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14. ABSTRACT Fibrosis in the joints of the extremities is due to the action of myofibroblasts, cells activated by the inflammatory cascade of injury to effect scarring and contracture. There is as yet no medical or pharmaceutical adjuvant therapy that can prevent or treat scar contracture in the joint. We postulate that molecular inhibition of myofibroblasts will mitigate scarring and contracture in a rabbit model of post-traumatic knee arthrofibrosis. The goal is to use small interfering RNAs as therapeutic agents, delivered through non-viral means. We have thus far established a baseline model of scar contracture across the knee joint in the rabbit hindleg that mimics a typical human clinical course: injury, followed by a period of fixation (8 weeks), followed by an extended period of release (16 weeks). We note marked contracture in the injured, operated joint, with none in the contralateral control hindlimb. Histologic and initial molecular analysis confirms dense intracapsular scar formation in the injured joints compared to contralateral control. These findings establish a significant baseline of injury against which our intervention may be measured.					
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Introduction

In recent conflicts, extremity injuries account for a very high percentage of all injuries suffered by military personnel, occurring in > 50% of all wounded warriors^{1,2}. These injuries typically require operative debridement and fixation/immobilization; thus, a very high percentage of wounded warriors are at risk for the development of post-traumatic joint contracture/fibrosis. In multiple studies in both humans^{3,4} and animals^{5,6} it has been demonstrated that post-traumatic joint contractures demonstrate very high levels of myofibroblasts. Myofibroblasts are the cells responsible for exerting deformational forces during healing after injury, and for the deposition of the hyperabundant collagenous matrix characteristic of fibrosis. Myofibroblasts are marked by their unique expression of α -smooth muscle actin (α -SMA), which, together with myosin, provides the cells with their high contractility and motility. These cellular properties, writ large on a tissue scale, are responsible for the fibrotic, contracted phenotype seen after injury. The central idea for this proposal is to administer agents, specifically, small interfering RNAs (siRNAs) or nonviral vectors expressing siRNAs that will inhibit myofibroblast function, and thereby prevent the development of post-traumatic joint contracture. In order to test this hypothesis, it is first necessary to establish an animal model system which manifests joint contracture and fibrosis and which faithfully recapitulates the events leading to joint contracture in humans, that is, injury, fixation, followed by release. Once this model of elevated scar/contracture is validated, our molecular therapies may be examined against it to determine their effectiveness.

Keywords

Scar, contracture, fibrosis, joint, extremity, trauma, myofibroblast, siRNA, minicircle

Overall Project Summary

We have established the baseline rabbit injury protocol in which our proposed interventions are tested. This involves a complicated series of surgical maneuvers: first an arthrotomy is made, then the anterior and posterior cruciate ligaments are directly transected. The joint capsule is then further ruptured by hyperextension of the leg to a 135 degree angle. The hindleg is then placed into a position of maximal flexion with internal fixation using a buried Kirschner wire. After a period of 8 weeks, a second survival surgery is carried out to remove the K-wire. The animal is then allowed to recover for a further 16 weeks to see if any degree of joint mobility may be regained.

At the end of said 16 weeks animals are examined to determine the degree of contraction that obtains at the knee joint. In the first group of completed animals we found that the unaffected, unoperated hindlimb shows complete extension, as expected. In contrast, the injured, operated hindlimb maintains a significant scar contracture, also consistent with expected results. Pictures showing the difference between the two are shown below in Figure 1 below.

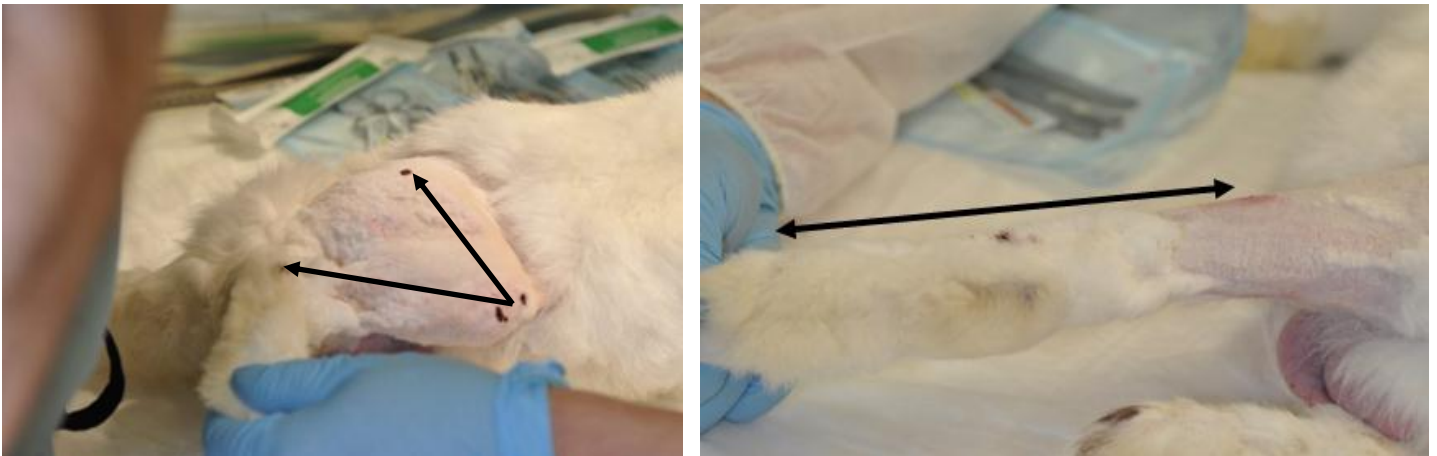


Figure 1: Photos of joint contracture after 24 weeks. (*left*) An operated hindlimb after 8 weeks fixation and then 16 weeks of release. The limb maintains an acute scar contracture. (*right*) The unoperated contralateral hindlimb of the same animal. Full extension is achieved.

Animals were then sacrificed and their joint/capsular tissues immediately harvested for histologic and molecular analysis. Operated knee joints showed dense fibrotic accumulations essentially obliterating the joint space. The contralateral unoperated joints showed essentially normal joint architecture, with normal appearing joint synovium which appeared to have some fatty component to its substratum as well. Samples from both were sent for hematoxylin and eosin as well as Masson's trichrome staining. Pictures of representative findings are shown in Figure 2 below.

Tissues were also stored in RNA later, then had total RNA extracted and characterized. Quantitative RT-PCR was carried out for α -smooth muscle actin (α -SMA) expression in scarred tissues versus the uninjured intracapsular ligamentous tissues of the unoperated hindlimb. Preliminary results are shown in Figure 3 below.

These results are all in accordance with expectations, specifically that injured limbs will heal with dense sclerotic scar formation and contracture. They give confidence of a significant pathological baseline against which our proposed molecular intervention can be measured.

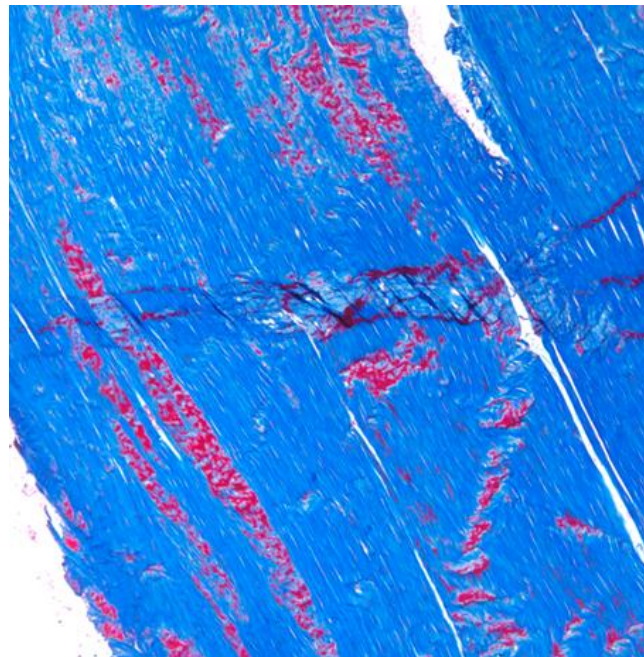
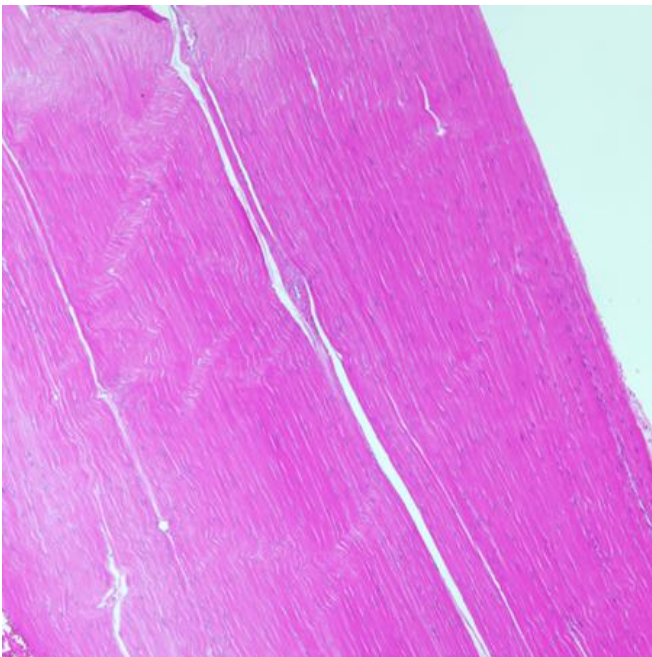
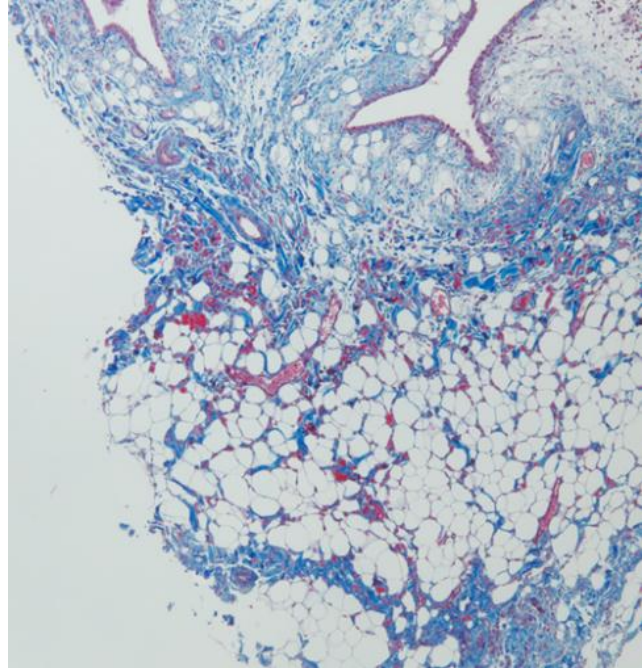
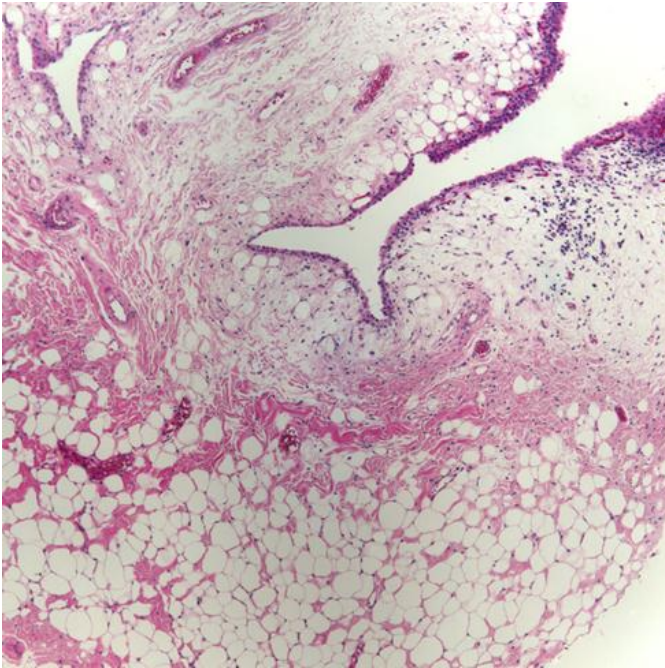


Figure 2. Histological examination of intracapsular tissue from unoperated and operated hindlimbs. (*top left*) H & E staining of uninjured, unoperated hindlimb tissue showing normal synovial and subsynovial architecture. (*top right*) Unoperated intracapsular tissue stained with Masson's trichrome. (*bottom left*) H & E staining of injured joint tissue. The normal tissue has been essentially completely replaced by scar. (*bottom right*) Masson's trichrome staining of injured joint tissue, again showing the dense collagen deposition of scar.

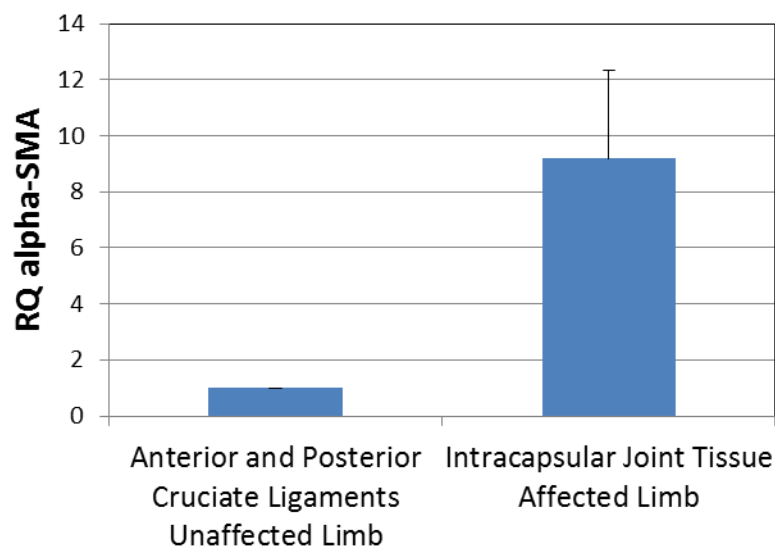


Figure 3. qRT-PCR determination of α -SMA in operated vs unoperated intracapsular tissue. Cruciate ligamentous tissue was assayed from unoperated limbs, versus dense scar tissue from operated limbs. Markedly elevated α -SMA was observed in the latter.

We have also made progress on the construction and testing of minicircle vectors for use in our studies. We have subcloned the RSV promoter/enhancer complex into the parent vector pMC-MCS2 and verified its position and orientation by confirmatory sequencing. We have subsequently added a 3' polyA signal sequence to increase message stability. This vector has now been transfected using ultrasound-assisted gene transfer into NIH 3T3 cells in vitro as a first attempt to examine its strength and durability of expression. The minicircle vector is able to drive expression of the luciferase reporter for a minimum of seven days, as shown in Figure 4 below.

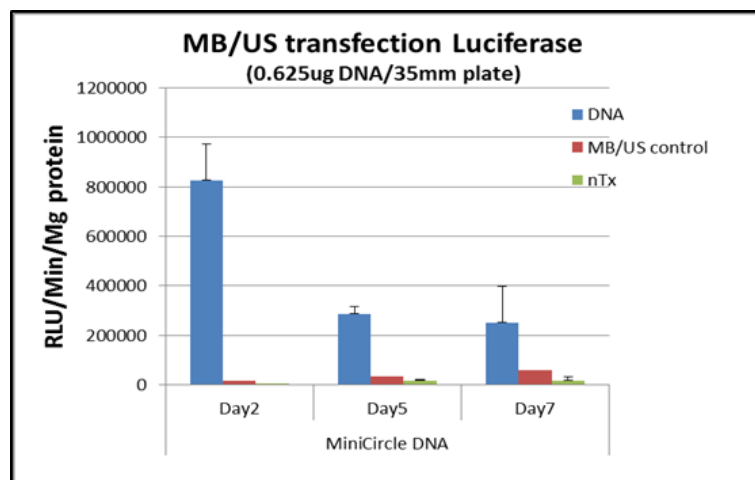


Figure 4. Luciferase reporter expression after ultrasound-assisted gene transfer into cells in vitro. Significant expression is seen when minicircle vector is used (DNA). Plasmid without reporter yields little expression (MB/US control). No transfection also gives little to no signal (nTx).

These are again encouraging preliminary results for the utility of our system.

Key Research Accomplishments

1. First demonstration that injury in this model of joint contracture yields a histological and molecular profile similar to that obtained in scirrhous cutaneous scar formation.
2. Successful demonstration of ultrasound-assisted gene transfer using a minicircle vector.

Conclusion

1. The animal model we have established offers an appropriately elevated level of scar and contracture against which molecular intervention can now be measured.

Publications, Abstracts and Presentations

None.

Inventions, Patents and Licenses

None.

Reportable Outcomes

None.

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