INTRODUCTION:
Total joint arthroplasty is an effective treatment for severe arthritis. Despite long-term clinical success, particulate wear debris can cause periprosthetic osteolysis and aseptic implant loosening. This process involves a number of different cells and inflammatory cytokines. Recently, we have demonstrated that titanium (Ti) particles can induce the expression of macrophage-colony stimulating factor (M-CSF), an essential cytokine in osteoclastogenesis, in MC3T3-E1 murine calvarial pre-osteoblastic cells and that this process is mediated by the Extracellular Signal-Regulated Kinase (ERK) signaling pathway. Inhibition of ERK resulted in the suppression of Ti particle-induced M-CSF expression.

The ERK signaling plays an important role in regulating various growth factors that affect cell proliferation, apoptosis, and post-mitotic differentiation. In osteoblasts, chronic treatment of osteoblast cultures with ERK inhibitors was reported to stimulate osteoblast differentiation, whereas ERK activation was inhibitory. In contrast, targeting ERK was shown to block osteoclast differentiation, function, and cytokine secretion in the setting of multiple myeloma. Further, others have reported that M-CSF-dependent induction of tumor necrosis factor-α (TNF-α) expression in macrophages is mediated by ERK, and this process can be suppressed with a pharmacologic ERK inhibitor.

In this study, we have investigated the role of ERK signaling in Receptor Activator for Nuclear Factor κ B Ligand (RANKL), another essential cytokine in osteoclastogenesis, expression in MC3T3-E1 cells upon Ti particle stimulation and have explored the possibility of using a pharmacologic ERK inhibitor as a treatment option for Ti particle-induced osteolysis using a murine calvarial osteolysis model.

MATERIALS AND METHODS:
Cell Culture: MC3T3-E1 murine calvarial pre-osteoblastic cells were grown in Minimum Essential Medium (MEM) α with 10% fetal bovine serum (FBS) and 1% antibiotic/antimycotic (abx). Bone marrow cells were harvested from the femur of C57BL/6J mice and placed in MEM α supplemented with 10% FBS and 1% abx. Non-adherent cells were collected and treated with 10 ng/µl of M-CSF so that only bone marrow-derived monocytes/macrophages (BMMs) would attach to the plate.

In vitro Study: The cells were starved for 1 hr. in serum-free media, after which endotoxin-free Ti particles (0.02% vol./vol.) were added to stimulate the cells. In the inhibition group, 20 µM of PD98059, ERK inhibitor, was added during the 1 hr. starvation period as well.

RNA extraction and RT-PCR: After 6 hrs., the cells were harvested and total RNA was extracted. cDNA was synthesized from 2 µg of RNA by extension of random primers. RT-PCR was performed for M-CSF, RANKL, TNF-α, IL-1β, and IL-6, which were all normalized to GAPDH.

Western blot: MC3T3-E1 cells were stimulated with Ti particles for 24 hrs. in 0.1% abx, 10% FBS media. 20 µM of PD98059 was added to the inhibition group. The cells were harvested, lysed, and probed with RANKL and β-actin antibodies.

In vivo Study: 15 C57BL/6J wild-type adult male mice (10 wks., 30 g) were divided into 3 groups (n=5/group): sham, Ti particle only, Ti particle+PD98059. After anesthesia, a 5 mm-long skin incision was made over the calvarium, and the peristium was scraped with a scalpel. 30 ml of saline was introduced onto the calvarium for the sham group, whereas the mice in the other two groups received 5 mg of Ti particles suspended in 30 ml of saline. For the treatment group, the mice were injected with PD98059 (6 mg/kg) mixed in 20 ml of DMSO directly on top of the calvarium 1 day prior to surgery and every other day following surgery. The mice in both the sham and Ti particle only groups received 20 ml of plain DMSO. The animals were sacrificed on post-operative day 1 and their calvaria retrieved.

Immunohistochemistry: After fixation and decalcification, the calvaria were sectioned in the coronal plane and immunohistochemical analysis was performed by probing the specimens with tartrate resistant acid phosphatase (TRAP), M-CSF, RANKL, and phosphorylated-ERK (p-ERK) antibodies.

RESULTS:
Ti particles induced the expression of M-CSF in MC3T3-E1 cells, and this was suppressed by PD98059 (Fig. 1, a). In contrast, Ti particles did not stimulate RANKL expression. However, ERK inhibition did suppress RANKL expression below the basal level (Fig. 1, b), which was confirmed with the Western blot (Fig. 2). Further, Ti particles increased TNF-α expression (Fig. 3, a) in BMMs, which was inhibited with PD98059, IL-1β and IL-6 levels were not effected (Fig. 3, b and c).

Ti particles increased the number of TRAP-positive cells in the sagittal suture of murine calvaria compared to the sham group, and PD98059 decreased it (Fig. 4, a-c). When TRAP-positive cells were counted, the PD98059-treated group had less TRAP-positive cells compared to the Ti particle only group in a statistically significant manner (p<0.01) (Fig. 5). As expected PD98059 decreased p-ERK expression (Fig. 4, f). Further, Ti particles stimulated both M-CSF and RANKL expressions (Fig. 4, h and k), but PD98059 suppressed them (Fig. 4, i and l).

DISCUSSION:
We have already demonstrated that Ti particles can directly stimulate the expression of M-CSF in MC3T3-E1 cells, and this process can be blocked with a pharmacologic ERK inhibitor, PD98059 (Fig. 1, a). In contrast, Ti particles did not increase the expression of RANKL, although PD98059 suppressed RANKL below the basal level (Fig. 1, b and 2). In BMM’s, Ti particles directly stimulated upregulation of TNF-α, while IL-1β and IL-6 were not effected (Fig. 3). As expected, PD98059 suppressed TNF-α expression.

Our in vivo data suggested that ERK inhibition is effective in decreasing the number of TRAP-positive cells in murine calvaria upon Ti particle stimulation (Figure 2, a-c). As expected, PD98059 suppressed M-CSF expression, which could be explained by our in vitro data using MC3T3-E1 cells (Figure 1, a). Further, our in vivo RANKL expression also decreased with PD98059 treatment (Figure 2, l), which concurs, at least in part, with the in vitro results (Fig. 1, b and 2). In conclusion, the use of a pharmacologic inhibitor, such as PD98059, to treat particle-induced osteolysis by targeting several important pro-inflammatory pathways may be possible in the near future.