell differentiation and activity. In the second aim, methylation-specific PCR (MSP) and bisulfite sequencing PCR (BSP) were performed to analyze DNA methylation in the promoters of *RUNX2* and *osterix* in MSCs or in the promoter of *IRF8* in preOCs. DNMT3A, 2DDR expression, and the integrin-PI3K/Akt signaling pathway were also examined in MSCs or preOCs. In the third objective, the correlations among TP expression, DNMT3A expression, and bone lesions were determined using samples from randomly selected myeloma patients. Additionally, the mechanism was confirmed using the mouse models. Myeloma-bearing mice were randomly selected for the treatment with two TP inhibitors.

[00112] All patient samples were obtained from the Myeloma Tissue Bank of The University of Texas MD Anderson Cancer Center. Bone lesions in humans were characterized by a radiologist who was blinded to the severity of clinical bone disease. The number of samples required to achieve a correlation coefficient ($r \geq 0.7$), a power of 80%, and the level of significance at 5% was determined to be at least 7 samples. In mouse studies, sample size, the composition of replicates, and an intermediate end point were based on prior knowledge of the mouse models (Yang *et al.*, 2012). The sample size was initially estimated using power analysis with our prior knowledge on the bone histomorphometric analysis in myeloma-bearing mice. To ensure a power of 80% to detect the changes in bone volume between the different TP expression groups with 2-sided type I error rate controlled at 0.05 level, we needed 5 mice per group.

[00113] The final end point prior to sacrifice was in accordance with the Institutional Animal Care and Use Committee policies and was predefined. All data were included in the analysis and the criteria for interpretation were established prospectively. Experiments were performed three to five times (as indicated in the figure legends). Animal results were verified by repetition over a 3-year period.

[00114] *Cell lines and primary cells:* Primary myeloma cells were isolated from the bone marrow aspirates of newly diagnosed myeloma patients using anti-CD138 antibody-coated magnetic beads (Miltenyi Biotec, Inc). The cells were maintained in RPMI 1640 medium with 10% fetal bovine serum. Normal plasma cells were isolated from the peripheral blood of healthy donors as previously described (Yaccoby *et al.*, 2004). Myeloma patient MSCs and monocytes were isolated from bone marrow aspirates and cultured as described before (Yang *et al.*, 2012). This study was approved by the Institutional Review Board of The University of Texas MD Anderson Cancer Center.

[00115] *DNMT3A activity analysis:* Nuclear extracts were isolated using the EpiQuik Nuclear Extraction Kit (Epigentek) and 3 µl of nuclear extracts from cells was added to each reaction well according to the manufacturer's protocol. DNMT3A activity was measured using the EpiQuik DNA Methyltransferase Activity/Inhibition Assay Kit (Epigentek) as described previously (Majid *et al.*, 2009).

[00116] *Measurement of 2DDR levels:* The relative levels of 2DDR in culture medium and mouse serum were measured as described previously (Garrett *et al.*, 1967). Briefly, the samples were degraded in 1.0 M HCl at 80°C, and the absorbance at 261 nm and at 277 nm was taken. The concentration was determined based on the calibration curve.

[00117] *Mouse models:* CB.17 SCID mice purchased from Harlan Laboratories were maintained in American Association of Laboratory Animal Care–accredited facilities. The mouse studies were approved by the Institutional Animal Care and Use Committee of The University of Texas MD Anderson Cancer Center. Cultured myeloma cells (5×10^5 cell/mouse) were injected into the femurs of 6- to 8-week-old SCID mice (Wan *et al.*, 2012). SCID-hu hosts were established as reported previously (Yang *et al.*, 2012). Human fetal bone chips were implanted subcutaneously into the right flanks of the mice. Primary myeloma cells (1×10^6 cell/mouse) were injected into the implanted human bone chips to establish myeloma. In some experiments, PBS or 7DX (200 µg/kg) or TPI (300 µg/kg) were injected into the

peritoneum of mice three times a week for two weeks beginning 3 weeks after myeloma cell injection.

[00118] *Monitoring tumor burden and bone lesions in mice:* Sera were collected from the mice weekly and tested for myeloma-secreted M-proteins or light chains by ELISA or for 2DDR levels by spectrophotometric analysis. To measure the size of lytic bone lesions, radiographs were scanned with a Faxitron cabinet X-ray system.

[00119] *Bone histomorphometry:* SCID mouse femurs or human bone chips of SCID-hu mice were fixed in 10% neutral-buffered formalin and de-calcified, and the bone sections were stained with toluidine blue or TRAP following standard protocols. To assess dynamic histomorphometric indices, mice were given two injections of 20 mg/kg calcein (Sigma Aldrich) at 6 and 3 days before dissection. The paraffin-fixed femurs were embedded and sectioned. Both analyses were done using BIOQUANT OSTEO software (BIOQUANT Image Analysis Co.). Mouse MSCs and monocytes were isolated and cultured as previously described (Soleimani and Nadri, 2009; Yang *et al.*, 2012).

[00120] *Statistical analysis:* In directly comparing two sets of quantitative data, statistical significance was determined with SPSS software (version 10.0) using unpaired Student *t*-tests. *P* value < 0.05 was considered statistically significant. All results were reproduced in at least three independent experiments.

[00121] *In vitro osteoblast formation and function assays:* MSCs were obtained from the bone marrow of healthy donors as described previously (Nakamura *et al.*, 1999). Mature osteoblasts were generated from MSCs with osteoblast medium (OB medium) as described (Nakamura *et al.*, 1999). The maturity of the osteoblasts was determined by measuring ALP activity using ALP assays, as described previously (Nakamura *et al.*, 1999). Alizarin red S staining was used to determine the bone formation activity of osteoblasts (Nakamura *et al.*, 1999). Briefly, cells were fixed with 2.5% glutaraldehyde and then stained with 2% Alizarin red S (Sigma-Aldrich) for 20 minutes at 37°C. The calcified minerals were extracted and quantified using a spectrophotometer at a wavelength of 490 nm.

[00122] *In vitro osteoclast formation and function assays:* Human monocytes were isolated from peripheral blood mononuclear cells (PBMCs) as previously described (Nakamura *et al.*, 1999) and cultured in medium with 25 ng/ml M-CSF (R&D Systems) for 7 days to obtain the precursors of osteoclasts. The precursors were then co-cultured with

myeloma cells in medium without or with a low dose of 10 ng/ml RANKL for an additional 7 days to induce mature osteoclast formation. TRAP staining for the detection of mature osteoclasts was performed with a leukocyte acid phosphatase kit (Sigma-Aldrich) according to the manufacturer's instructions. TRAP isoform 5b was quantified using a Bone TRAP Assay (Immunodiagnostic Systems Inc.) according to the manufacturer's instructions.

[00123] *Transfection of siRNA or lentiviral infection of shRNA and ORF expression clone in vitro:* Cells were infected with lentivirus containing human *TP*, *DNMT3A*, or *IRF8* shRNAs (Sigma-Aldrich) or with lentivirus carrying human *TP* ORF (Genecopoeia) to knockdown or overexpress specific genes according to the manufacturer's protocol. Stable cell lines were selected with 0.7 µg/ml of puromycin (Sigma-Aldrich) for 4 weeks. MSCs and preOCs were transfected with 50 nM siRNA targeted against human integrin α_5/α_v or pooled siRNA duplexes against human Akt1/2 (Santa Cruz Biotechnology) using the Lipofectamine 2000 (Life technologies). A scrambled siRNA served as control. Cells were examined 48 hours after the transfection.

[00124] *Western blot analysis:* Cells were harvested and lysed with 1× lysis buffer (50 mM Tris, pH 7.5, 140 mM NaCl, 5 mM EDTA, 5 mM NaN₃, 1% Triton-X-100, 1% NP-40, and 1× protease inhibitor cocktail). Cell lysates were subjected to SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and immunoblotted with antibodies against phosphorylated or non-phosphorylated ERK1/2, FAK, Paxillin, p130Cas, Akt, Akt1, Akt2, TP, IRF8, NFATc1, RUNX2, β -Actin (Cell Signaling Technology), and osterix (R&D Systems).

[00125] *RT-PCR and real-time quantitative PCR:* Total RNA was isolated with the RNeasy kit (Qiagen). An aliquot of 1 µg of total RNA was subjected to reverse transcription (RT) with a SuperScript II (Invitrogen) RT-PCR kit according to the manufacturer's instructions. RT-PCR was performed using a Veriti PCR system (Life Technologies). qPCR was performed using SYBR Green Master Mix (Life Technologies) with the StepOnePlus real-time PCR system (Life Technologies). The primers used in RT-PCR and qPCR are in Table 1.

[00126] *Cell proliferation and apoptosis assays:* The proliferation of myeloma cells was assessed via the CellTiter 96Aqueous One Solution Cell Proliferation Assay (Promega) following the manufacturer's instructions. An annexin V-binding assay was used

to detect cell apoptosis, according to the manufacturer's instructions, and analyzed by a BD LSRFortessa flow cytometer.

[00127] *Immunohistochemistry:* Formalin-fixed, paraffin-embedded sections of bone marrow biopsy specimens from myeloma patients and tissue arrays containing bone marrow biopsy specimens (US Biomax) from patients with multiple myeloma and healthy donors were deparaffinized as described previously (Marie, 2013). Slides were stained with anti-TP (R&D Systems) or anti-CD138 antibodies (LifeSpan BioSciences, Inc) using the EnVision System (DAKO) following the manufacturer's instructions and counterstained with hematoxylin.

[00128] *Chromatin immunoprecipitation assays:* Cells were fixed with 4% formaldehyde and sonicated to prepare the chromatin fragments. Chromatin samples were immunoprecipitated with antibodies against RUNX2 or osterix or control immunoglobulin (IgG) at 4°C for 3 hours. Immunoprecipitates and total chromatin input were reversed cross-linked; DNA was isolated and analyzed by PCR with primers specific for the promoter regions of *BGLAP* and *COL1A1*. The primer sequences are in Table 2. The ChIP-PCR products were separated via gel electrophoresis and quantified using Image J software. Relative Fold enrichments were calculated by determining the immunoprecipitation efficiency (ratios of the amount of immunoprecipitated DNA to that of the input sample).

[00129] *Methylation-specific PCR and bisulfite sequencing PCR:* Genomic DNA was extracted and treated with bisulfite as previously described (Rodan *et al.*, 1997). Primers targeting the promoter regions of RUNX2, osterix, and IRF8 are listed in Table 3. Methylation-specific PCR (MSP) was performed in CpG-rich regions of promoters and examined by DNA gel electrophoresis. PCR products using primers designed for bisulfite sequencing PCR (BSP) were cloned using the TOPO TA Cloning Kit (Life Technologies) according to the manufacturer's instructions.

[00130] Table 1. Primers for RT-PCR and qPCR.

Gene	RT-PCR (Forward)	RT-PCR (Reverse)
<i>GAPDH</i>	ATCATCCCTGCCTCTACTGG (SEQ ID NO: 1)	GTCAGGTCACCACTGACAC (SEQ ID NO: 2)
<i>DNMT3A</i>	TTTGAGTTCTACCGCCTCCT (SEQ ID NO: 3)	CAGGGTTGGA CTGAGAAAT (SEQ ID NO: 4)
<i>DNMT3B</i>	CCATTCGAGTCCTGTCATTG (SEQ ID NO: 5)	GCAATGGACTCCTCACACAC (SEQ ID NO: 6)
<i>DNMT1</i>	AGTACCTGGACGACCTGAC (SEQ ID NO: 7)	GGAAGCGCCTCATAACTCTC (SEQ ID NO: 8)
	qPCR (Forward)	qPCR (Reverse)
<i>GAPDH</i>	CTGGGCTACACTGAGCACC (SEQ ID NO: 9)	AAGTGGTCGTTGAGGGCAATG (SEQ ID NO: 10)
<i>TRAP</i>	AGATCCTGGGTGCAGACTTC (SEQ ID NO: 11)	AAGGGAGCGGTCAGAGAATA (SEQ ID NO: 12)
<i>CALCR</i>	GGGAATCCAGTTTGTCTCT (SEQ ID NO: 13)	ACAAAGAAGCCCTGGAAATG (SEQ ID NO: 14)
<i>CTSK</i>	CCATATGTGGGACAGGAAGA (SEQ ID NO: 15)	CCTCTTCAGGGCTTTCTCAT (SEQ ID NO: 16)
<i>BGLAP</i>	ACTGTGACGAGTTGGCTGAC (SEQ ID NO: 17)	AAGAGGAAAGAAGGGTGCCT (SEQ ID NO: 18)
<i>ALP</i>	TCCCAGTTGAGGAGGAGAAC (SEQ ID NO: 19)	CCCAGGAAGATGATGAGGTT (SEQ ID NO: 20)
<i>COL1A1</i>	TGTTTCAGCTTTGTGGACCTC (SEQ ID NO: 21)	GGTGATTGGTGGGATGTCTT (SEQ ID NO: 22)
<i>TP</i>	ACAGGAGGCACCTTGATAA (SEQ ID NO: 23)	GCTCACTCTGACCCACGATA (SEQ ID NO: 24)
<i>DNMT3A</i>	CAACGATTGCTAGACTGGGA (SEQ ID NO: 25)	AAACTAAGGCCCAACCAG (SEQ ID NO: 26)
<i>Gapdh</i>	CCTCCTGCAGACAGACGTAA (SEQ ID NO: 27)	AGCATCGACCACTGCTACAG (SEQ ID NO: 28)
<i>Dnmt3a</i>	GAGTGGCCATAGAGTGGGTT (SEQ ID NO: 29)	CCGGGCAATCAAATTAGTCT (SEQ ID NO: 30)

[00131] Table 2. Primers for ChIP-PCR.

Primers for binding site of:	Forward	Reverse
RUNX2 on <i>COL1A1</i> promoter	CTTCCAACCTCCAACCTCAGC (SEQ ID NO: 31)	CTGGACCTCCCTTCTCTCCT (SEQ ID NO: 32)
RUNX2 on <i>COL1A1</i> promoter-ctrl	TCTTGAACCCACCAAAGTCC (SEQ ID NO: 33)	GAAGCAAGGAAGTGGACAGG (SEQ ID NO: 34)
RUNX2 on <i>BGLAP</i> promoter	GGGGGTCTCTGAGGAAGAGT (SEQ ID NO: 35)	GCCAAACCCCAAAGGATATT (SEQ ID NO: 36)
RUNX2 on <i>BGLAP</i> promoter-ctrl	CCTGAGGTCAGGAGTTCGAG (SEQ ID NO: 37)	CCCGAGTAGCTGGGATTACA (SEQ ID NO: 38)
Osterix on <i>COL1A1</i> promoter	CTTCCAACCTCCAACCTCAGC (SEQ ID NO: 39)	CTGGACCTCCCTTCTCTCCT (SEQ ID NO: 40)
Osterix on <i>COL1A1</i> promoter-ctrl	CCCAAGGAGGCATATGAAGA (SEQ ID NO: 41)	AGTCCAGAAAGGATGCAGGA (SEQ ID NO: 42)
Osterix on <i>BGLAP</i> promoter	ACCTCTGAGAACGGCTGAAA (SEQ ID NO: 43)	GGTGTATGTCCAACCCCAAG (SEQ ID NO: 44)
Osterix on <i>BGLAP</i> promoter -ctrl	GCCTCCAGCTTTGTTCTTTG (SEQ ID NO: 45)	ACAATTGTGGCATGGAGGAT (SEQ ID NO: 46)

[00132] Table 3. Primers for MSP and BSP. M, methylated; U, unmethylated.

Gene	BSP (Forward)	BSP (Reverse)
<i>RUNX2</i>	TAGGGGAGTTTGTAAATTGTAGGA (SEQ ID NO: 47)	ATATAAATTCAACAATCCAACCCC (SEQ ID NO: 48)
<i>Osterix</i>	GGTAGGTGGTAGTGTAGGTGT (SEQ ID NO: 49)	GGTAGGTGGTAGTGTAGGTGT (SEQ ID NO: 50)
<i>IRF8</i>	TTAGTTTTTGGTTGTGGATTTGA (SEQ ID NO: 51)	TACAAAAAACTTTCCCAAAAATTC (SEQ ID NO: 52)
	MSP (Forward)	MSP (Reverse)
<i>RUNX2</i> (M)	TATTATGGTGGAGATTATCGTCGA (SEQ ID NO: 53)	AAAATCTTATTACAACGCCAATACG (SEQ ID NO: 54)
<i>RUNX2</i> (U)	GTATTATGGTGGAGATTATTGTTGA (SEQ ID NO: 55)	AATCTTATTACAACACCAATACAAA (SEQ ID NO: 56)
<i>Osterix</i> (M)	TTTTTAGAAGGATAGGATAGGTCGT (SEQ ID NO: 57)	CACTAAAACACGAAATAAAAACGAA (SEQ ID NO: 58)
<i>Osterix</i> (U)	TTTTTAGAAGGATAGGATAGGTTGT (SEQ ID NO: 59)	CACTAAAACACAAAATAAAAACAAA (SEQ ID NO: 60)
<i>IRF8</i> (M)	TTTTTGGGAAAGTTTTTTGTAGC (SEQ ID NO: 61)	GACCAATAACGTCAACTTAACTCG (SEQ ID NO: 62)
<i>IRF8</i> (U)	ATTTTGGGAAAGTTTTTTGTAGT (SEQ ID NO: 63)	CAACCAATAACATCAACTTAACTCAC (SEQ ID NO: 64)

Example 3 – Efficacy of TP Inhibitors

[00133] **The efficacy of TP inhibitors on the treatment of multiple myeloma-induced bone lesions.** In *in vitro* studies, the progenitors of osteoblasts were co-cultured with multiple myeloma (MM) cells ARP-1 or RPMI8226, and it was observed that MM cells inhibited osteoblast differentiation. When adding the TP inhibitor (TPI) to the cultures, TPI could significantly enhance the levels of Alizarin red staining, indicating that TPI treatment reverses the effect of MM cells on suppression of osteoblastogenesis (FIG. 17A). Moreover, when TPI was added to the co-culture of the progenitors of osteoclasts with MM cells, it was found that TPI reduced MM-induced increased numbers of multinuclear TRAP-positive osteoclast like cells, clearly suggesting that TPI treatment inhibits osteoclast differentiation in MM (FIG. 17B).

[00134] In the *in vivo* studies, the MM cell line ARP-1 cells were injected into SCID mice to establish a xenografted MM mouse model. In this mouse model, injection of ARP-1 cells caused severe bone lesions, which were observed in mouse femurs via X-ray radiography. Administration of MM-bearing mice with the TP inhibitors 7DX or TPI significantly enhanced the number (FIG. 18A) and thickness (FIG. 18B) of trabecular bones, indicating that TPI treatment improves bone mass or density in mice with MM. Overall, these results suggest that inhibition of TP enhances osteoblastogenesis, and reduces osteoclastogenesis and osteolytic bone lesions.

[00135] **The action mechanism of TP inhibitor on MM-induced bone lesions.** Recent studies have shown that the mature bone cell, osteocyte, is a major source to produce osteolytic cytokines which can regulate osteoblast and osteoclast differentiation and function. It was next determined whether MM-expressed TP inhibits osteoblastogenesis and activates osteoclastogenesis through upregulation of the expression of osteolytic cytokines in osteocytes. Since TP can catalyze the conversion of thymidine to thymine and the small molecule 2-deoxy-D-ribose (2DDR), 2DDR was added to a culture of osteocytes. The results showed that in the presence of 2DDR, the levels of *Tnfsf11* (FIG. 19A) and *Sost* (FIG. 19B) gene expression in osteocytes were increased in a dose-dependent manner, when compared with those in osteocytes without 2DDR. Interestingly, both genes are important in regulation of bone remodeling: the *Tnfsf11* gene encodes RANKL, which is a major stimulator of osteoclast differentiation and activity of bone resorption; the *Sost* gene encodes sclerostin, which binds to LRF5/LRF6 receptors and blunts Wnt signaling or antagonizes the activity of

bone morphogenetic proteins, leading to suppressed osteoblastogenesis. These findings indicate that TP/2DDR can upregulate the production of these osteolytic cytokines, resulting in bone lesions in MM. To confirm the *in vitro* observation, the levels of these cytokines were examined in the serum of mice implanted with MM cells (ARP-1 and RPMI8226). An increased serum level of RANKL and sclerostin was observed in MM-bearing mice, in line with the *in vitro* observation. When the MM-bearing mice were treated with the TP inhibitors 7DX or TPI, significantly reduced levels of RANKL (FIG. 20A) and sclerostin (FIG. 20B) were found in mouse sera, compared with those in sera of mice with PBS. In summary, these results suggest that inhibition of TP can reduce the production of osteolytic cytokines from osteocytes, leading to the recovery of bone resorption in MM.

[00136] The clinical relevance of TP expression in MM-induced bone lesions. The serum levels of RANKL and sclerostin were examined in newly diagnosed MM patients. Twenty MM patient samples were collected, and a strong positive correlation was found between the levels of the osteolytic cytokines RANKL and sclerostin in patient sera and the levels of TP expression in CD138-positive MM cells. Because both RANKL and sclerostin are biomarkers of lytic lesions, these findings suggest that assessment of TP levels can be used for the clinical prognosis and diagnosis of the status of bone lesions in patients.

* * *

[00137] All of the methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

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The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference.

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CLAIMS

WHAT IS CLAIMED:

1. A method of treating a bone disease in a subject comprising administering an effective amount of an inhibitor of (a) thymidine phosphorylase (TP) and/or (b) a product of thymidine metabolism to the subject.
2. The method of claim 1, wherein the bone disease is cancer-induced bone disease.
3. The method of claim 2, wherein the subject is diagnosed as having cancer.
4. The method of claim 3, wherein the cancer is multiple myeloma, breast cancer, colorectal cancer, or lung cancer.
5. The method of claim 3, wherein the cancer is multiple myeloma.
6. The method of claim 5, wherein the multiple myeloma is further defined as CD138-positive multiple myeloma.
7. The method of any one of claims 1-6, wherein the bone disease is further defined as osteolytic bone lesions.
8. The method of any one of claims 1-7, wherein the inhibitor of thymidine phosphorylase is tipiracil hydrochloride (TPI), 7-deazaxanthine (7DX), 5'-O-tritylinosine (KIN-59), or 9-(8-phosphonooctyl)-7-deazaxanthine (TP65).
9. The method of any one of claims 1-8, wherein the product of thymidine metabolism is 2DDR.
10. The method of any one of claims 1-9, wherein the subject has an increased serum level of 2DDR as compared to the serum level of 2DDR in a healthy subject.
11. The method of any one of claims 1-10, wherein the subject has a decreased serum level of 2DDR after administration of the inhibitor of TP and/or product of thymidine metabolism.

12. The method of any one of claims 1-11, wherein the subject has a decreased expression of *DNMT3A*, *TRAP*, *CALCR*, and/or *CTSK* after administration of the inhibitor of TP and/or product of thymidine metabolism.
- 5 13. The method of any one of claims 1-12, wherein the subject has increased expression of *RUNX2*, *osterix*, and/or *IRF8* after administration of the inhibitor of TP and/or product of thymidine metabolism.
14. The method of any one of claims 1-13, wherein the subject has reduced osteoclast differentiation, decreased osteolytic bone lesions, and/or increased osteoblast-mediated bone formation after administration of the inhibitor of TP and/or product of thymidine metabolism.
- 10 15. The method of any one of claims 1-14, wherein the inhibitor of TP and/or product of thymidine metabolism is administered intravenously, intraperitoneally, intratracheally, intratumorally, intramuscularly, endoscopically, intralesionally, percutaneously, subcutaneously, regionally, or by direct injection or perfusion.
- 15 16. The method of any one of claims 1-15, further comprising administering at least a second therapeutic agent.
17. The method of claim 16, wherein the at least a second therapeutic agent is an anti-resorptive agent and/or an anti-cancer agent.
18. The method of claim 16, wherein the at least a second therapeutic agent is a DNMT inhibitor.
- 20 19. The method of claim 17, wherein the anti-resorptive agent is an anti-RANKL antibody, anti-DKK-1 antibody, bisphosphonate, or hormone therapy.
20. The method of claim 19, wherein the bisphosphonate is alendronate, risedronate, ibandronate, or etidronate.
- 25 21. The method of claim 19, wherein the hormone therapy is a selective estrogen receptor modulator (SERM) or calcitonin.
22. The method of claim 19, wherein the anti-cancer therapy is chemotherapy, immunotherapy, surgery, radiotherapy, or biotherapy.

23. The method of claim 19, wherein the anti-cancer therapy is a proteasome inhibitor.
24. The method of claim 19, wherein the proteasome inhibitor is bortezomib or carfilzomib.
25. The method of claim 19, wherein the anti-cancer therapy is anti-integrin alpha-v antibody or anti-integrin alpha-5 antibody.
26. The method of claim 19, wherein the anti-cancer therapy is a nucleoside analog.
27. The method of claim 26, wherein the nucleoside analog is trifluridine.
28. The method of any one of claims 1-27, wherein the subject is a human.
29. A pharmaceutical composition comprising an inhibitor of TP or 2DDR and a pharmaceutically acceptable carrier for use in the treatment of a bone disease.
30. A composition comprising an effective amount of an inhibitor of TP or 2DDR for the treatment of a bone disease in a subject.
31. A method of treating a bone disease in a subject comprising administering an effective amount of an inhibitor of thymidine phosphorylase (TP) to the subject.
32. The method of any one of claims 29-31, wherein the bone disease is cancer-induced bone disease.
33. The method of claim 32, wherein the subject is diagnosed as having cancer.
34. The method of claim 33, wherein the cancer is multiple myeloma, breast cancer, or lung cancer.
35. The method of claim 33, wherein the cancer is multiple myeloma.
36. The method of any one of claims 29-31, wherein the bone disease is further defined as osteolytic bone lesions.
37. The method of any one of claims 29-31, wherein the inhibitor of thymidine phosphorylase is tipiracil hydrochloride (TPI), 7-deazaxanthine (7DX), 5'-O-tritylinosine (KIN-59), or 9-(8-phosphonooctyl)-7-deazaxanthine (TP65).

38. The method of any one of claims 29-31, wherein the subject has an increased serum level of 2DDR as compared to the serum level of 2DDR in a healthy subject.
39. The method of any one of claims 29-31, wherein the subject has a decreased serum level of 2DDR after administration of the inhibitor of TP.
- 5 40. The method of any one of claims 29-31, wherein the subject has a decreased expression of *DNMT3A*, *TRAP*, *CALCR*, and/or *CTSK* after administration of the inhibitor of TP.
41. The method of any one of claims 29-31, wherein the subject has increased expression of *RUNX2*, *osterix*, and/or *IRF8* after administration of the inhibitor of TP.
- 10 42. The method of any one of claims 29-31, wherein the subject has a decreased level of RANKL and/or sclerostin after administration of the inhibitor of TP.
43. The method of any one of claims 29-31, wherein the subject has reduced osteoclast differentiation, decreased osteolytic bone lesions, and/or increased bone volume after administration of the inhibitor of TP.
- 15 44. The method of any one of claims 29-31, wherein the inhibitor of TP and/or product of thymidine metabolism is administered intravenously, intraperitoneally, intratracheally, intratumorally, intramuscularly, endoscopically, intralesionally, percutaneously, subcutaneously, regionally, or by direct injection or perfusion.
45. The method of claim 31, wherein the inhibitor of TP is injected directly into a bone of
20 the subject.
46. The method of claim 31, further comprising administering at least a second therapeutic agent.
47. The method of claim 46, wherein the at least a second therapeutic agent is an anti-resorptive agent and/or an anti-cancer agent.
- 25 48. The method of claim 46, wherein the at least a second therapeutic agent is a DNMT inhibitor.

49. The method of claim 47, wherein the anti-resorptive agent is an anti-RANKL antibody, anti-DKK-1 antibody, bisphosphonate, or hormone therapy.
50. The method of claim 49, wherein the bisphosphonate is alendronate, risedronate, ibandronate, or etidronate.
- 5 51. The method of claim 49, wherein the hormone therapy is a selective estrogen receptor modulator (SERM) or calcitonin.
52. The method of claim 49, wherein the anti-cancer therapy is chemotherapy, immunotherapy, surgery, radiotherapy, or biotherapy.
53. The method of claim 49, wherein the anti-cancer therapy is a proteasome inhibitor.
- 10 54. The method of claim 49, wherein the proteasome inhibitor is bortezomib or carfilzomib.
55. The method of claim 49, wherein the anti-cancer therapy is anti-integrin alpha-v antibody or anti-integrin alpha-5 antibody.
56. The method of claim 49, wherein the anti-cancer therapy is a nucleoside analog.
- 15 57. The method of claim 56, wherein the nucleoside analog is trifluridine.
58. The method of claim 31, wherein the subject is a human.
59. A pharmaceutical composition comprising an inhibitor of TP and a pharmaceutically acceptable carrier for use in the treatment of a bone disease.
- 20 60. A method of treating bone disease in a subject comprising administering an effective amount of an inhibitor of TP to the subject, wherein the subject is identified to have an increased level of TP as compared to a control.
61. The method of claim 60, wherein the increased level of TP identifies the presence of bone lesions in said subject.
- 25 62. The method of claim 60 or 61, wherein the subject has increased levels of RANKL and/or sclerostin as compared to a control.

63. The method of any one of claims 60-62, wherein the administration of an inhibitor of TP results in decreased levels of RANKL and/or sclerostin as compared to levels prior treatment.
- 5 64. The method of any one of claims 60-62, wherein the level of TP is measured in the serum of said subject.
65. The method of any one of claims 60-64, wherein the bone disease is cancer-induced bone disease.
66. The method of any one of claims 60-65, wherein the subject is diagnosed as having cancer.
- 10 67. The method of claim 66, wherein the cancer is multiple myeloma, breast cancer, or lung cancer.
68. The method of claim 66, wherein the cancer is multiple myeloma.
69. The method of any one of claims 60-68, wherein the bone disease is further defined as osteolytic bone lesions.
- 15 70. The method of any one of claims 60-69, wherein the inhibitor of thymidine phosphorylase is tipiracil hydrochloride (TPI), 7-deazaxanthine (7DX), 5'-O-tritylinosine (KIN-59), or 9-(8-phosphonooctyl)-7-deazaxanthine (TP65).
71. The method of any one of claims 60-70, wherein the inhibitor of TP and/or product of thymidine metabolism is administered intravenously, intraperitoneally, 20 intratracheally, intratumorally, intramuscularly, endoscopically, intralesionally, percutaneously, subcutaneously, regionally, or by direct injection or perfusion.
72. The method of any one of claims 60-71, wherein the inhibitor of TP is injected directly into a bone of the subject.
73. The method of any one of claims 60-72, further comprising administering at least a 25 second therapeutic agent.
74. The method of claim 73, wherein the at least a second therapeutic agent is an anti-resorptive agent and/or an anti-cancer agent.

75. The method of claim 73, wherein the at least a second therapeutic agent is a DNMT inhibitor.
76. The method of claim 74, wherein the anti-resorptive agent is an anti-RANKL antibody, anti-DKK-1 antibody, bisphosphonate, or hormone therapy.
- 5 77. The method of claim 76, wherein the bisphosphonate is alendronate, risedronate, ibandronate, or etidronate.
78. The method of claim 76, wherein the hormone therapy is a selective estrogen receptor modulator (SERM) or calcitonin.
79. The method of claim 76, wherein the anti-cancer therapy is chemotherapy,
10 immunotherapy, surgery, radiotherapy, or biotherapy.
80. The method of claim 76, wherein the anti-cancer therapy is a proteasome inhibitor.
81. The method of claim 76, wherein the proteasome inhibitor is bortezomib or carfilzomib.
82. The method of claim 76, wherein the anti-cancer therapy is anti-integrin alpha-v
15 antibody or anti-integrin alpha-5 antibody.
83. The method of claim 76, wherein the anti-cancer therapy is a nucleoside analog.
84. The method of claim 83, wherein the nucleoside analog is trifluridine.
85. The method of any one of claims 60-84, wherein the subject is a human.

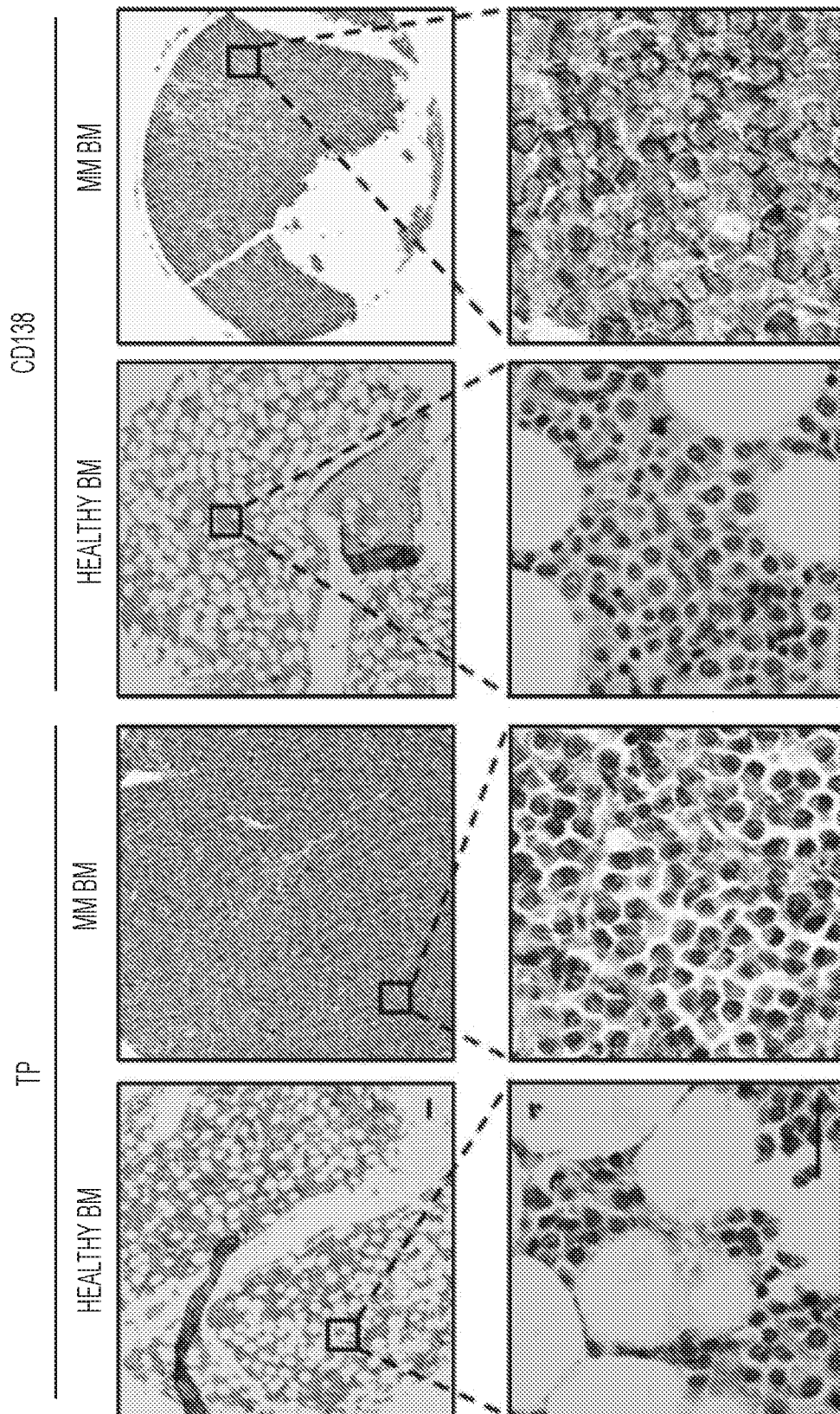


FIG. 1A

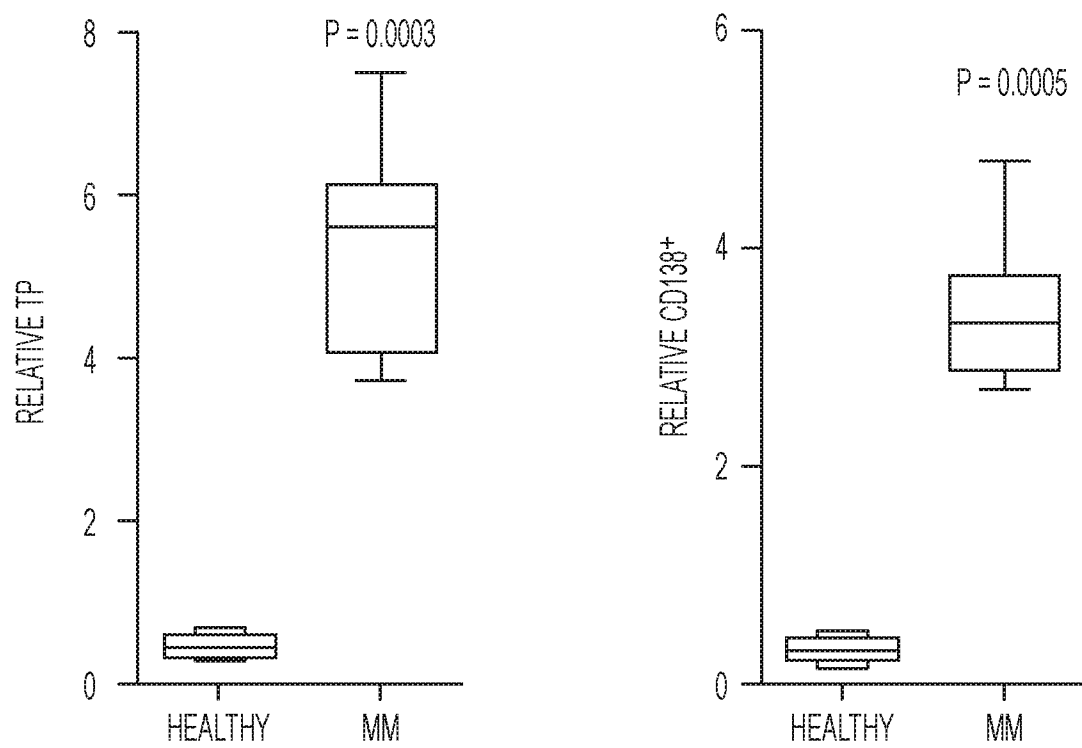
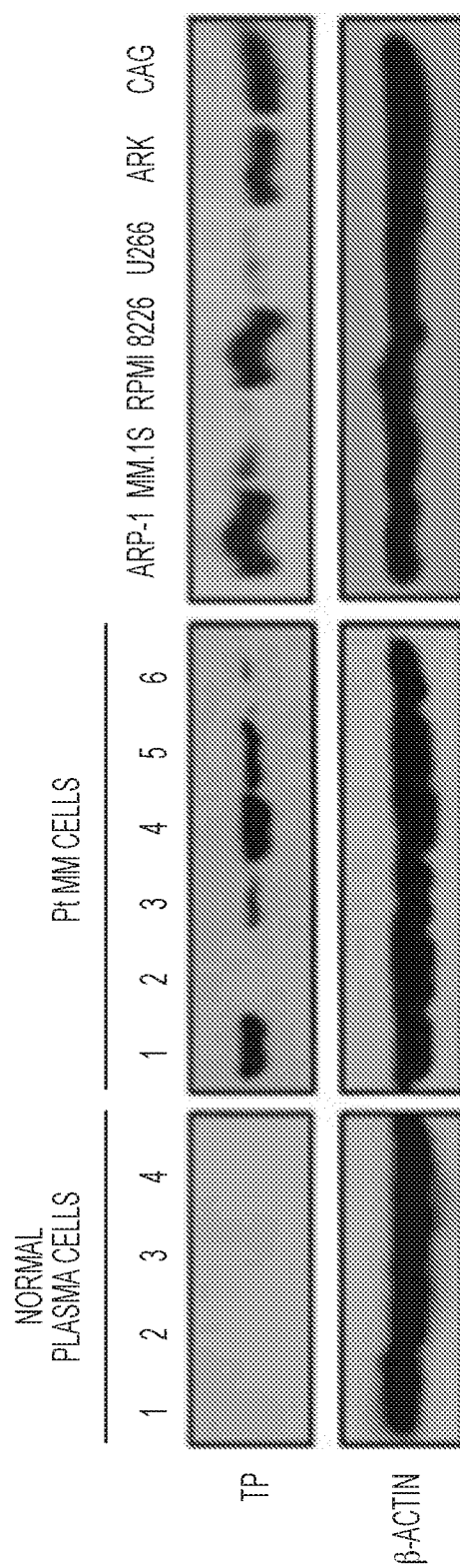


FIG. 1B



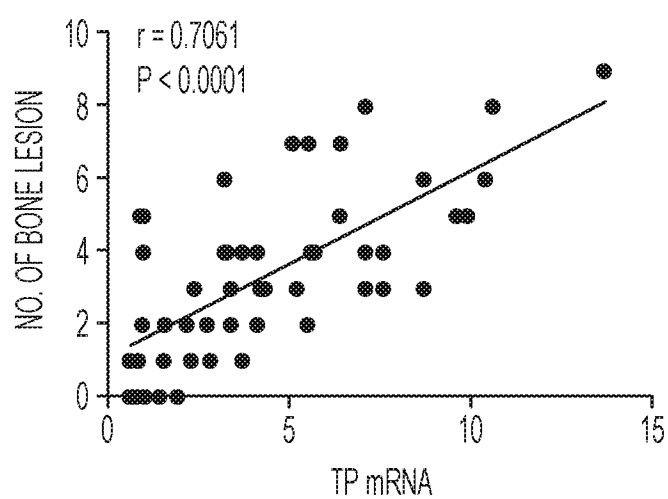


FIG. 2A

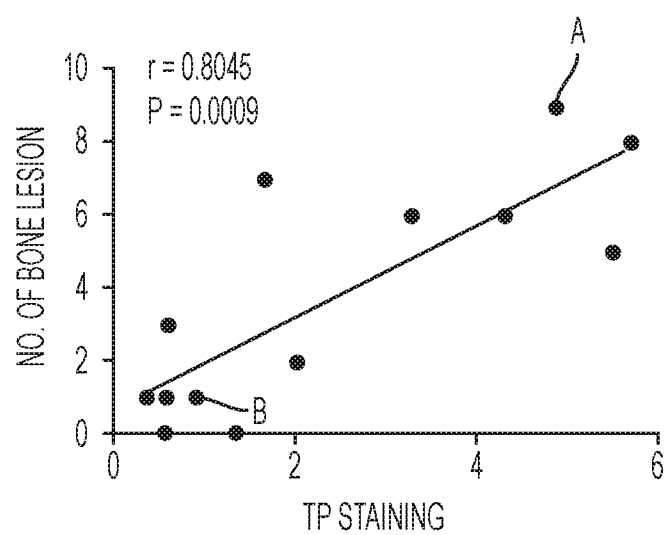


FIG. 2B

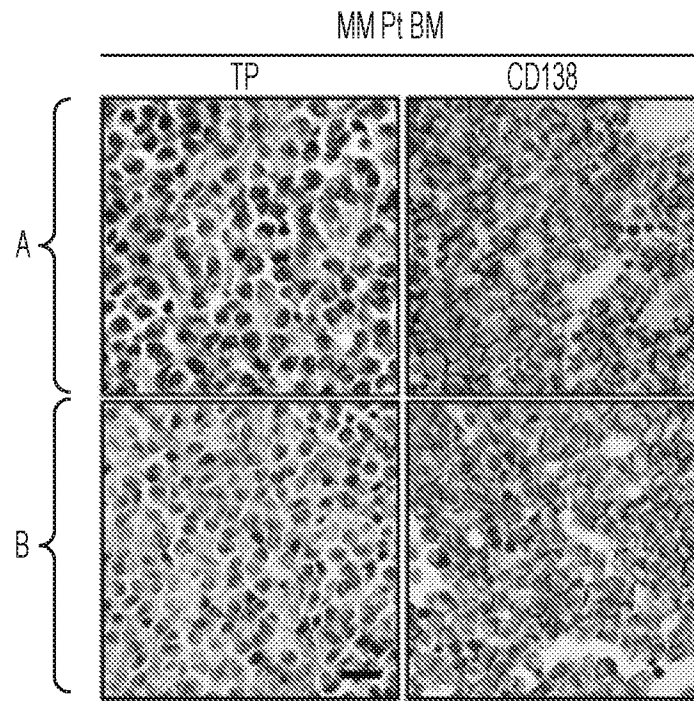


FIG. 2C

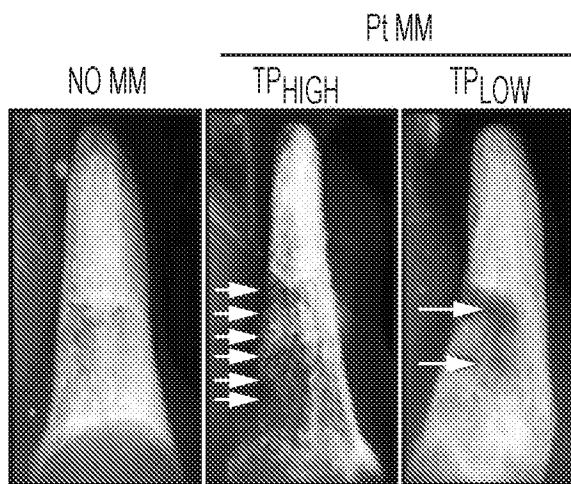


FIG. 2D

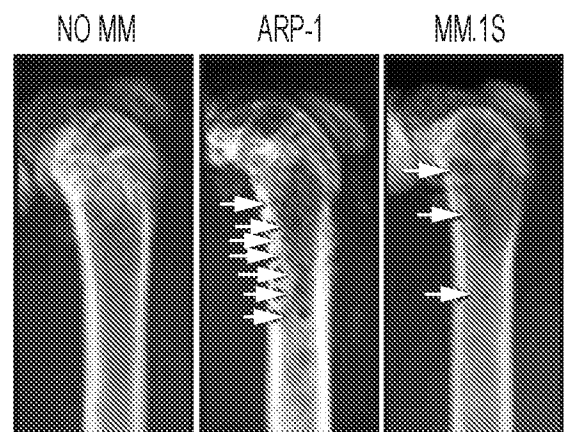


FIG. 2E

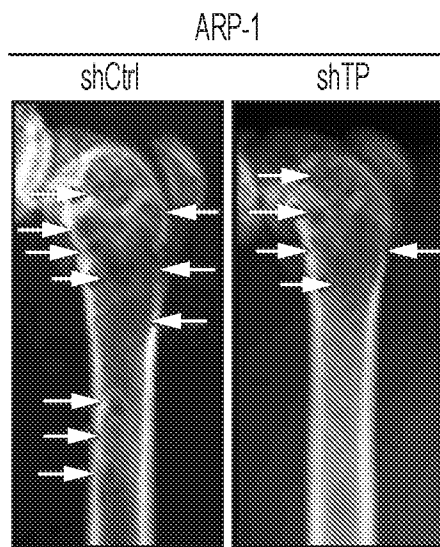


FIG. 2F

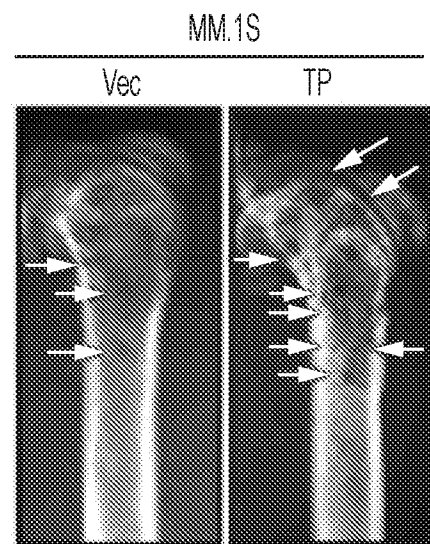


FIG. 2G

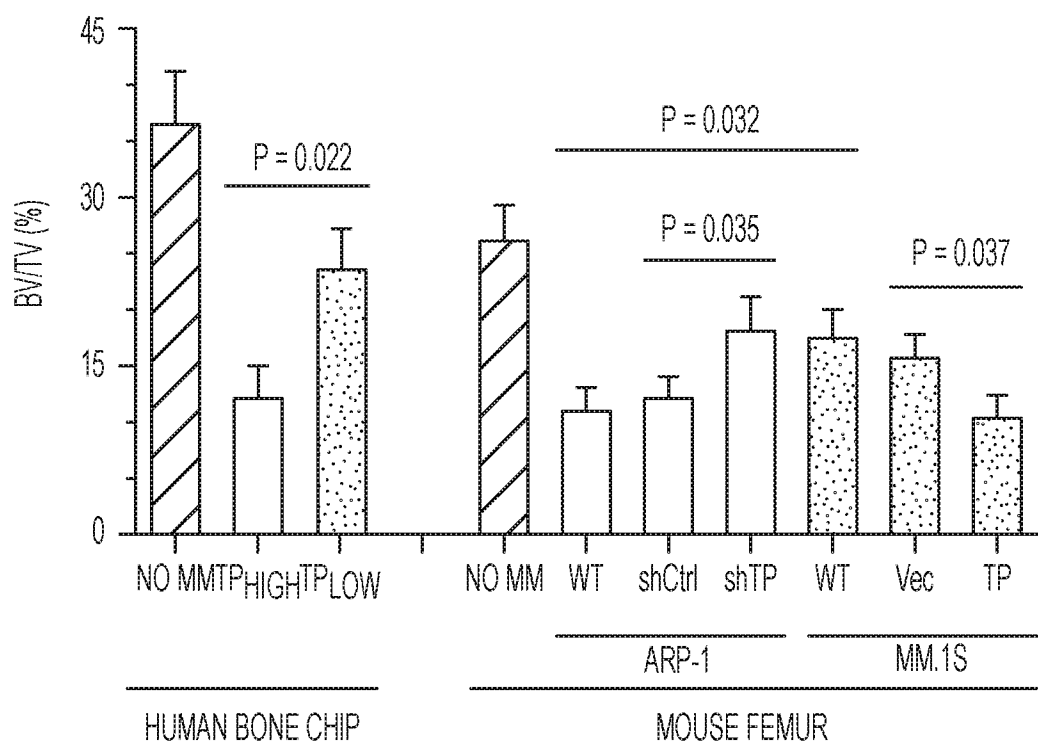


FIG. 2H

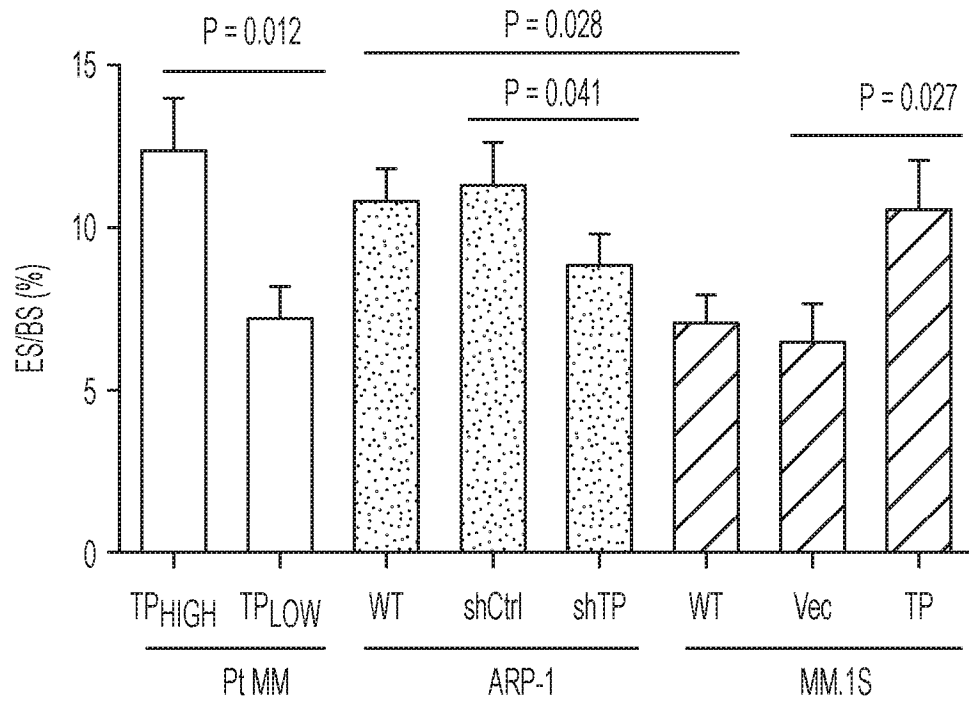


FIG. 3A

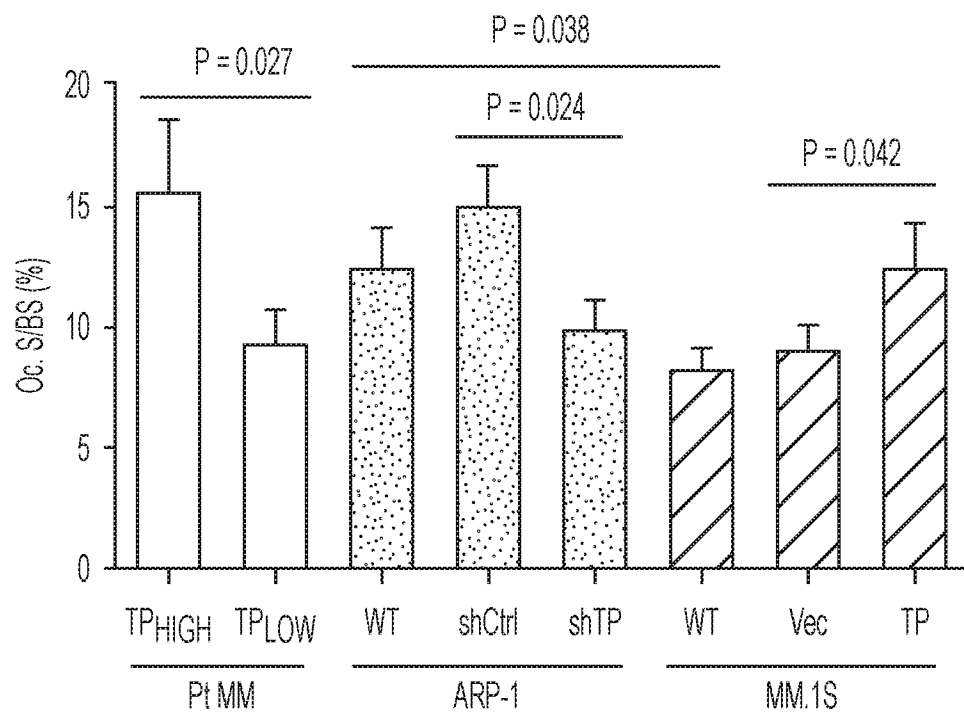


FIG. 3B

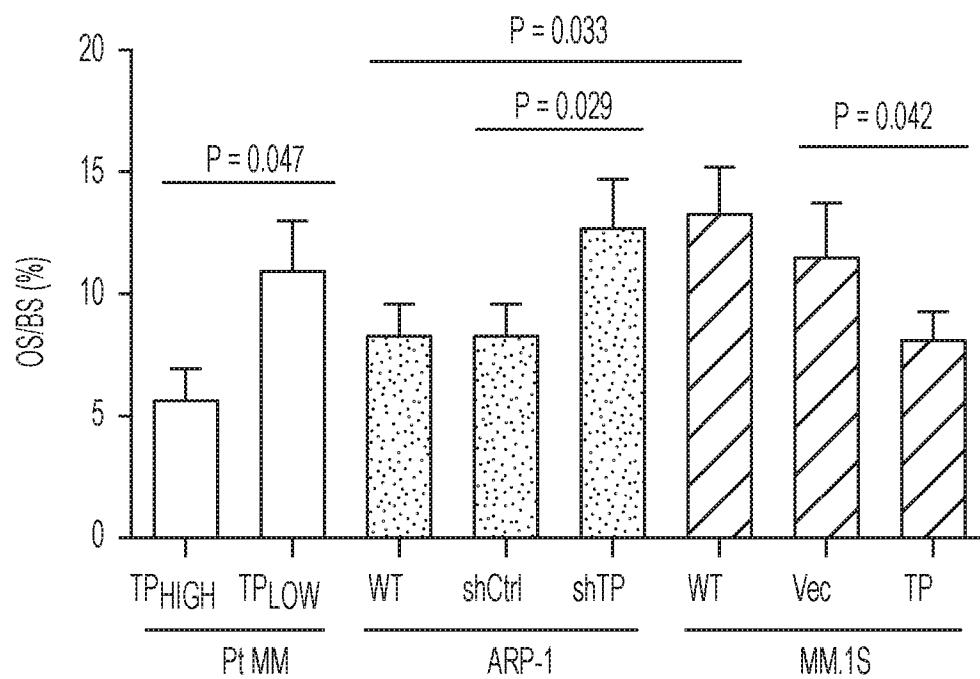


FIG. 3C

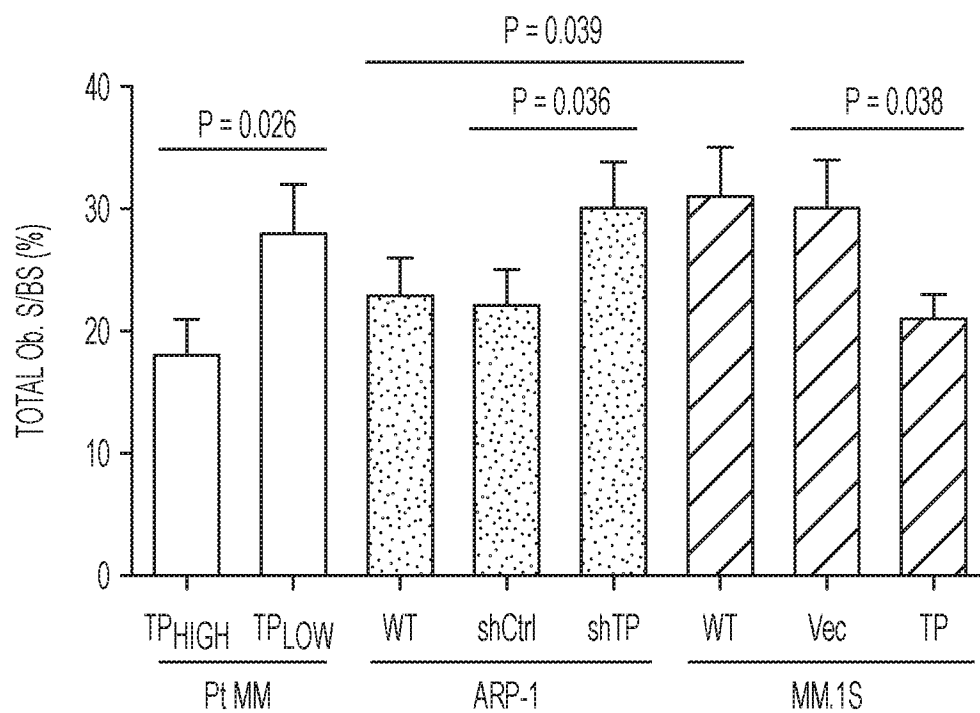


FIG. 3D

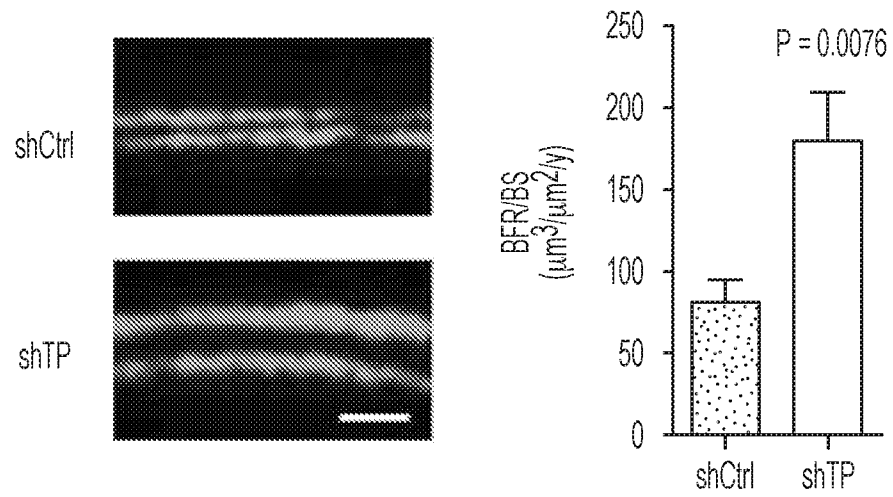


FIG. 3E

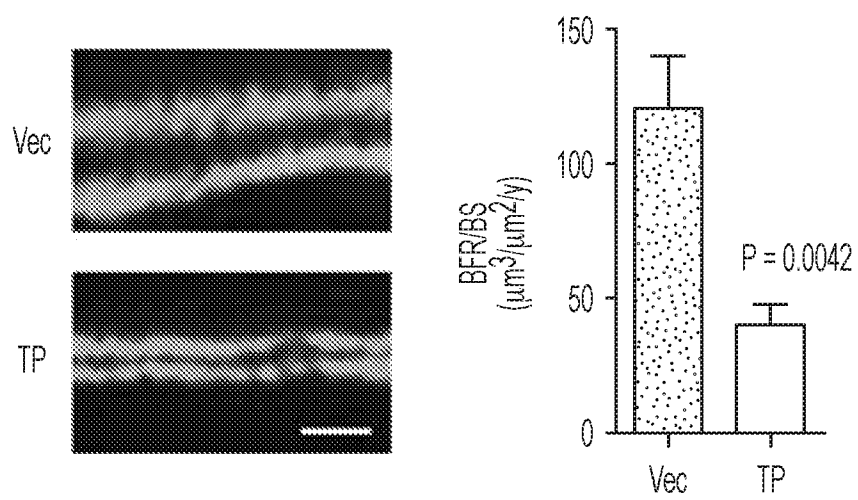


FIG. 3F

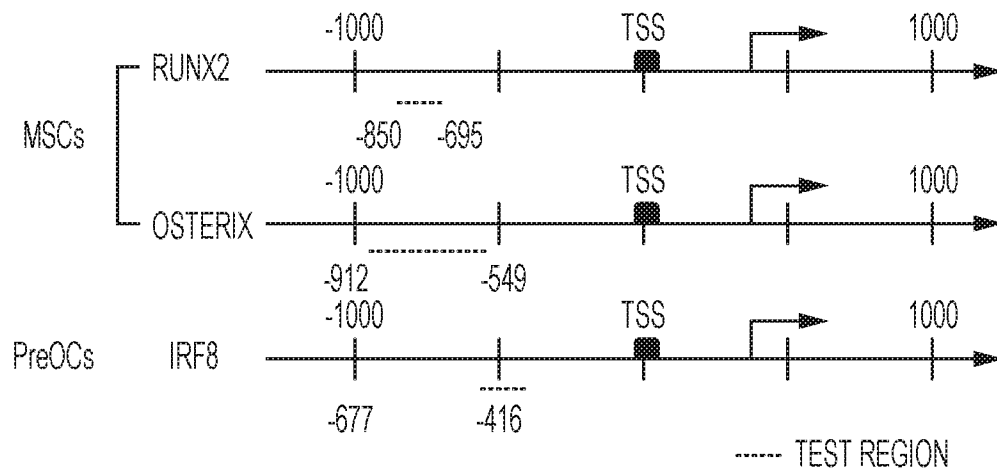


FIG. 4A

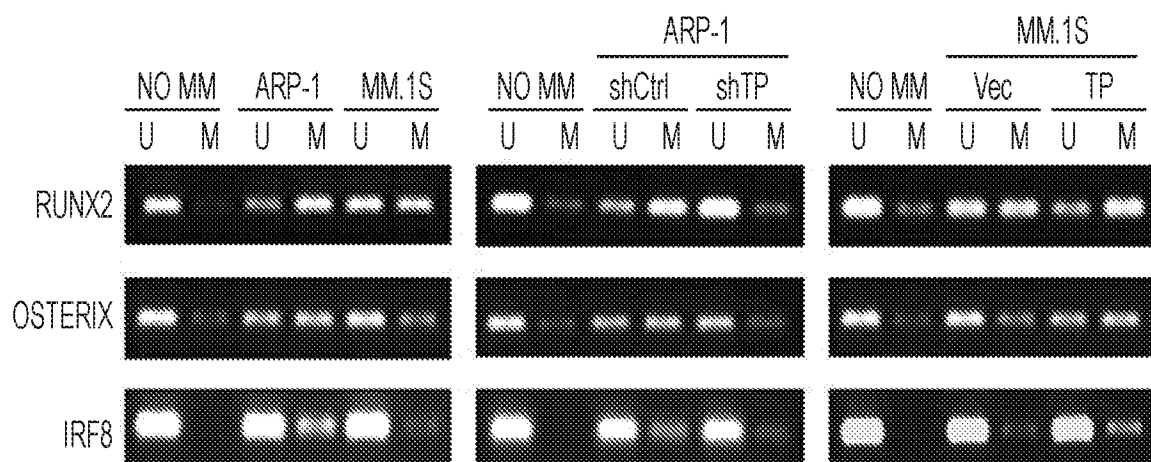


FIG. 4B

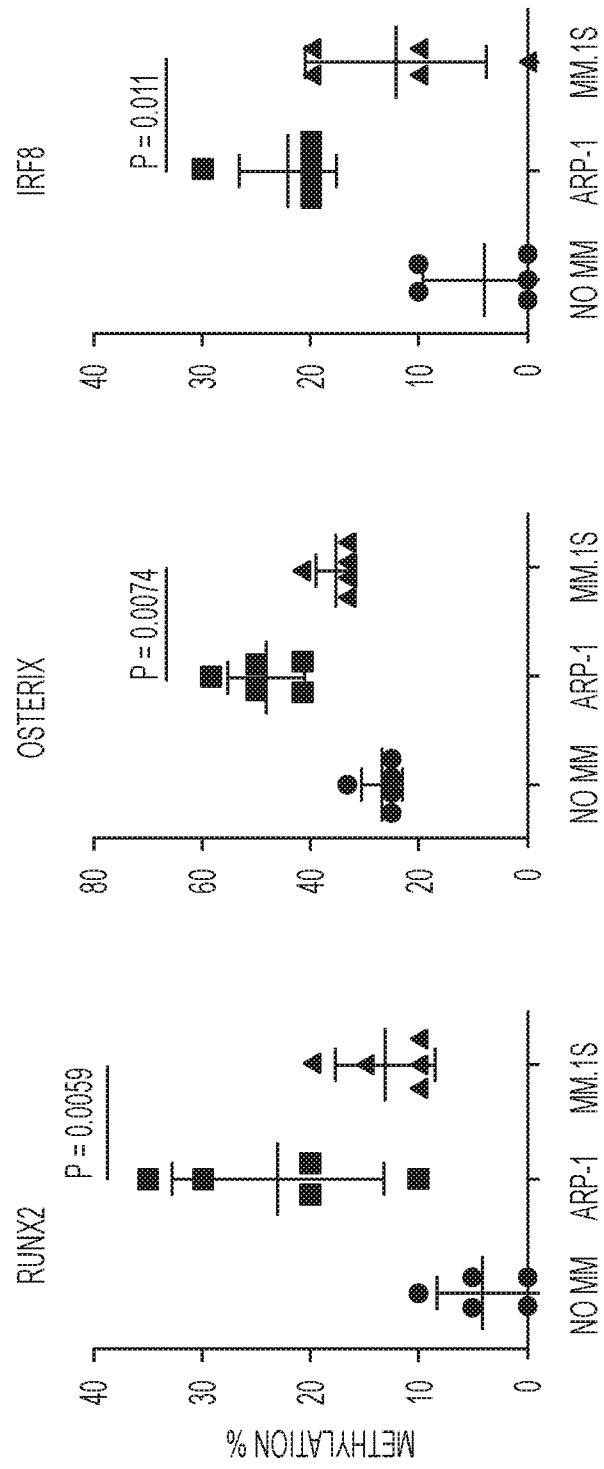
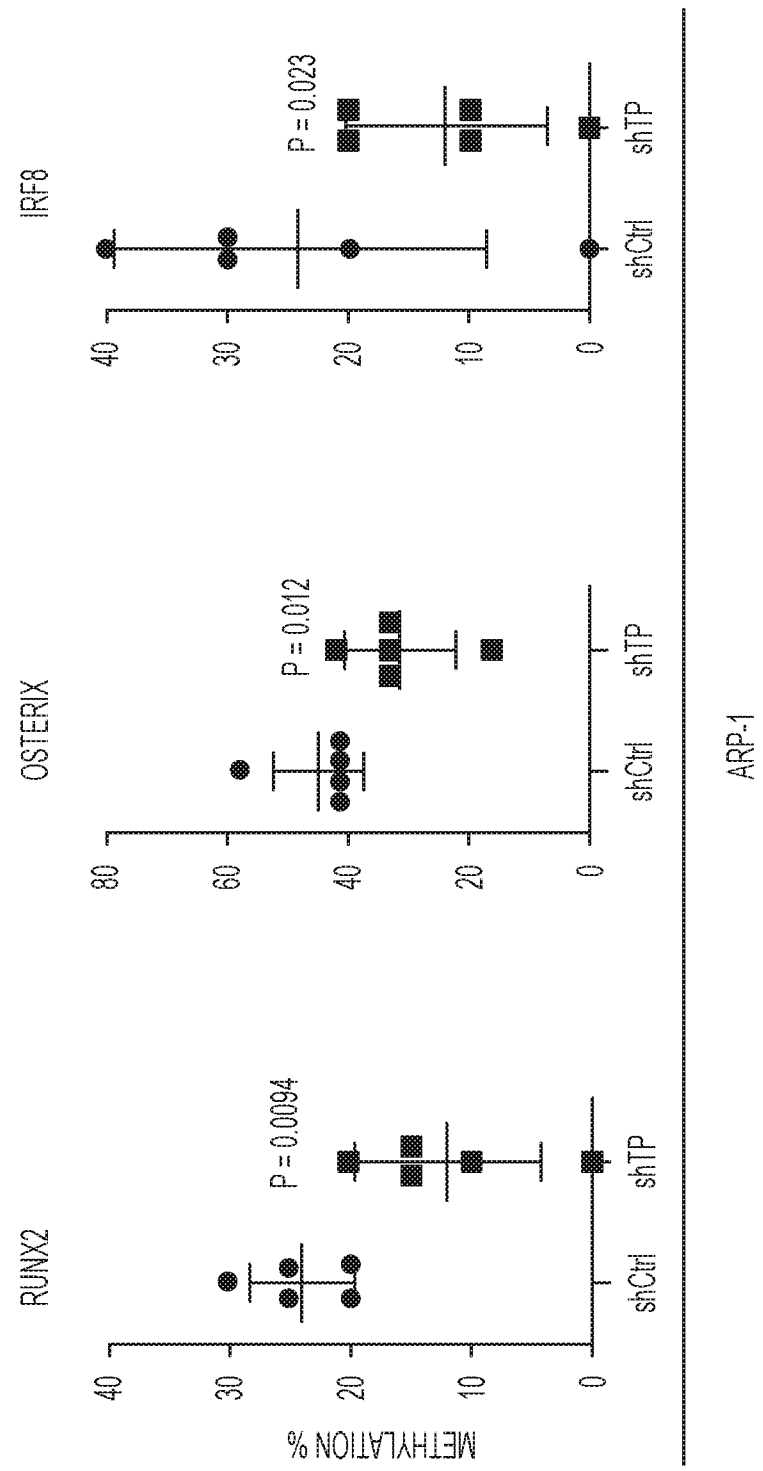


FIG. 4C



ARP-1

FIG. 4D

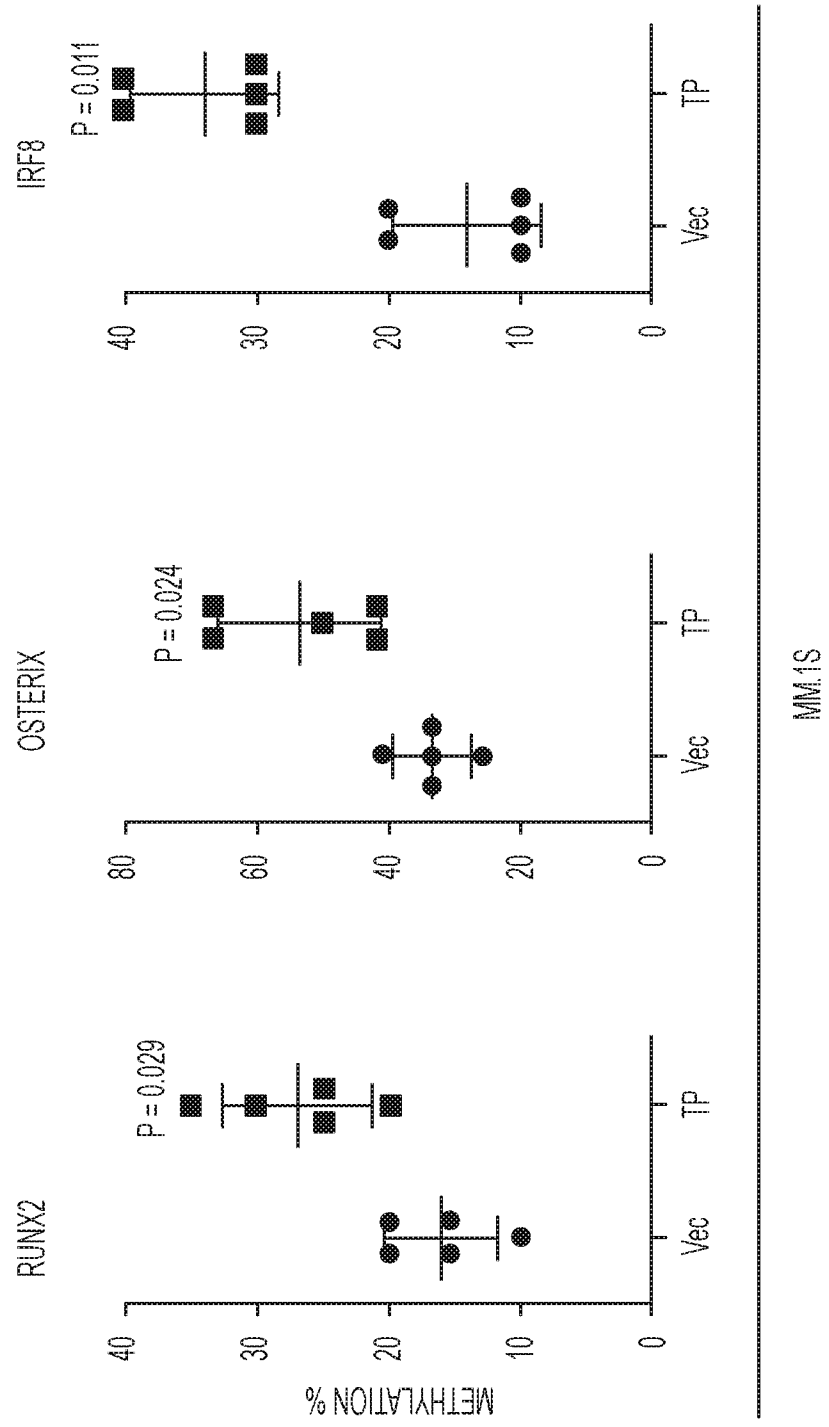


FIG. 4E

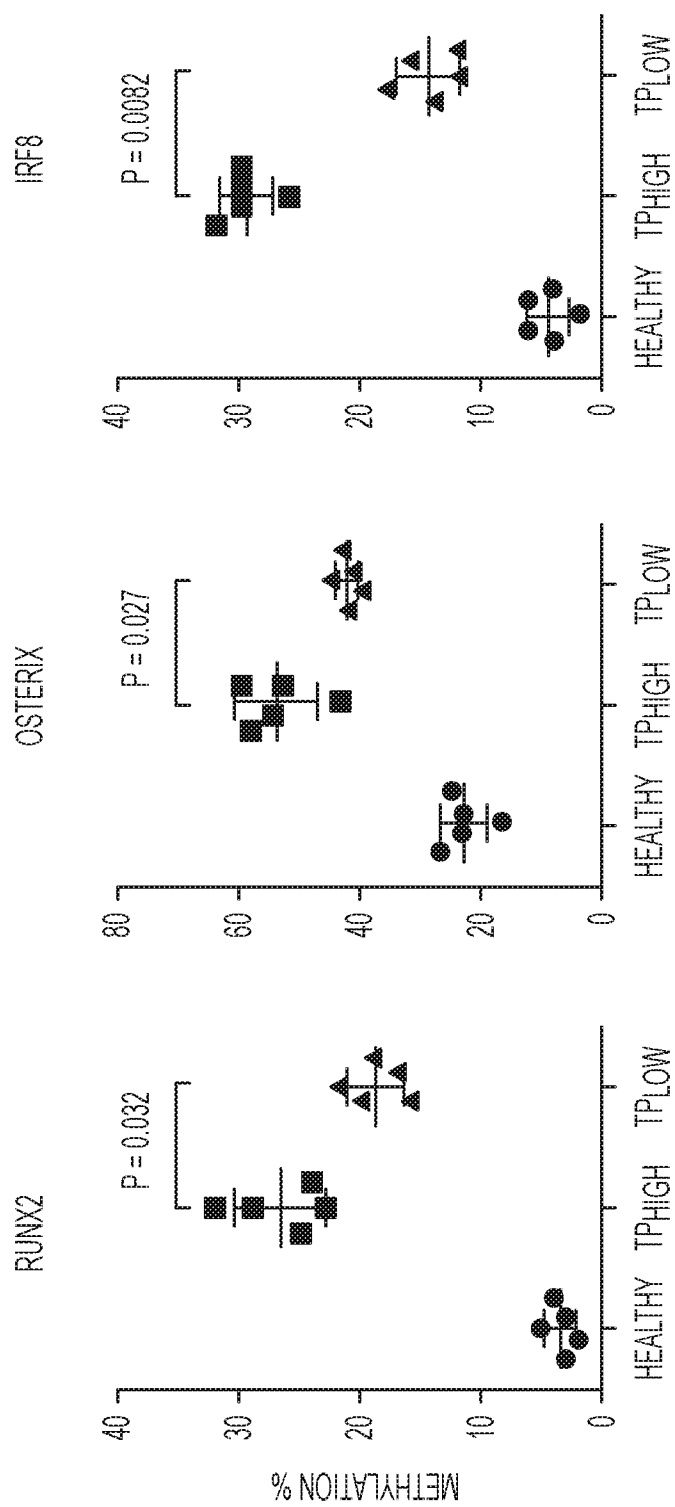


FIG. 4F

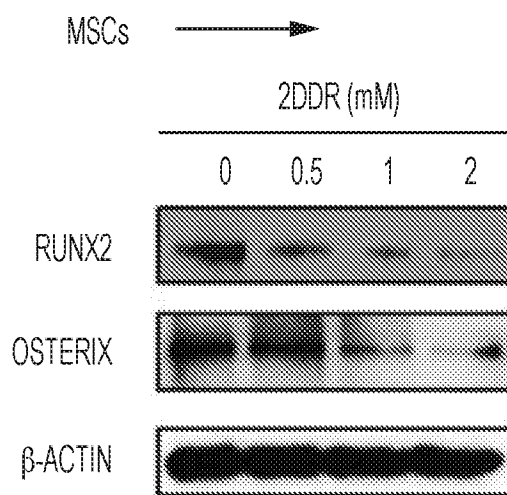


FIG. 5A

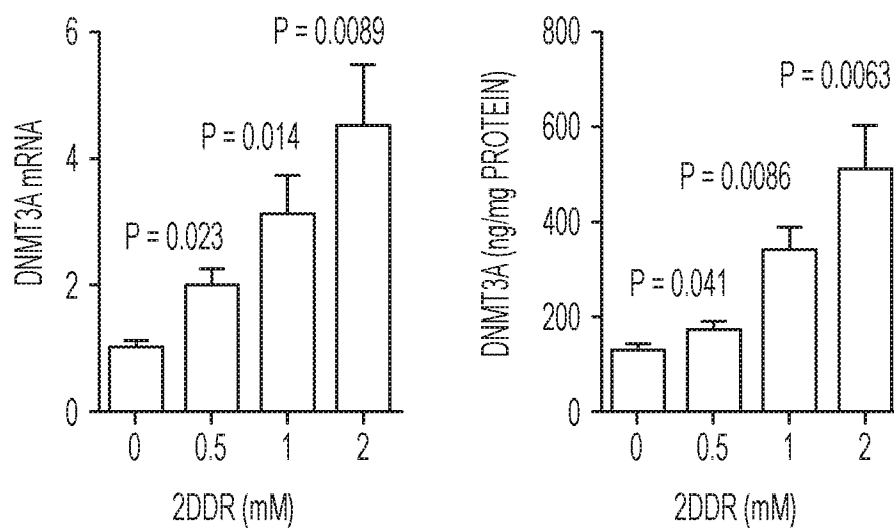


FIG. 5B

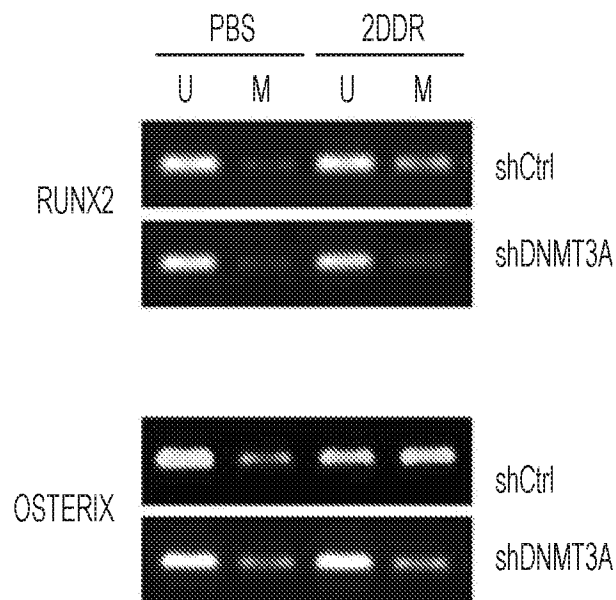


FIG. 5C

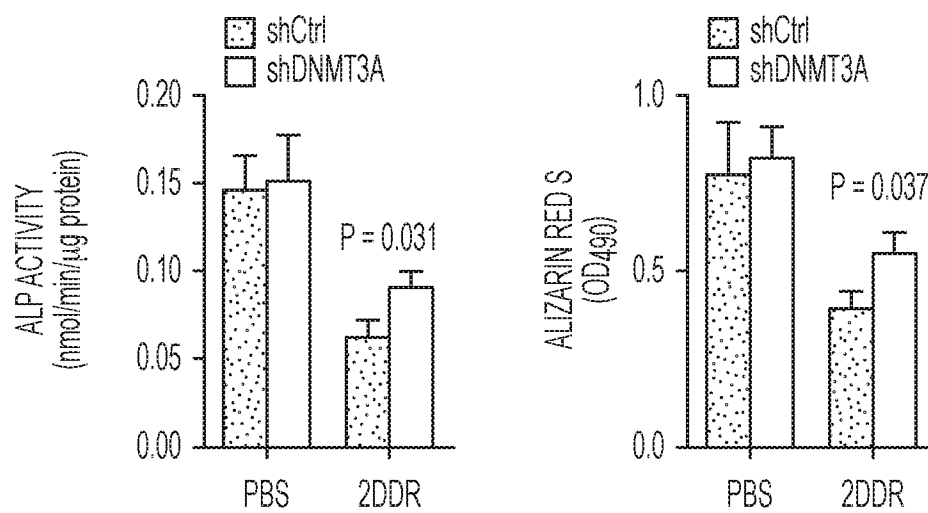


FIG. 5D

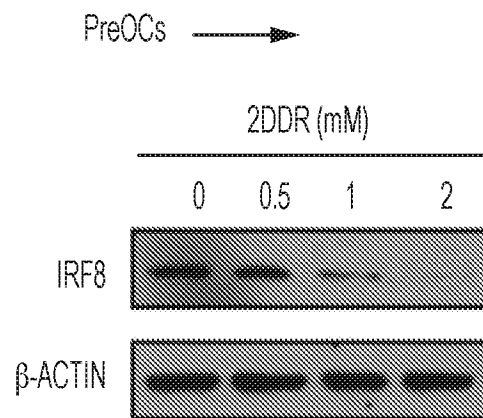


FIG. 5E

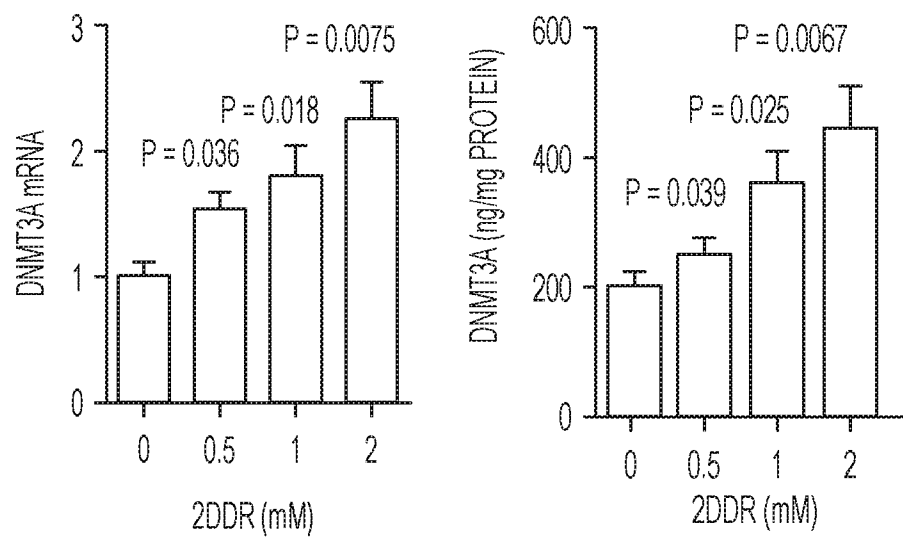


FIG. 5F

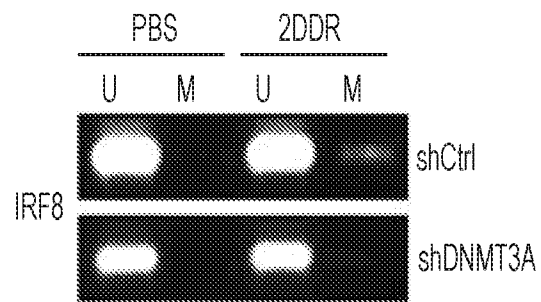


FIG. 5G

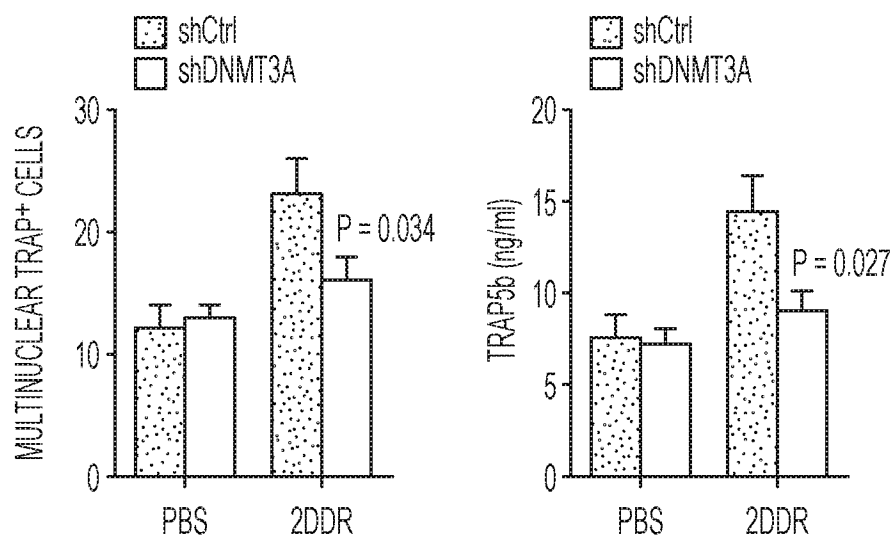


FIG. 5H

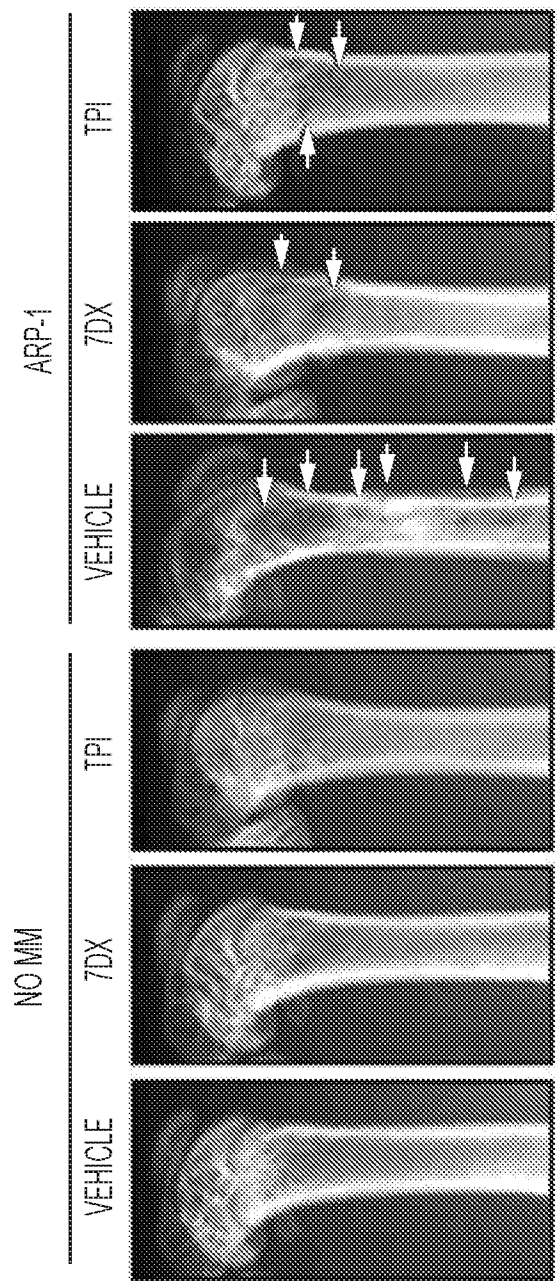


FIG. 6A

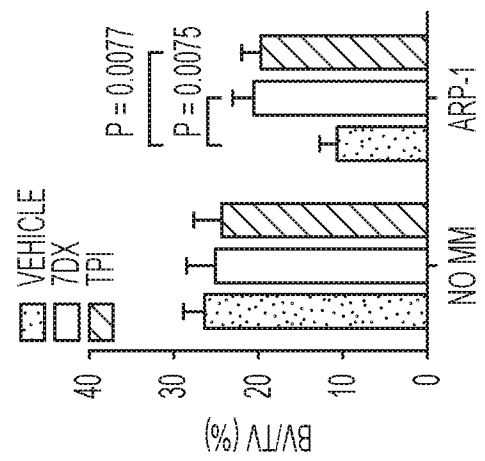


FIG. 6B

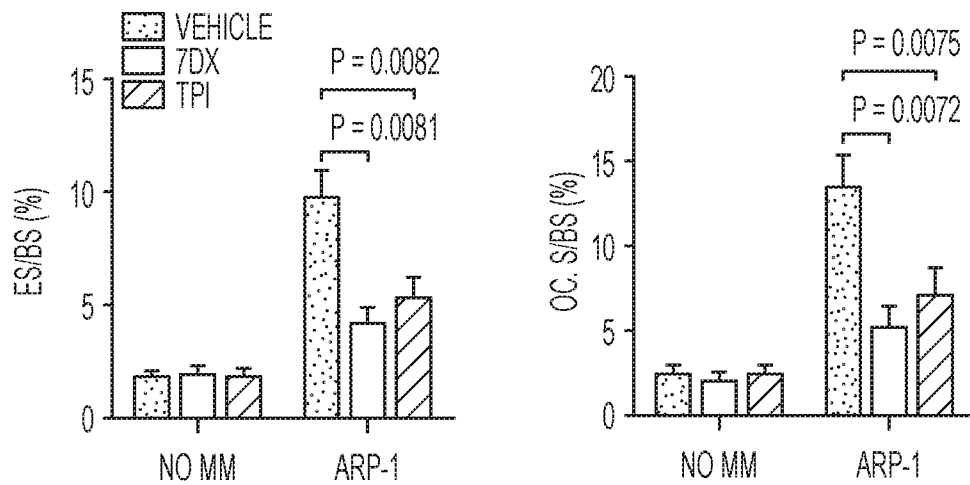


FIG. 6C

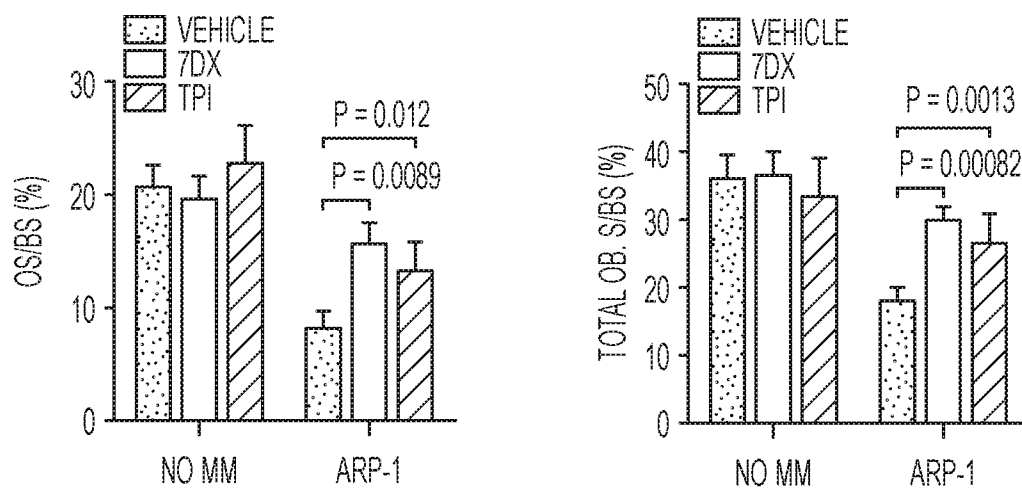


FIG. 6D

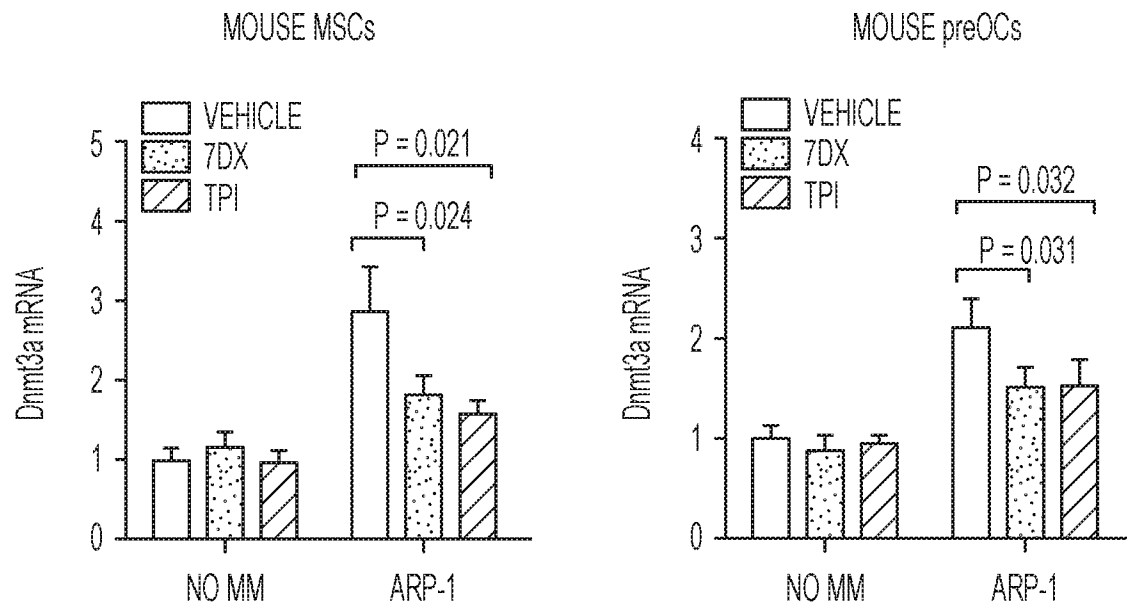


FIG. 6E

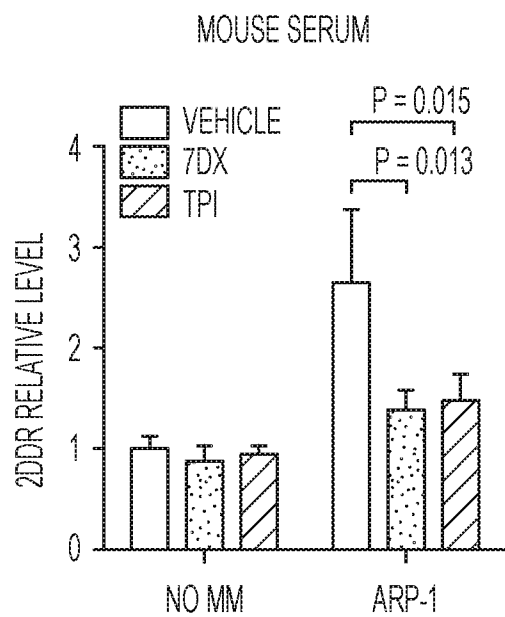


FIG. 6F

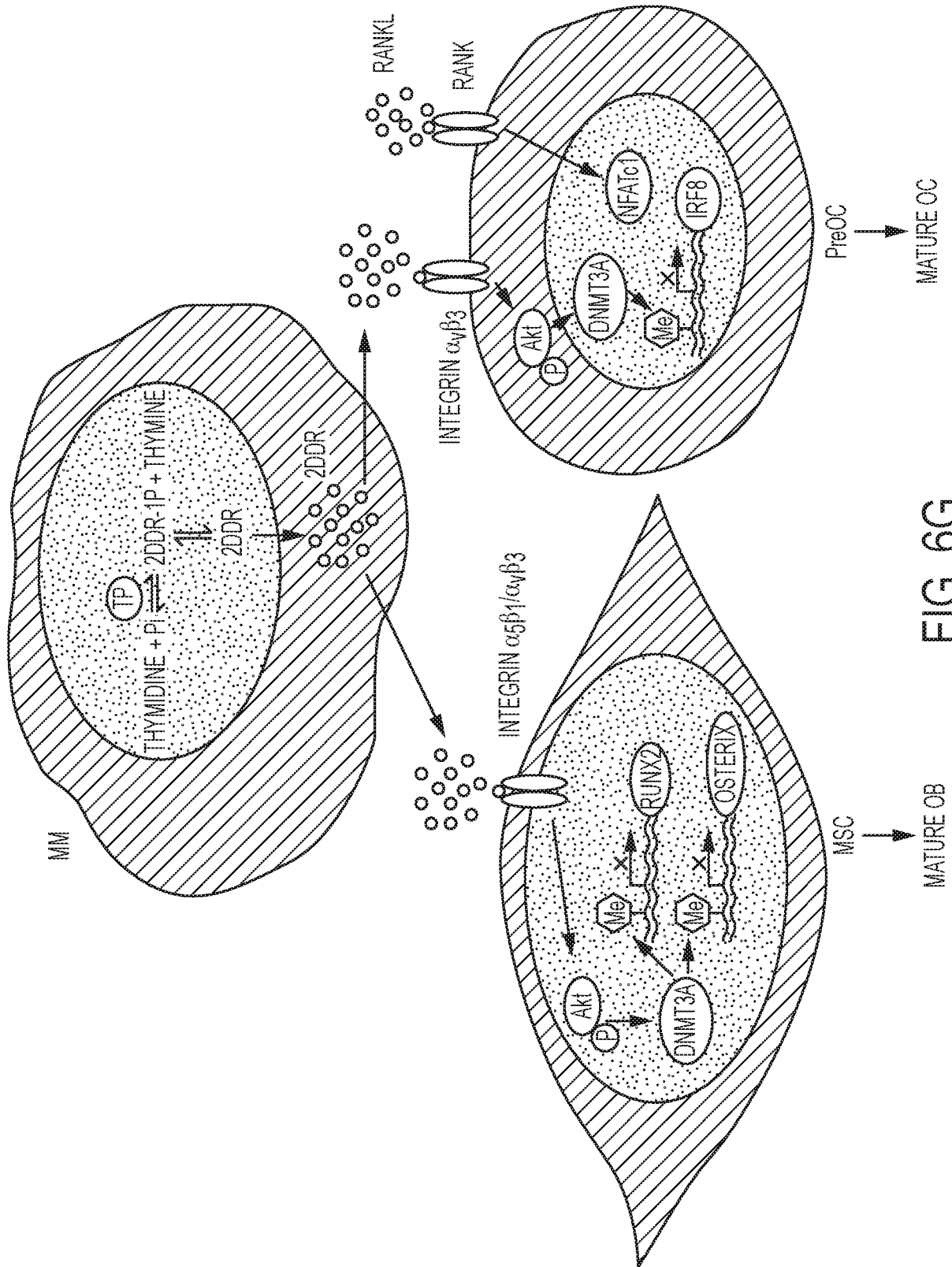


FIG. 6G

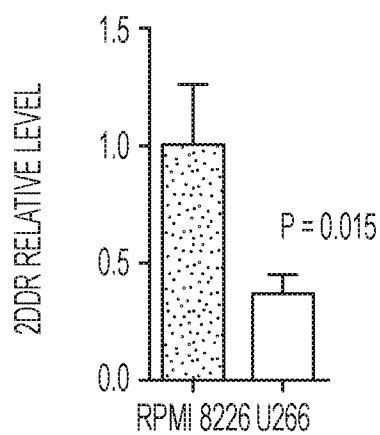


FIG. 7A

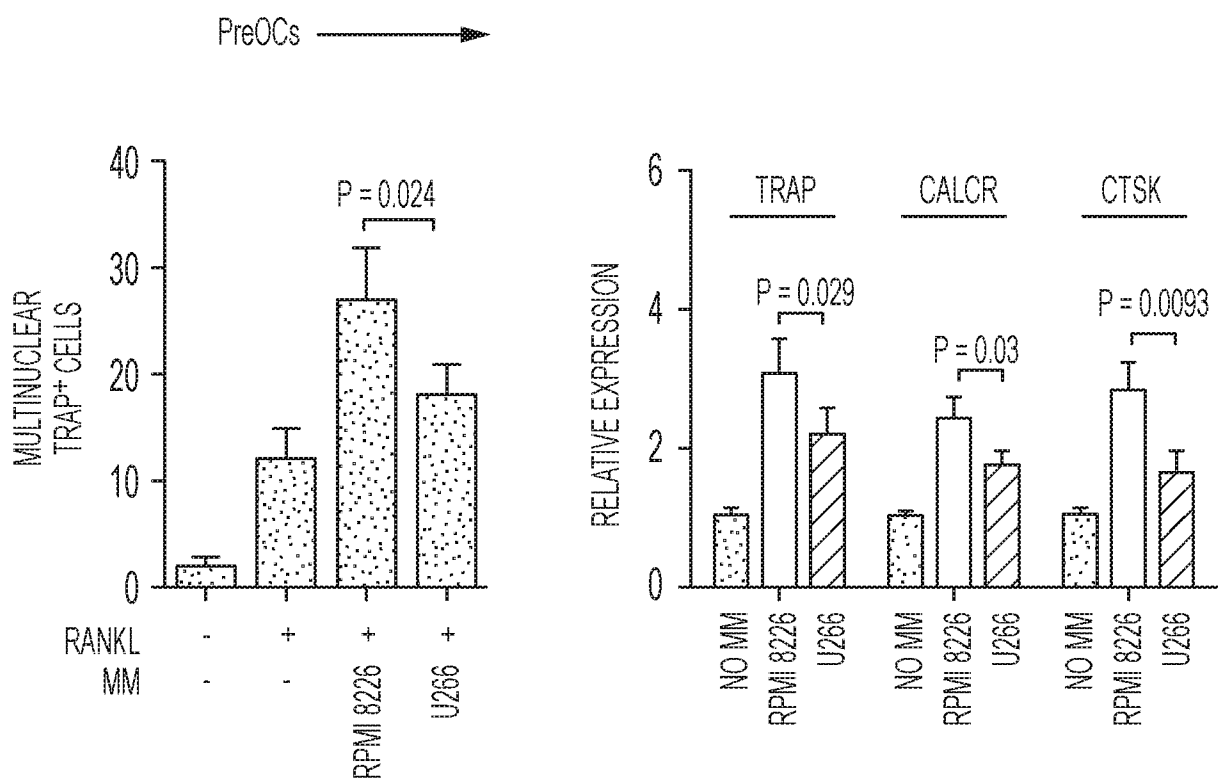


FIG. 7B

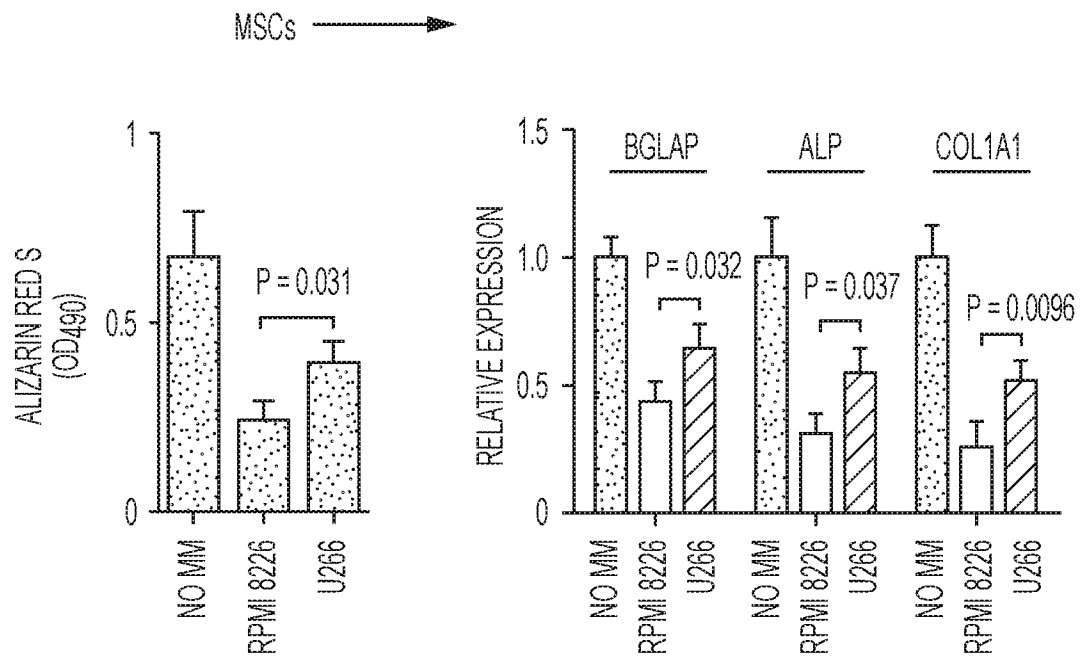


FIG. 7C

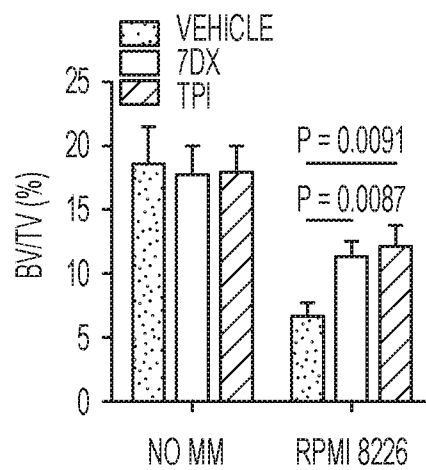


FIG. 7D

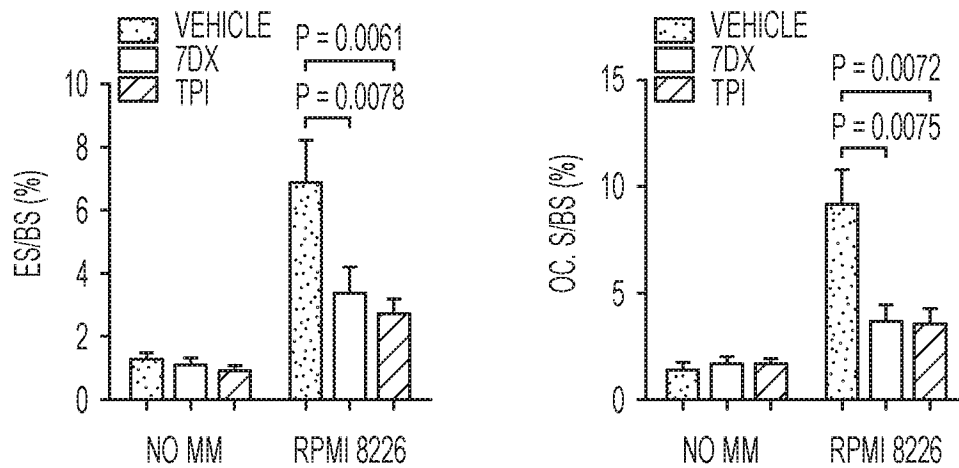


FIG. 7E

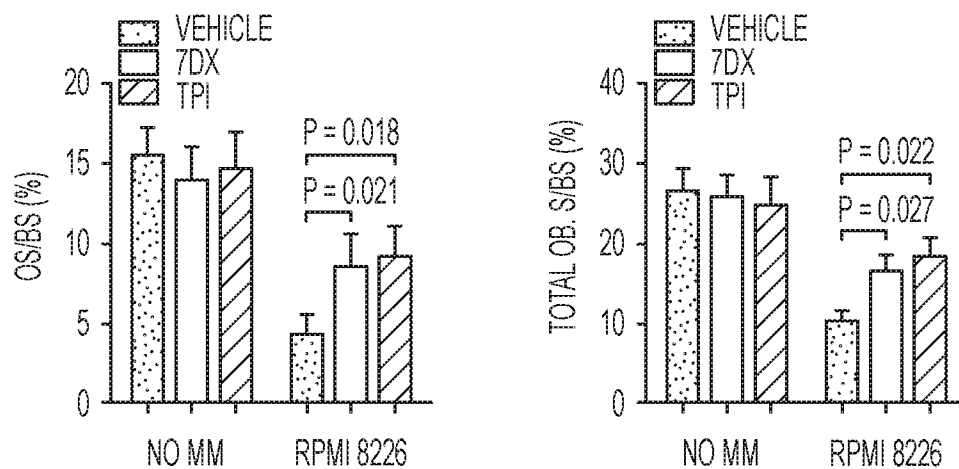


FIG. 7F

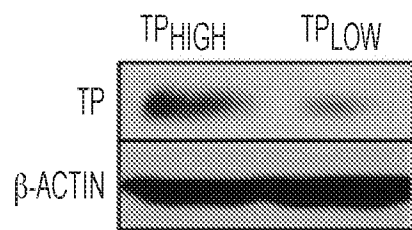


FIG. 8A

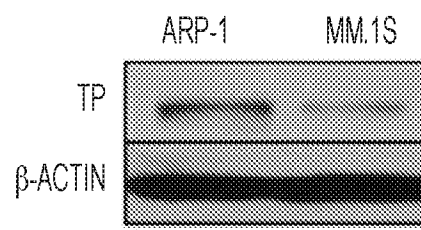


FIG. 8B

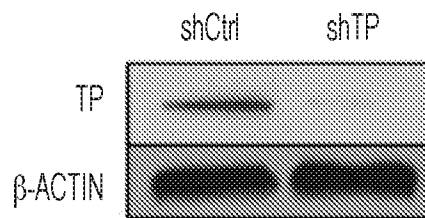


FIG. 8C

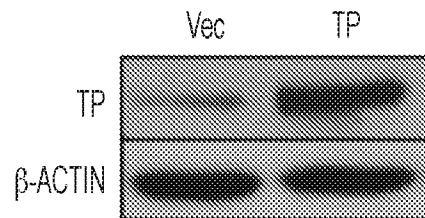


FIG. 8D

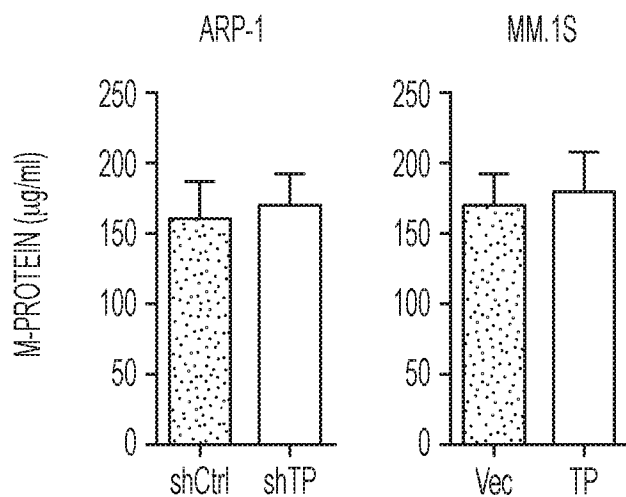


FIG. 8E

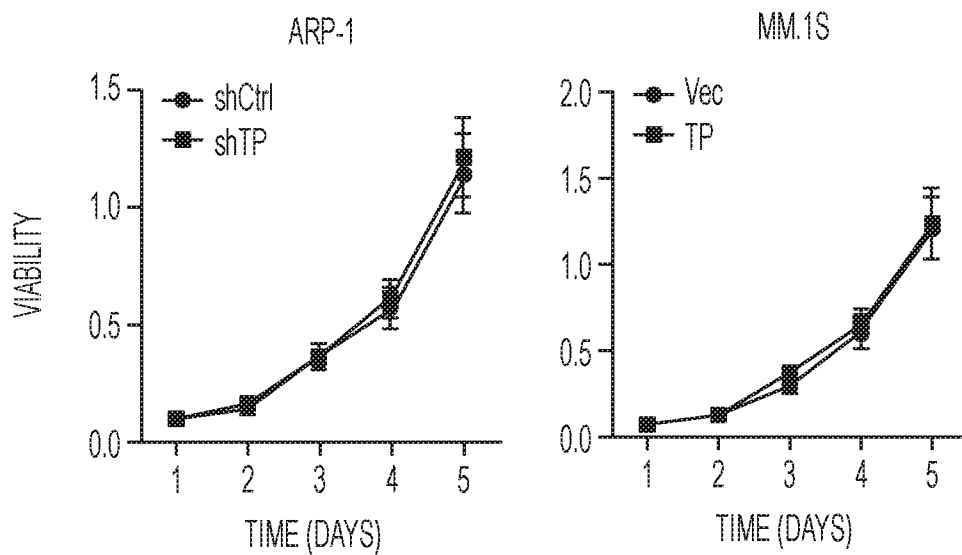


FIG. 8F

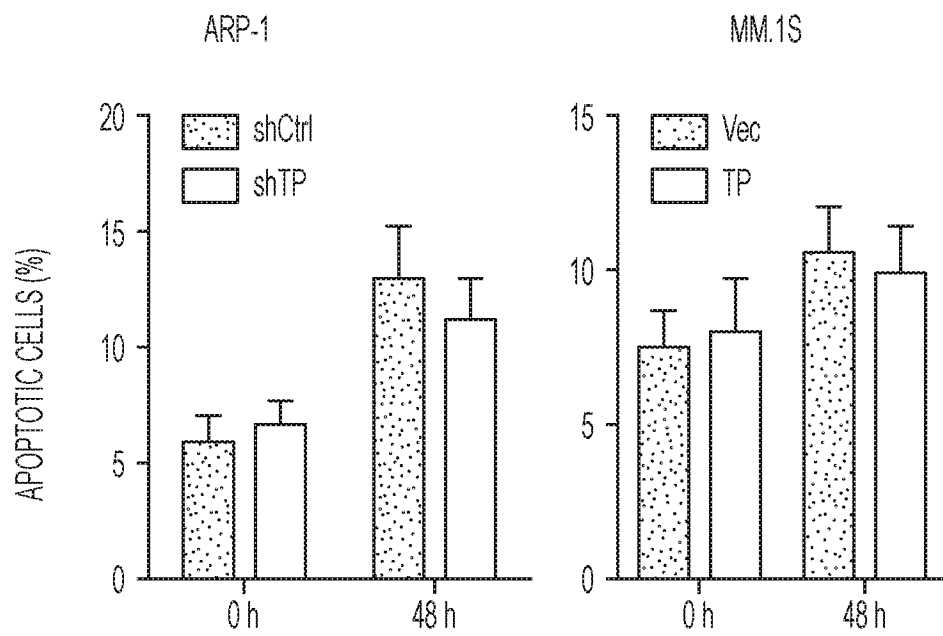


FIG. 8G

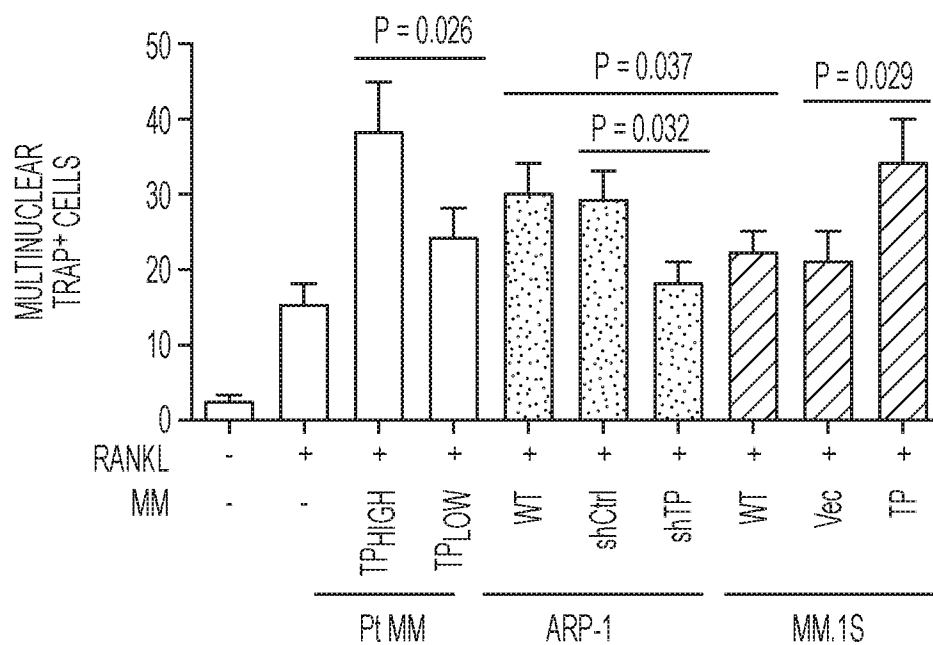


FIG. 9A

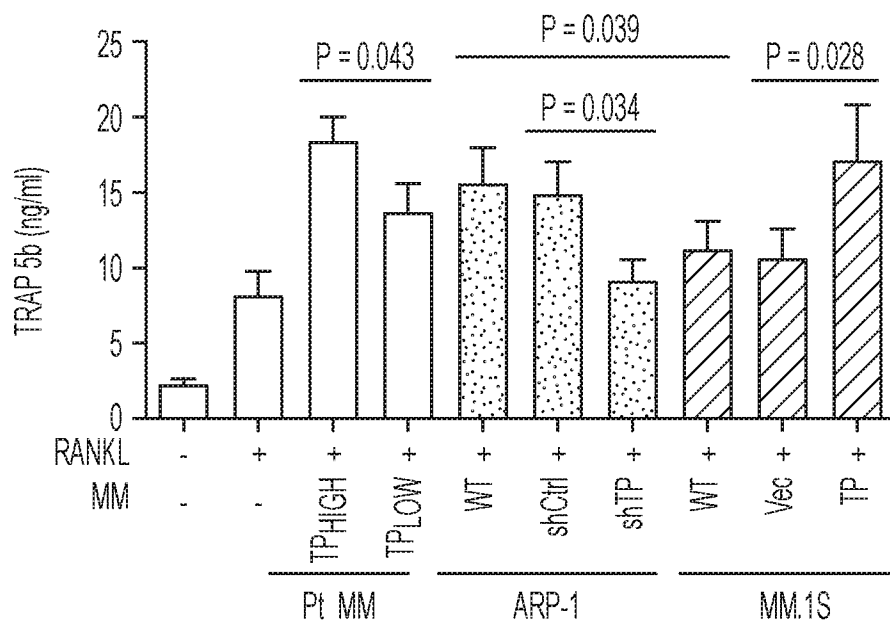


FIG. 9B

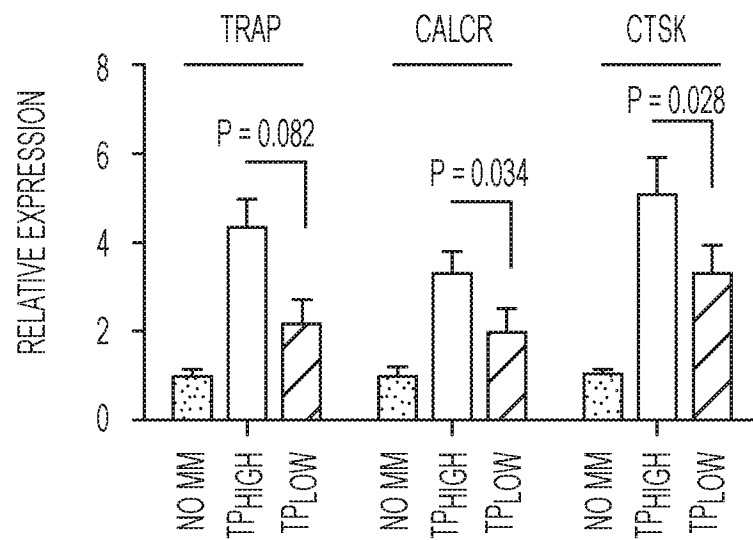


FIG. 9C

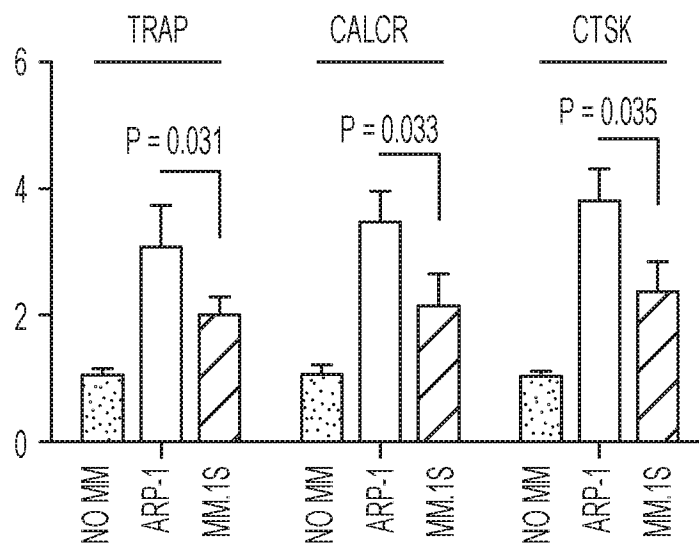


FIG. 9D

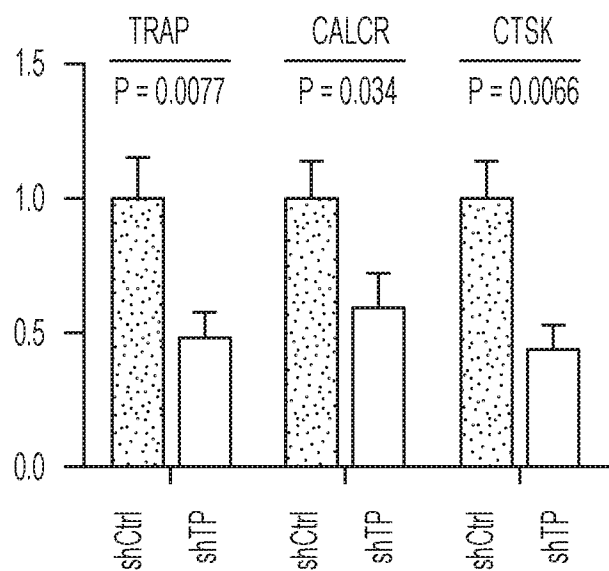


FIG. 9E

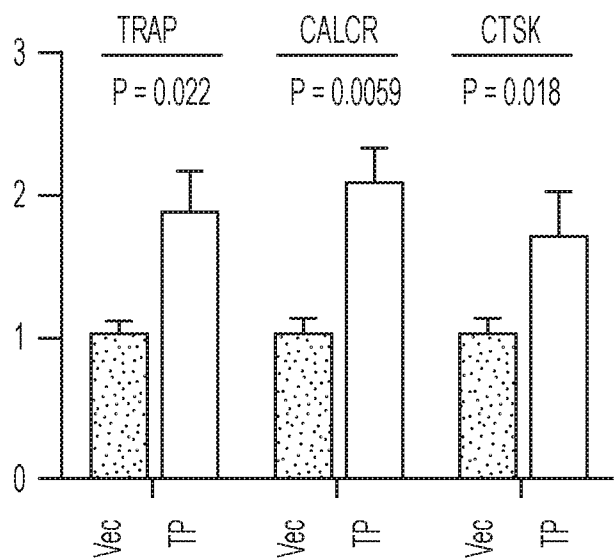


FIG. 9F

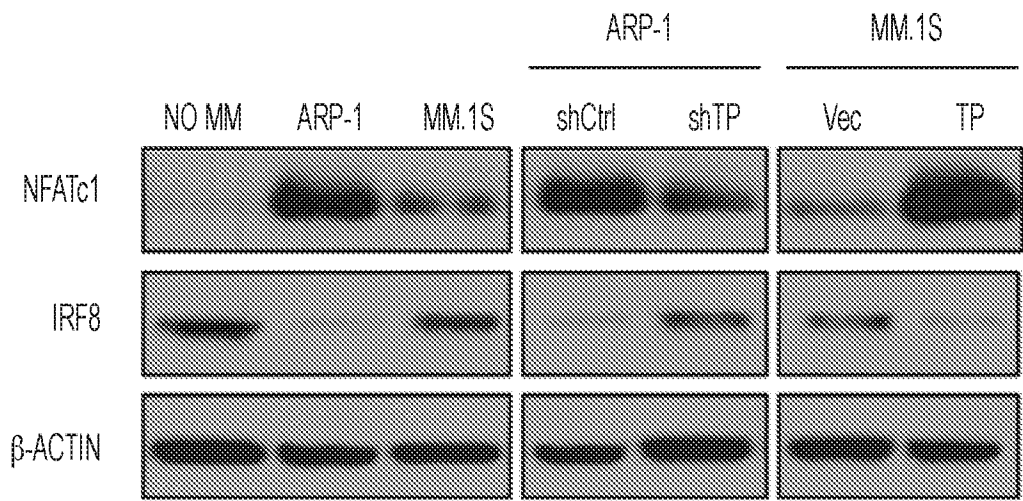


FIG. 10A

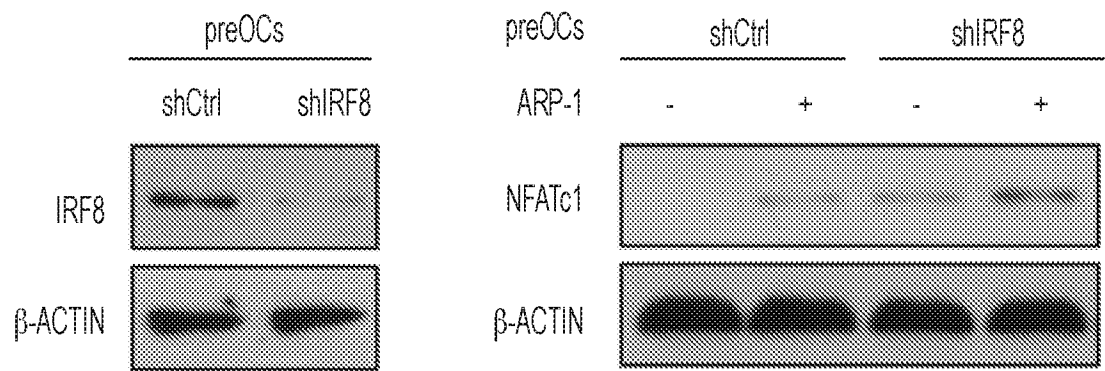


FIG. 10B

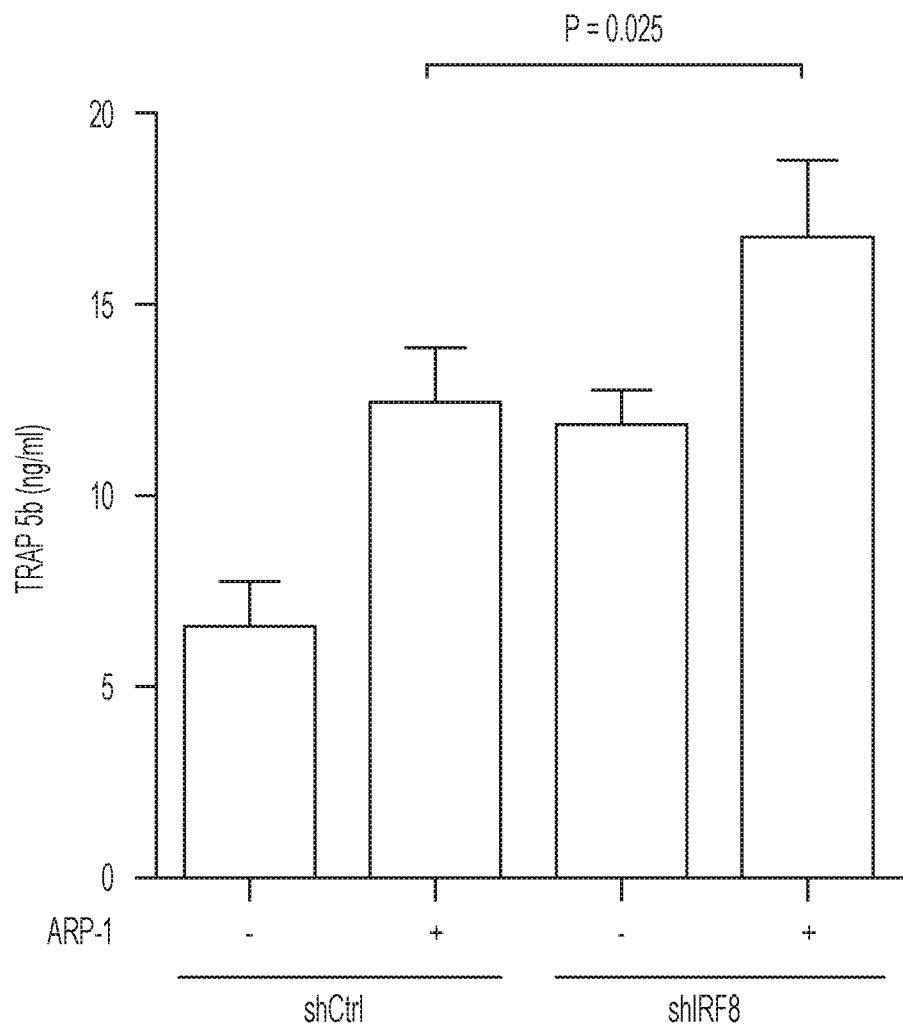


FIG. 10C

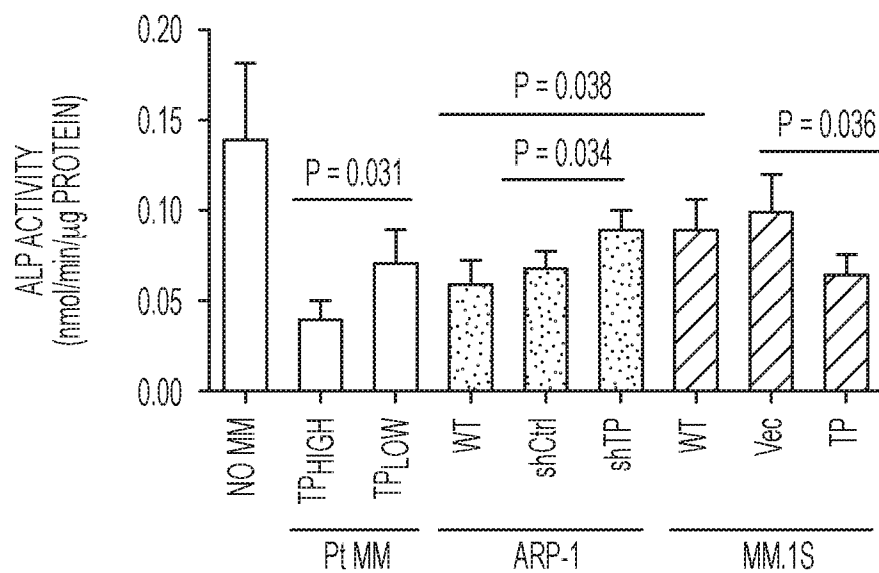


FIG. 11A

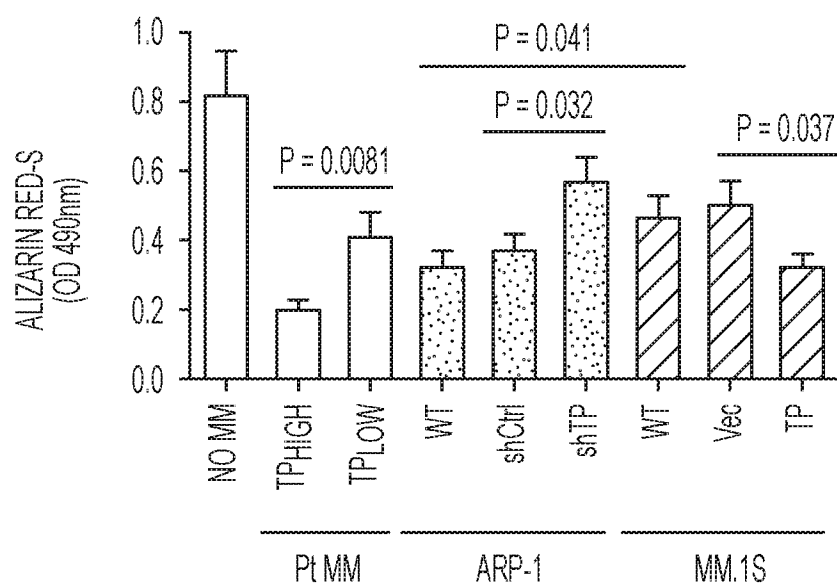


FIG. 11B

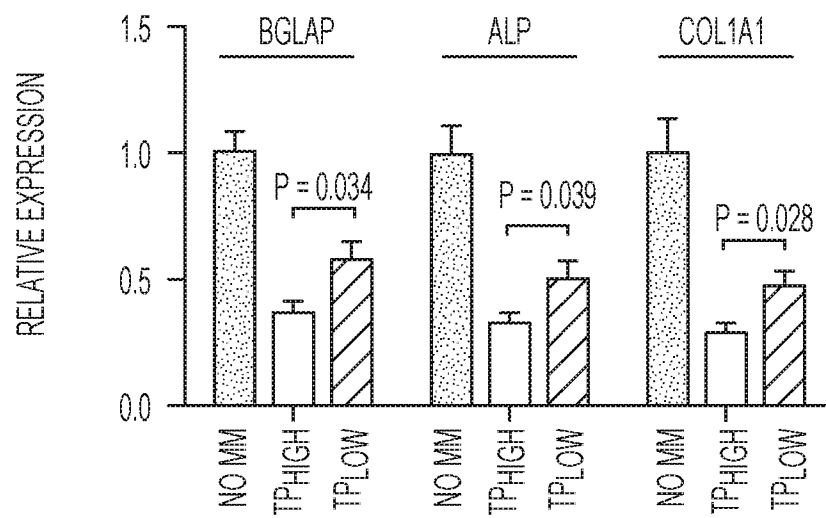


FIG. 11C

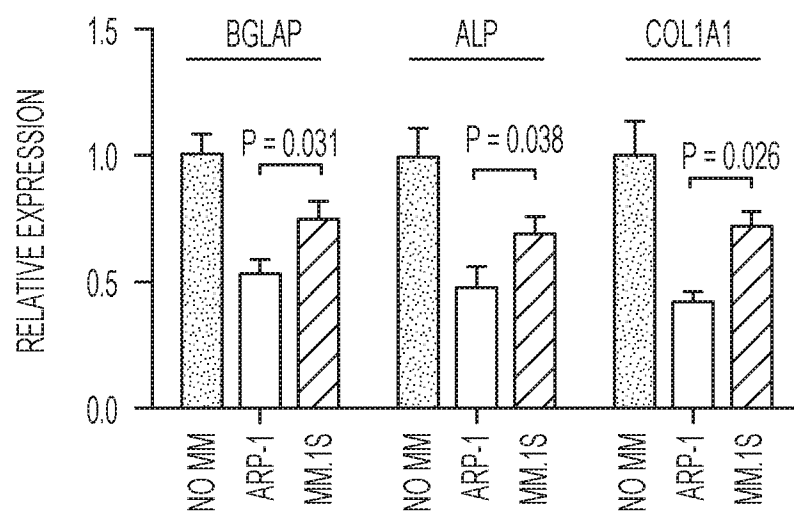


FIG. 11D

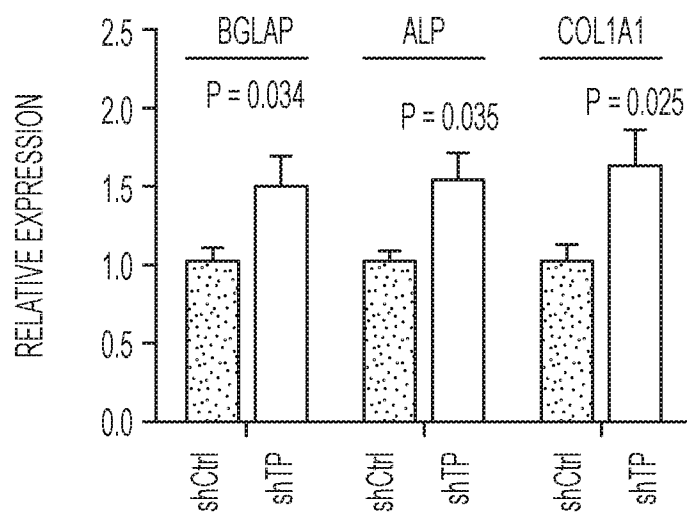


FIG. 11E

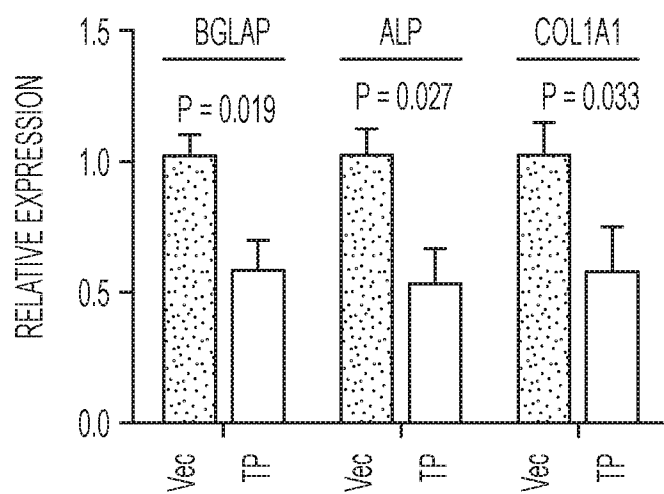


FIG. 11F

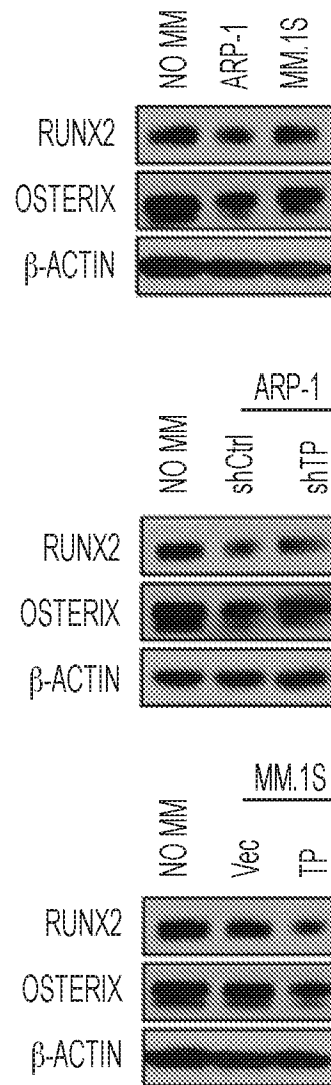


FIG. 12A

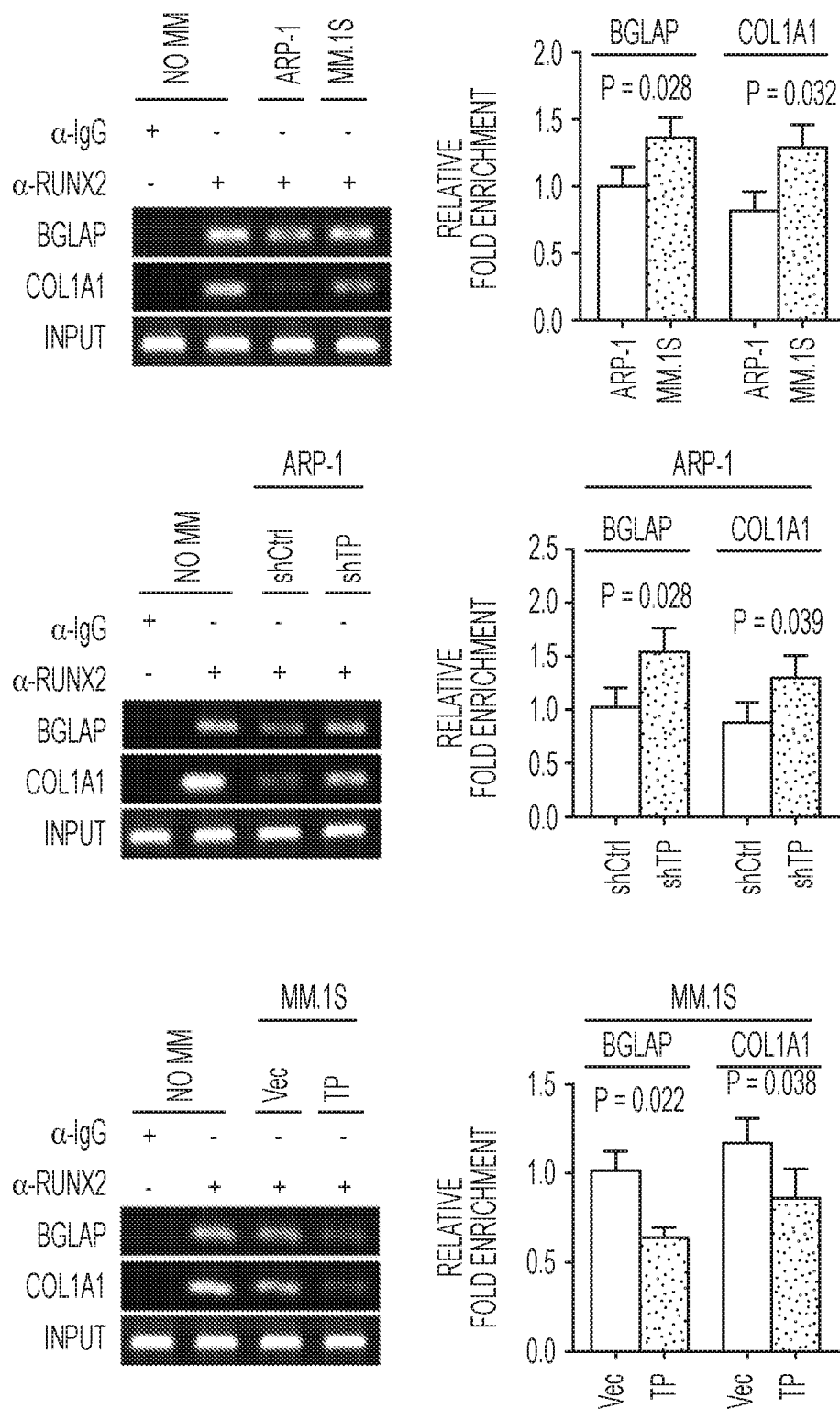


FIG. 12B

38/56

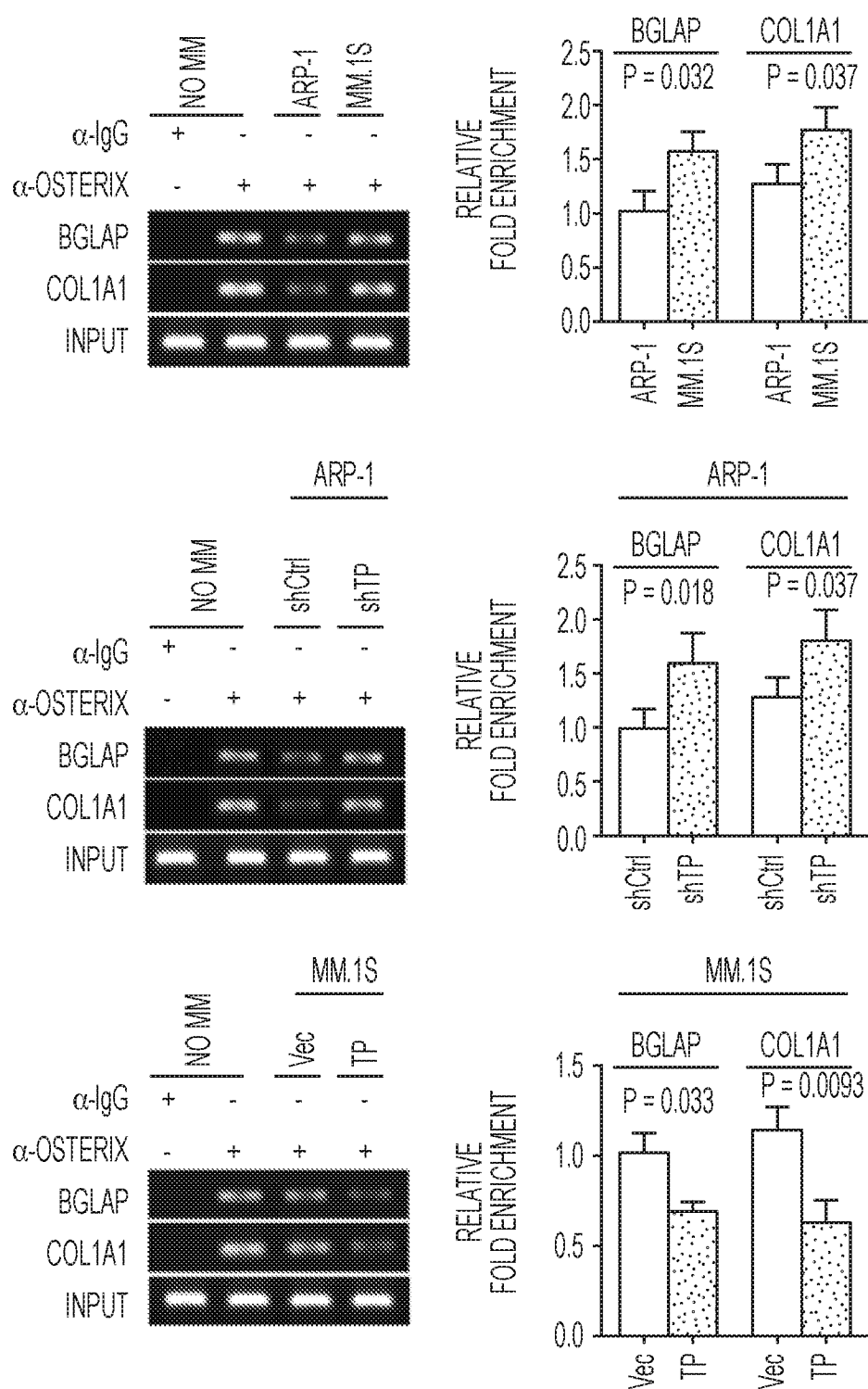


FIG. 12C

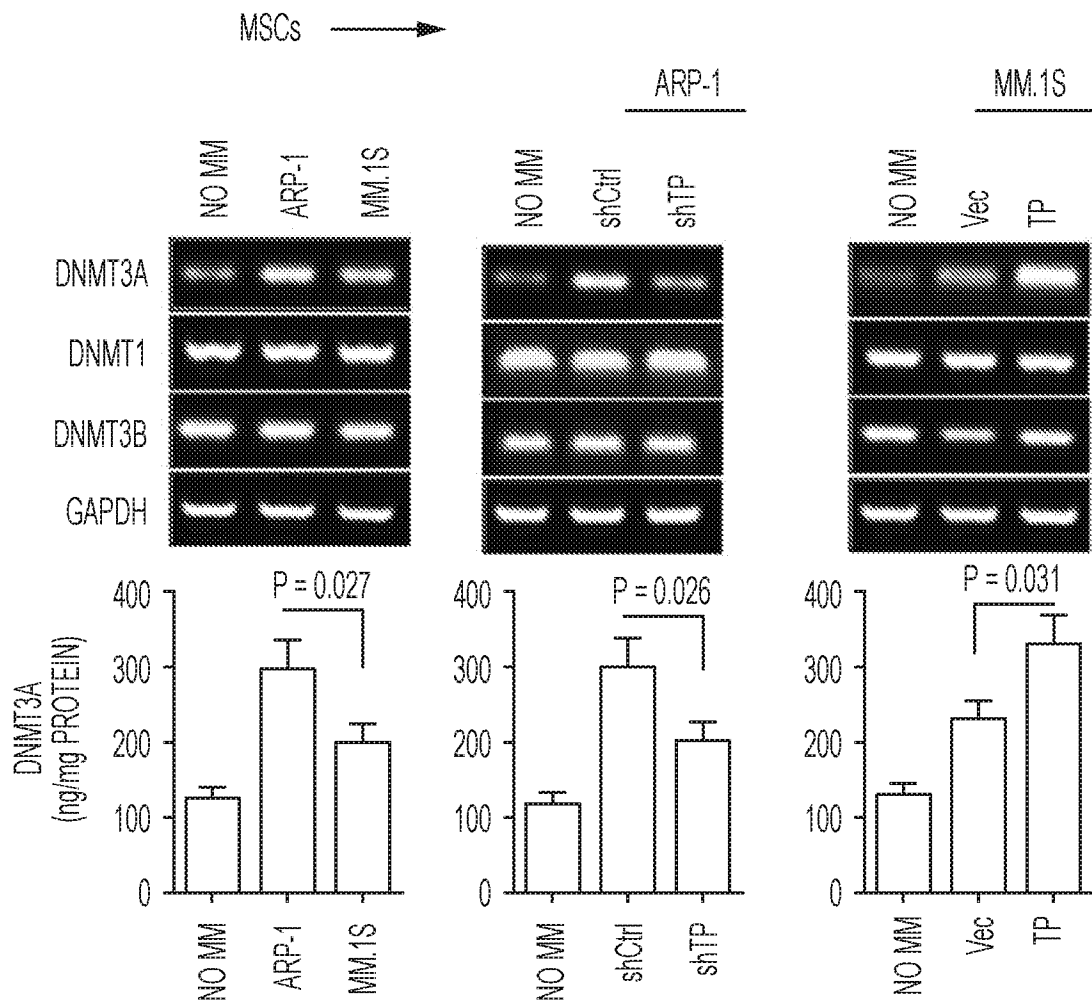


FIG. 13A

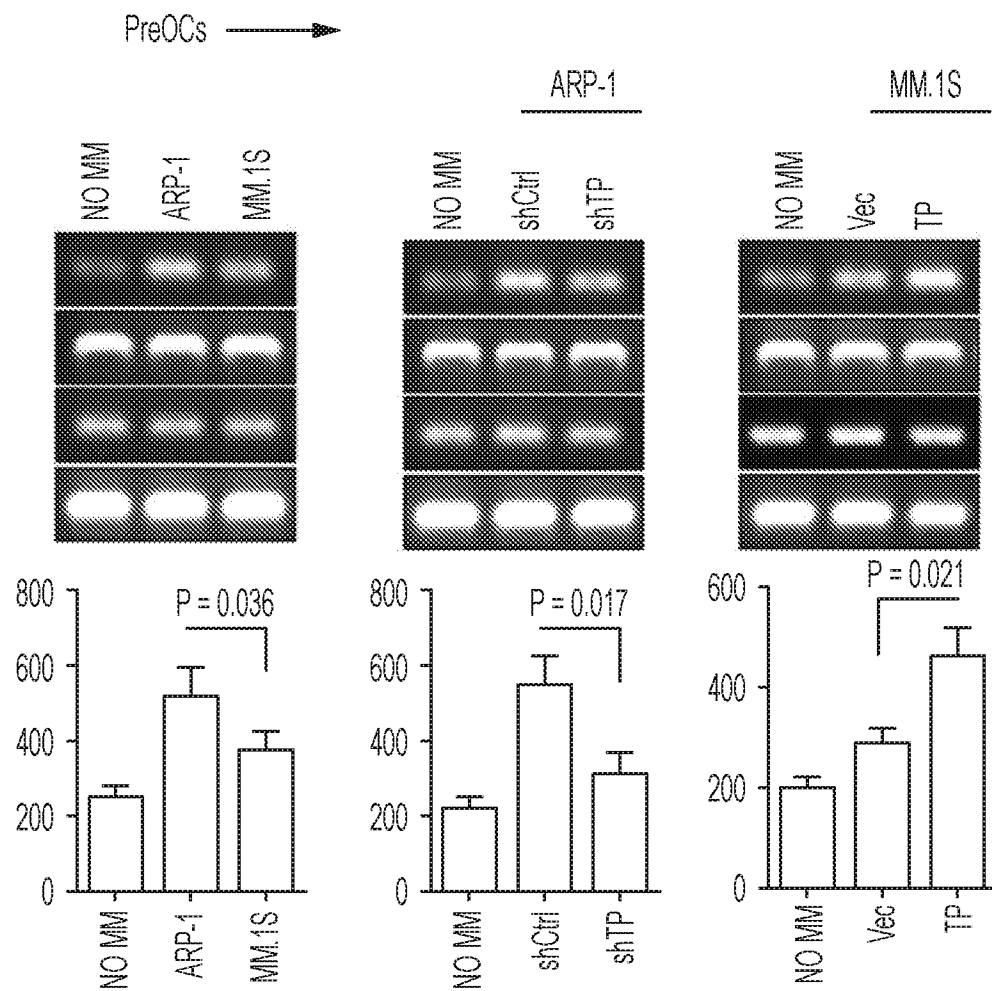


FIG. 13B

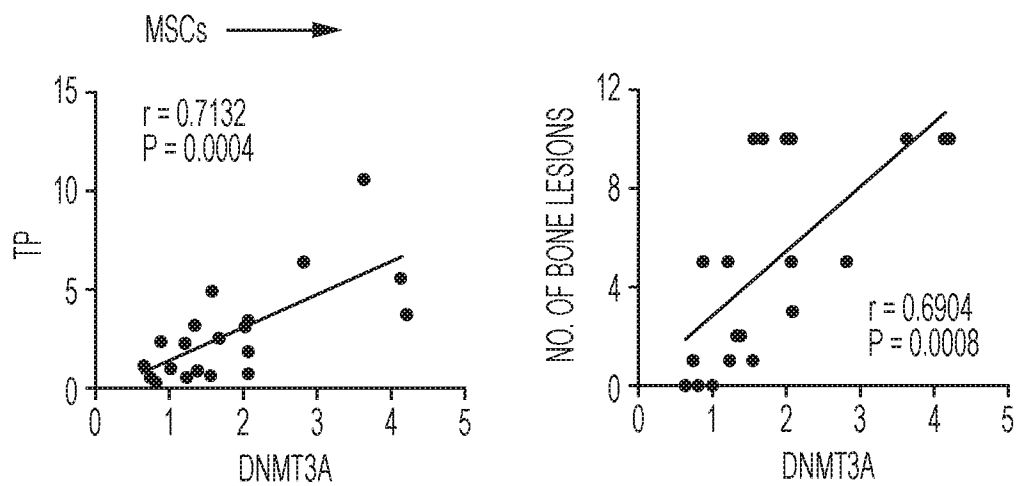


FIG. 13C

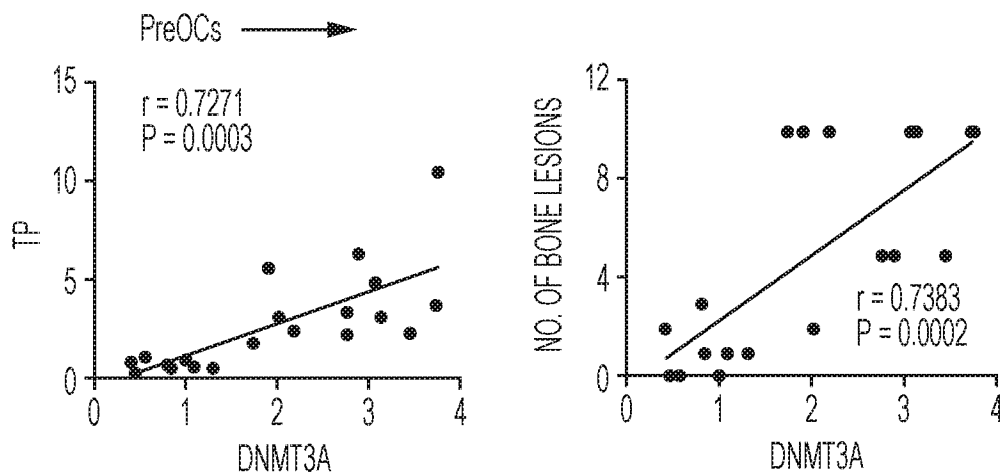


FIG. 13D

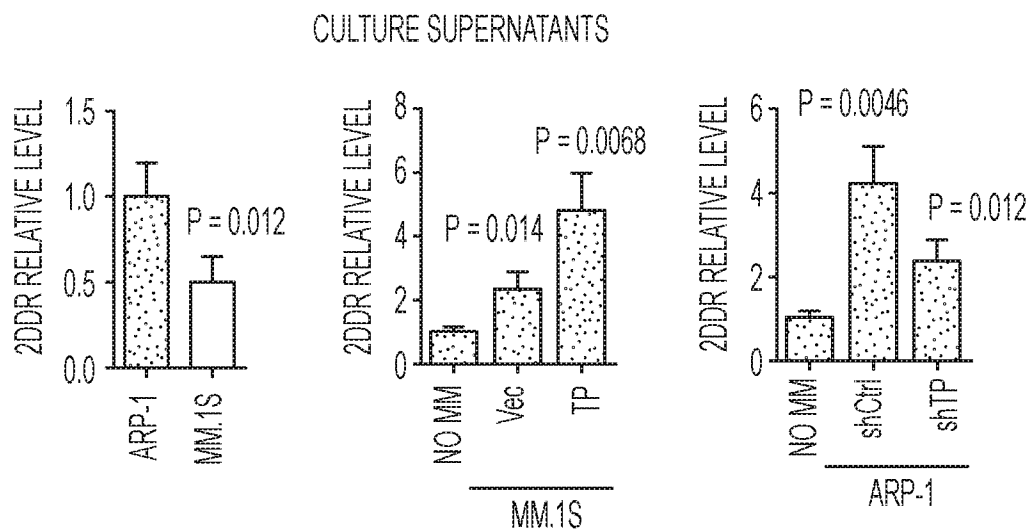


FIG. 14A

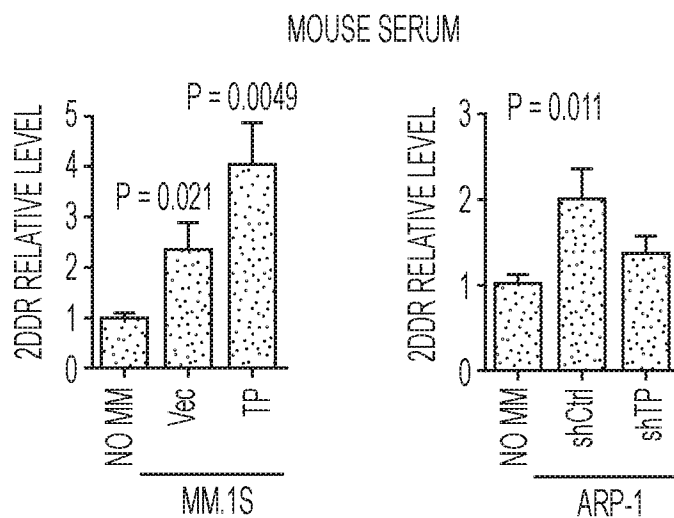


FIG. 14B

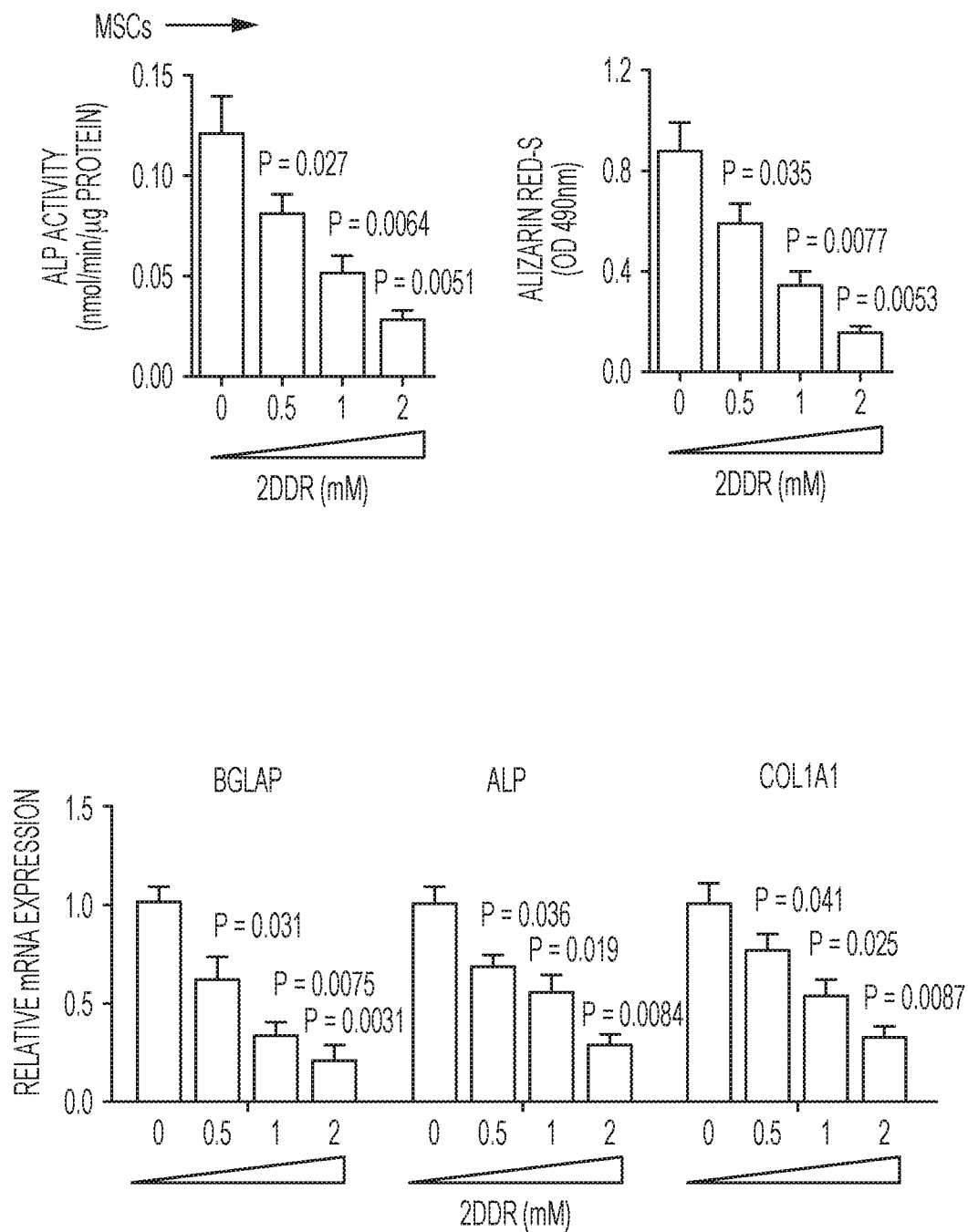


FIG. 14C

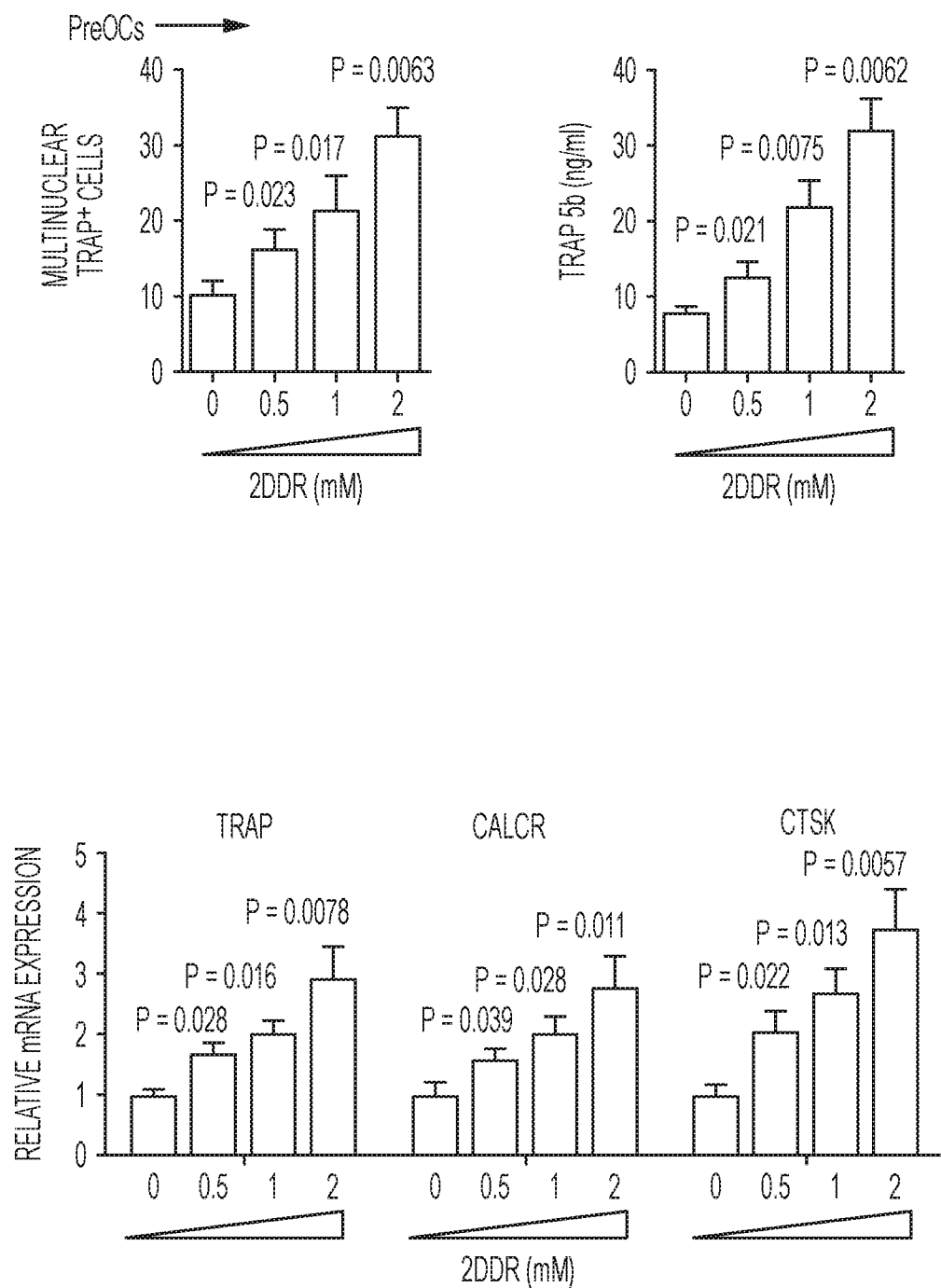


FIG. 14D

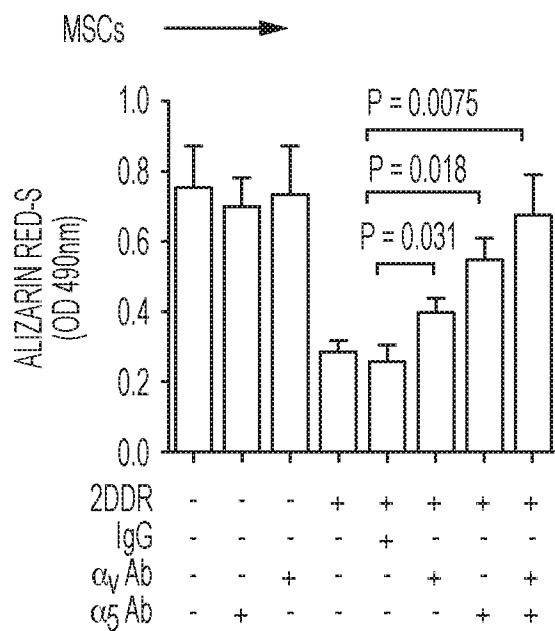


FIG. 15A

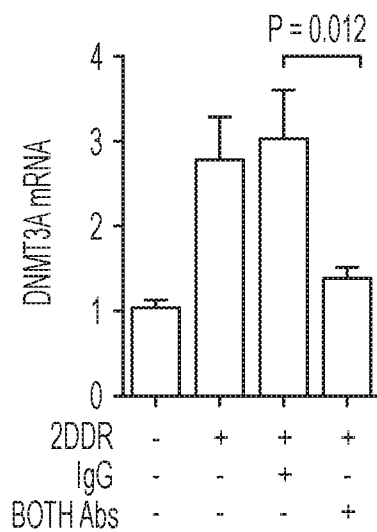


FIG. 15B

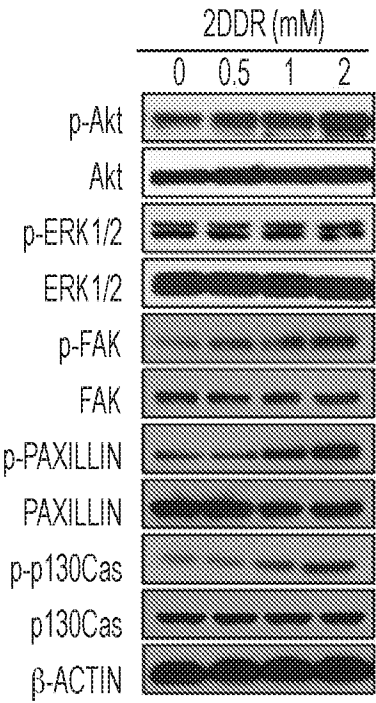


FIG. 15C

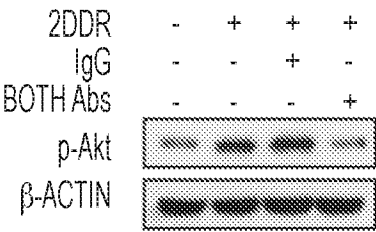


FIG. 15D

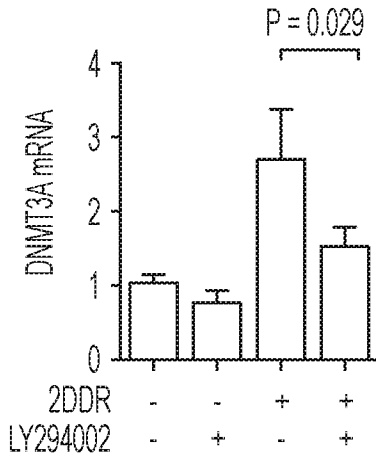


FIG. 15E

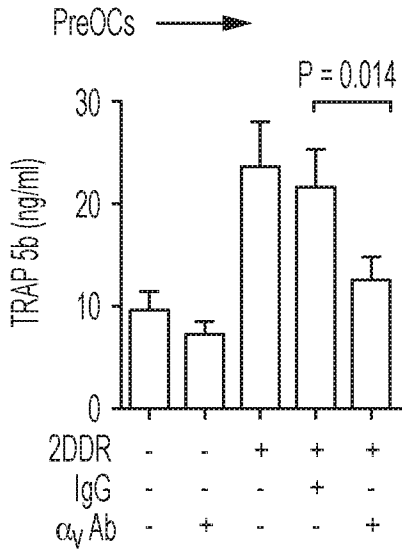


FIG. 15F

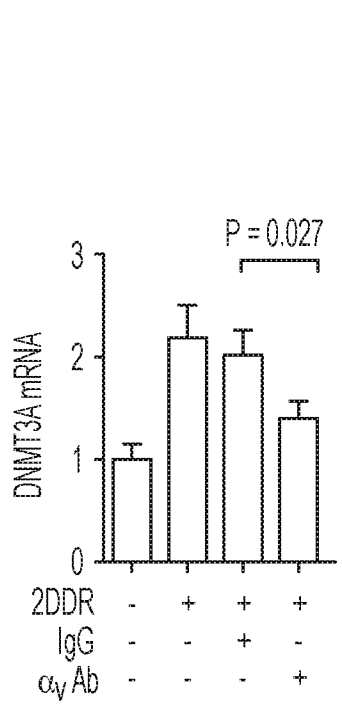


FIG. 15G

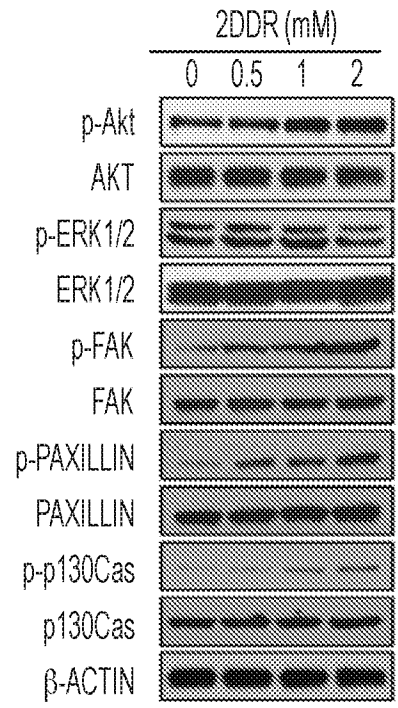


FIG. 15H

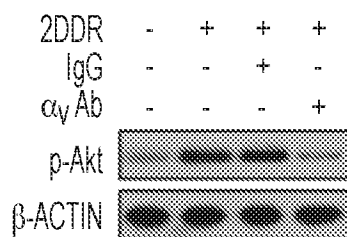


FIG. 15I

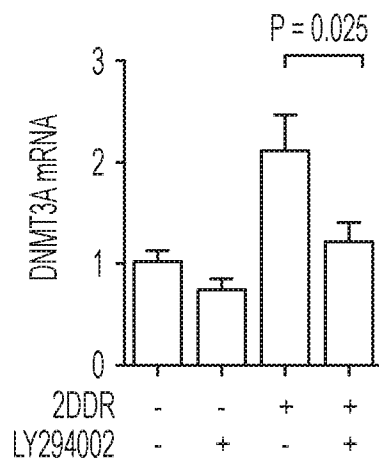


FIG. 15J

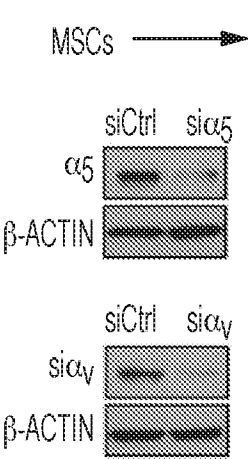


FIG. 16A

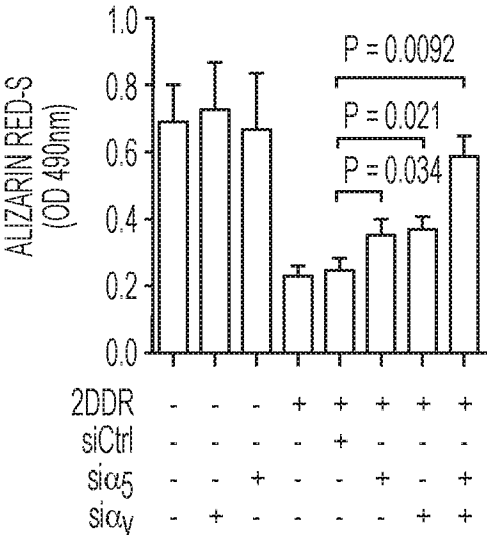


FIG. 16B

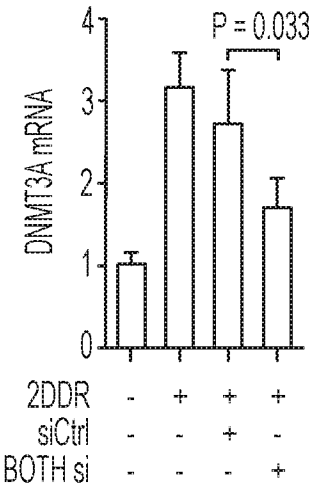


FIG. 16C

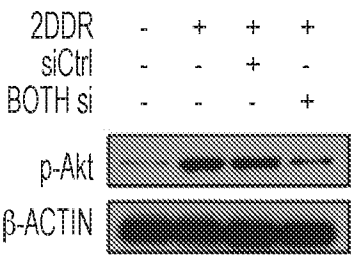


FIG. 16D

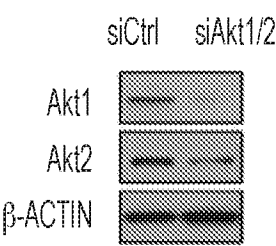


FIG. 16E

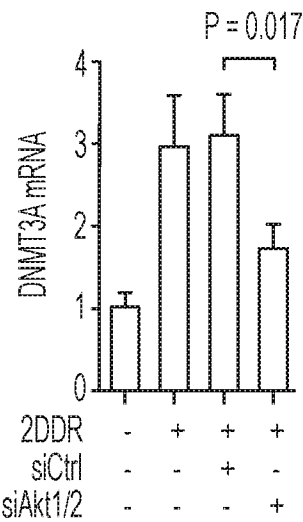


FIG. 16F

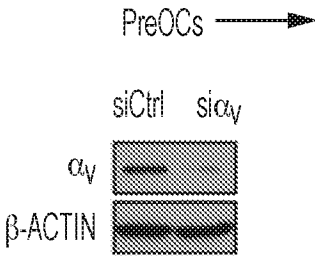


FIG. 16G

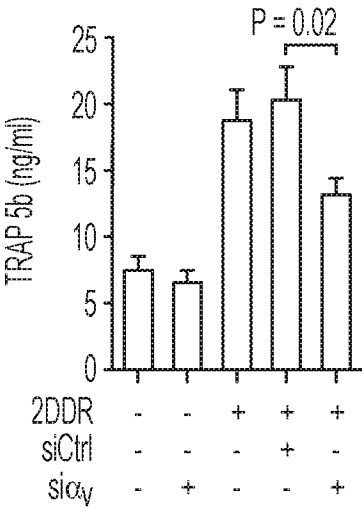


FIG. 16H

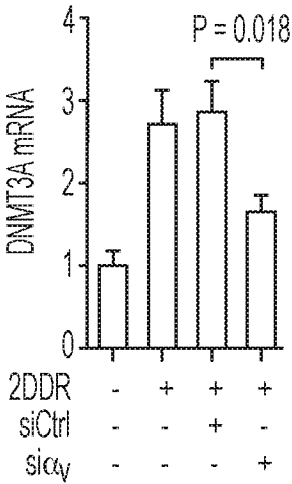


FIG. 16I

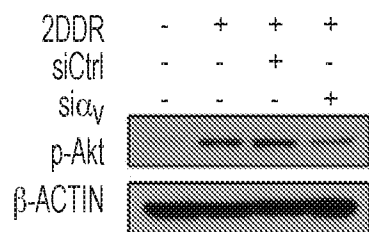


FIG. 16J

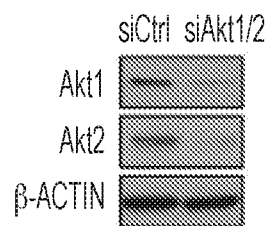


FIG. 16K

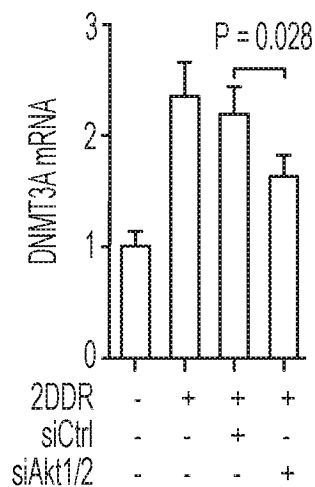


FIG. 16L

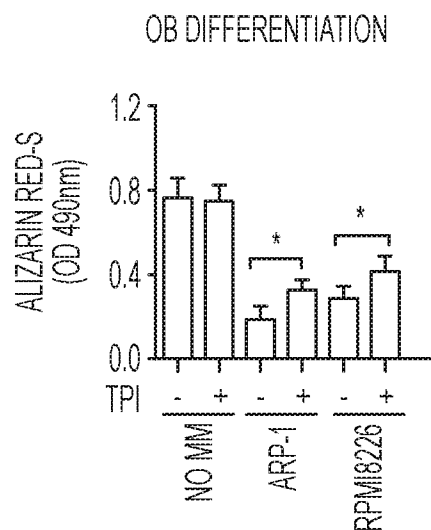


FIG. 17A

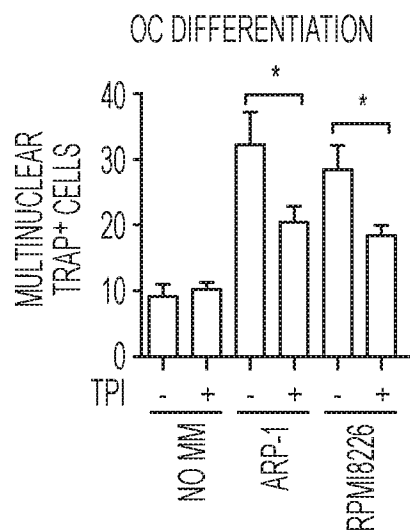


FIG. 17B

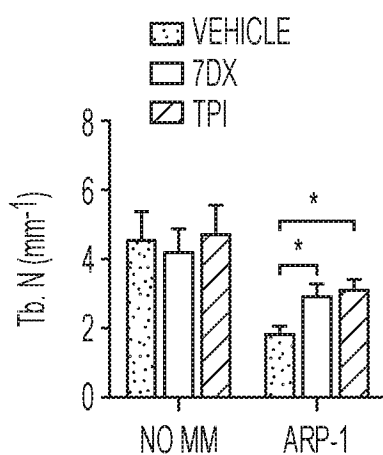


FIG. 18A

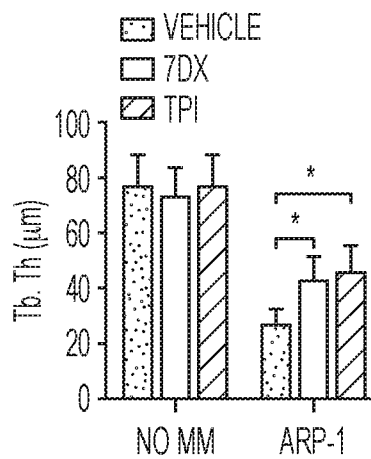


FIG. 18B

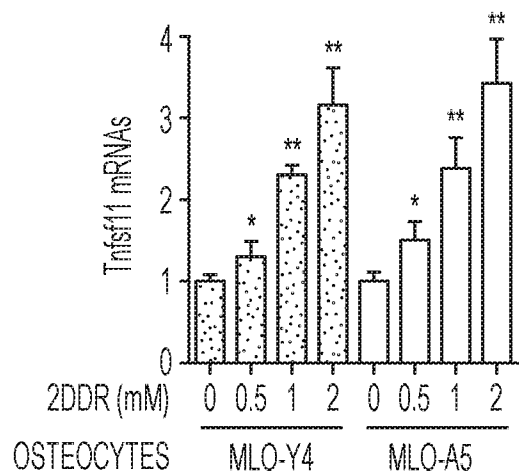


FIG. 19A

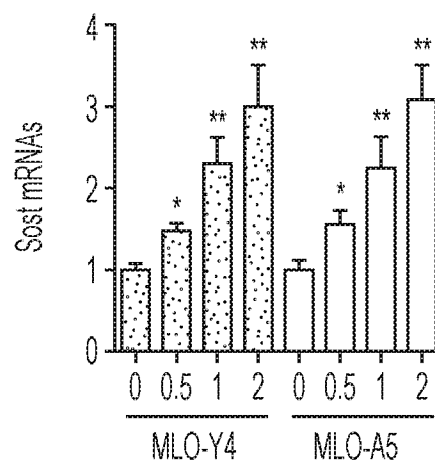


FIG. 19B

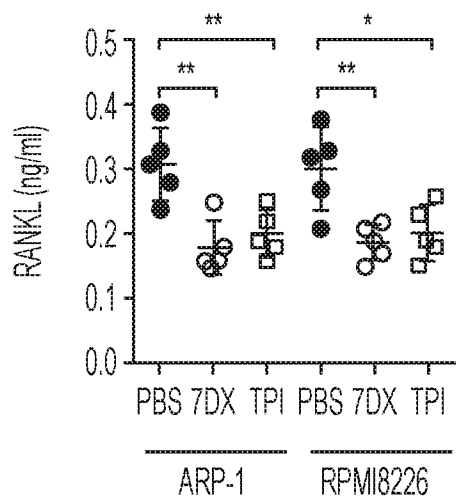


FIG. 20A

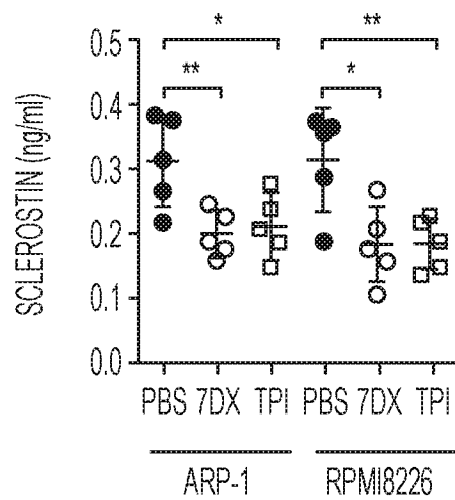


FIG. 20B

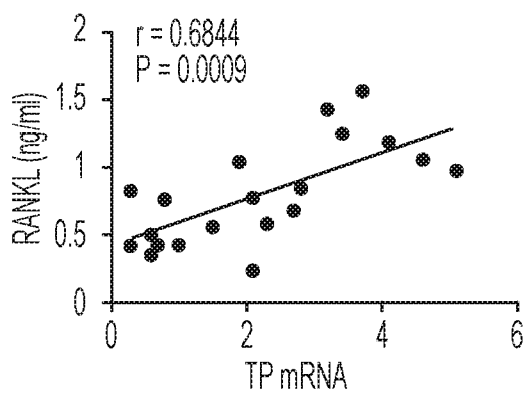


FIG. 21A

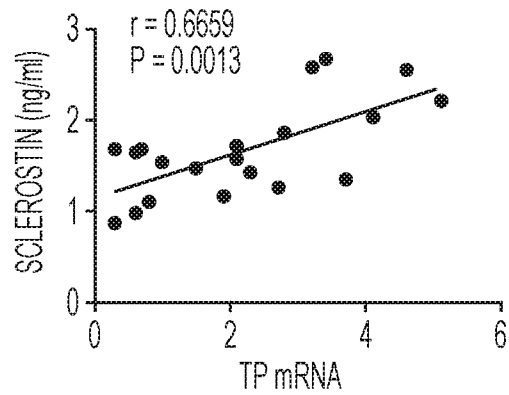


FIG. 21B

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 17/48232

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: 8-28, 63-85
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 17/48232

A. CLASSIFICATION OF SUBJECT MATTER
 IPC(8) - A61K 31/535, A61K 31/513 (2017.01)
 CPC - A61K 31/513

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History Document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

See Search History Document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History Document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- A	US 2006/0167031 A1 (Emura, et al.) 27 July 2006 (27.07.2006) claim 1, para [0004], [0014]-[0016], [0028]	29, 30, 59 ----- 1-7, 31-58, 60-62
X	US 2016/0145241 A1 (Taiho Pharmaceutical Co., Ltd.) 26 May 2016 (26.05.2016) claims 1, 14	29, 30, 59
A	✓ Bronckaers, et al. The dual role of thymidine phosphorylase in cancer development and chemotherapy. Med Res Rev. 2009, 29(6):903-53; pg 912, Figure 5	1-7, 31-58
A	✓ Nakajima, et al. Inhibition of metastasis of tumor cells overexpressing thymidine phosphorylase by 2-deoxy-L-ribose. Cancer Res. 2004, 64(5):1794-1801; Abstract	1-7, 31-58
A	✓ Tabata, et al. Thymidine phosphorylase activates NFκB and stimulates the expression of angiogenic and metastatic factors in human cancer cells. Oncotarget 2014, 5(21):10473-85; Abstract	1-7, 31-58
A	✓ Canalis, et al. Effects of endothelial cell growth factor on bone remodelling in vitro. J Clin Invest. 1987, 79(1):52-8; Abstract	1-7, 31-58, 60-62

☒ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier application or patent but published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

09 October 2017 (09.10.2017)

Date of mailing of the international search report

08 NOV 2017

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Authorized officer:

Lee W. Young

PCT Helpdesk: 571-272-4300
 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 17/48232

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Kawaguchi, et al. The JAK Inhibitor (Tofacitinib) Inhibits TNF-Induced Gliostatin/thymidine Phosphorylase Expression in Human Fibroblast-like Synoviocytes. <i>Annals of the Rheumatic Diseases</i> 10 June 2016, 75:441; Abstract FRI0041 in entirety	1-7, 31-58, 60-62
A	Schett, et al. Bone erosion in rheumatoid arthritis: mechanisms, diagnosis and treatment. <i>Nat Rev Rheumatol.</i> 2012, 8(11): 656-664; pg 2 [according to the posted document], pg 5	1-7, 31-58, 60-62
X,P	Liu, et al. Thymidine phosphorylase exerts complex effects on bone resorption and formation in myeloma. <i>Sci Transl Med.</i> 24 August 2016, 8(353):353ra113; in entirety	1-7, 31-58, 60-62