INTRODUCTION
Most clinically significant injuries involving the rotator cuff muscle are diagnosed and treated in a chronic setting. Those rotator cuff injuries requiring surgical repair of the tendon have a surprisingly high failure rate when analyzed with imaging; failure rates for massive tears have been reported to be as high as 80%. These failures are due in part to atrophy of the chronically injured rotator cuff muscle. Muscle atrophy is characterized by fatty infiltration, loss of muscle volume and a decrease in the elasticity of the muscle-tendon unit.

We have previously described a chronic rotator cuff injury and repair model in sheep. We found that chronic (six weeks) detachment of the tendon from the bone resulted in a 50% decrease in the force of muscle contraction and a twelve-fold increase in fat concentration within both the muscle cells and the interstitial spaces. Repair of the tendon partially reversed these findings. We hypothesize that the injection of stem cells directly into the infraspinatus muscle at the time of surgical repair will improve muscle function and decrease fat content within the muscle compared to controls. We use both muscle derived and marrow derived adult sheep stem cells in our experiments.

MATERIALS AND METHODS
Rotator Cuff Injury Model
The infraspinatus tendon was released in twenty-four skeletally mature ewes. Biopsy specimens were obtained from the muscle and were analyzed for fat content. The force generated by the muscle with supramaximal stimulation was recorded intra-operatively. The tendon was wrapped in a dura substitute (Preclude, W.L. Gore) to prevent scarring; the tendon was repaired six weeks after release. The infraspinatus muscle was injected at the time of surgical repair using an 18 gauge spinal needle with cell medium (8 sheep, control group); marrow derived stem cells (8 sheep, marrow MSC group); and muscle derived stem cells (8 sheep, muscle MSC group).

Animals were sacrificed at three months following surgical repair. Muscle biopsies and muscle testing was repeated at the time of repair and again at the time of sacrifice. Six specimens were prepared for MR analysis. The remaining shoulders were fixed with 10% formalin for histological analysis.

Stem Cell Isolation
Bone marrow cells: 1-2 ml of bone marrow was aspirated from the iliac crest of three donor animals into heparin solution. The nucleated cells were isolated via centrifugation, and then were seeded into tissue culture flasks at a concentration of 1x10^6 cells/cm² in low glucose DMEM containing 10% FBS. The medium was changed once after 24 hours to remove non-adherent cells. Adherent bone marrow mesenchymal stem cells (BM-MSCs) formed colonies over the subsequent 5-7 days. These cells were trypsinized and reseeded at a concentration of 12x10^3 cells/cm² in AlphaMEM supplemented with 10% FBS and 1 ng/ml FGF-2 for further expansion. Each culture was passaged three times prior to injection.

Muscle cells: 2-3 grams of muscle was harvested from three donor animals. Muscle cells were collected following enzymatic digestion and then seeded into collagen-coated tissue culture flasks in low glucose DMEM supplemented with 10% FBS, 1% horse serum, and 1% penicillin/streptomycin, and grown in an embryo extract. Cells that adhered between 48-96 hours isolation were collected and expanded at an initial seeding density of 12x10^5 cells/cm². Each culture was passaged three times prior to injection.

Histological Analysis
Tissues were washed thoroughly and were post-fixed with 1% aqueous osmium tetroxide for two to four hours for preservation of lipids. Tissues were washed overnight, sequentially dehydrated in a series of alcohols, and embedded in paraffin. Sections were collected at a 5 to 8-μm thickness and were stained with hematoxylin and eosin or Masson trichrome stain. The percentage of lipid within the tissue volume was determined with use of an image analysis system (Metamorph).

Magnetic Resonance Imaging
Six sheep shoulders were scanned on a 3.0 Tesla clinical imaging system (GE Healthcare) using an 8-channel phased-array knee coil. Morphologic imaging was performed utilizing a fat-sensitive T1-weighted fast spin-echo sequence in the oblique sagittal and axial planes, aligned with the distal infraspinatus tendon. The images were assessed for relative fat infiltration of the distal infraspinatus muscle based upon the scoring system as originally described by Goutallier.

RESULTS
Muscle testing
At the initial surgery, the infraspinatus muscle generated an average load (and standard deviation) of 37.68 lbs (+/- 0.57 lbs) for the twenty-four animals. After six weeks of detachment, the muscle generated an average load of 18.66 lbs (+/- 0.19 lbs) for the twenty-four animals; this represents a 50.5% decrease in the force of contraction following the 6 weeks of detachment (p<0.05). At the time of sacrifice, 3 months after surgical repair of the tendon and injection of the muscle, the infraspinatus generated the following average loads: 21.18 lbs (+/- 0.27 lbs) for the control group; 24.07 lbs (+/- 0.32 lbs) for the muscle MSC group; and 26.12 lbs (+/-0.18 lbs) for the marrow MSC group. These values, compared to 6 weeks after injury, represent a 14% increase for the control group (p<0.05), a 29% increase for the muscle MSC group (p<0.05) and a 40% increase for the marrow MSC group (p<0.05).

Histology
At the index procedure, biopsy specimens from the muscles of the twenty-four animals contained an average lipid concentration (and standard deviation) of 0.35% (+/- 0.12%). Six weeks after tendon detachment, the lipid concentration in the biopsies from all specimens averaged 2.82% (+/- 0.42%). Following repair, the average lipid content in the muscle biopsies from the three groups was as follows: 2.64% (+/- 0.23%) in the control group; 2.28% (+/- 0.16%) in the muscle MSC group; and 2.09% (+/- 0.19%) in the marrow MSC group.

Cross-sections of the infraspinatus muscle from the marrow MSC group demonstrated an increase in vascularity compared to both the control and the muscle MSC groups. Cross-sections from both the marrow and muscle MSC groups demonstrated an increase in the ratio of myosites to adiposities compared to specimens from the control group.

MRI
All of the specimens from the marrow MSC and muscle MSC groups scored a “2”, representing less fat than muscle as seen on MR analysis. One of the control specimens scored a “3”, representing an equal amount of fat and muscle.

DISCUSSION
Our animal model allows us to study the physiology of the rotator cuff muscle in a chronically injured setting. Six weeks of tendon detachment resulted in a 50% reduction in the force of muscle contraction and an eight fold increase in fat concentration within the muscle; surgical repair of the tendon partially reverses these physiologic changes. Injection of mesenchymal stem cells directly into the infraspinatus muscle at the time of surgical repair resulted in an increased improvement in the force of contraction compared to controls. This improvement is greater with marrow derived stem cells compared to muscle derived stem cells. Histological and MR analysis suggests that the addition of stem cells results in an increased ratio of myosites to adiposities and, in the case of the marrow derived stem cells, an increase in vascularity within the muscle.

While rotator cuff research has historically focused on the healing of the tendon-bone interface, our research supports the concept that the atrophy of the muscle and the resulting physiologic changes contribute to failures of surgical repair. Impaired muscle function appears to correlate with an increase in lipid concentration and a decrease in elasticity. In particular, the loss of elasticity and the inability of the muscle to recover function most likely contribute to the tendon pulling away from the bone after repair. As we have previously suggested, there may be a point of no return after which time the muscle will not recover despite repair. Injection of stem cells into the muscle at the time of repair may improve muscle function, help restore elasticity and ultimately increase the rates of success for rotator cuff surgery. Some types of mesenchymal stem cells might be more effective than others.