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(54) **TMD1 PROTEIN FOR TREATING BONE LOSS DISEASES**

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**A61P 19/08** (2006.01)  
**A61K 38/36** (2006.01)

(52) **U.S. Cl.**  
CPC ..... **A61K 38/366** (2013.01)

(58) **Field of Classification Search**  
None  
See application file for complete search history.

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(57) **ABSTRACT**

The present invention relates to a method for treating a bone loss disease, condition, or disorder in a subject in need thereof, comprising administering to said subject a pharmaceutically effective amount of a composition comprising thrombomodulin lectin-like domain (TMD1).

**6 Claims, 7 Drawing Sheets**  
**(4 of 7 Drawing Sheet(s) Filed in Color)**

Fig. 1

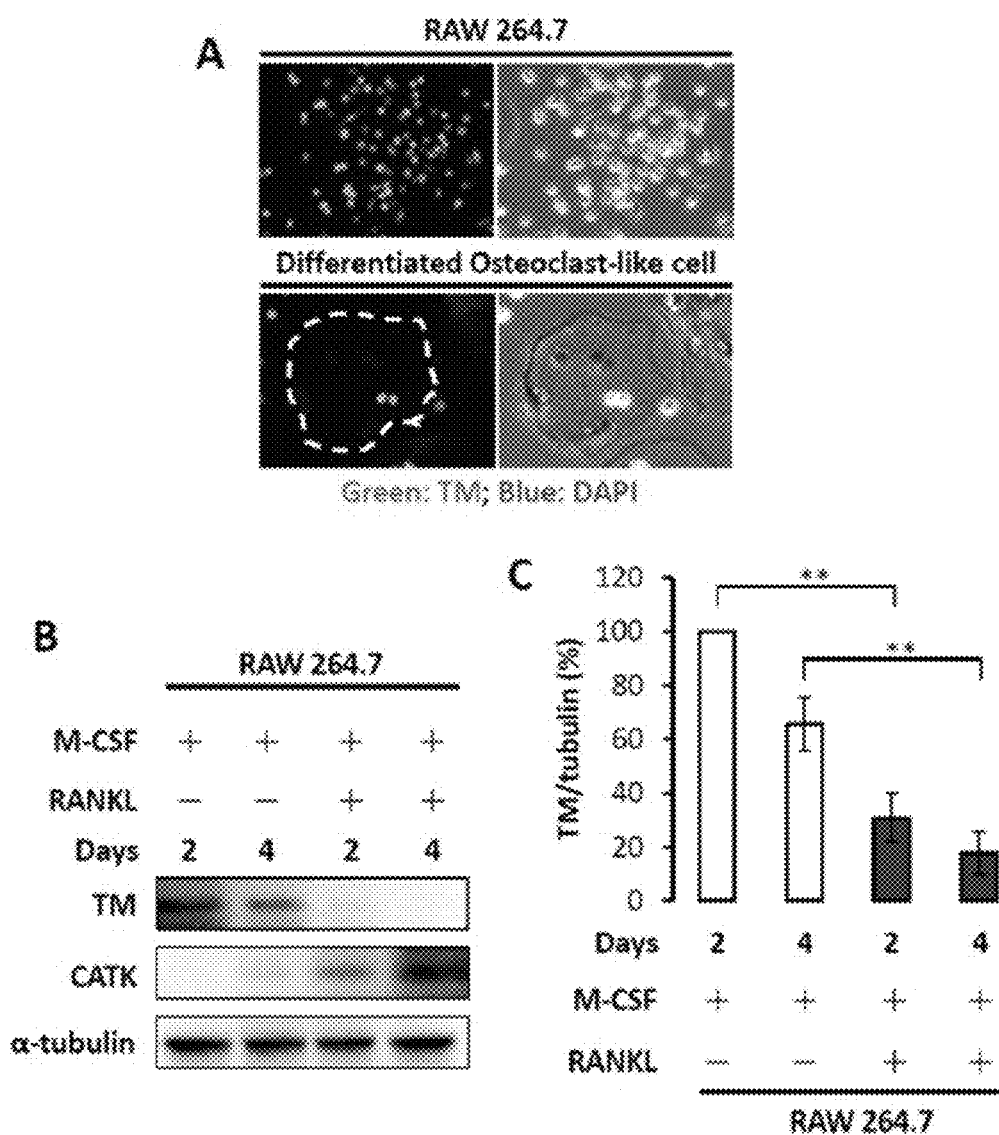


Fig. 2

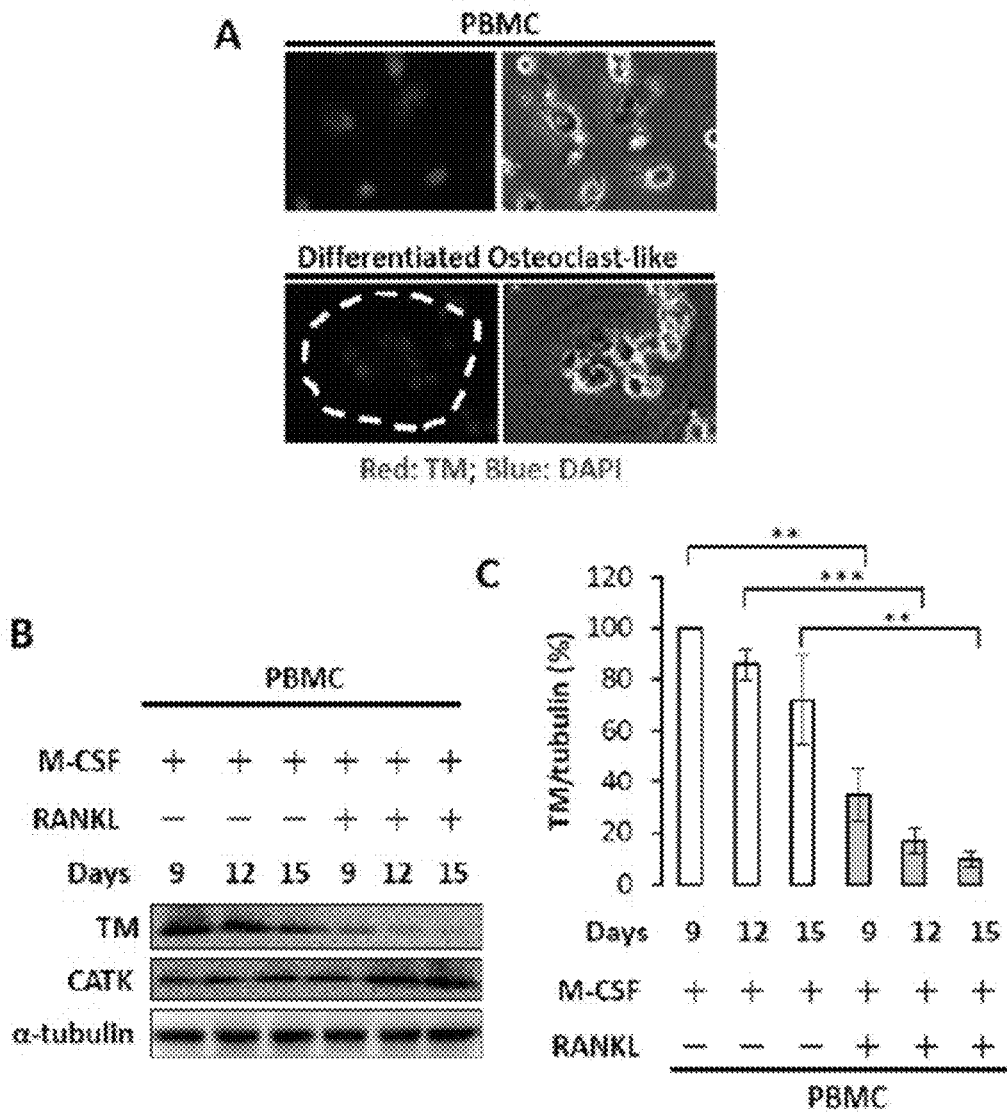


Fig. 3

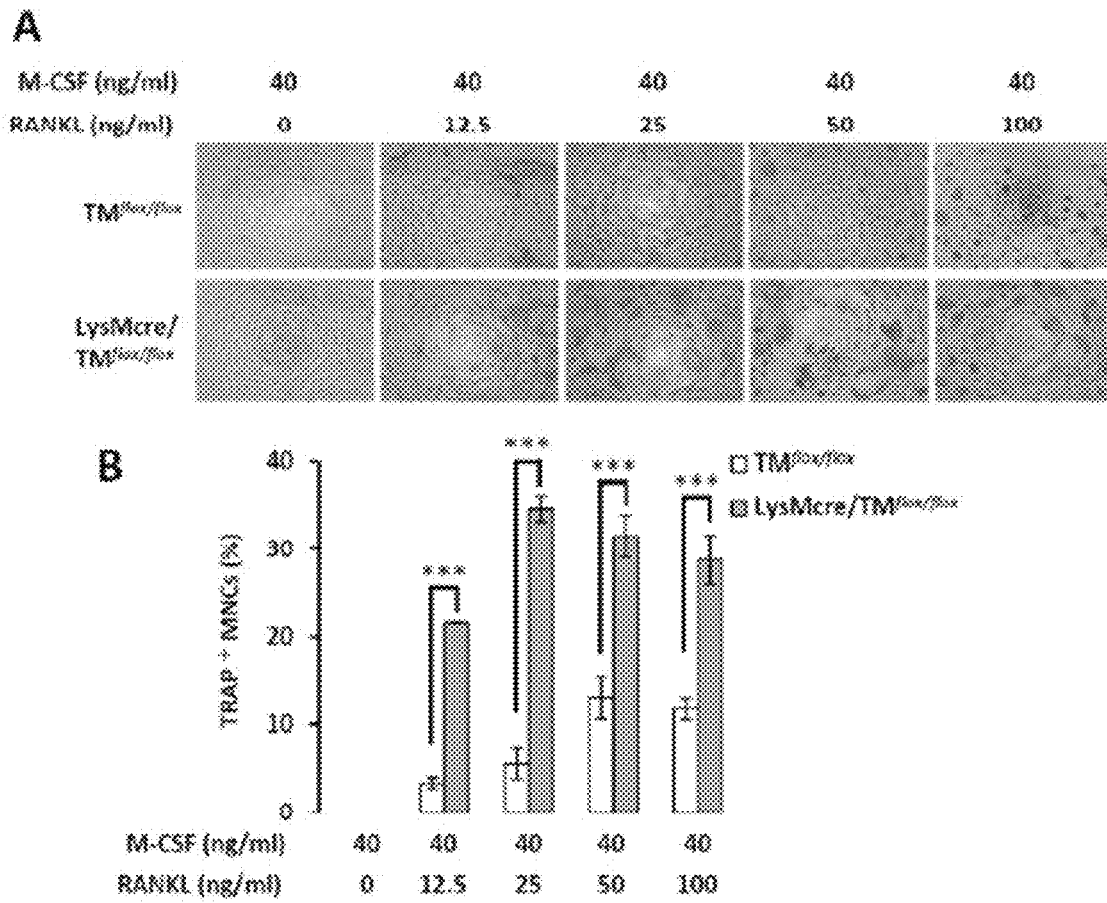
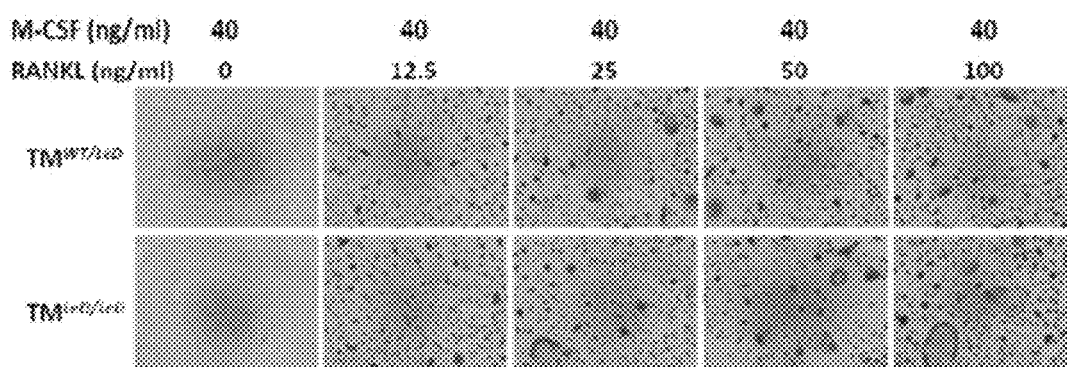


Fig. 4

**A**



**B**

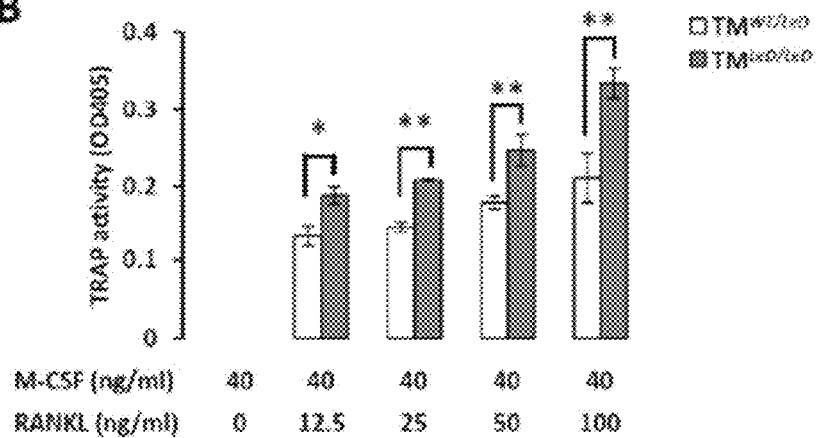


Fig. 5

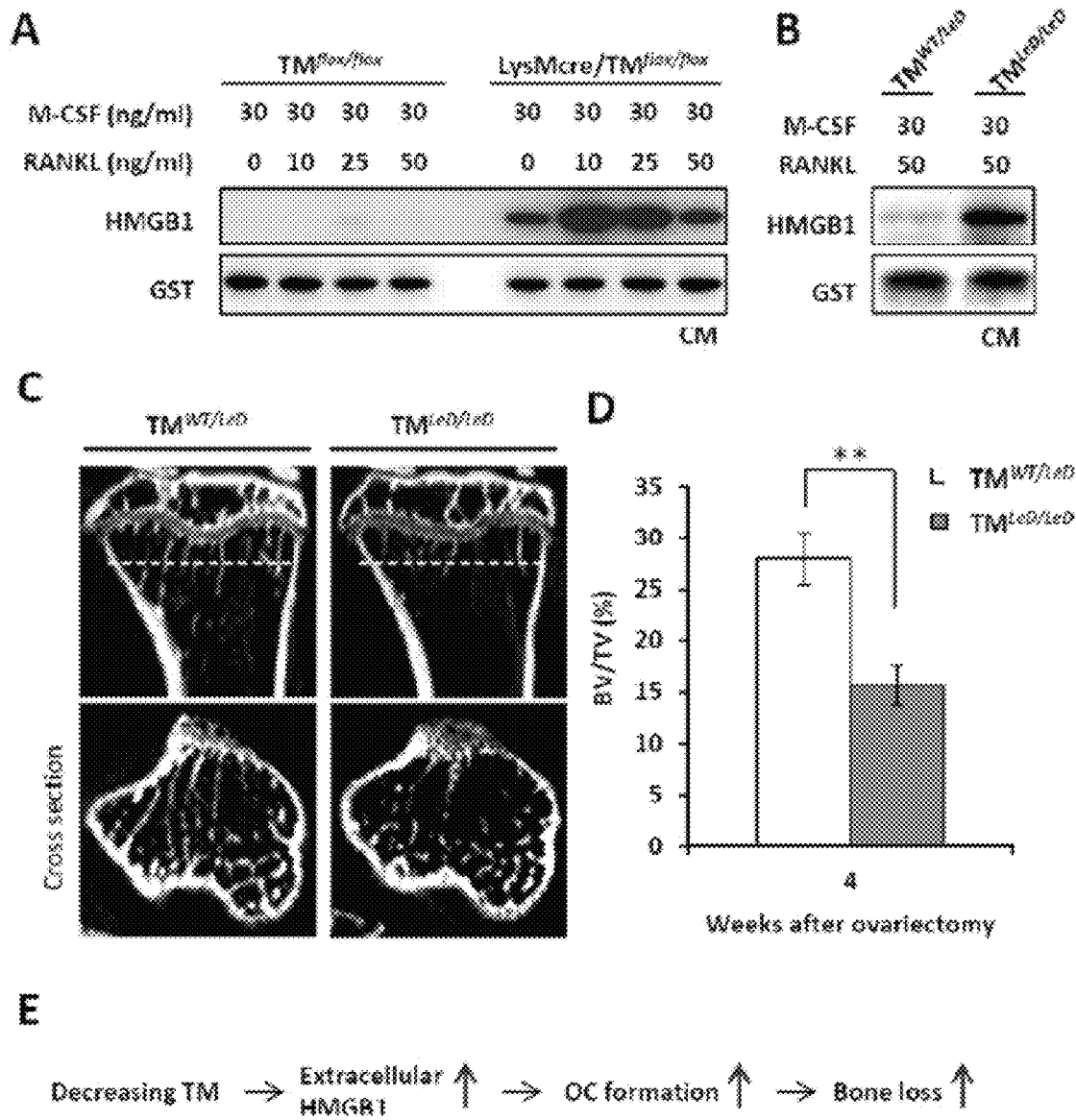


Fig. 6

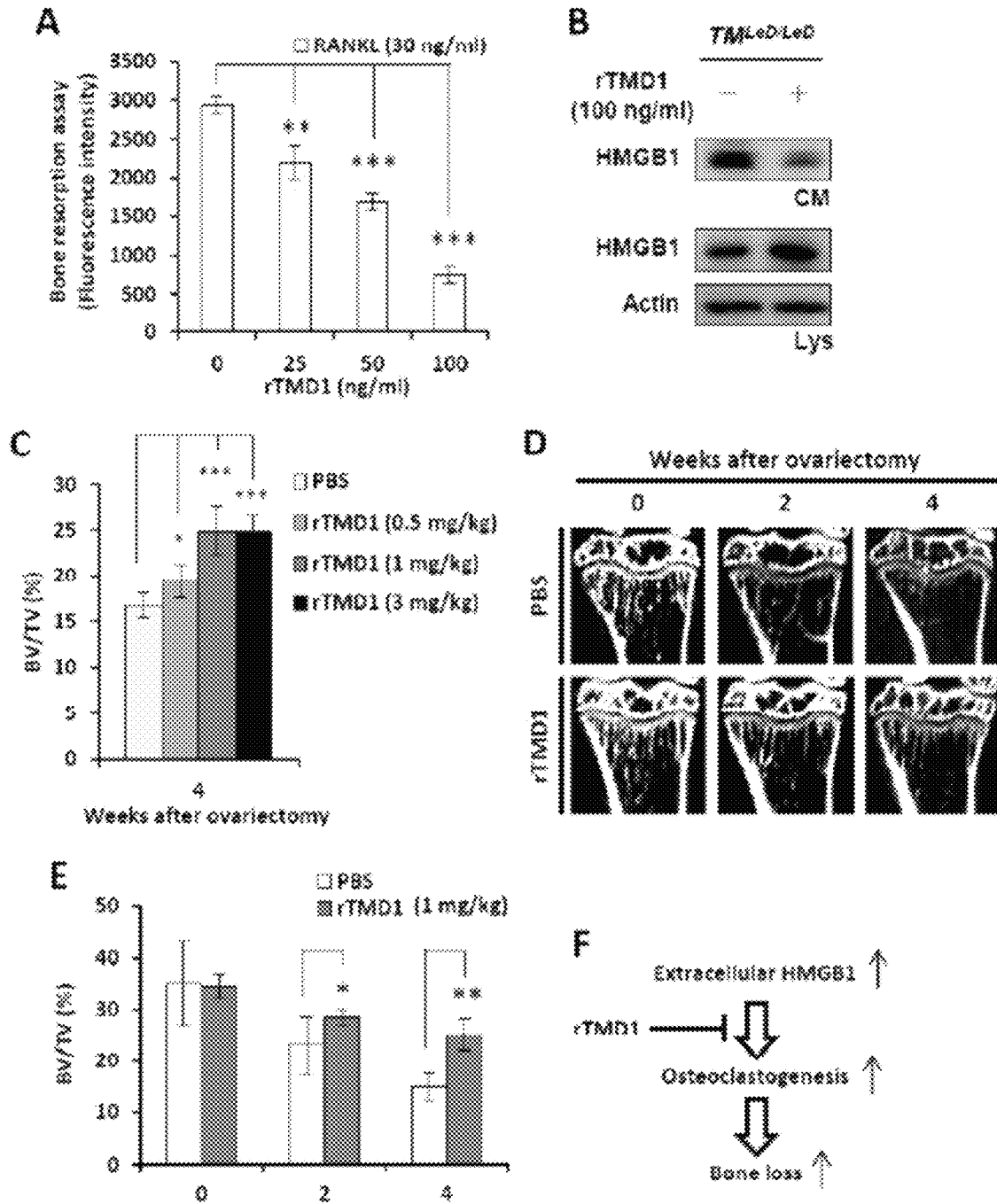
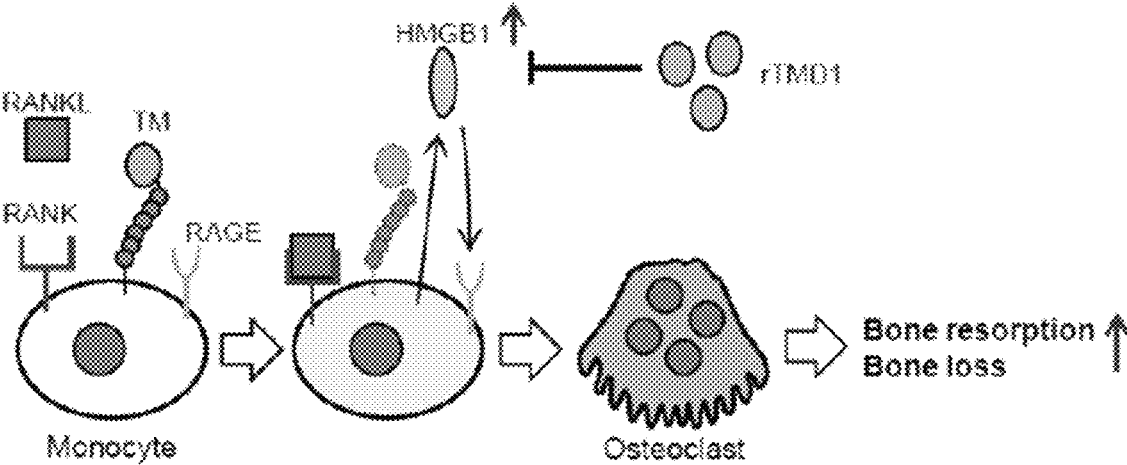


Fig. 7





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## TMD1 PROTEIN FOR TREATING BONE LOSS DISEASES

The present application claims priority to U.S. Provisional Appl. No. 62/288,430, filed Dec. 29, 2016, incorporated herein by reference in its entirety.

### FIELD OF THE INVENTION

The present invention relates to a method for treating bone loss diseases by inhibiting receptor activator of nuclear factor- $\kappa$ B ligand (RANKL)-induced osteoclast differentiation.

### BACKGROUND OF THE INVENTION

Bone is a living organ that is continuously being reshaped in a process called remodeling. In this process, cells called osteoclasts resorb bone tissues whereas cells called osteoblasts deposit new bone tissue. Osteoblasts can become trapped within the bone matrix they secrete, promoting their differentiation into osteocytes. These cells are thought to play an important role in sensing bone load. Under high loading conditions, osteoblasts increase bone mass, whereas under low loading conditions, osteoclasts remove bone tissue, optimizing its structure. Therefore, osteoclasts are closely related to bone loss or decreased bone density.

People may have bone loss or decreased bone density due to physiological factors or other external factors, which leads to osteoporosis. The signs of osteoporosis may include fracture, back pain, limited ability to act (even unable to move). Currently the treatment methods for osteoporosis include nutrition improvement, exercise, medication, etc. Regarding medication, drugs for clinical use comprise: estrogen or estrogen analogues, drugs for treatment of osteoporosis induced by glucocorticoid hormones, diphosphate, or calcitonin, etc. However, all these drugs described above have side effects, for example, diphosphate may cause nausea, indigestion, diarrhea, or constipation, and calcitonin may cause facial flushing, frequent urination, nausea, or itchy skin. Most of all, the drugs described above are unable to suppress the generation of osteoclasts cells, thus are unable to effectively cure osteoporosis.

Receptor activator of nuclear factor- $\kappa$ B ligand (RANKL) is a critical factor to induce the differentiation of osteoclasts from bone marrow-derived macrophages (BMM).

### BRIEF DESCRIPTION OF THE DRAWINGS

The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Office upon request and payment of the necessary fee.

FIG. 1 shows down-regulated TM expression in RAW 264.7-differentiated osteoclasts. A. TM protein expression detected using IF staining in RAW 264.7 cells and differentiated osteoclast-like cells treated with M-CSF (20 ng/ml) and RANKL (30 ng/ml) for 4 days (original magnification  $\times 200$ ). B. Western blot analysis of TM and CATK expression in RAW 264.7 cells treated with M-CSF and RANKL. Representative figures from three independent experiments are shown. C. Quantitative representation of (B). \*\*P<0.01

FIG. 2 shows down-regulated TM expression in PBMC-differentiated osteoclasts. A. TM protein expression detected using IF staining in PBMCs and differentiated osteoclast-like cells treated with M-CSF (20 ng/ml) and RANKL (30

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ng/ml) for 1 week (original magnification  $\times 200$ ). B. Western blot analysis of TM and CATK expression in PBMCs treated with M-CSF and RANKL. Representative figures from three independent experiments are shown. C. Quantitative representation of (B). \*\*P<0.01, \*\*\*P<0.001.

FIG. 3 shows that myeloid-specific deletion of TM enhances osteoclasts formation. A. TRAP staining analysis in osteoclasts differentiated from primary cultured macrophages obtained from TM<sup>fllox/fllox</sup> and LysMcre/TM<sup>fllox/fllox</sup> mice with treatment of M-CSF and various concentrations of RANKL for differentiation (original magnification  $\times 200$ ). B. Quantitation of TRAP positive multi-nucleated osteoclasts (MNCs) per well. \*\*\*P<0.001. Experiments were repeated three times.

FIG. 4 shows that TM lectin-like domain on macrophages retards RANKL-induced osteoclast formation. A. TRAP staining of osteoclasts differentiated from primary cultured macrophages obtained from TM<sup>WT/LeD</sup> and TM<sup>LeD/LeD</sup> mice with treatment of M-CSF and various concentrations of RANKL for differentiation (original magnification  $\times 200$ ). B. Quantitation of TRAP activity using 4-NPP as substrate. \*P<0.05, \*\*P<0.01. Experiments were repeated three times.

FIG. 5 shows that TM deficiency enhances the production of extracellular HMGB1 and ovariectomy-induced bone loss. Evaluation of extracellular HMGB1 production by western blot analysis in primary cultured macrophages from TM<sup>fllox/fllox</sup> and LysMcre/TM<sup>fllox/fllox</sup> mice A., and from TM<sup>WT/LeD</sup> and TM<sup>LeD/LeD</sup> mice B. Glutathione-S-transferase (GST) was used as an internal control. CM: conditioned medium. Representative figures from three independent experiments are shown. C. Bone loss in tibia was detected by  $\mu$ CT scanning after 4 weeks of ovariectomy. Dashed lines indicate the cross sections. D. Quantitative results of trabecular bone volume fraction (BV/TV). BV, trabecular bone volume; TV, total bone volume. \*\*P<0.01, n=5. E. Potential mechanisms of TM deficiency in promotion of bone loss.

FIG. 6 shows that recombinant TM lectin-like domain (rTMD1) suppresses RANKL-induced bone resorption and reduces ovariectomy-induced bone loss. A. Results of bone resorption assay in RAW264.7 cells treated with RANKL and rTMD1. Experiments were repeated three times. B. Production of extracellular HMGB1 in bone marrow macrophages/monocytes from TM<sup>LeD/LeD</sup> mice in response to rTMD1 treatment. CM, conditioned medium. Lys, cell lysates. C. Quantitative results of bone volume fraction (BV/TV) in ovariectomized wild-type mice treated with various doses of intraperitoneal rTMD1 injection. BV, trabecular bone volume; TV, total bone volume. n=5. D. Bone loss in tibia was detected by  $\mu$ CT scanning in wild-type mice with and without rTMD1 treatment within 4 weeks of ovariectomy. E. Quantitative results of D. n=5. F. Proposed mechanism of rTMD1 in the reduction of ovariectomy-induced bone loss. \*P<0.05, \*\*P<0.01, \*\*\*P<0.001.

FIG. 7 shows a schematic model of TM in osteoclastogenesis. During osteoclastogenesis, RANKL reduces TM expression and promotes extracellular HMGB1 secretion in monocytes, which results in the enhancement of bone resorption and bone loss. In addition, rTMD1 treatment can inhibit HMGB1 secretion, bone resorption, and bone loss.

### SUMMARY OF THE INVENTION

The present invention relates to a method for treating a bone loss disease, condition, or disorder in a subject in need thereof, comprising administering to said subject a pharma-

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ceutically effective amount of a composition comprising thrombomodulin lectin-like domain (TMD1).

#### DETAILED DESCRIPTION OF THE INVENTION

The object of the present invention is to treat a disease, condition, or disorder associated with bone loss. In the present invention, receptor activator of nuclear factor- $\kappa$ B ligand (RANKL)-induced osteoclast differentiation is inhibited to achieve therapeutic effect of osteoporosis. The thrombomodulin (TM) used in the present invention is a naturally occurring enzyme. The thrombomodulin lectin-like domain (the first domain of TM, TMD1) can be formulated into a protein drug for inhibiting activation of RANKL, thereby achieve treatment of osteoporosis by inhibition of osteoclast differentiation.

Using the model of RANKL-induced osteoclast differentiation in RAW264.7 cells, the present invention shows for the first time that supplementation of TMD1 significantly attenuates the osteoclast differentiation and bone resorption in a dose-dependent manner. In summary, as shown in the illustrated model in FIG. 7, the present invention demonstrates that TM expressed by monocytes/macrophages is a negative regulator of osteoclastogenesis, and treatment of rTMD1 can inhibit OVX-induced bone loss. These observations provide a new therapeutic strategy for bone loss disorders. Thus, TMD1 may be a new therapeutic treatment for bone loss diseases.

Therefore, the present invention provides a method for treating a bone loss disease, condition, or disorder in a subject in need thereof, comprising administering to said subject a pharmaceutically effective amount of a composition comprising thrombomodulin lectin-like domain (TMD1).

In an embodiment, the method inhibits osteoclast differentiation in the subject.

In a further embodiment, the method inhibits activation of receptor activator of nuclear factor- $\kappa$ B ligand (RANKL) in the subject.

In an embodiment, the bone loss disease, condition, or disorder includes but not limited to osteoporosis or rheumatoid arthritis.

In an embodiment, the subject is a mammal. In a further embodiment, the subject is a human.

#### EXAMPLES

The examples below are non-limiting and are merely representative of various aspects and features of the present invention. Therefore, the method of the present invention should not be limited to only for diagnosing neurodegenerative disease.

##### Example 1

#### Methods and Materials

##### Experimental Animals

LysMcre/TM<sup>flox/flox</sup> mice with myeloid specific TM deletion were generated as previously reported. Compared with their controls (TM<sup>flox/flox</sup> mice), TM expression in the myeloid lineage (macrophages and neutrophils), but not in other tissues and organs, was suppressed in LysMcre/TM<sup>flox/flox</sup> mice. Mice lacking the lectin-like domain of TM (TM<sup>LeD/LeD</sup> mice) and their controls (TM<sup>WT/LeD</sup> mice) were a gift from Dr. Conway. These B6-background mice were maintained in a pathogen-free animal facility at the Center

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of the National Cheng Kung University (NCKU). All animal protocols were approved by the Animal Care Committee of NCKU. Bilateral ovariectomy (OVX), as described previously, was performed at 8 weeks of age.

##### Cell Isolation

Human PBMCs were separated from whole blood using a Ficoll-Paque PLUS (GE Healthcare, Brussels, Belgium) gradient based on the manufacturer's instructions. Mouse bone marrow macrophages/monocytes (BMMs) were isolated from the femur and tibia (6 to 12 weeks old) as described previously with some modifications. Briefly, isolated total bone marrow cells were incubated overnight in complete Minimum Essential Medium (Alpha modification;  $\alpha$ -MEM). Then, nonadherent cells were collected and mononuclear cells were prepared using a Ficoll-Hypaque (GE Healthcare, Piscataway, N.J., USA) density gradient centrifugation. The BMMs were evenly scattered across the interface between Ficoll-Hypaque and medium.

##### Osteoclast Culture and TRAP Staining

Cells (RAW264.7, PBMCs, or BMMs) were cultured in 96-well plates ( $1 \times 10^5$  cells/well). RANKL and M-CSF were added to stimulate osteoclast generation. Media were replenished every 2 days. On day 8, cells were fixed with 3% formaldehyde and were stained with tartrate acid phosphatase (TRAP) (Sigma, St. Louis, Mo., USA). TRAP-positive cells with three or more nuclei were counted as osteoclasts.

##### TRAP Activity Assay

TRAP activity was measured using 4-NPP as substrate, based on the microplate assay method with modifications. A 50  $\mu$ L sample was incubated with 150  $\mu$ L of substrate consisting of 8 mM 4-NPP in 100 mM sodium acetate buffer containing 500 mM sodium tartrate at 37° C. for 40 min with pH 5.0. The reaction was terminated by the addition of 50  $\mu$ L 3 M NaOH. Absorbance was measured at 405 nm in a microplate reader (SPECTRAMax™ 340; Molecular Devices, Palo Alto, Calif., USA).

##### Western Blot Analysis

Cells were lysed and western blot analysis was performed as previously described. Approximately 50  $\mu$ g of total protein was separated in a 10% sodium dodecyl sulfate-polyacrylamide gel and was transferred onto a polyvinylidene difluoride membrane. After probing with a primary and a secondary antibody, the signal was detected using an enhanced chemiluminescence reagent (Amersham Pharmacia Biotech).

##### Bone Resorption Assay

Bone resorption activities were determined by the bone resorption assay kit (Cosmo Bio. Co. Ltd., Tokyo, Japan) based on the manufacturer's instructions. In brief, RAW264.7 cells were incubated on fluoresceinated calcium phosphate-coated plates with M-CSF (30 ng/ml) and RANKL (30 ng/ml) in the presence or absence of various concentrations of rTMD1 for 7 days. The RANKL-induced bone resorption activity was evaluated by measuring the fluorescence intensity of the conditioned medium with an excitation wavelength of 485 nm and an emission wavelength of 535 nm.

##### Micro-Computed Tomography ( $\mu$ CT)

All  $\mu$ CT analyses were consistent with the current guidelines. 29 Bone samples from all groups were imaged using a SkyScan-1076 Micro-CT System (Skyscan, Belgium). For trabecular bone analysis, the  $\mu$ CT scanner was operated at 45 kV, 220  $\mu$ A, 0.4 $\mu$  rotation step, 0.5 mm aluminum filter, and a scan resolution of 18 pin/pixel. The following parameters

were measured: total bone volume (TV, mm<sup>3</sup>), trabecular bone volume (BV, mm<sup>3</sup>), and trabecular bone volume fraction (BV/TV, %).

#### Results

#### Decreased TM Protein Expression in Monocytes/Macrophages During Osteoclastogenesis

Osteoclastogenesis in mouse RAW 264.7 cells and human PBMCs was induced using RANKL and M-CSF to evaluate the levels of TM protein during the process. Immunofluorescence staining revealed TM expression dramatically decreased as RAW 264.7 cells differentiated into osteoclast-like cells (FIG. 1A). Western blot analysis showed that treatment of RAW 264.7 cells with RANKL reduced TM and increased CATK, a marker of osteoclasts, in a time-dependent manner (FIG. 1, (B)-(C)). Similar results were observed in human PBMCs (FIG. 2). Collectively, these results suggest that TM expression in monocytes/macrophages may be inversely related to osteoclastogenesis.

#### Deficiency of Full-Length TM in Macrophages Enhances the RANKL-Induced Osteoclastogenesis

To investigate whether TM might be a negative regulator in osteoclastogenesis, macrophages from myeloid-specific transgenic mice were isolated. TRAP staining analysis showed that RANKL can dose-dependently induce osteoclastogenesis in macrophages from *LysMcre/TM<sup>flox/flox</sup>* and *TM<sup>flox/flox</sup>* mice. However, the cell areas in the *LysMcre/TM<sup>flox/flox</sup>* mice were larger than those from *TM<sup>flox/flox</sup>* mice (FIG. 3, (A)). Quantification of the results showed that the ratio of differentiated TRAP positive multinucleated cells (TRAP+ MNC) in the *LysMcre/TM<sup>flox/flox</sup>* group was at least 3-fold higher compared with that in the *TM<sup>flox/flox</sup>* group (FIG. 3, (B)). These results indicate that the existence of full-length TM in macrophages may hinder RANKL-induced osteoclastogenesis.

#### TM Lectin-Like Domain in Macrophages Retards RANKL-Induced Osteoclastogenesis

It has been shown that osteoblast-derived C-type lectin can inhibit osteoclast formation. To further investigate whether the lectin-like domain of TM might contribute to the inhibition of RANKL-induced osteoclastogenesis, primary macrophages from *TM<sup>WT/LeD</sup>* and *TM<sup>LeD/LeD</sup>* mice were examined. TRAP staining analysis showed that RANKL can induce osteoclastogenesis in primary cells from both *TM<sup>WT/LeD</sup>* and *TM<sup>LeD/LeD</sup>* mice. However, the cell area of some OCs in the *TM<sup>LeD/LeD</sup>* group appeared larger than in the *TM<sup>WT/LeD</sup>* group (FIG. 4, (A)). Moreover, the detected activities of TRAP in the *TM<sup>LeD/LeD</sup>* group were significantly higher than those in the *TM<sup>WT/LeD</sup>* group (FIG. 4, (B)). These results suggest that the lectin-like domain of TM may have a critical effect in inhibiting RANKL-induced osteoclastogenesis.

#### Deletion of the Full-length TM or Deletion of the TM Lectin-like Domain Enhanced Extracellular HMGB1 Production in Macrophages and Ovariectomy-induced Bone Loss.

Since the release of HMGB1 was required for RANKL-induced osteoclastogenesis, the effects of TM deficiency on the production of extracellular HMGB1 were further evaluated. As shown in FIG. 5, HMGB1 protein production in the conditioned medium from RANKL-treated *LysMcre/TM<sup>flox/flox</sup>* cells was substantially higher than that from *TM<sup>flox/flox</sup>* cells (FIG. 5, (A)). Likewise, the extracellular HMGB1 produced by *TM<sup>LeD/LeD</sup>* macrophages was also higher than that from *TM<sup>WT/LeD</sup>* (FIG. 5, (B)). In addition,  $\mu$ CT scanning showed that tibia bone loss induced by ovariectomy was significantly more severe in *TM<sup>LeD/LeD</sup>* mice than in *TM<sup>WT/LeD</sup>* mice (FIG. 5, (C)-(D)). These data

suggested that TM deficiency in macrophages, likely in association with production of extracellular HMGB1, enhanced osteoclast formation and bone loss (FIG. 5, (E)). Treatment with rTMD1 Inhibits Bone Resorption and Bone Loss

The effects of human rTMD1 on bone resorption in vitro and bone loss in vivo were investigated. Results of the bone resorption assay indicated that rTMD1 treatment inhibited RANKL-induced bone resorption in a dose-dependent manner (FIG. 6, (A)) and also decreased the production of extracellular HMGB1 in BMMs from *TM<sup>LeD/LeD</sup>* mice (FIG. 6, (B)). In addition, rTMD1 treatment dose-dependently increased the bone volume fraction (BV/TV) in ovariectomized wild-type mice (FIG. 6, (C)). Consistent with these results, rTMD1 treatment inhibited bone loss across a 4-week period (FIG. 6, (D)-(E)). These results suggest that rTMD1 inhibits bone loss, at least in part, through blockade of HMGB1-induced osteoclastogenesis (FIG. 6, (F)).

One skilled in the art readily appreciates that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The methods and uses thereof are representative of preferred embodiments, are exemplary, and are not intended as limitations on the scope of the invention. Modifications therein and other uses will occur to those skilled in the art. These modifications are encompassed within the spirit of the invention and are defined by the scope of the claims.

It will be readily apparent to a person skilled in the art that varying substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention.

All patents and publications mentioned in the specification are indicative of the levels of those of ordinary skill in the art to which the invention pertains. All patents and publications are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

The invention illustratively described herein suitably may be practiced in the absence of any element or elements, limitation or limitations, which are not specifically disclosed herein. The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention that in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the concepts herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention as defined by the appended claims.

What is claimed is:

1. A method for treating a bone loss disease, condition, or disorder in a subject in need thereof, comprising administering to said subject a pharmaceutically effective amount of a composition comprising thrombomodulin lectin-like domain (TMD1), provided that the bone loss disease, condition, or disorder does not comprise rheumatoid arthritis or arthritis.

2. The method of claim 1, which inhibits activation of receptor activator of nuclear factor- $\kappa$ B ligand (RANKL) in the subject.

3. The method of claim 1, which inhibits osteoclast differentiation in the subject.

4. The method of claim 1, wherein the bone loss disease, condition, or disorder comprises osteoporosis.

5. The method of claim 1, wherein the subject is a mammal.

6. The method of claim 1, wherein the subject is a human.

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