## Appendix E-1

*Determination of Optimal in Vitro Concentration of Zoledronate*

A preliminary study was performed to determine an optimal concentration range of zoledronate, which effectively reduces the preosteoclast population, but is least harmful to osteoblasts. Cell death assays were conducted using mouse osteoblasts and osteoclast precursor cells at different concentrations of zoledronate (Bedford Laboratories, Bedford, Ohio). MC3T3 cells, which are well-established mouse calvarial cells of osteoblastic lineage, and RAW 264.7 mouse macrophage-like cells, which are well known to form osteoclasts, were purchased from American Type Culture Collection (Manassas, Virginia). MC3T3 and RAW 264.7 cells were grown in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 mg/mL streptomycin, and 0.1% Fungizone (amphotericin B). The cells were kept at 37°C in a humidified atmosphere of 5% $CO_2$ and 95% air. After the cells were treated with zoledronate at concentrations of 0, 5, 10, 50, and 100 µM for twenty-four hours, the cells were pelleted by centrifugation and resuspended with 5 mL of phosphate-buffered saline solution (PBS). Cell number was measured, and the cells were then centrifuged and resuspended in binding buffer (A.G. Scientific, San Diego, California) to a final cell density of $5 \times 10^5$ cells/mL. Five milliliters of annexin V-FITC (A.G. Scientific) were added to 195 mL of cell suspension and incubated for ten minutes. The cells were centrifuged and washed with phosphate-buffered saline solution and were resuspended in 190 mL of binding buffer. Ten milliliters of 20 mg/mL propidium iodide (A.G. Scientific) were added to the cell suspension, and flow cytometry was performed with use of a FACSCalibur (Becton Dickinson, Franklin Lakes,

New Jersey). Monoparametric cytograms of annexin V-FITC fluorescence (FL1) versus number of events were created with the CellQuest program, gating for living cells and excluding dead cells on the basis of their propidium iodide uptake[14].

The in vitro experiments with RAW 264.7 and MC3T3 cells showed that 50 μM of zoledronate induced 100% apoptosis in RAW 264.7 cells, whereas only 20% of MC3T3 cells underwent apoptosis (Fig. E-1). Therefore, a 50-μM zoledronate solution was used for all subsequent animal studies.

*Effect of Zoledronate on Binding of Human Mesenchymal Stem Cells to Hydroxyapatite in Vitro*

Different concentrations of zoledronate (0, 10, 50, and 100 μM) were applied to a twelve-well plate with hydroxyapatite-coated disks in the wells (BD Biosciences, Franklin Lakes, New Jersey). The disks were soaked in zoledronate for five minutes at room air and were air-dried. Excess bisphosphonate was removed by washing the disks with phosphate-buffered saline solution twice. Human mesenchymal stem cells (hMSCs), purchased from Lonza Bioscience (Walkersville, Maryland), were seeded onto the wells (50,000 cells/well) and grown in osteogenic growth media (Lonza Bioscience) for four days at 37°C in a humidified atmosphere of 5% $CO_2$ and 95% air. After four days, hMSCs were stained for alkaline phosphatase (ALP) with use of a commercially available kit (Sigma-Aldrich, St. Louis, Missouri). The number of ALP-positive cells was manually counted. The experiments were performed in triplicates. One-way analysis of variance was conducted, followed by a Scheffe test using SPSS version 11.5 ($p < 0.05$ was considered to be significant).

Zoledronate did not exhibit any deleterious effects on hMSC survival or differentiation (Fig.

E-2). In fact, the number of ALP-positive cells was slightly increased in wells that contained the highest concentration (100 μM) of zoledronate ($p = 0.02$).

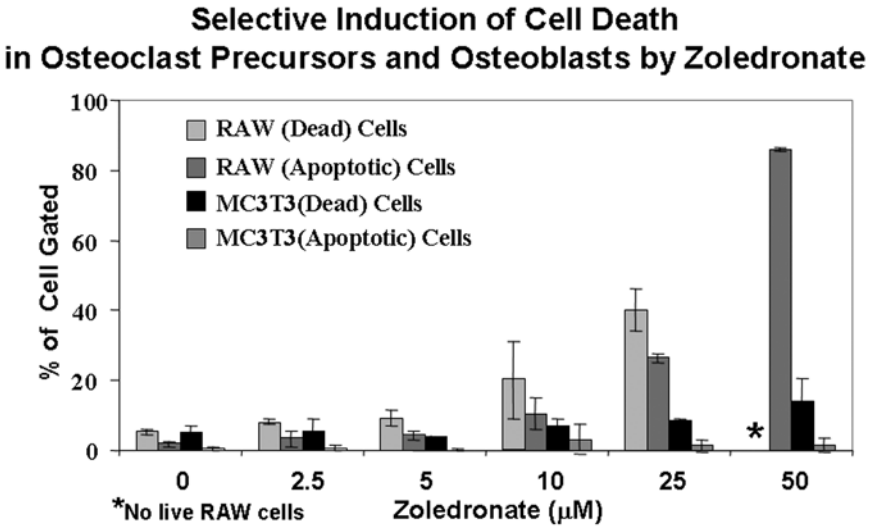


Fig. E-1

The percentage of cells gated, showing selected induction of cell death for osteoclast precursors and osteoblasts as a function of zoledronate concentration. No live RAW cells at a zoledronate concentration of 50 μM.

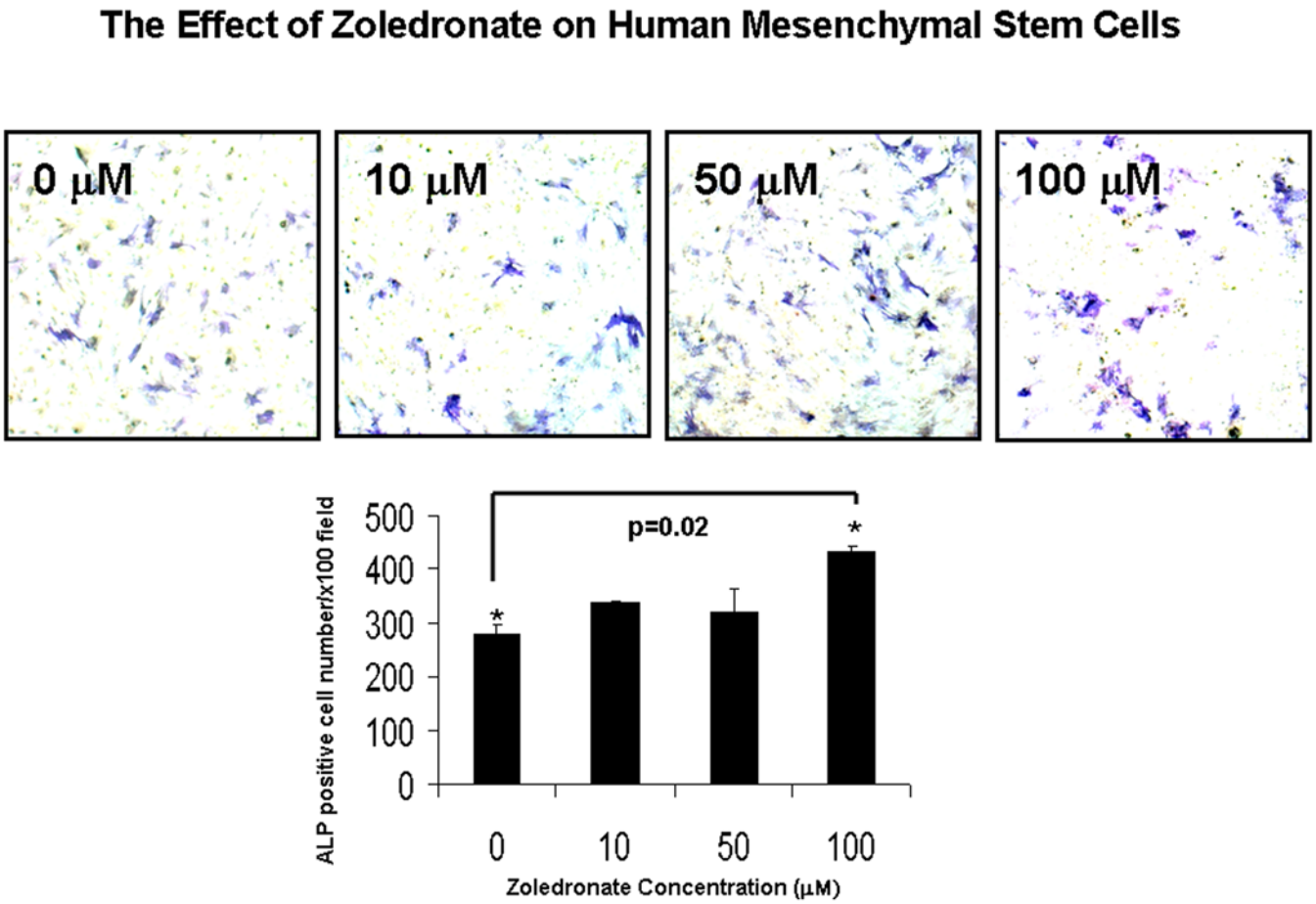


Fig. E-2

Images of human mesenchymal stem cells at four concentrations of zoledronate, showing no effect of zoledronate concentration on human mesenchymal cell proliferation and differentiation both visually and quantitatively by the number of alkaline phosphatase-positive (ALP) cells present.