INTRODUCTION: Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases that aid in tissue remodeling through degradation of various components within the extracellular matrix (ECM) (1). Previously, we have detected that MMP1 is effective in overcoming the barrier of fibrosis, which can hinder successful restoration of the damaged tissue, and ultimately lead to loss of function (2, 3). To oversee the mechanism behind MMP1 in the muscle healing process, the purpose of this experiment is to investigate stem cell population/regenerative processes and fibrosis formation in injured muscle by using the synthetic MMP inhibitor, GM6001.

C2C12 myoblasts were evaluated using immunocytochemistry to assess for stem cell markers (Sca1, CD34) after treatment with MMP1 or GM6001. Lacerations to the Gastrocnemius muscle (GM) were used to represent a skeletal muscle injury in a murine model, where the sites of injury were treated with MMP1 and GM6001.

METHODS: Immunocytochemistry: C2C12 myoblasts were cultured as previously described (2). At determined time periods, cells were fixed with 4% paraformaldehyde (PFA) for 8 minutes and rinsed 3 times for 10 min with phosphate buffered saline (PBS). The cells were then treated with 0.2% Triton for 10 min to allow for cell permeabilization. To block unspecific binding, the cells were then treated with 10% H2O in PBS for 1 hour. The primary antibodies were then added at a concentration of 1:300 in 2% HS overnight at 4°C. The cells were then washed 3 times for 10 min with PBS before the secondary antibody was added at a concentration of 1:500 for 1 hour at room temperature. The cells were then washed twice for 10 min with PBS. The nuclei of the cells were then stained with 4',6-diamidino-2-phenylindole (DAPI) at a concentration of 1:1000 for 3 min. The cells were then washed 2 times for 10 min with PBS.

Immunohistochemistry: Tissues were fixed with 5% formalin for 5 minutes at room temperature. The tissues were then rinsed 2 times for 10 min with PBS. Unspecific binding was then blocked with 10% H2O in PBS for 1 hour at room temperature. The primary antibodies were then added at a concentration of 1:300 in 2% HS overnight at 4°C. The tissues were then washed 2 times for 10 min with PBS. The secondary antibodies were then added at a concentration of 1:300 for 1 hour at room temperature. The tissues were then washed twice for 10 min with PBS. The nuclei of the cells in the tissues were then stained with DAPI at a concentration of 1:1000 for 3 min. The tissues were then washed 2 times for 5 min with PBS followed by mounting with Cytoseal 60™ XYL. The trichrome staining was performed using the Masson Trichrome Staining Kit from International Medical Equipment, Inc. (IMEB).

Mouse Model: All of the animal work was performed under an approved IACUC protocol. The GM of both hind legs of the mice received a laceration, which cut through the skin and muscle, followed by stitching. The mice then received either 200ng of MMP1, 200µg of GM6001, or an injection of PBS with 10% ethyl alcohol (EtOH) as a control. The mice were then sacrificed and the two GMs of each mouse were harvested. The harvested muscle was then cryosectioned 8µm thick at -27°C.

RESULT:
1. More fibrosis is evident when treated with GM6001 in comparison with the control: Trichrome staining was performed to reveal collagen deposition in the injured muscle. The muscle treated with the GM6001 (Fig. 1B) had a greater area of fibrosis then the control (Fig 1A). There was also a greater number of center nucleated cells in the inhibited tissue compared to the control.
2. Lower percentage of cells expressing stem cell markers within a population when treated with GM6001 in comparison to MMP1 treated and control: C2C12 myoblasts were cultured followed by treatment of either 100ng of MMP1 (Fig 2a) or they received no treatment (Fig 2b). Some cells that were pretreated with MMP1 were then treated with 20mM of GM6001 (Fig 2c). Cells treated with MMP1 showed a higher percentage of cells expressing Sca1 (green) and CD34 (red), as well as a higher percentage of co-localization than the control and the inhibited cells. Cells that were treated with GM6001 showed the lowest percentage of cells expressing stem cell markers within the population.

DISCUSSION: MMPs play an important role in tissue remodeling through the degradation of the components of the ECM. By doing so, MMPs are effective in aiding the restoration of damaged tissue. We have determined that MMP1 can improve muscle healing through its effect on muscle cell migration and differentiation as well as digesting fibrous scar tissues. However, the mechanisms behind how the MMPs function directly and indirectly are still unclear. MMP1 is a collagenase which allows for degradation of collagens (type I and III) which are components fibrous scar tissues. As evidence in Figure 1, inhibiting MMP1 leads to a greater area of fibrosis. It is also possible that GM6001 delays regeneration of muscle fibers as seen by the many center nucleated cells in the inhibited tissue as opposed to the less frequent center nucleated cells of the more mature and healed muscle fibers of the control. Our lab has previously found evidence that GM6001 promotes stem cell like cell populations, and this is further developed in Figure 2. The MMP1 treated population contained more cells positive for stem cell markers, as well as a greater amount of co-localization between the two stem cell markers (Sca1, CD34) in comparison to the MMP1 inhibited cells.

The application of synthetic MMP inhibitor, GM6001, resulted in increased fibrosis, slowing down of regenerative processes, as well as reducing the percentage cells expressing stem cell markers within a population in vitro. These findings have implications in furthering the knowledge of the roles of MMP1 in muscle healing and regeneration as well as providing possible insight into the mechanisms behind these processes.

REFERENCES: