Video Article

A Novel *In Vivo* Gene Transfer Technique and *In Vitro* Cell Based Assays for the Study of Bone Loss in Musculoskeletal Disorders

Dennis J Wu[1], Neha Dixit[1], Thanh Nguyen[2], Erika Suzuki[1], Hyun Seock Shin[1], Jack Davis[2], Emanual Maverakis[3], Iannis E Adamopoulos[1,2]

[1]Division of Rheumatology, Allergy and Clinical Immunology, University of California, Davis

[2]Institute for Pediatric Regenerative Medicine, Shriners Hospitals for Children - Northern California

[3]Department of Dermatology, University of California, Davis

Correspondence to: Iannis E Adamopoulos at iannis@ucdavis.edu

URL: http://www.jove.com/video/51810
DOI: doi:10.3791/51810

Keywords: Osteoclast, arthritis, minicircle DNA, macrophages, cell culture, hydrodynamic delivery

Date Published: 4/14/2014

Citation: Wu, D.J., Dixit, N., Nguyen, T., Suzuki, E., Shin, H.S., Davis, J., Maverakis, E., Adamopoulos, I.E. A Novel *In Vivo* Gene Transfer Technique and *In Vitro* Cell Based Assays for the Study of Bone Loss in Musculoskeletal Disorders. *J. Vis. Exp.* (), e51810, doi:10.3791/51810 (2014).

Abstract

Differentiation and activation of osteoclasts play a key role in the development of musculoskeletal diseases as these cells are primarily involved in bone resorption. Osteoclasts can be generated *in vitro* from monocyte/macrophage precursor cells in the presence of certain cytokines, which promote survival and differentiation. Here, both *in vivo* and *in vitro* techniques are demonstrated, which allow scientists to study different cytokine contributions towards osteoclast differentiation, signaling, and activation. The minicircle DNA delivery gene transfer system provides an alternative method to establish an osteoporosis-related model is particularly useful to study the efficacy of various pharmacological inhibitors *in vivo*. Similarly, *in vitro* culturing protocols for producing osteoclasts from human precursor cells in the presence of specific cytokines enables scientists to study osteoclastogenesis in human cells for translational applications. Combined, these techniques have the potential to accelerate drug discovery efforts for osteoclast-specific targeted therapeutics, which may benefit millions of osteoporosis and arthritis patients worldwide.

Video Link

The video component of this article can be found at http://www.jove.com/video/51810/

Introduction

Musculoskeletal diseases affect millions of people in the United States and present severe consequences for national and local health systems [1]. These disorders are characterized by loss of bone and joint function that require extensive treatment and long periods of recovery. Commonly, a relative increase in the number and/or activity of osteoclasts, cells specialized to resorb bone, in osteoporosis and arthritis is observed [2]. Under physiological conditions the number and activity of osteoclasts is regulated by receptor activator of nuclear factor κ-B ligand (RANKL), which is produced by osteoblasts. Osteoprotegerin (OPG), a decoy receptor for RANKL is also produced by osteoblasts[3]. *In vivo* animal models that involve systemic overexpression of sRANKL, or deletion of OPG are very valuable in osteoporosis research; however, these methods require the generation of transgenic mice [4,5]. Here, a novel alternative method of overexpressing sRANKL for the study of musculoskeletal-related disorders is described. Specifically, minicircle (MC) DNA technology and hydrodynamic delivery methods were used to achieve gene transfer of sRANKL *in vivo* and overexpress mouse sRANKL systemically [6].

This method is also complementary to other *in vivo* models of osteoporosis, such as hormonal modulation of osteoclasts following ovariectomy [7] and dietary intervention by low-calcium diet [8]. These models are very useful to study different aspects of musculoskeletal-related disorders however they require surgical procedures and may take up to several months, at a significant cost [9]. Ovariectomized (OVX) rodent model is an experimental animal model where removal of ovaries leads to estrogen deficiency thereby mimicking human postmenopausal osteoporosis [10]. Human post-menopausal osteoporosis, a condition where estrogen deficiency leads to increased risk of bone fractures and osteoporosis affects approximately eight million women in the United States alone. Although the OVX model is useful for post-menopausal osteoporosis it offers limited advantages in studying osteoporosis in general. Estrogen suppresses bone loss, by inducing osteoclast and inhibiting osteoblast apoptosis, therefore in its absence an increased osteoclast activity is observed [10, 11, 12]. A RANKL-OPG ratio imbalance that favors bone resorption is also observed [13]. However, estrogen deficiency *in vivo* is also accompanied by decreased levels of transforming growth factor β (TGF β), increased interleukin-7 (IL-7) and TNF, IL-1 and IL-6 [14,15]. As these cytokines have known bone remodeling modulatory functions independent of the RANKL pathway, it is impossible to attribute any osteoclast activation solely to the RANKL-RANK axis. The model described in this paper enables researchers to study *in vivo* RANKL-RANK axis in osteoclastogenesis and bone loss without pro-inflammatory cytokines compared to OVX rodent models.

Additionally, *in vitro* osteoclastogenesis techniques are essential tools to study osteoclast activation for potential therapeutic treatments of musculoskeletal diseases. Previous studies have also shown that culturing mouse bone marrow derived macrophages (BMMs) with mouse macrophage colony-stimulating factor (M-CSF) and mouse sRANKL can lead to osteoclast differentiation [3,16,17]. Here, the protocols to generate multinucleated osteoclast-like cells from mouse bone marrow as well as from human peripheral blood mononuclear cells (PBMCs) *in vitro* [18] are

described. The cell-based assays required to define a mature terminally differentiated and fully functional osteoclast are also briefly described. These *in vitro* techniques complement the novel *in vivo* approach and together serve as powerful investigative tools to study osteoclast differentiation and activation. Using these systems, scientists are able to generate osteoclasts *in vivo* and *in vitro* and define the stimuli and signals required for their proliferation and activation as well as test the efficacy of pharmacological and biological inhibitors.

Protocol

1. Hydrodynamic Delivery of sRANKL MC DNA

1.1) Hydrodynamic Delivery via mouse tail vein

1. Weigh the mouse before the tail vein injection. Dilute sRANKL or green fluorescent protein (GFP) MC in Ringer's solution (pre-warm at 37°C) in a total volume of ~10% of the mouse's body weight.
2. Warm up the mouse in a cage for 10 minutes prior to injection in order to dilate blood vessels and make lateral veins (LVs) visible. Monitor the mouse carefully to avoid dehydration and hyperthermia.
3. As soon as the LVs are dilated and visible, transfer the mouse to the restrainer and insert the plug far enough into the barrel to restrain movement. Monitor the mouse for normal activity such as breathing. Adjust the plug of restrainer if needed.
4. Disinfect the injection area 3/4 from the tip of the tail with an alcohol wipe. Use a 27-30 gauge needle for mouse tail vein injection. Hold the tail firmly with one hand and insert the needle into the tail vein. Apply pressure on the syringe and complete the injection within 5-7 seconds.
5. Remove the needle from the tail vein and apply pressure with a cotton ball on the injection site to stop bleeding. Monitor themouse for 15-30 minutes, then transfer the mouse back to vivarium once the breathing rate is reduced to its normal rate.

1.2) Confirmation of *systemic* mouse sRANKL expression and quantification of mouse serum TRACP5b post gene transfer

1. Warm up the mouse in a cage for 5-10 minutes in order to dilate blood vessels and make LVs visible. Monitor the mouse carefully to avoid dehydration and hyperthermia.
2. Make a small incision with a blade to the mouse's tail at a site one forth from the tip of the tail.
3. Collect approximately 100 µl of blood in serum separation tubes.
4. Incubate serum separation tubes at room temperature for 30 minutes.
5. Centrifuge samples at 10,000 x g for 5 minutes and collect serum. Store serum in -80°C for further analysis.
6. Perform sRANKL ELISA and mouse serum TRACP5b assay on serum samples using a commercial available kit.

2. In vitro osteoclast generation from mouse BMMs

2.1) Isolation and culturing of BMMs

1. Isolation of the tibia and femur bones: Euthanize the donor mouse with CO_2, and disinfect the legs with 70% ethanol. Dissect from the pubic bone to the calcaneus bone, to remove the femur and tibia bones intact.
2. Remove the skin and muscle carefully without damaging the bone. Place processed femur and tibia bone in a petri dish containing phosphate buffered saline (PBS).
3. Removal of bone marrow : Cut off the tip on one side of the femur and tibia bones and flush out the bone marrow into a 50ml tube using a 1ml syringe with a 25 gauge needle, loaded with α-MEM containing 1% penicillin/streptomycin (Osteoclast Culture Medium/OCM).
4. Process bone marrow : Mix and transfer bone marrow suspension to a new 50 ml tube by passing it through a 70 µm nylon cell strainer. Centrifuge the cells at 300 x g and re-suspend in α-MEM containing 1% penicillin/streptomycin and 10% FBS (Osteoclast Culture medium/ OCM (+)).

2.2) Culture of macrophages

1. Count cells using a hemocytometer or automatic cell counter.
2. Plate $1x10^6$ cells per well for a 6-well plate or $3x10^5$ cells to glass coverslips (5mm) or dentine slices placed in 96-well plate and incubate at 37°C.
3. Aspirate all media containing non-adherent cells and incubate cells at 37°C with fresh OCM (+) containing 25ng/ml mouse M-CSF for 48 hours.
4. Aspirate all media and incubate cells at 37°C with fresh OCM (+) media containing 25ng/ml mouse M-CSF, and 30ng/ml mouse sRANKL to initiate osteoclastogenesis. Replenish media every 3 days as required.
5. Grow cells for approximately 6-8 days to form giant multi-nuclear cells capable of resorbing bone. As soon as multinucleated cells appear, perform TRAP (tartrate resistant acid phosphatase) staining using a commercial available kit, and when fully matured, perform functional assays. Visualize F-actin rings on coverslips using phalloidin stain and image the cells attached to dentine slices by scanning electron microscopy (SEM) as described previously [19].

3. *In vitro* osteoclast generation from human PBMCs

3.1) Isolation of human PBMCs

1. Add 10ml of pre-warmed Histopaque-1077 into 50ml tube.
2. Empty leukocyte filter obtained from blood bank with sterile PBS into a 50ml tube (1:1 ratio of blood with PBS). Layer the diluted blood over the histopaque solution very slowly, making sure that the blood does not mix with the histopaque.

3. Centrifuge samples at 1000 x g for 20 minutes at 18°C. Following centrifugation, carefully isolate the white buffy coat layer containing the white blood cells and transfer to a new 50ml tube.
4. Dilute cells with PBS and centrifuge again at 650 x g for 5 minutes to collect the cells.
5. Remove supernatant and resuspend cells in OCM (-) media and count cells using cell counter.

3.2) Plating and culturing PBMCs

1. Resuspend the cells in OCM (+) media and plate 8 x 10^6 cells/ml per well in a 6 well plate or 1 x 10^6 cells/ml per well in 96 well plate. Plate cells on glass coverslips (5mm) as required for immunofluorescence imaging as well as on bone slices suitable for bone resorption assays as previously described[20].
2. Change culture media, replenishing the cells with human M-CSF every 2-3 days or as required. Then proceed to differentiation step on day 5 by adding 30ng/ml of human sRANKL along with 25ng/ml human M-CSF in OCM (+) media to initiate osteoclastogenesis.
3. Grow cells for approximately 14 to 21 days to form giant multi-nuclear cells. As soon as multinucleated cells appear, these cells can then be labeled for TRAP, and when fully matured, functional assays can be performed. F-actin rings can be visualized on coverslips using phalloidin stain. The cell morphology can be examined in cells attached to dentine slices by SEM.

Representative Results

Here, a novel gene transfer technique for differentiation of osteoclasts *in vivo* and cell culture protocols for differentiating precursor cells into osteoclasts *in vitro* as a method to study the effects of cytokines on osteoclastogenesis are described. In Figure 1, the representative results of successful gene transfer of GFP and mouse sRANKL MC in mice are shown. In Figure 2, the representative images of mouse bone marrow or human PBMC cell differentiation time course cultures of precursor cells to osteoclasts by bright field microscopy are shown. Figure 3, the representative images from three independent methods for characterizing mouse bone marrow derived osteoclasts are shown. The presence of mouse sRANKL activates the expression of TRAP (Figure 3A and B) and the formation of F-actin rings (Figure 3C and D). Scanning Electron Microscopy (SEM) reveals that mouse sRANKL induces the formation of giant cells capable of resorbing bone (Figure 3E and F). Figure 4, the same characterization methods on human PBMC derived osteoclasts are shown.

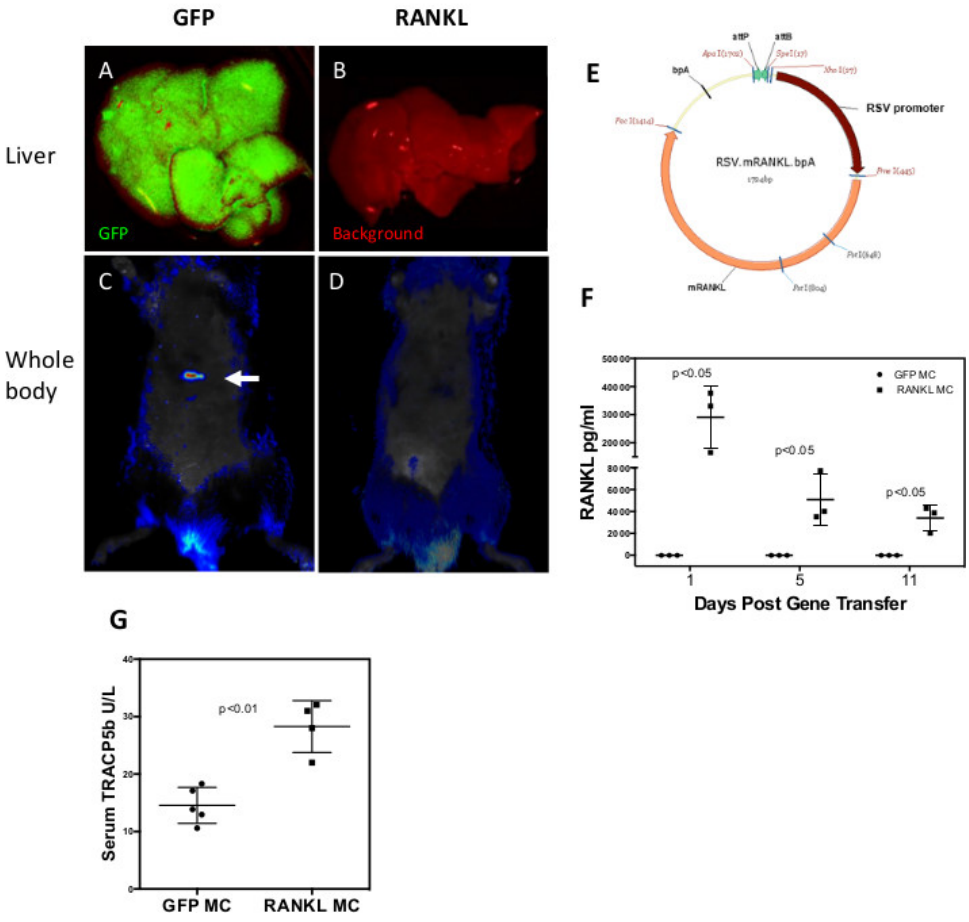

Figure 1. sRANKL *in vivo* gene-transfer leads to increased osteoclastogenesis.

Anterior view of **A, B)** mouse liver explants and **C, D)** whole body images of 12-week old C57BL/6 male mice injected with 5 µg of GFP or sRANKL MC DNA. The arrow indicates GFP expression *in vivo* in GFP gene transfer mouse. RANKL gene transfer mice serve as no background fluorescence. Images captured by Maestro 2 Imager, one-day post gene-transfer (red and green denote background and GFP

respectively). **E)** Plasmid map of sRANKL MC-DNA construct and **F)** sRANKL level in the serum analyzed at 1, 5 and 11 days post GFP or sRANKL gene transfer. G) Mouse serum TRACP5b level analyzed 11 days after GFP or sRANKL gene transfer. Scale bars indicate 5 mm in A, B, and 10 mm in C, D.

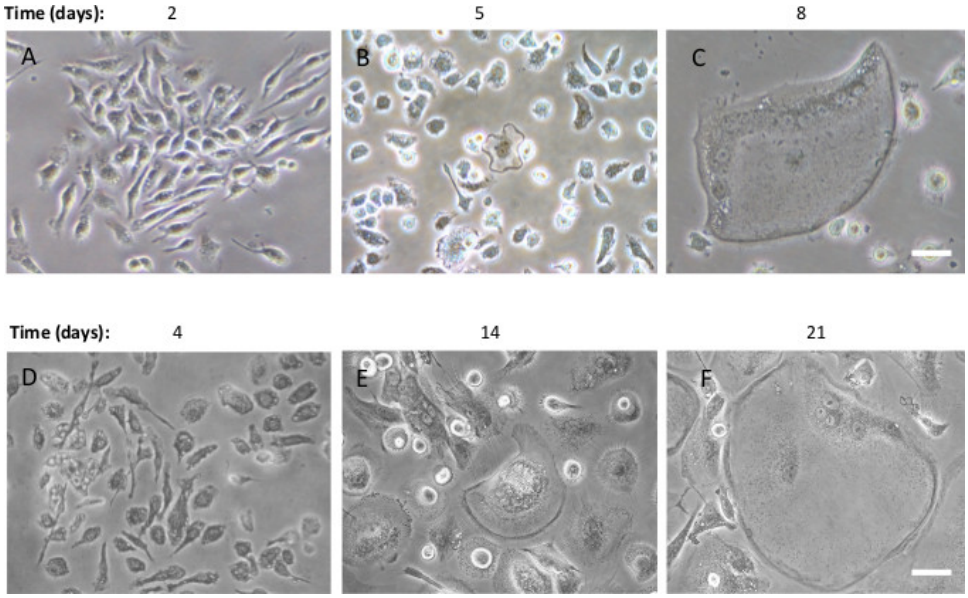

Figure 2. *In vitro* osteoclastogenesis in both mouse and human cell culture systems.

Bright-field microscopy of mouse cells isolated from bone marrow and cultured in the presence of 25 ng/ml mouse M-CSF and 30 ng/ml mouse sRANKL showing a differentiation time course at days **A),** 2 **B)** 5 and **C)** 8. Bright-field microscopy of human cells isolated from peripheral blood and cultured in the presence of 25 ng/ml human M-CSF and 30 ng/ml human sRANKL showing a differentiation time course at days **D),** 4 **E)** 14 and **F)** 21. Scale bars indicate 20 µm.

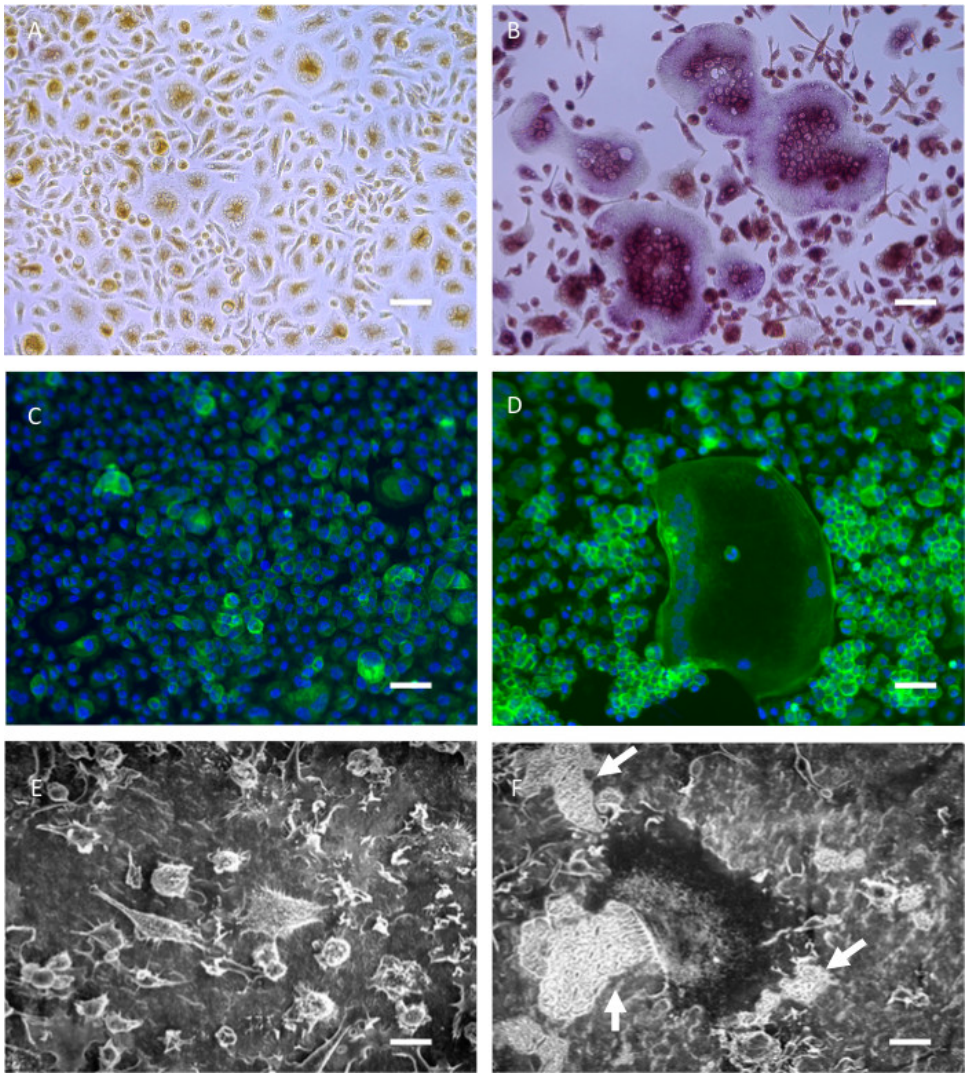

Figure 3. Generation and characterization of mouse osteoclasts.

TRAP staining (pink) of mouse cells isolated from bone marrow and cultured for 6 days in the presence of **A)** 25 ng/ml mouse M-CSF or **B)** 25 ng/ml mouse M-CSF and 30 ng/ml mouse sRANKL. Phalloidin (green) and DAPI (Blue) staining of mouse cells isolated from bone marrow and cultured for 8 days in the presence of **C)** 25 ng/ml mouse M-CSF or **D)** 25 ng/ml mouse M-CSF and 30 ng/ml mouse sRANKL showing the formation of F-actin ring in multinucleated cells. SEM photomicrographs of mouse cells isolated from bone marrow and cultured for 8 days in the presence of **E)** 25 ng/ml mouse M-CSF or **F)** 25 ng/ml mouse M-CSF and 30 ng/ml mouse sRANKL showing the formation of resorption areas on bone as indicated by the arrows. Scale bars indicate 40 μm in A-D, and 30 μm in E, F.

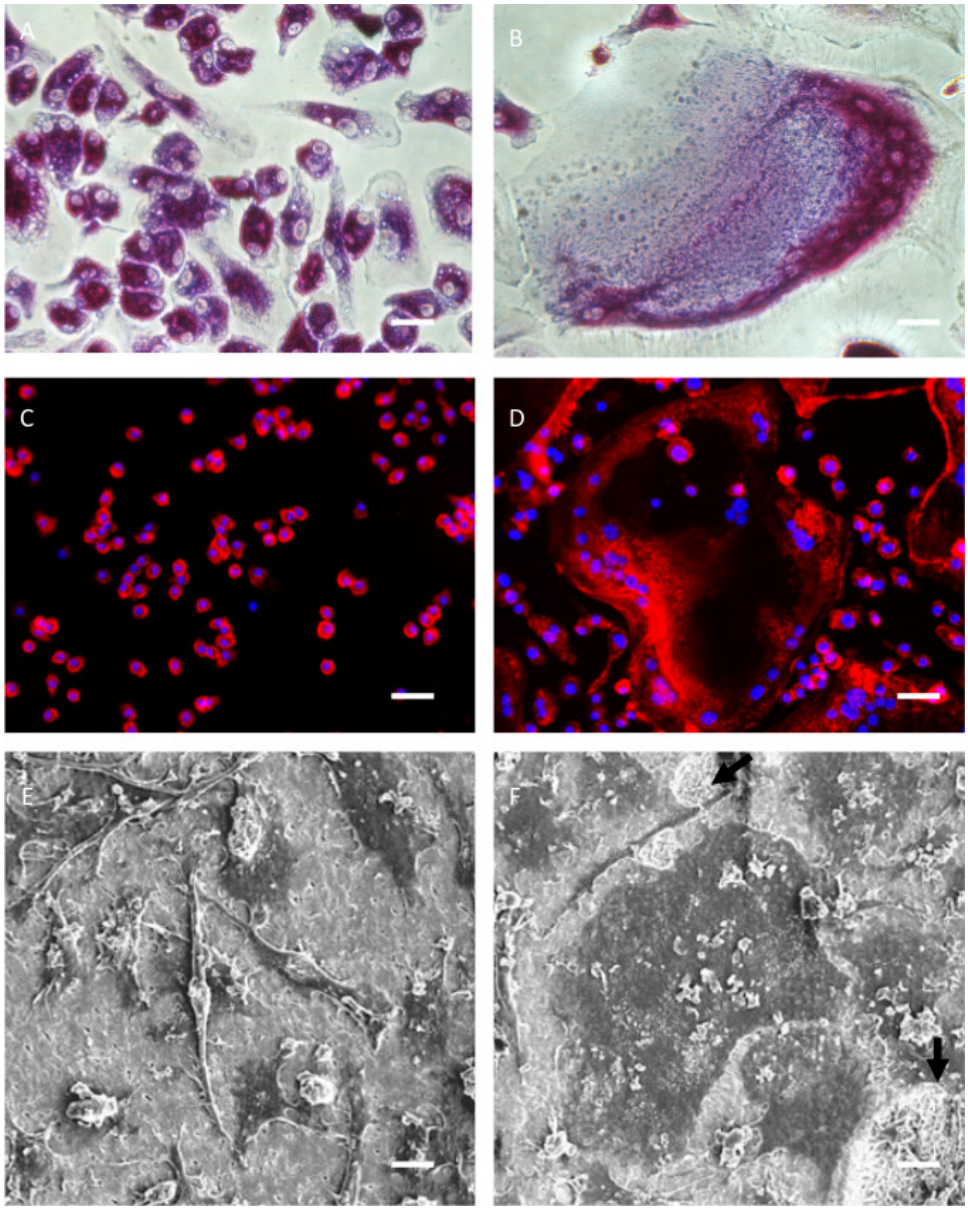

Figure 4. Generation and characterization of human osteoclasts

TRAP staining (pink) of human cells isolated from PBMCs and cultured for 15 days in the presence of **A)** 25 ng/ml human M-CSF or **B)** 25 ng/ml human M-CSF and 30 ng/ml human sRANKL. Phalloidin (green) and DAPI (Blue) staining of human cells isolated from PBMC and cultured for 19 days in the presence of **C)** 25 ng/ml human M-CSF or **D)** 25 ng/ml human M-CSF and 30 ng/ml human sRANKL showing the formation of F-actin rings in multinucleated cells. SEM photomicrographs of human cells isolated from PBMC and cultured for 18 days in the presence of **E)** 25 ng/ml human M-CSF or **F)** 25 ng/ml human M-CSF and 30 ng/ml human sRANKL showing the formation of resorption areas on bone as indicated by the arrows. Scale bars indicate 10 µm in A, B, 20 µm in C, D 15 µm in E, F.

Discussion

Musculoskeletal conditions are leading causes of morbidity and disability and are comprised of over 150 diseases and syndromes; affecting approximately 90 million Americans today. Joint inflammation and bone destruction are predominant features of musculoskeletal conditions, including arthritis and osteoporosis. Osteoporosis is a condition that weakens bone integrity, often leading to fractures of the bone. Arthritis is a chronic, debilitating disease characterized by inflammation of the joints that become swollen, tender and stiff–restricting normal movement and can lead to disability. Although the pathogenesis of musculoskeletal diseases greatly differs from one another, bone destruction is the overarching common feature. Bone destruction in musculoskeletal conditions is primarily caused by an imbalance between bone resorption and bone formation [21,22]. One therapeutic approach is to attempt to block the capacity of these cells to destroy bone [23]. The techniques and procedures outlined here allow the study of osteoclasts both *in vivo* and *in vitro.*

The *in vivo* model described here uses minicircles, which are small episomal DNA vectors (~4kb) freed from bacterial backbone, that also serve as transgene carriers [24]. Minicircles express transgenes 10-1,000 fold higher then normal bacterial plasmids with a stable expression for up to several months. The smaller molecular size of minicircles contributes to more efficient transfection rate and substantial expression compare to normal bacterial plasmids [24]. Therefore, minicircles are now widely applied in pre-clinical gene transfer studies [25,26]. Hydrodynamic delivery is a well-developed method based on hydrodynamic pressure in the capillaries to deliver plasmid DNA into the whole body of a mouse. Hydrodynamic gene delivery to hepatocytes via tail vein injection in mice is well-established to express transgene by plasmid DNA [6]. In these studies, overexpressing mouse sRANKL in the *in vivo* mouse model is achieved by sRANKL MC and hydrodynamic delivery via tail vein injections. Mouse sRANKL ELISA can be used to detect serum levels of RANKL in mice. Significant elevation of mouse serum TRACP5b, a bone resorption marker [27], is observed in sRANKL gene transfer mice. Notably, this technique is not limited to overexpression of sRANKL; it can also be further applied to overexpress other secreted proteins [28]. The primary technical difficulty in this procedure is the hydrodynamic delivery via the tail vein. Apart from regular tail vein injection, hydrodynamic delivery requires delivery of minicircles in large volumes of physiological solution into the mice within 5-7 seconds. Thus, steady handling and injection techniques are essential for success with this procedure. Generating sRANKL transgenic mice is another approach for *in vivo* osteoclastogenesis. However, a high level of sRANKL can lead to embryonic lethality [4] and generating transgenic mice is also expensive and time-consuming.

In OVX rodent model, both inflammatory cytokines such as TNF, IL-1 and IL-6 and the non-inflammatory cytokine sRANKL contribute to *in vivo* osteoclastogenesis and bone loss. sRANKL gene transfer model leads to overexpression of only the non-inflammatory cytokine sRANKL. Therefore, the sRANKL gene transfer model described here offers researchers a powerful tool to examine the contribution of the RANKL-RANK axis in bone remodeling in the absence of inflammation.

An *in vitro* osteoclast culture method, which involves co-culturing bone marrow cells and osteoblasts, was established in the late 1980s prior to the discovery of RANKL in late 1990's [29,30]. Recombinant M-CSF and RANKL induce the osteoclast differentiation efficiently *in vitro* and the method has been previously described in other studies [3,31]. M-CSF is known to stimulate RANK expression in early osteoclast precursors [32] and to act as a survival factor for osteoclast precursors and mature osteoclasts [33].RANKL is essential for osteoclast differentiation and activation [3]. Here, the method of *in vitro* osteoclastogenesis is described. Successful culturing and differentiation of precursor cells to osteoclasts require sterile cell isolation and proper plating techniques as well as careful understanding of timed cell harvest. Calculated plating of the cells is crucial because osteoclast differentiation requires fusion of osteoclast precursor cells [34] and an inadequate number of osteoclast precursor cells will not facilitate fusion and differentiation of precursor cells into osteoclasts. Similarly, an excess number of osteoclast precursor cells, leads to clump formation and eventual cell death. Removal of non-adherent cells two hours after plating the cells can help maintain an optimal cell number. Lastly, the time of cell harvest is crucial as osteoclasts are terminally differentiated short-lived cells with a lifespan of approximately 48 hours [35]. Thus, cells should be harvested within 48 hours after the first observation of multinucleated cells. These cells can then be characterized by TRAP and F-actin staining by immunohistochemistry and bone resorption activity can be detected by scanning electron microscopy (SEM). These experimental approaches enable clear definition of osteoclasts in terms of their phenotype and function.

The *in vivo* and *in vitro* techniques described here will facilitate the development of osteoclast inhibitors currently in pre-clinical studies, leading to efficacious therapeutic strategies for the millions of osteoporosis and arthritis patients worldwide.

Disclosures

The authors have nothing to disclose.

Acknowledgements

Research was partly supported by NIH research grants R01 AR062173 and SHC 250862 to IEA. ES is the recipient of NIH T32 CTSC predoctoral fellowship.

References

1. Yelin, E. Cost of musculoskeletal diseases: impact of work disability and functional decline. *The Journal of rheumatology. Supplement.* **68**, 8-11 (2003).
2. Boyce, B. F., Rosenberg, E., de Papp, A. E. & Duong le, T. The osteoclast, bone remodelling and treatment of metabolic bone disease. *European journal of clinical investigation.* **42**, 1332-1341, doi:10.1111/j.1365-2362.2012.02717.x (2012).
3. Lacey, D. L. *et al.* Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. *Cell.* **93**, 165-176 (1998).
4. Mizuno, A. *et al.* Transgenic mice overexpressing soluble osteoclast differentiation factor (sODF) exhibit severe osteoporosis. *Journal of bone and mineral metabolism.* **20**, 337-344, doi:10.1007/s007740200049 (2002).
5. Bucay, N. *et al.* osteoprotegerin-deficient mice develop early onset osteoporosis and arterial calcification. *Genes & development.* **12**, 1260-1268 (1998).
6. Suda, T. & Liu, D. Hydrodynamic gene delivery: its principles and applications. *Molecular therapy : the journal of the American Society of Gene Therapy.* **15**, 2063-2069, doi:10.1038/sj.mt.6300314 (2007).
7. Wronski, T. J., Dann, L. M., Scott, K. S. & Cintron, M. Long-term effects of ovariectomy and aging on the rat skeleton. *Calcified tissue international.* **45**, 360-366 (1989).
8. Seto, H., Aoki, K., Kasugai, S. & Ohya, K. Trabecular bone turnover, bone marrow cell development, and gene expression of bone matrix proteins after low calcium feeding in rats. *Bone.* **25**, 687-695 (1999).
9. Lelovas, P. P., Xanthos, T. T., Thoma, S. E., Lyritis, G. P. & Dontas, I. A. The laboratory rat as an animal model for osteoporosis research. *Comparative medicine.* **58**, 424-430 (2008).

10. Sherman, B. M., West, J. H. & Korenman, S. G. The menopausal transition: analysis of LH, FSH, estradiol, and progesterone concentrations during menstrual cycles of older women. *The Journal of clinical endocrinology and metabolism.* **42**, 629-636, doi:10.1210/jcem-42-4-629 (1976).
11. Hughes, D. E. *et al.* Estrogen promotes apoptosis of murine osteoclasts mediated by TGF-beta. *Nature medicine.* **2**, 1132-1136 (1996).
12. Kousteni, S. *et al.* Nongenotropic, sex-nonspecific signaling through the estrogen or androgen receptors: dissociation from transcriptional activity. *Cell.* **104**, 719-730 (2001).
13. Ominsky, M. S. *et al.* RANKL inhibition with osteoprotegerin increases bone strength by improving cortical and trabecular bone architecture in ovariectomized rats. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research.* **23**, 672-682, doi:10.1359/jbmr.080109 (2008).
14. Kitazawa, R., Kimble, R. B., Vannice, J. L., Kung, V. T. & Pacifici, R. Interleukin-1 receptor antagonist and tumor necrosis factor binding protein decrease osteoclast formation and bone resorption in ovariectomized mice. *The Journal of clinical investigation.* **94**, 2397-2406, doi:10.1172/JCI117606 (1994).
15. Weitzmann, M. N. & Pacifici, R. Estrogen deficiency and bone loss: an inflammatory tale. *The Journal of clinical investigation.* **116**, 1186-1194, doi:10.1172/JCI28550 (2006).
16. Suda, T., Nakamura, I., Jimi, E. & Takahashi, N. Regulation of osteoclast function. *J Bone Miner Res* **12**, 869-879 (1997).
17. Asagiri, M. & Takayanagi, H. The molecular understanding of osteoclast differentiation. *Bone.* **40**, 251-264, doi:10.1016/j.bone.2006.09.023 (2007).
18. Matsuzaki, K. *et al.* Osteoclast differentiation factor (ODF) induces osteoclast-like cell formation in human peripheral blood mononuclear cell cultures. *Biochemical and biophysical research communications.* **246**, 199-204, doi:10.1006/bbrc.1998.8586 (1998).
19. Adamopoulos, I. E. *et al.* Synovial fluid macrophages are capable of osteoclast formation and resorption. *The Journal of pathology.* **208**, 35-43, doi:10.1002/path.1891 (2006).
20. Adamopoulos, I. E. *et al.* Interleukin-17A upregulates receptor activator of NF-kappaB on osteoclast precursors. *Arthritis research & therapy.* **12**, R29, doi:10.1186/ar2936 (2010).
21. Jones, D., Glimcher, L. H. & Aliprantis, A. O. Osteoimmunology at the nexus of arthritis, osteoporosis, cancer, and infection. *J Clin Invest.* **121**, 2534-2542, doi:10.1172/JCI46262 (2011).
22. Sato, K. & Takayanagi, H. Osteoclasts, rheumatoid arthritis, and osteoimmunology. *Curr Opin Rheumatol.* **18**, 419-426, doi:10.1097/01.bor.0000231912.24740.a5 (2006).
23. Das, S. & Crockett, J. C. Osteoporosis - a current view of pharmacological prevention and treatment. *Drug design, development and therapy.* **7**, 435-448, doi:10.2147/DDDT.S31504 (2013).
24. Chen, Z. Y., He, C. Y., Ehrhardt, A. & Kay, M. A. Minicircle DNA vectors devoid of bacterial DNA result in persistent and high-level transgene expression in vivo. *Molecular therapy : the journal of the American Society of Gene Therapy.* **8**, 495-500 (2003).
25. Kay, M. A., He, C. Y. & Chen, Z. Y. A robust system for production of minicircle DNA vectors. *Nature biotechnology.* **28**, 1287-1289, doi:10.1038/nbt.1708 (2010).
26. Chen, Z. Y., He, C. Y. & Kay, M. A. Improved production and purification of minicircle DNA vector free of plasmid bacterial sequences and capable of persistent transgene expression in vivo. *Human gene therapy.* **16**, 126-131, doi:10.1089/hum.2005.16.126 (2005).
27. Halleen, J. M. *et al.* Tartrate-resistant acid phosphatase 5b: a novel serum marker of bone resorption. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research.* **15**, 1337-1345, doi:10.1359/jbmr.2000.15.7.1337 (2000).
28. Adamopoulos, I. E. *et al.* IL-23 is critical for induction of arthritis, osteoclast formation, and maintenance of bone mass. *J Immunol.* **187**, 951-959, doi:10.4049/jimmunol.1003986 (2011).
29. Suda, T., Takahashi, N. & Martin, T. J. Modulation of osteoclast differentiation. *Endocrine reviews.* **13**, 66-80 (1992).
30. Takahashi, N. *et al.* Osteoblastic cells are involved in osteoclast formation. *Endocrinology.* **123**, 2600-2602 (1988).
31. Bradley, E. W. & Oursler, M. J. Osteoclast culture and resorption assays. *Methods Mol Biol.* **455**, 19-35, doi:10.1007/978-1-59745-104-8_2 (2008).
32. Arai, F. *et al.* Commitment and differentiation of osteoclast precursor cells by the sequential expression of c-Fms and receptor activator of nuclear factor kappaB (RANK) receptors. *The Journal of experimental medicine.* **190**, 1741-1754 (1999).
33. Fuller, K. *et al.* Macrophage colony-stimulating factor stimulates survival and chemotactic behavior in isolated osteoclasts. *The Journal of experimental medicine.* **178**, 1733-1744 (1993).
34. Edwards, J. R. & Mundy, G. R. Advances in osteoclast biology: old findings and new insights from mouse models. *Nature reviews. Rheumatology.* **7**, 235-243, doi:10.1038/nrrheum.2011.23 (2011).
35. Weinstein, R. S. *et al.* Promotion of osteoclast survival and antagonism of bisphosphonate-induced osteoclast apoptosis by glucocorticoids. *The Journal of clinical investigation.* **109**, 1041-1048, doi:10.1172/JCI14538 (2002).

