INTRODUCTION:
Non-healing bone defects or non-unions present significant orthopaedic challenges. A commonly used model is the critical size calvarial defect, which is defined as a defect that will not heal without intervention during the lifetime of the animal. Over time, without intervention, critical size calvarial defects fill with fibrous tissue but not with functional bone. Retrovirally transduced muscle-derived stem cells (MDSCs) have been shown to mediate healing of a critical size bone defect in the calvaria when applied at the time of creation of the defect. This study examines the effect of MDSCs applied to an established non-healing defect to determine if MDSCs can also influence existing fibrous tissue to remodel properly into bone.

METHODOLOGY:
MDSCs were isolated from three week old male C57BL/10J mice using a modified preplate technique and cultured in proliferation medium (DMEM, 10% fetal bovine serum, 10% horse serum, 5% chick embryo extract, and 1% penicillin streptomycin). Cells were then transduced with a CLB2/4G retroviral vector to express human bone morphogenetic protein 4 and GFP (MDSC-B4G) or a CLB2/4G retroviral vector to express GFP only (MDSC-G). All experiments were carried out on mature, male C57BL/10J mice, with n=10 animals per group. First, all animals underwent surgical procedure to create the critical size cranial defect. Briefly, the scalp was dissected and a 5 mm trephine was used to create the circular bone defect. The wound was closed, and all animals were allowed to recover and heal for 21 days. At that time, all groups were evaluated with a microCT scanner (VivaCT40, Scanco, Switzerland), and all groups except the non-treatment group underwent a second procedure. The scalp was again dissected and either MDSC-B4G in fibrin, MDSC-G in fibrin or fibrin only was applied to the defect area. 100,000 cells were applied and the volume of fibrin was 40 μL. All groups were again scanned at 35 days and 49 days (14 and 28 days post treatment respectively). All animal experiments were conducted with the approval of the IACUC of University of Pittsburgh.

Area of defect was computed by analyzing the 3D reconstructions computed by the Scanco software with Northern Eclipse, and efficiency was calculated using volumetric measurements (total and within defect) from Scanco software (efficiency = within defect volume/total new bone volume). All statistical analysis (either 2 or 3 way ANOVA) was performed with SPSS, and differences were considered significant if p<.05.

RESULTS:
Figure 1: Representative microCT reconstructions of treatment groups. MDSC-B4G shows extracranial and intracranial views at 49 days; all other groups and timepoints are extracranial views. Visually, MDSC-G, fibrin and the no treatment groups showed similar, minimal healing, while MDSC-B4G group showed partial healing at 35 days. At 49 days, 2 of 10 animals in MDSC-B4G group showed complete visual healing with near complete healing in all other animals; all other groups remained as they were at 35 days.

Figure 2: Area of defect at 21, 35 and 49 days. § significantly different from MDSC-B4G at 21d. # significantly different from fibrin at 21d. * significantly different from MDSC-B4G at 49d.

Figure 3: Efficiency of bone formation at 35 and 49 days. # significantly different from MDSC-B4G at 35 days and * significantly different from MDSC-B4G at 49 days. Efficiency of 1 denotes that all bone formed was within the defect area, and a smaller number represents more bone was formed outside the defect than within it.

DISCUSSION:
The objective of this study was to determine the effect of MDSCs in an established non-healing bone defect, i.e. can MDSCs mediate healing in a defect filled with fibrous tissue in the same way as a newly created defect? As seen in Figures 1 and 2, MDSC-B4G can indeed mediate formation of bone that covers an established calvarial defect. Quantification of this coverage reveals that MDSC-B4G treatment is significantly better than MDSC-G, fibrin only or no treatment groups. In fact, MDSC-G and fibrin were not different from the no treatment group, indicating these therapies were ineffective. However, as seen in Figures 1 and 3, although the bone formed in the MDSC-B4G group covered the defect (which may be functionally acceptable), it did not fill the defect. More specifically, at 49 days, MDSC-B4G group had an average efficiency of .055, meaning that only 5.5% of the bone formed was within the defect.

Limitations of this study include size of mice and control of MDSC-B4G mediated bone formation. Due to the extremely thin nature of the mouse dura, during the treatment procedure, it was not possible to thoroughly debride the defect area. In a clinical situation, debridement and removal of fibrous tissue would precede treatment of a non-healing defect. Debridement may possibly reinjure the tissue and release blood cells, blood vessel related cells, and/or inflammatory cells, effectively “renewing” the defect, allowing MDSCs to function in the same way as in a newly created defect. Previous work by our group has shown that delivery method (fibrin gel vs collagen gel vs gelatin sponge) can affect calvarial healing. Additional control of bone formation can also be implemented by using combinations of BMP4, VEGF (vascular endothelial growth factor) and Noggin (BMP antagonist). Improving efficiency and spatial control of bone formation is the highest future priority.

This study demonstrated that MDSC-B4G can induce bone formation which covers an established non-healing defect. Future studies will examine control of this bone formation and explore delivery methods and combinations with other growth factors to achieve optimal healing.