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Flexor tendon adhesions

A mouse model of flexor tendon injury and repair

PhD dissertation

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PREFACE

The present PhD dissertation, Flexor Tendon Adhesions: A mouse model of flexor tendon injury and repair, is based on experimental work performed in the laboratories of Professor Hani Awad, the Center for Musculoskeletal Research, University of Rochester, NY, USA, and in the laboratories of Associate Professor Lars Aagaard and Professor Thomas G. Jensen, Department of Biomedicine, Aarhus University, Denmark.

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LIST OF PAPERS

The thesis is based on the following papers:

Paper I:

"A Mouse Model of Flexor Tendon Repair." Hasslund, S., O'Keefe, R. J., Awad, H. A. Skeletal Development and Repair: Methods and Protocols, Methods in Molecular Biology, vol. 1130, chapter 6. DOI 10.1007/978-1-62703-989-5_6. (Accepted for publication).

Paper II:

"Adhesions in a Murine Flexor Tendon Graft Model: Autograft versus Allograft Reconstruction." Hasslund, S., Jacobsen, J., Dadali, T., Basil, P., Vinther, M. Søballe, K., Schwartz, E. M., O'Keefe, R. J., Mitten, D., Awad, H. A. (2008). Journal of Orthopaedic Research. 26(6), pp. 824–33. DOI 10.1002/jor.20531

Paper III:

"Freeze-dried Tendon Allografts as Tissue-engineering Scaffolds for Gdf5 Gene Delivery." Basile, P., Dadali, T., Jacobsen, J., Hasslund, S., Vinther, M., Søballe, K., Yasuhiko, N., Hicham Drissi, M., Mitten, D., O'Keefe, R., Schwarz, E., Awad, H. (2008). Molecular Therapy. 16(3), pp. 466–72. DOI 10.1038/ sj.mt.6300395.

Paper IV:

"Freeze-dried Allografts-Mediated Gene or Protein Delivery of GDF-5 Improves Murine Flexor Tendon Healing." Hasslund, S., Dadali, T., Vinther, M., Soballe, K., Awad, H. (In review, Journal of Tissue Engineering).

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TABLE OF CONTENTS

ABBREVIATIONS

AAV	Adeno-associated virus
BMP	Bone morphogenic protein
bFGF	Basic fibroblastic growth factor
ECM	Extracellular matrix
FAK	Focal adhesion kinase
FDL	Flexor digitorum longus
FDP	Flexor digitorum profundus
FDS	Flexor digitorum superficialis
GC	Gliding coefficient
GDF	Growth and differentiation factor
GFP	Green fluorescent protein
IGF-1	Insulin-like growth factor-1
IP	Interphalangeal
MMP	Matrix metalloproteinase
mRNA	Messenger RNA
MTP	Metatarsophalangeal
PDGF	Platelet-derived growth factor
rAAV	Recombinant adeno-associated virus
rmGDF-5	Recombinant mouse GDF-5
ROM	Range of motion
TGF-β	Tumor necrotic factor beta
VEGF	Vascular endothelial growth factor

SUMMARY (English)

The flexor tendons of the human hand are prone to injuries due to their superficial anatomical position and the multiple functions of the hand. The repair of flexor tendon injuries is complicated by the formation of fibrotic adhesions, which restrict tendon gliding and flexion of the injured digits. Even a small reduction in digits' range of motion can be disabling and complicate everyday activities. Despite implementation of modern suture techniques and post-operative motion protocols, the rehabilitation following these injuries is unpredictable. Further improvement and consistency of the outcome following flexor tendon injuries is likely to warrant manipulation of the biological tendon-healing response. For further understanding of the molecular mechanisms contributing to adhesion formation, *in vivo* screening models are needed. In this thesis I describe the first mouse model of flexor tendon injury and reconstruction. We have developed a functional test of tendon gliding and adhesions, and a subsequent test of tendon healing strength.

Tendon reconstruction by the use of tendon autografts is a common secondary procedure, when primary repair is not possible or has failed. Autografts are limited, however, by availability and donor site morbidity, and an attractive alternative may be tendon allografts. Allografts are moderately studied in flexor tendon reconstruction; but despite their advantages, their use has been limited by concerns of impaired healing capacity and long-term side effects. In our mouse model we have compared live autograft and freeze-dried allograft. We have found that the mechanical advantages of the autograft over the allograft are minimal in our model. Furthermore, we have observed no increase in adhesion formation over that found in autografts, but rather a decrease in adhesions and an increase in digit range of motion.

Growth and differentiation factor 5 (GDF-5) is known to be involved in tendon development, and it has been demonstrated to increase tendon healing strength in several animal models. GDF-5's effect on tendon adhesions, however, has not previously been investigated. In our experimental model, we have reconstructed flexor tendons by freeze-dried allografts coated with recombinant protein GDF-5 or with viral vectors encoding GDF-5 (rAAV-*Gdf5*). We have found that the GDF-5-loaded allografts have an anti-fibrotic effect on tendon healing and demonstrate a significantly improved range of motion in the digits. The anti-fibrotic effect seems to be independent of delivery method (protein vs. rAAV).

The mouse model of flexor tendon injury and repair has, despite its limitations, proven to be a valuable screening tool for evaluating the molecular, cellular and biomechanical effects of specific genes and molecules on the tendon-healing process.

SUMMARY (Danish)

Den superficielle anatomiske placering af håndens flexorsener resulterer ofte i skader på disse. Behandlingen af flexorsenelæsioner medfører ofte dannelse af adhærencer, der forhindrer senens glidning og flexionen af den pågældende finger. Selv en mindre nedsættelse af bevægeligheden kan have store konsekvenser for håndens funktion og betydning for udførelsen af daglige gøremål. Til trods for udviklingen af suturteknikker og mobiliseringsprogrammer er den efterfølgende rehabilitering og genoprettelse af håndens funktion stadig uforudsigelig og ofte fulgt af nedsat fleksion. Yderligere forbedring og stabilitet i behandlingen af flexorsenelæsioner kræver formentligt manipulering af det biologiske senehelingsrespons. Der er derfor behov for at afdække de biologiske processer, der ligger til grund for dannelsen af adhærencer, hvilket kræver in vivo screeningsmodeller. I denne afhandling beskrives den første musemodel for flexorsenelæsioner og behandling heraf. Vi har desuden udviklet en test til bestemmelse af fingerens fleksion samt en efterfølgende test af senens helingsstyrke.

Flexorsenelæsioner behandles ofte ved transplantation af en senegraft, når primær suturering ikke er mulig eller er mislykkedes. Autografter er den hyppigst anvendte grafttype, men er begrænset af tilstedeværelsen af egnede donorsener hos patienten samt efterfølgende komplikationer ved donorstedet. Allografter kunne udgøre et attraktivt alternativ, men erfaringen med brugen af allografter ved flexorsenerekonstruktioner er begrænset på grund af frygten for et dårligere naturligt helingspotentiale. Vi har i vores model sammenlignet autograft og allograft senerekonstruktion. Vi konstaterede, at de biomekaniske fordele ved autograft- sammenlignet med allograftrekonstruktion var minimale. Desuden observerede vi en reduktion af adhærencedannelsen og en forbedring af fleksionen ved allograftrekonstruktion.

GDF-5 (Growth and differentiation factor-5) er kendt for at være involveret i dannelsen af senevæv. Desuden har flere eksperimentelle studier i dyremodeller vist, at GDF-5 kan forbedre senehelingsstyrken. Effekten af GDF-5 på seneadhærencer er ikke tidligere blevet undersøgt. I vores musemodel har vi udført en senerekonstruktion med en frysetørret seneallograft, som var behandlet med enten GDF-5 protein eller en viral vektor kodende for GDF-5 genet (rAAV-*Gdf-5*). Vi konstaterede, at senerekonstruktion med en GDF-5 behandlet allograft havde en anti-fibrotisk effekt og resulterede i en signifikant øget fingerfleksion. Den anti-fibrotiske effekt var uafhængig af behandlingsmetode (protein vs. genterapi).

Vores model for flexorsenelæsioner og behandling heraf har, til trods for sine begrænsninger, vist sig at være et værdifuldt screeningsværktøj til undersøgelse af de molekylære, cellulære og biomekaniske effekter af specifikke molekyler og gener i helingsprocessen.

1.0 INTRODUCTION

1.1 Flexor tendon injuries

The human flexor tendons and their aiding structures make up an ingenious biomechanical system, enabling us to carry out complex hand and finger movements. Today most people depend on this biomechanical system to perform everyday activities -not to mention that numerous professions require sophisticated hand and finger movements, including both crane operators and hand surgeons. Thus, when these structures are injured, it is of utmost importance to restore function in order to resume everyday activities and possibly even to sustain a career. Even a small reduction in the digits' range of motion can be disabling. Despite implementation of modern suture techniques and post-operative motion protocols, the rehabilitation following these injuries is highly unpredictable, and adhesions remain a frequent complication.

1.1.1 Anatomy

In the digits the flexor tendons (the flexor digitorum profundus (FDP) and the flexor digitorum superficialis (FDS)) run through a fibrous tunnel of pulleys (Figure 1) [1,2]. The hand and its flexor mechanism have been divided into five zones (Figure 2) [3]. In the course of the flexor tendons through the hand, the tendons have intrasynovial passages in Bunnell's zone II and IV (Figure 2) [4]. The composition of the synovial sheath lowers the tendons' gliding friction in the fibrous tunnel [5]. The blood supply for these tendons runs through the vincular system (Figure 1B), and the synovial fluid serves as an additional source of nutrition [6]. Zone II is rich in vincula, and damage to these structures complicate the healing [7].

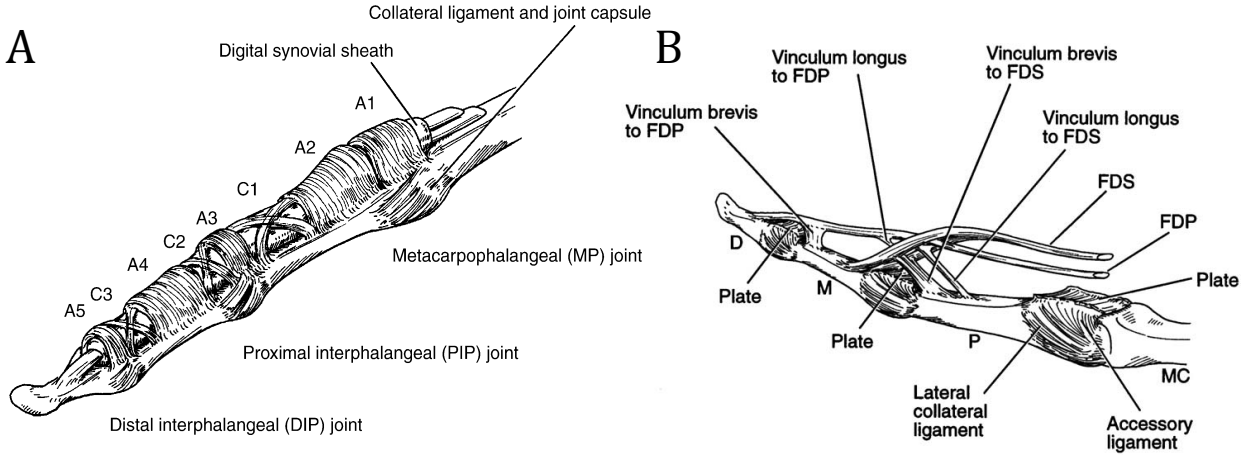

Figure 1: A: Digital flexor sheath. Annular (A1–A5) and crusiate (C1–C3) pulleys. B: The flexor tendons and vincula. Adapted from [1].

1.1.2 Epidemiology and Etiology

Little has been published to describe the incidence of flexor tendon injuries. Incidences have been reported ranging from 4.8 to 14.3 in 100,000 individuals per year [8,9]. Flexor tendon injuries are most often seen in younger individuals, in males more than females, and more in the dominant hand [8–13]. Tendon injuries happen in all five zones of the hand (Figure 2). Injuries in zone II are both the most common and, unfortunately, the most problematic [10].

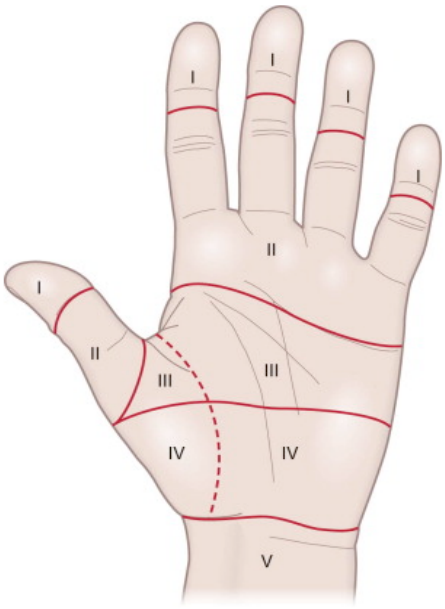

Figure 2: Bunnell's zones of the hand. Zone 1 contains FDP. Zone 2 is intrasynovial, FDP passes through the FDS at chiasma tendinum. This zone is rich in vincula. It is the most critical zone for flexor tendon injuries. Zone 3 is extrasynovial and contains FDP and FDS. The lumbrical muscles arise here. In zone 4 the tendons pass through the carpal tunnel. Zone 5 is extrasynovial. Adapted from [3].

The etiology of flexor tendon injuries can be categorized as traumatic or degenerative, and in rare cases, without clear etiology, as spontaneous [14]. The traumatic injuries can be subdivided into sharp lacerations, crush injuries and avulsion injuries. Avulsion injuries are caused by forced extension during active flexion (baseball, rugby, handball, basketball) [15]. Degenerative flexor tendon ruptures occur secondary to cortisone injections, infections and rheumatoid arthritis, and also frequently in rock climbers who expose their flexor tendons to intensive stress and overuse [9,16,17]. Degenerative injuries have an unfavorable prognosis, and tendon grafting will often be considered [18,19].

1.1.3 Treatment of flexor tendon injuries

Current practice in treatment of flexor tendon injuries is still subject to a great deal of variation, depending on traditions in the department and personal preferences of the surgeon. There seems to be no agreement on a golden standard in the choice of suture material, suture technique or post-operative rehabilitation program. Several surgeons have stressed that every patient must be treated according to the characteristics of the injury and the patient's individual needs and lifestyle [20,21]. The great variation in surgical procedure makes it difficult to compare and evaluate new methods. The following section will provide a short summary of the general improvements in the treatment of flexor tendon injuries. The section is based primarily on studies of flexor tendon injuries in zone II, since this is the most frequent and problematic, and therefore the most investigated type of injury.

Surgical treatment:

Flexor tendon injuries need surgical repair to bring the tendon ends together and ensure healing [22]. The aim of flexor tendon repair is to restore tendon strength and tendon gliding and thereby regain satisfactory digital function. Originally it was recommended by Bunnell (considered the father of hand surgery) that injuries in zone II should not be primary-repaired, but rather bypassed by a tendon graft sutured in zones I and III [4,23]. Today, the technical improvements of surgical material, along with advances in surgical technique and the implementation of early-motion protocols, have made primary repair the standard treatment for flexor tendon injuries. Tendon grafting, however, is still a common secondary procedure, when the injury is not suitable for primary repair or when primary repair has failed [24–27].

Suture technique: An enormous amount of research has increased our knowledge of the various surgical techniques and their effects on the surgical outcome. According to these investigative efforts, Strickland has listed the characteristics of an ideal primary flexor tendon repair (Table 1) [20]. Furthermore, the tendon repair should consist of both core and peripheral sutures, since both will contribute to the strength of the repair and diminish gab formation [28]. In the effort to optimize the surgical method, numerous core and peripheral repair techniques have been proposed and tested (Figure 3) [29]. Traditionally, surgeons have used two-strand core suture techniques such as the modified Kessler (Figure 3BC). Today, it has been demonstrated that multi-strand repair techniques experience less gapping and increase the strength of the repair in proportion to the number of suture strands [22,23,30–33]. But the ideal number of strands is a balance between the strength and the technical difficulty of placing the suture. The increased handling of the tendon will increase the risk of tissue crushing, which has been correlated to increased adhesion formation [34,35].

• Sutures should be easily placed in the tendon. • Suture knots should be secure. • Tendon ends should be smoothly united. • The repair site should experience minimal gapping. • Tendon vascularity should be minimally affected. • Sutures should provide sufficient strength to permit early motion of the tendon.

Table 1: Strickland's characteristics of an ideal flexor tendon repair.

Tendon grafts: The routinely used graft type for flexor tendon reconstruction is autografts, typically of the palmaris longus (when it is present), the plantaris or the toe extensors [36]. Autografts are limited by availability and donor site morbidity. Furthermore, autografts are most commonly extrasynovial, and it has been demonstrated that extrasynovial grafts are associated with more adhesions [37–39]. Tendon allografts may be an interesting alternative for flexor tendon reconstruction [40]. Little is known of the actual healing potential of flexor tendon allografts, and their clinical use in reconstructing flexor tendons has been only sporadically reported in the literature [41,42]. On the other hand, tendon allografts

have become widely popular for other types of tendon and ligament injuries [43,44]. In fact, allograft reconstruction of the anterior cruciate ligament of the knee has been advocated for routine use, by some [45]. Allografts pose several advantages over autografts, including increased versatility and selection, decreased operating and tourniquet time, elimination of donor site morbidity and eased post-operative recovery [43]. Furthermore, allograft reconstruction opens possibilities for a variety of pre-surgical manipulations, including surface modification and cell seeding by stem cells [46–48].

Despite the advantages of the allografts, their use in flexor tendon reconstruction has been limited by the fear of a potentially less robust healing response and a slower biological incorporation. Whether tendon allografts hold the same reconstructive potential as live autografts in flexor tendon reconstruction is poorly investigated [49–51].

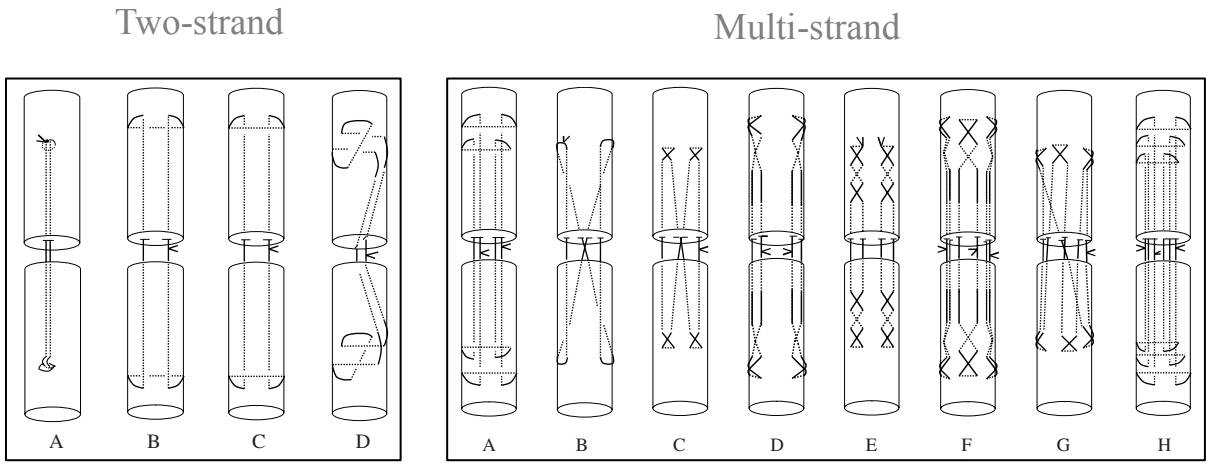

Figure 3: Two-strand core suture techniques: A: Tsuge, B: Modified grasping Kessler, C: Modified locking Kessler, D: Modified Pennington. Multi-strand core suture techniques: A: Double modified locking Kessler, B: Cruciate non-locked, C: Cruciate cross-stitch locked, D: 4-strand Savage, E: Augmented Becker, F: 6-strand Savage, G: Modified Savage, H: Triple modified Kessler. Adapted from [29].

In the use of tendon allografts, some general concerns need to be taken into account. One concern of using tendon allografts is the risk of an immune reaction. Cellular antigens are recognized by the host as foreign, and so they may induce an inflammatory response or an immune-mediated rejection of the foreign tissue. Nevertheless, components of the extracellular matrix (ECM), including collagen, are generally conserved among species and are tolerated similarly by recipients from the same species [52]. The immunogenicity of a decellularized allograft is not a major concern, and decellularization by freezing and thawing has been demonstrated to have a strong effect on decreasing the immunological antigenicity [53,54]. Although disease transmission following allograft reconstruction is uncommon, it does occur [55]. Nevertheless, the continuous development of better sterilization, screening and testing procedures has reduced safety concerns dramatically [56].

Post-operative rehabilitation

The most-used clinical classification system for evaluating the recovery following flexor tendon injury and repair is the adjusted Strickland classification system [57,58]. Strickland has classified the outcome following flexor tendon injury and repair into 4 categories (Table 2). Although the classification purports to be functional, it is questionable whether a patient experiencing 75% of normal flexion considers this to be an excellent hand function.

Score	Adjusted Strickland (%)
Excellent	75-100
Good	50-74
Fair	24-49
Poor	<24

Table 2: Percentage of normal flexion = (active flexion PIP + DIP) - (extension deficit PIP + DIP) / 175° x 100

Historically, flexor tendon surgery was followed by at least 3 weeks of immobilization [13]. This measure, however, was radically changed by the discovery of the beneficial effects of early passive motion, regarding tendon gliding and healing strength [59]. Compared with digits' not being mobilized, passive motion improved the number of digits categorized to have good or excellent flexion by 40% [58]. It has been reported that a tendon excursion of 3-5 mm is needed to prevent adhesion formation [59]. Kleinert et al. introduced an early passive-motion program based on active extension and passive flexion [60]. Various studies have contributed with modifications of the early passive-motion programs to achieve optimal tendon excursion [61]. Stronger tendon repairs have enabled implementation of active-motion programs [62]. The effect of these programs is still controversial, and they have been suspected to result in higher rupture rates than passive-motion protocols [21,23,63]. Not all patients are suitable for a post-operative rehabilitation program. Furthermore, a compliant patient is a prerequisite to any successful rehabilitation program.

1.1.3 Complications and prognosis

The most frequent complication following flexor tendon injury and repair is the formation of tendon adhesions [27]. Adhesions restrict tendon gliding and compromise the digit's range of motion. In cases of mild adhesions, aggressive post-operative therapy can be sufficient to regain some function; if not, tenolysis or even revision surgery must be performed [64,65]. Half a century of research has resulted in a number of improvements that have led to increased post-operative hand function. Nevertheless, it still takes a skilled and experienced surgeon, a whole team of occupational therapists and a well-informed and motivated patient to achieve acceptable recovery. Despite these optimized conditions, digit range of motion and hand function are categorized as poor or fair in approximately 25% of patients [66–68]. Furthermore, the reported rates of poor or fair have been ranging from 10% to 57%, and thus the functional outcome following flexor tendon injury and repair is highly unpredictable [68]. In general, the outcome following flexor tendon grafting is reported as inferior to primary repair [36,69,70]. The patients

treated by tendon grafting, however, are a selected group not suited for primary repair, and this difference makes comparison difficult.

Another feared complication is tendon rupture, which will require revision surgery. But clinical reports from the prime hand centers around the world have been reviewed, and the rupture rates have declined from about 10% to 3% over a 20-year period [68]. Other, less frequent complications are infections, joint contracture, triggering and pulley failure [27].

1.2 Flexor tendon healing

It is well documented that tendons have a poor healing capacity, and it is questionable whether a healed tendon will ever match the mechanical properties of an intact tendon [71]. Knowledge of tendon healing is derived predominantly from studies of transected animal tendons, and the translational value of this knowledge remains unclear. Nevertheless, it is based on these studies that the processes of tendon healing are partially uncovered.

1.2.1 Tendon structure

Tendons consist of dense regular connective tissue and are composed primarily of an extracellular matrix. Collagen composes up to 80% of the dry mass of tendons. Type I collagen is by far the most

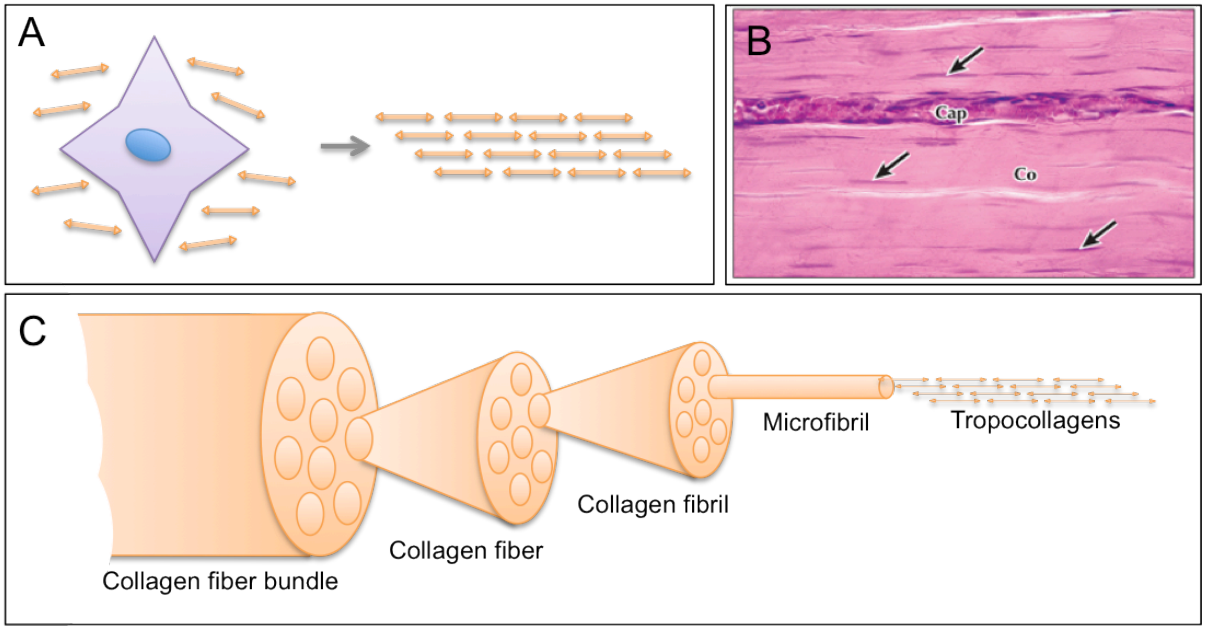

Figure 4: A) Procollagen molecules are produced individually and then undergo post-transcriptional modification and develop into triple-helical tropocollagen. Tropocollagen is exported by the fibroblast and self-assembles into microfibrils. B) Tendon in longitudinal section. Collagen fiber fasciles (Co) and inactive tenocytes; only the oval dense nuclei are visible (arrows). A capillary is crossing the field of view (Cap). Adapted from [76]. C) Simplified tendon structure. Tendons are bundles of collagen fibers. The collagen fibers consist of collagen fibrils, which are made of closely packed microfibrils. The microfibrils consist of tropocollagen molecules.

prevalent, although type III collagen can compose up to 10% in healing tendons [72,73]. A collagen fiber is the smallest tendon unit that can be seen under light microscopy [74], and collagen fibers are covered with a thin cellular layer called the endotenon. The septa of the endotenon join together and form a fine outer layer, the epitenon, surrounding the entire tendon. The vascular, lymphatic and nervous supply for the tendon is contained within these sheaths of cells and connective tissue.

1.2.2 Cellular tendon healing

The tendon cells are a type of fibroblasts, also called tenoblasts or tenocytes, and can be found in rows of cells, between the fiber bundles (Figure 4b). In a healthy tendon, only 5% of the volume is occupied by cells [75]. In mature, healthy tendon tissue, the tenocytes are relatively inactive and immobile; but, when the tendon is injured, the tenocytes proliferate and become active collagen-synthesizing tenoblasts [76]. Collagen is synthesized in the form of tropocollagen and self-assembles into microfibrils. Microfibril bundles are organized to form collagen fibrils that are closely packed to collagen fibers (Figure 4a, 4c) [72].

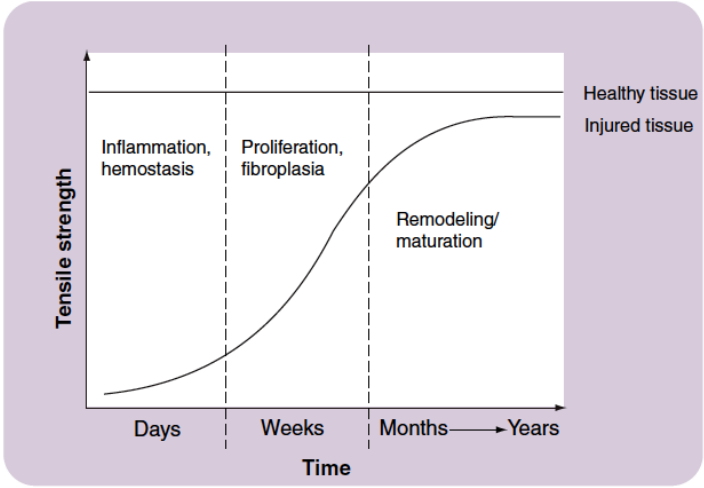

Figure 5: Healing phases during tendon regeneration. This figure is schematic, and the indicated increase in strength during the first days can indeed be discussed. Others believe that the healing process is characterized by an initial no-gain period in strength. Adapted from [77].

Like wound healing in other tissues, tendon healing can be divided into three main processes or phases (Figure 5) [77]. The phases of tendon healing in primary repair and graft reconstruction are comparable [78]. The phases are not well-defined but overlapping stages defined by the dominating process [67]. The inflammatory phase: Within the first 24 hours, inflammatory cells accumulate at the site of injury. Necrotic material will be phagocytized by macrophages. Fibroblasts will be activated by proliferative signals, and gradually migrate to the injury site and begin synthesizing collagen III [74]. The proliferative phase: Within days, the proliferative phase sets in. This phase is characterized by increased cellularity and collagen III production [74]. The remodeling phase: Four to six weeks after tendon repair, the remodeling phase dominates. Cellularity will decrease, and the repair tissue will change from cellular to fibrous. Collagen replacement and organization will take place, and collagen III will be converted to collagen I.[79] The collagen fibers become aligned in the direction of the tension. In the later stages of the remodeling phase, the formed fibrous scar-like tissue will undergo a gradual

change into more tendon-like tissue. The process, however, may take up to one year and may never be completed [74,80,81].

Two mechanisms of tendon healing have been proposed: an intrinsic healing process, whereby the cells active in the healing process originate from within the tendon, endotenon, or epitenon [82,83], or alternatively an extrinsic healing process, whereby fibroblasts and inflammatory cells from the surrounding tissue invade the healing site [34,82]. Today most think that the healing response observed clinically is a combination of extrinsic and intrinsic healing mechanisms [35]. The balance between the two mechanisms is most likely not constant but dependent on the tendon's environment and the degree to which the surrounding structures are injured too.

1.2.3 Molecular tendon healing

During tendon healing, a number of growth factors and cytokines are involved in activating and regulating the cellular healing. The molecules bind to cell surface receptors and activate a certain intracellular pathway. Despite the accumulating research in this field, the molecular process of tendon healing is only partially understood. In this section, therefore, I will briefly outline the growth factors and cytokines that have been best characterized in regard to tendon healing. Transforming growth factor beta (TGF-β), insulin-like growth factor one (IGF-1), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) have all been demonstrated to be up-regulated in response to tendon injury and healing (Figure 6) [84–88]. IGF-1 is highly up-regulated in the inflammatory phase. IGF-1 seems to induce proliferation and migration of fibroblasts [89]. Later, as the tendon heals, IGF-1 is believed to increase collagen production [89]. TGF-β expression is increased during the inflammatory phase. TGF-β is also a regulator of cellular proliferation and migration, but it is also a stimulator of collagen production [90]. PDGF is also a molecule of the inflammatory phase and helps stimulate the expression of additional growth factors, including IGF-1. Furthermore, PDGF has a role in tissue remodeling [91,92]. VEGF expression is up-regulated during the proliferative phase and

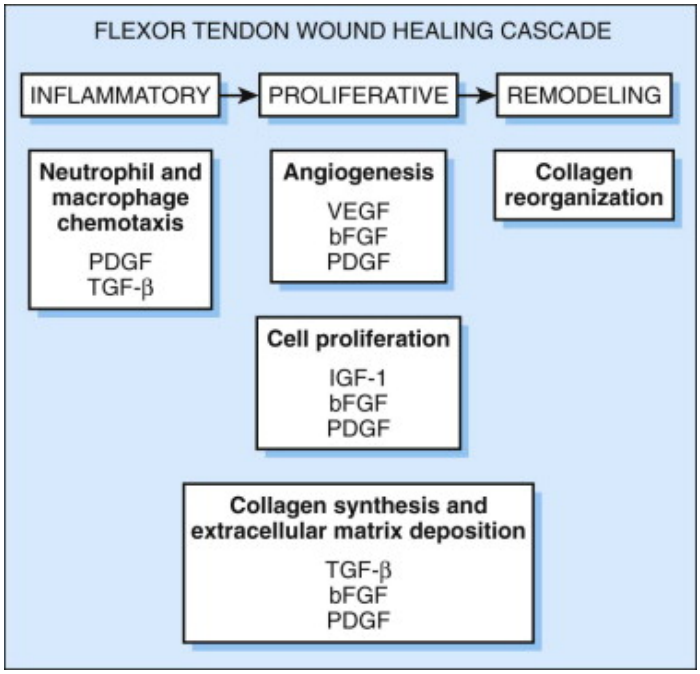

Figure 6: Cytokines and growth factors in the phases of tendon healing. Adapted from [97].

is a stimulator of angiogenesis [93,94]. Another stimulator of angiogenesis is bFGF, which is also a regulator for proliferation and migration [95–97].

In addition, matrix metalloproteinases (MMPs) are important regulators of ECM remodeling and degradation. It is accepted that a general increase in MMP activity likely indicates matrix degeneration, which is believed to be a part of the remodeling process [98]. A number of MMPs have been identified and have been demonstrated to play different roles. For example, in a rat flexor tendon injury model, MMP-9 and MMP-13 have been suggested to be active during the inflammatory phase, whereas MMP-2, MMP-3 and MMP-14 participate later in the tendon healing, presumably both in the process of degradation and in the remodeling of collagen [99].

1.3 Flexor tendon adhesions

Tendon rupture is a significant problem in tendon injuries in general. In the last decade, however, the rupture rate following repair of flexor tendon injuries has decreased [68], leaving adhesions as the major complication and concern after flexor tendon injury and repair. Many animal and human studies have been performed to illuminate the factors affecting tendon adhesion formation. Even more studies have investigated methods for preventing adhesion formation after flexor tendon repair. These efforts notwithstanding, existing research has not provided a method for adhesion-free flexor tendon healing. It may be speculated whether adhesion-free flexor tendon healing is possible at all.

Historically, adhesions have been considered an integrated and necessary part of the healing response. The concept of adhesion-free tendon healing, therefore, has been deemed impossible, or at least not achievable without severe sacrifice of tendon healing strength [34,82]. Several studies, however, have demonstrated that tendons have an intrinsic healing capacity [100,101]. Fetal wounds in various tissues have been demonstrated to heal more quickly and without scar formation [102–104], and the same has recently been documented to be true for fetal tendon healing [105,106]. Importantly, scar-free fetal tendon healing has been demonstrated to restore the biomechanical properties to the level of the uninjured tendon at 3 weeks after injury [106]. One of the mechanisms for scar-free wound healing in fetuses is considered to be an almost complete absence of an acute inflammatory response [104]. Accordingly, low levels of TGF-β were found in injured tendon tissues in fetuses compared with the same tissues in adults [105,106]. These findings show that adhesions are not an indispensable part of tendon healing, and grant promise that biologic modulation of flexor tendon repair may lead to adhesion-free healing.

1.3.1 Factors influencing the adhesion formation

This section will summarize the current knowledge and speculations about the factors influencing the formation of tendon adhesions. A lot of effort has been put into understanding factors associated with adhesion formation and these factors can be divided into four categories.

1) Factors inherent to the tendon injury: The type of injury and the degree of involvement of the tendon sheath and nutritional system [67]. It has been established that damage to the surrounding tissue (skin, sheath, subcutaneous tissue, and vincular system) alone is sufficient to induce adhesion formation [83]. Furthermore, it has been demonstrated that disruption of the vincular system leads to a decrease in total active digital range of motion [7]. Additionally, injury to the synovial tendon sheath has been observed to result in dense and well developed adhesions [6]. The synovial tendon sheath contains aggressive fibroblasts, which play a central role in adhesion formation [107].

2) Factors related to the repair procedure: The choice of tendon repair type (primary or grafting), the suture technique, and whether the sheath is repaired are all factors that will influence the magnitude of gliding restricting adhesions [59]. Although controversial, it has been argued that the presence of repair site gaps caused an increase in tendon adhesion formation [83,108,109]. Furthermore, the degree of tendon surface injuries and tissue crushing inflicted by the surgical manipulation of the tendon has been shown correlated with the degree of post-operative adhesions [34,59]. It could be suggested that the underlying mechanism for this correlation could be insult and disruption of a recently discovered basement membrane (BM) located at the outermost surface of the tendon [110]. The BM is primarily constructed of collagen IV and a mouse model holding a mutated collagen IV gene and a defective BM has been demonstrated to generate spontaneous adhesions of the flexor tendons [110].

3) The post-repair management: Immobilization of the involved digits following surgery will lead to a higher degree of adhesions compared to mobilized digits [58]. The application of mechanical force has been noted to increase fibroblast migration and collagen deposition, and to improve cell alignment in the direction of the applied force [111].

4) Factors involved in the healing response: The balance between intrinsic and extrinsic healing mechanisms has been suggested to determine the degree of post-operative adhesion formation, and when extrinsic healing dominates, adhesions are supposed to be inevitable [5,67,112–114]. Inflammatory processes might induce the extrinsic healing process leading to adhesions and it is believed that the degree of inflammation is correlated to the degree of adhesions [115]. It has been observed that modulation of the inflammatory response by anti-inflammatory drugs (NSAIDs) has shown potential

to reduce adhesion formation [116–118]. Likewise the absence of inflammation is believed to be an important factor responsible for scar-free fetal tendon healing [35,104,119,120]. Furthermore, ischemia in the tendon healing area is believed to increase adhesion formation [101].

1.3.2 Prevention of adhesion formation

Efforts to manipulate the biological tendon healing response to diminish adhesions have been investigated by the use of mechanical, chemical and molecular methods of manipulation. Several of these techniques hold some promise, but no method has reached general clinical use.

Mechanical methods:

Studies of mechanical methods to prevent adhesions range from a variety of more or less promising mechanical barriers to the successful implementation of early motion protocols. The implementation of early motion protocols has been covered by section 1.1.2, p. 15.

Surgical technique: Meticulous surgical techniques inflicting minimal additional trauma to the surrounding structures can decrease adhesion formation [7,34,59].

Mechanical barriers: The purpose of the mechanical barriers is to act as a separator between the tendon and the surrounding tissue. The tendon will be allowed to heal solely by the intrinsic mechanism and the surrounding tissue will be prevented from migrating into the tendon. Different types of barriers have been tested: rods, tubes, ribbons, membranes and gels. The barrier can be either permanent or biodegradable. Great creativity in applied materials has been seen, including silver, stainless steel, siliastic, alumina, cellophane, vaseline, latex, gelatin, polyethylene, expanded polytetrafluoroethylen (e-PTFE) and even autogenous materials (fascia, paratenon, veins, amniotic membrane) [121–124]. More recent research has focused on the anti-adhesion membrane barriers developed for use in the peritoneal cavity. These membranes are well established for gynecologic, colorectal and general surgery. The barrier products (Seprafilm or Interceed) consist of hyaluronic acid and methylcellulose. The anti-adhesion membranes have demonstrated an ability to improve range of motion in several animal models of flexor tendon repair [125–129]. The anti-adhesion membranes are considered safe for use in human hand surgery and might actually have potential to decrease tendon adhesions, although statistically significant improvement has still not been documented [130,131]. The ongoing development in tissue engineering techniques has led to the generation of increasingly advanced materials. Recently, an ibuprofen-loaded poly (l-lactic acid)-polyethylene glycerol fibrous membrane reduced adhesions in a chicken model [132]. It is even possible to engineer a synoviocyte–collagen membrane that synthesizes endogenous hyaluronic acid [133]. The shift from barriers to more

membrane-like structures leads to an increasingly biological effect of the otherwise mechanical barrier methods.

Ultrasound therapy: Ultrasound therapy has been demonstrated to advance scar maturation as well as to increase range of motion in animal flexor tendon models [134–136]. The effect of ultrasounds might be caused by an earlier resolution of inflammation, an increased blood flow or a better organization of collagen fibers [134].

Chemical methods:

Several other chemical substances have been tested in a very limited number of studies and with varying promise. The chemical modifications that have demonstrated the most promise will be outlined below.

Hyaluronic acid and lubricin: As previously described, the flexor tendons glide within the tendon sheaths in a fibrous tunnel. In normal healthy hands, the tendon-pulley system has a low coefficient of friction, providing optimal conditions for tendon gliding [137]. Hyaluronic acid (HA) and lubricin are present on the surface of the flexor tendons, and it is believed that HA and lubricin play a role in the innate tendon lubrication mechanism, comparable to their role in cartilage [138]. The effect of exogenously applied lubricin or HA has been investigated in many studies and has demonstrated promising results. Lubricin and chemical modifications of HA have improved digital function following flexor tendon repair and grafting in vivo [47,139–144]. Initial human studies have failed to demonstrate beneficial effects of HA-based treatments [145,146]. However, results from a recent preliminary clinical study revealed that repetitive injections of HA through a catheter at the repair site increased the active range of motion by approximately 15% (25 degrees) 3 months after surgery [147]. Similar results have previously been reported in another clinical trial using an HA-based gel–product [148], although the surgeon was not blinded to the treatment. Although repetitive injections through an inserted catheter might not be acceptable widely for clinical use, these promising results suggest that HA holds potential to reduce flexor tendon adhesions. In fact, a current clinical trial is testing an HA-based product, but no preliminary results have been reported yet [149].

The reduction of fibrotic adhesions could be explained by increased lubrication increasing the initial tendon gliding following surgery, thus preventing adhesion formation. Alternatively, the high viscoelastic properties of HA could create a scaffold around the repair site, forming a mechanical barrier. Furthermore, the high concentrations of HA will alter the diffusion in the extracellular matrix (ECM) and could affect the molecular healing response. Additionally, it is known that the fetal ECM is rich in HA, and it has been suggested that this could be one of the factors that leads to scar-free fetal healing [104].

5-fluorouracil: A chemotherapeutic antimetabolite, 5-fluorouracil (5-FU) has been found to decrease scar formation. Topical application of 5-FU in a single 5-minute exposure during tendon repair has been reported to reduce post-surgical adhesions in a few animal studies [150–152]. In one clinical trial, 5-FU exposure during flexor tendon repair has also been demonstrated to improve the functional outcome. The rate of excellent or good outcomes was increased by 17.5% in the 5-FU-treated group compared to the control [153]. The anti-adhesive effect of 5-FU could be explained by an inhibitory effect on fibroblast proliferation. This inhibition has been demonstrated to exhibit a greater effect on synovial fibroblasts than on endotenon fibroblasts, which might lead to a shift in the balance between extrinsic and intrinsic healing mechanisms [154].

Steroids and Nonsteroidal anti-inflammatory drugs: Treatments with NSAIDs have been demonstrated to improve tendon excursion in animal models [116–118,155]. However, one of the studies reported an unfavorable effect on tendon healing strength [117], and whether NSAIDs are beneficial or detrimental to tendon healing remains controversial [156,157]. Corticosteroids have been demonstrated to decrease the strength and density of adhesions [158], but they are also associated with weaker tendons, diminished wound healing and decreased resistance to infection, which has limited their use in flexor tendon repair. A potentially beneficial effect would probably be caused by the anti-inflammatory properties of these drugs.

Molecular methods:

Theoretically, adhesion-free tendon healing could be achieved by blocking the cytokines or growth factors exhibiting pro-adhesive mechanisms, or by enhancing those with anti-adhesive mechanisms. This is of course a simplification, and most of these molecules will probably perform contradictory effects depending on the location, timing and size of their activity. The mechanisms are complex and the addition of one growth factor will affect the secretion of several others [159]. Nevertheless, encouraging results have already been obtained and the most promising targets seem to be transforming growth factor-β (TGF-β), basic fibroblast growth factor (bFGF) and members of the bone morphogenic protein (BMP) family.

Transforming growth factor beta (TGF-β): TGF-β is a cytokine with numerous biological activities. Practically all cells in the body produce and have receptors for TGF-β. Three isoforms exist, TGF-β 1, 2 and 3, all three are highly conserved in mammals. In all probability, the isoforms have slightly different functions. In general, three biologic functions are associated with TGF-β. First, TGF-β is critical to cell growth [160]. Second, TGF-β is a very potent regulator of the extracellular matrix [161]. Third, TGF-β functions as a modulator of the immune system [162]. TGF-β executes its regulatory functions on gene expression primarily through the activation of the Smad signaling pathway. The activation of this pathway is initiated by phosphorylation of Smad2 or Smad3, which will bind to Smad4. The resulting

Smad complex will then move to the cell nucleus and regulate gene transcription. TGF-β's biological activities are essential to wound healing, in general, since it has been demonstrated to induce angiogenesis, recruit fibroblasts and macrophages, stimulate collagen production, down-regulate proteinase activity and increase the activity of metalloproteinase inhibitors [162]. TGF-β has also been found to be up-regulated after tendon injury and repair [161], but the exact role and function of TGF-β in tendon healing is still unclear [161–163]. In addition to TGF-β 's multiple roles in wound healing, it has become widely appreciated that TGF-β is a key player in the pathogenesis of scar formation and fibrosis [164]. TGF-β activates an aggressive inflammatory response in the local environment of the wound. However, this effect may proceed uncontrollably and result in pathologic fibrosis, with excessive collagen deposition. [164,165]. Therefore TGF-β is suspected to also be an actor in tendon adhesion formation. Furthermore, low levels of TGF-β were found in adhesion-free fetal tendon healing [105,106]. The adhesion inductive effects of TGF-β1 have been supported by several in vivo flexor tendon studies, where inhibition of TGF-β1 has led to improved range of motion [166–170]. This effect has also been demonstrated in our murine model. Knock out of the TGF-β transcription factor (Smad3) improved range of motion at 14 and 21 days following injury and repair in the Smad3$^{-/-}$ mice compared to wild type controls [171]. Currently, a clinical trial is testing a product containing a natural TGF-β inhibitor (mannose-6-phosphate) in regard to safety, tolerability and potential to improve range of motion following flexor tendon injury and repair [172]. No preliminary results have been reported.

Growth and differentiation factor-5 (GDF-5): The growth and differentiation factors 5, 6, and 7 (GDF-5, 6 and 7, also known as bone morphogenic proteins (BMPs) –14, -13 and –12, respectively) are members of the BMP family. GDF-5, 6 and 7 have all been shown to induce the production of tendon-like tissue in vivo [173]. Therefore, these factors have received increasing attention in regard to improvement of tendon healing. Studies investigating GDF-5-deficient mice have demonstrated that GDF-5 is essential for normal tendon development, as an example GDF-5 deficiency led to disruption of tail tendon form and function [174]. Furthermore, GDF-5 can stimulate proliferation of messenchymal stem cells and regulate differentiation to tenocytes [175]. GDF-5 deficiency has also been shown to alter the ultrastructure, mechanical properties and composition of the Achilles tendon, and to significantly delay its healing in an injury model [176]. Exogenous GDF-5 has been demonstrated to increase the tensile strength in Achilles tendon injury models in several studies [177–180]. Similarly, recent studies have also shown that GDF-5 protein coated onto sutures has an early beneficial effect on tendon healing in zone II flexor tendon repairs in a rabbit flexor tendon injury model [181]. However, the effects of GDF-5 on flexor tendon fibrotic adhesions are less studied, and no previous in vivo experiments have been conducted.

Basic fibroblast growth factor (bFGF): Recently, bFGF has also been given attention in the search for adhesion preventing molecules. Proliferation of tenocytes and increased collagen expression in vitro have been accelerated by bFGF [182,183]. Furthermore, bFGF coated onto a nylon suture increased the healing strength of rabbit flexor tendons at 3 weeks after injury and repair, but not at 6 weeks [184]. The in vivo effect of bFGF was investigated in two studies of flexor tendon injury and in both studies bFGF was demonstrated to improve the flexor tendon healing strength and to decrease the degree of adhesions, where bFGF was delivered by repeated direct application [185] or via viral gene therapy [186].

1.4 Tendon gene therapy

Achievement of adhesion-free tendon healing is likely to require manipulation of the molecular tendon healing response. There are two critical components; one is the identification of a suitable growth factor or cytokine, and the other is the development of an efficient method of local delivery to the site of injury. The growth factor can be administered by local direct application, which has been done with varying success [95,187]. This approach is limited by the clearance of the molecule from the local milieu. Growth factors seem to have a short half-life in vivo [188,189], and thus local administration presumably only enables short-term modulation of biological effects. Various slow-release carrier systems have been developed to overcome this limitation [177,190,191]. However, gene therapy allows local production of the desired molecule and obviates the need for slow-release carriers or repeated protein injections. Gene therapy consequently offers a method to solve the delivery issue of growth factor therapy. Gene therapy has traditionally aimed at curing congenital or metabolic diseases, necessitating a long-term gene expression. Prolonged gene expression is still a challenge to gene therapy, although for manipulation of post-injury tendon healing, long-term gene expression is neither necessary nor desired.

1.4.1 Vector systems

Somatic gene therapy can be performed either on patient cells extracted from the body (ex vivo) or on cells while they are in the body (in vivo). Gene therapy works by a transfer of genetic information to the patient's cell. Unprotected (naked) DNA is quickly degraded and has a very limited ability to cross both the cell and nuclear membranes. Therefore, various carrier systems have been developed to protect the DNA and to facilitate the entry into the cell nucleus. The carrier systems can be divided into viral (e.g., adenoviruses, adeno-associated viruses, herpes simplex viruses, retroviruses, lentiviruses) and non-viral vectors (e.g., liposomal, electroporation, sonoporation, shots from a "gene gun"). The viral vectors compose two-thirds of approved clinical trials [192].

The major drawback of the viral vectors is their safety and production [193,194], whereas the non-viral vectors are considered to be safe generally, but have a lower efficiency, and especially in connective tissue, the achievement of high transfection rates has been difficult [195]. In tendon healing, only a few non-viral methods have been reported for in vivo use. These methods include both liposome-based methods, ultrasound-assisted microbubbles and naked plasmid transfer by electroporation [196–198]. However, these methods have only been used for transfection of a marker gene and have not yet proven potential in delivery of therapeutic genes. Viral vector-based gene therapy has been the preferred method for efficient tendon gene therapy [199]. Viruses are capable of transferring their genome to the host cell and exploiting the cellular mechanisms for viral propagation. Viral vectors are capable of gene transfer, defined as transduction, but the part of the viral genome that leads to replication and propagation has been deleted. The gene of interest (the transgene) can be included in the viral vector genome and will be delivered to the cell by the natural mechanisms exploited by the virus. Viral vectors have been developed from different types of naturally occurring viruses and have been named according to their origin. Each type of vector has advantages and limitations. Some vectors can integrate into the host cell genome and thereby ensure prolonged expression of the transgene. Other vectors are non-integrating and will result in a transient expression, which depends on the mitotic activity of the cell [193]. Viral tendon gene therapy has been demonstrated to improve tendon healing strength in two studies in a rat Achilles tendon injury model. Both studies used an adenoviral vector encoding GDF-7 (BMP-12) or GDF-5 (BMP-14) for gene transfer [179,200]. Adenoviral and adeno-associated viral vectors have been the most used for tendon gene therapy [199]

Adenoviral vectors are derived from a pathogenic virus, frequently causing human infections (conjunctivitis, gastroenteritis and respiratory tract infections) [193]. Consequently, most people have neutralizing antibodies against adenovirus and administration of the vector can result in a severe immune reaction [201]. Despite these safety concerns, adenoviral vectors are among the most frequently used vectors in clinical trials, and they have been used in the majority of the animal studies of tendon gene therapy [192,199]. Adenoviral vectors are non-integrating and transduce both dividing and non-dividing cells.

Recombinant adeno-assosiated viral vectors are derived from a non-pathogenic parvovirus not associated with human disease and are regarded as “safe.” The natural virus (AAV) integrates in a site-specific manner on chromosome 19, without any known interference to the functional genome [202]. The recombinant AAV vector (rAAV) integration is less than 1% [203,204]. Furthermore, the risk of immune response has been diminished by deletion of the major part of the viral genome; thus, only the viral capsid and the transgene are a potential antigen target. Different serotypes of AAV differ in their cell target specificity (tropism), and for each type of tissue, a comparison between serotypes should be conducted to identify the most efficient. An important limitation to rAAV vectors is the presence of

preexisting neutralizing antibodies against the capsid [205]. This can reduce the transduction efficiency and make re-administration even more problematic. Capsid-engineering or serotype-switching can potentially overcome this problem [206]. The rAAV vector is the most attractive vector for somatic gene therapy in vivo, primarily because it holds a good safety profile [204,205]. For tendon healing, the high degree of safety in the vector system is clearly important, since tendon injury is local and not life threatening.

1.4.2 Flexor tendon gene therapy

In flexor tendon models, as in other tendon studies, viral vectors have been the dominant in vivo delivery method. In fact, only one non-viral method has been tested specifically for flexor tendon gene therapy, a three-step liposomal-based method for transduction of a marker gene [196]. Adenoviral-based vectors have been demonstrated as an efficient means of gene delivery in rabbit flexor tendons in vivo, where dose-dependent transduction was observed [207]. Furthermore, different serotypes of recombinant adeno-associated vectors (rAAV1 to rAAV8) have been compared in flexor tendon tenocytes in vitro. The only serotype that enabled efficient transduction was rAAV2, and this vector demonstrated increased expression of the encoded gene over 3 weeks [208]. Gene expression duration has been observed to peak at 2 weeks and to last up to 4 weeks in an in vivo tendon model [209]. Lou and colleagues were the first to demonstrate the potential of viral gene therapy to modify the flexor tendon healing response. They demonstrated that adenoviral gene transfer of FAK (focal adhesion kinase) mediated significantly increased adhesion formation, but unfortunately, FAK antisense failed to reduce adhesion formation [210]. In a later study, the adenoviral vector was used to deliver GDF-7 (BMP-12), and succeeded in improving tendon healing strength [211]. These studies demonstrated proof of principle and gene delivery for manipulation of the flexor tendon healing response has been demonstrated to be possible in regard to both tendon healing strength and adhesion formation.

1.5 Mouse model rationale

Mouse models have become a powerful research tool. The use of mouse models has increased rapidly, concurrently with the development of techniques for genomic manipulation. Laboratory mice have become a billion-dollar industry, and each year over 25 million mice are bred and shipped to research laboratories [212]. Mouse models have provided important insights in many fields of medicine.

A number of large animal models for studying flexor tendon healing and adhesion formation exist and have been used for decades. These models include non-human primates [213], canines [137,214–217], chickens [218–220], rabbits [166,221–223] and rats [115,224,225]. In many studies, large animal models, closely resembling the

anatomy and size of the human hand, are preferable. Large animal models have contributed to our knowledge of the effects of physical treatment options (surgical technique, passive motion protocols, anti-adhesion modification of the tendon surface). However, to investigate the molecular mechanisms involved in flexor tendon adhesion formation, large animal models lack several of the advantages of a murine model.

One major advantage of a murine model is the detailed knowledge of the murine genome and the availability of knock-out and knock-in models. This can help us to understand the role of a single gene in tendon adhesion formation. These genetically modified mouse models have already been used to illuminate other aspects of tendon healing [163,226–228]. Another advantage of a murine model is its accessibility and low costs. In combination, this makes the model a great screening tool for testing the effects of some of the many growth factors that have been uncovered as either up or down-regulated in response to tendon healing.

Numerous in vitro studies have been performed to study the molecular mechanisms of flexor tendon healing [90,114,165,229–232]. However, in regard to the effect on adhesion formation, the in vitro studies in this field have an innate limitation. Since the mechanisms that generate the adhesion formation are still indefinite, the in vitro results are difficult to interpret. For instance, a treatment or modification can be found to increase [229], decrease [233] or change the ratio of collagen I and III [234], but whether this can be translated to leading to an increase or decrease of tendon adhesions remains debatable. Collagen I and III are the main components of not only scar tissue and adhesions, but also of healing tendon tissue. Furthermore, when tendons are injured, a collateral injury will occur in the surrounding tissue and in the vascular supply. In a recent study, it has been demonstrated that the cellular reaction from the injured surrounding tissue played a considerable role in tendon healing [113]. In vitro studies will obviously lack the influence of the surrounding tissue reaction and the pathobiology of tendon healing would, in this respect, be inaccurately represented. The general limitations of in vitro studies, with the lack of systemic functions such as immune response, might be of particular importance in flexor tendon healing, as it has been suggested that the inflammatory phase of the healing response is of significance to the degree of adhesion formation [115,120]. Since the translational value of data from in vitro studies can be questioned, there is a need for in vivo screening models.

2.0 AIMS AND HYPOTHESIS

Our overall aim was to establish a mouse model of flexor tendon injury and repair, along with functional tests of joint flexion and tendon healing strength. Furthermore, our aim was to use the model to develop a delivery system of therapeutic molecules to modify the flexor tendon healing response. Freeze-dried allografts have previously been used as a delivery system for bone healing with success [235,236]. We wanted to take advantage of the same technique in tendon reconstruction. Growth and differentiation factor 5 (GDF-5) is known to play a role in tendon development and healing [173,177,181]. However, the effects of GDF-5 on tendon adhesions and joint flexion have not been investigated previously. Therefore, we chose to investigate GFD-5 as a therapeutic molecule in these studies. We aimed to investigate the effect of GDF-5 on tendon healing, when coated to a freeze-dried tendon allograft, as either recombinant protein (rmGDF-5) or viral gene expression vectors encoding GDF-5 (rAAV-*Gdf5*).

We hypothesized that it would be possible to establish a model of flexor tendon injury and repair in the mouse, and that tendon reconstruction by GDF-5-coated, freeze-dried allografts would improve healing in regard to strength and adhesions in a delivery and dose-dependent manner.

The specific aims and hypothesis for each paper can be found in the result section.

3.0 METHODOLOGICAL CONSIDERATIONS

3.1 The murine model

In this section, an overview of the model-related considerations will be provided. A detailed description of the model can be found in Paper I. The advantages of establishing a murine model have been outlined in the introduction.

3.1.1 The surgical method

Location: Many of the advantages of a murine model are related to the small size of the mouse; unfortunately, this also poses the greatest challenge. The surgical procedure is technically demanding and requires sufficient training to achieve reproducible and reliable results. Recent work has documented that despite its small size, the hind paw of the mouse has structures that are comparable to zone II of the human hand and thus represents a good model to study flexor tendon injury [237]. However, the small size of the mouse digits has forced us to abandon the idea of a zone II model because the miniaturizing of the anatomical structures causes the gliding resistance of the repair to exceed that of the adhesions. Thus, it will not be possible to perform a mechanical test of adhesions. A consequence of abandoning zone II is that our injury site will be extrasynovial, which probably influences the healing mechanisms and limits the translational value to intrasynovial injury and healing. Our model, although not in zone II, allows reconstruction of the flexor tendons and subsequent testing of the digital flexion. The reproducibility of our results highly supports the feasibility of the model. Furthermore, fibrotic tendon adhesions in the palm have been described as reducing tendon gliding and digit flexion clinically [238].

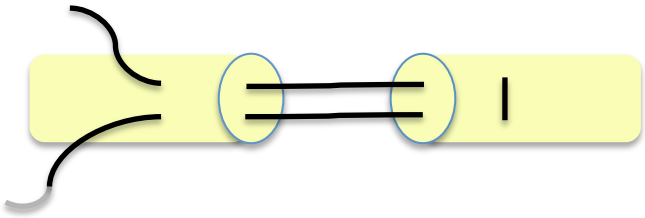

Figure 7: Illustration of the suture technique.

Injury: The tendon injury in our model is generated by sharp transection of the tendon and simulates a traumatic sharp laceration injury. The injury is performed in a controlled environment and with minimal trauma to the nervous and vascular supply. Although not fully resembling a traumatic injury, this procedure allows generation of comparable and reproducible injuries. In cases of tendon rupture, the tendon almost always has frayed ends and a degenerative background. The translational value of the model can be questioned for these kinds of injuries. However, tendon transection is also commonly used in models of typical rupture injuries, most commonly Achilles tendon models [176,179,239]

Suture technique: Our main purpose was to develop a standardized method to bring the tendon ends together. The technique should provide sufficient strength to prevent rupture of the repair. Furthermore, the technique should be as simple as possible to minimize the technical challenges and to ensure reproducibility. Our first approach was based on the Kessler technique, a commonly used technique for tendon repair [240]. However, concerns about the effect of a relatively large intratendinous knot led to modifications of the technique, and we decided on a horizontal mattress suture pattern (Figure 7).

Immobilization: Sufficient and standardized immobilization is essential to the model and is needed both to protect the repair site and to induce adhesions [58,59,113]**.** All our attempts at exterior fixation failed. To ensure proper immobilization, we introduced severing of the muscle during the surgical procedure, which results in a complete loss of deliberate tendon gliding. Corresponding methods have been used in other models of flexor tendon healing [113,241]. Even though we observed healing of the muscle to the surrounding tissue and recovery of muscle traction, the loading of the tendon was expected to have been significantly reduced during the entire healing period. The application of mechanical force has been noted to increase collagen synthesis [242] and to result in increased tendon healing strength [243]. To overcome this limitation, we developed an alternative procedure. To induce a transient paralysis, Botox was injected into the muscle 24 hours prior to surgery (Paper IV). Others have demonstrated that Botox, in the dosage used, can eliminate up to 90% of the grip strength in the first 3 days. In the same study, it was demonstrated that the mice regained grip strength at a rate of approximately 2% per day [244]. Another study documented that unloading by Botox reduces the tendon healing strength compared to loaded healing in a rat Achilles tendon model [245]. However, the muscle paralysis in this study was achieved by injecting 6 times the amount of Botox used in our model, and no regain of muscle function was observed up to 6 weeks [246]. A major pitfall of this method is insufficient and unequal immobilization, which could cause differences in adhesion formation and thus confound the results. Therefore, we introduced a grip test on the day of surgery to qualitatively assess that sufficient paralysis had been achieved. Mice that still demonstrated gripping action, as a sign of failed injection, were excluded from the experiment (less than 5%).

We compared the two methods for immobilization and verified that both methods protected the repair site from rupture and induced adhesions (demonstrated by decreased joint flexion) (Paper IV-supplementary data). We noticed that the degree of decreased flexion was similar at 14 and 28 days post-surgery in the group immobilized by muscle transection. In contrast, the Botox-immobilized group experienced a significant improvement in joint flexion at 28 days post-surgery, compared to 14 days post-surgery. We suggest that this difference is caused by the gradual regain in grip strength, mimicking an active motion protocol in the Botox-immobilized group. We still need to demonstrate the long-term effects of the Botox protocol on joint flexion and tendon healing strength. Furthermore,

it would be advantageous to change the qualitative pre-operative test of grip action to a quantitative test to ensure standardized immobilization.

To minimize the risk of immobilization bias, the mice were randomized prior to the surgery. When possible, the mice were also blinded to the operator (which was not possible in Paper II due to differences in the surgical procedure). All specimens were blinded and randomized prior to the adhesion test and the biomechanical testing.

3.1.2 The flexion test

Test set up: In the production of the holding device for the flexion test, attention must be given to the fixation of the foot in order to generate consistent results. Proper fixation is required to avoid plantar flexion of the foot, rotation of the tibia and changes in the position of the angle. Furthermore, the camera must be perpendicular to the foot and the tendon must be loaded in the direction of the anatomical pull. If these circumstances are not fulfilled, the determination of the joint flexion angle could be confounded. Evaluation of test reproducibility was challenged by interruption of some of the adhesions during loading and because repetition of the flexion test was not possible. To compensate, the subsequent measurements of the flexion angles were performed in triplicate. Furthermore, we performed an intra- and inter-observer analysis on the measurements and found less than 1% variability (Paper II). The maximal loading during the flexion test corresponds to approximately 10% of the maximum repair strength. We have conducted experiments to validate that the test is in fact non-destructive and will not affect the outcome of a subsequent test of biomechanical properties (Paper II).

Data analysis: The flexion angles were plotted against the load and a curve fit was made based on a one-phase exponential association. The gliding coefficient (GC), calculated from the association, expresses a rate constant for joint flexion. The GC is dependent on the accuracy of the curve fit. More advanced non-linear associations may provide a better curve fit. However, good correlation between the GC and the more widely reported outcome measure, range of motion (ROM), was documented (Paper II). An optimization of the curve-fit model could potentially increase the sensitivity of the flexion test. Another improvement of the test could be to measure and report the sum of the metatarsophalangeal (MTP) and the interphalangeal (IP) joint flexion angle. The flexor digitorum longus (FDL) tendon is responsible for the flexion of both of these joints and reporting the total flexion could increase the sensitivity of the test. This protocol change has already been implemented in more-recent studies (unpublished).

Interpretation: The nature of adhesions makes accurate quantification difficult and our flexion test faces the same challenges and pitfalls as other functional tests of adhesions. ROM tendon gliding and

work of flexion are all commonly reported outcome measures of functional adhesion tests. All of these measures are an indirect measure of adhesions restricting tendon gliding and digit flexion. However, other factors could potentially contribute to the reduction in tendon gliding and digit flexion. These factors include joint stiffness, tissue edema and the resistance of the tendon repair. In our model, we observed that the grafted tendons that were allowed to heal for up to 28 days demonstrated a significantly decreased joint flexion compared to grafted tendons not allowed to heal (Paper II), indicating that the resistance of the adhesions exceeds the resistance of the repair. Supported by our histological findings, we claim that this difference is caused primarily by the formation of adhesion. However, we have not quantitatively investigated the nature of this correlation and we cannot exclude or estimate a contribution from tissue edema and joint stiffness. A potential contribution of these factors must be assumed to be relatively constant and would only constitute a problem if the investigated intervention affects these factors. Further investigation of the correlation between decrease in digit flexion and the degree of adhesions should be conducted. This would require visual inspection by quantitative gross observations and/or stereological histomorphometry. However, visual inspection does not reveal the flexion resistance of the adhesions, nor does it discriminate among adhesions with and without significant restrictive capacity.

3.1.3 The biomechanical test

Preparation and mounting: Prior to mechanical testing, the proximal end of the tendon was dissected free of the surrounding tissue with the risk of damaging the tendon tissue. Meticulous dissection under magnification was performed to minimize this risk. During the tissue preparation and mounting, the tendon was kept moist using gauze soaked in saline. Dehydration can potentially change the outcome of the biomechanical test and keeping the tissue sufficiently hydrated through the test is an important challenge of this test [247,248]. In the test device, the tendon must be mounted axial to the direction of the applied force to ensure equal application of force to all tendon fibers. The clamp force must be sufficient to avoid slippage of the strongest specimens tested. A constant clamp distance and consistency in the positioning will ensure that the length of the tested specimen is standardized.

Testing: The recorded failure modes, in our test set up, revealed that tendon failure most frequently happened at the proximal graft repair, and only rarely at the distal repair or oblique through the graft. This can probably be explained by a protective effect of the adhesions on the distal repair. Consequently, in cases of excessive adhesions, we will not have tested fully the strength of the graft or the distal repair site, but primarily the proximal repair. However, this is what corresponds to the biological conditions in vivo, where the adhesions will contribute to the healing strength. If the tendon specimens were completely isolated prior to the mechanical test, we would eliminate this confounder.

However, we would risk removing granulation tissue contributing to the strength and thus introduce another potential confounder. The preferred approach must depend on the issue requiring investigation.

3.2 Translational value of mouse models

In addition to the model-specific consideration outlined above, some general considerations are necessary when working with animal models.

There are obvious size differences among different animals, and size considerations are important when choosing an orthopedic disease model. Since cellular processes and signaling pathways are highly conserved between species, size is of less importance with regard to these processes [212].

Furthermore, it is of great importance that the animal model adequately represents the human disease in question. Whether we have succeeded to sufficiently achieve this is discussed above. Despite the large proportion of our genome that we share with mice, there are important phenotypical, physiological and metabolic differences [249]. The heart rate of the mouse is almost 10 times that of humans, and its metabolic rates are also faster [249]. Consequently, various processes, including healing processes, are faster in small animals than humans, as is known from bone healing [250]. This needs to be taken into account when planning animal experiments. When choosing a mouse strain, attention must be given to the fact that mouse strains have different healing capacities. The C57BL/6J strain, used in the studies of this thesis, has demonstrated a mediocre wound-healing rate [251]. We have focused on the 14- and 28-day time points in our studies, which correspond to the observed adhesion peak in our model for both the grafting procedure (Paper II) and the simple repair [252]. The peak of adhesions have been reported in the same time span in other rodent models of flexor tendon healing [224,253]. Furthermore, 14 to 28 days is the most commonly reported evaluation time of adhesions in animal models of flexor tendon healing [6,144,254].

The translational value of results obtained from animal studies also depends on the study group in question. In our studies, young and healthy female mice were used. In other disease models, the age could constitute a translational limitation, but since flexor tendon injuries are most frequent in younger active individuals, this is not a limitation to our model. However, the gender could be of concern since differences in male and female tendon healing have been observed [242]. The translational value can be increased by including randomization and blinding in the experimental design, as we have done in these studies. It has been demonstrated that animal studies lacking both randomization and blinding were more likely to report a difference between study groups [255] and that inclusion improves the concordance between animal experiments and clinical trials [256]. Based on the

shortcomings of animal studies, it is recommendable to remain critical and cautious about the applicability of animal data to the clinical domain.

3.3 Quantitative real-time PCR (qRT-PCR)

Reverse transcription of RNA to its complementary DNA and subsequent qRT-PCR is routinely used as a quantification method to analyze gene expression. There are limitations related to the analysis as well as the interpretation of the results.

We used SYBR Green as fluorescent dye in the qRT-PCR experiments (papers II and IV). SYBR Green is nonspecific and will bind to all double-stranded DNA molecules, including primer dimers and off-target amplicons. Hence, high primer specificity is critical to obtain reliable results. Gel electrophoresis was conducted on all qRT-PCR products to ensure that correct amplicons were being amplified.

The expression of a gene is normalized to a "stable" reference gene (housekeeping gene), allowing for a relative measure of the expression. We used beta-actin as the reference gene in Paper II. However, the expression of reference genes is not always stable [257,258], and in fact it is recommendable to use a combination of different genes to diminish the risk of reference gene errors. Optimally, stable and reliable reference genes should be determined prior to the experiment.

The interpretation of mRNA levels as a measure of protein levels obviously relies on a stabile correlation between mRNA and the processes of translation and post-translational modifications. Unfortunately, this correlation is not always predictable [259], and the results need to be verified, such as by immunohistochemistry.

3.4 Bioluminescence imaging

Bioluminescence imaging is a semi-quantitative technique that allows monitoring of reporter gene expression in live animals. We have used the technique with firefly luciferase. D-luciferin was injected prior to imaging, and light emission was recorded as the substrate was oxidized.

The substrate can be administered to the animal using intravenous (IV), intraperitoneal (IP), or subcutaneous (SC) injection. Importantly, the route of administration must be considered, and the optimal choice will depend on the model. Since our tissue of interest is not located in close proximity to any of the routes, all three routes could be used for our model. In the studies included in this thesis, we used IP injection. The IP route is the most common and has been used in these studies. However, the risk of bowel injection has been reported to be 3–10% even among experienced staff [260]. The subcutaneous route is recommended to avoid injection failure, and we changed the protocol.

Quantification of bioluminescent signals requires knowledge of the kinetics of the signal. Following injection, D-luciferin will be distributed in the body. The distribution is complex and dependent on several factors including administration route, metabolism, and the location of the tissue. To obtain reliable quantification and to be able to compare signals during longitudinal studies, a kinetic analysis must be made to identify the plateau of the signal [261]. As several animals can be scanned simultaneously, attention must be paid to whether large differences in signal intensity occur. The strongest signal is at risk of saturating the camera before weaker signals are fully recorded, and an animal emitting a significantly stronger signal must be removed from the session to allow reliable measurement of the remaining signals. The signal intensity should be obtained from a standardized region of interest (ROI) and results reported as radiance (photons/cm^2/sec/sr). Radiance allows comparison between different setups and systems since camera settings are taken into account.

4.0 RESULTS

We have established the first murine model of flexor tendon repair and successfully developed functional tests of joint flexion and tendon healing strength (Paper I and II). We have demonstrated that using freeze-dried allografts for tendon grafting results in at least a similar functional outcome compared to autografts in our murine model (Paper II). Thus, freeze-dried allografts may be an attractive alternative to live autografts in flexor tendon reconstruction. Next, we exploited the hydrophilic capacity of the freeze-dried tendon allograft and demonstrated that GDF-5 gene delivery via recombinant adeno-associated viral (rAAV) vectors could improve digital flexion (Paper III). These results suggested an anti-fibrotic effect of GDF-5 in flexor tendon healing, and the effect was confirmed in later studies (Paper IV). We found similar anti-fibrotic effects of GDF-5 regardless of whether the protein (rmGDF-5) or the gene (rAAV-*Gdf5*) was used for allograft delivery (Paper IV). Furthermore, we found that growth factor therapy with GDF-5 warrants careful dosage considerations, since a dose-dependent positive effect may not exist (Paper IV). The results from papers II, III, and IV demonstrate the applicability and potential of the murine model of flexor tendon repair as a tool for investigating the effect of different manipulators on the formation and remodeling of fibrotic tendon adhesions.

The specific aims, hypothesis and main results of the papers of this thesis will be summarized here:

Paper I: "A Mouse Model of Flexor Tendon Repair." Hasslund, S., O'Keefe, RJ., Awad, HA. Skeletal Development and Repair: Methods and Protocols, Methods in Molecular Biology, vol. 1130, chapter 6. (Review accepted for publication).

Aims and hypothesis: The aim of Paper I was to describe the methods of the murine model in detail along with its potential, advantages and limitations.

Main results: The paper contained a detailed description of the surgical procedure (Figure 8) and the adhesion test. The details of the method can be found in the paper and will not be summarized here. Our model, although not a zone II model, does experience adhesions. The performance of the flexion test prior to the test of biomechanical properties did not alter the results.

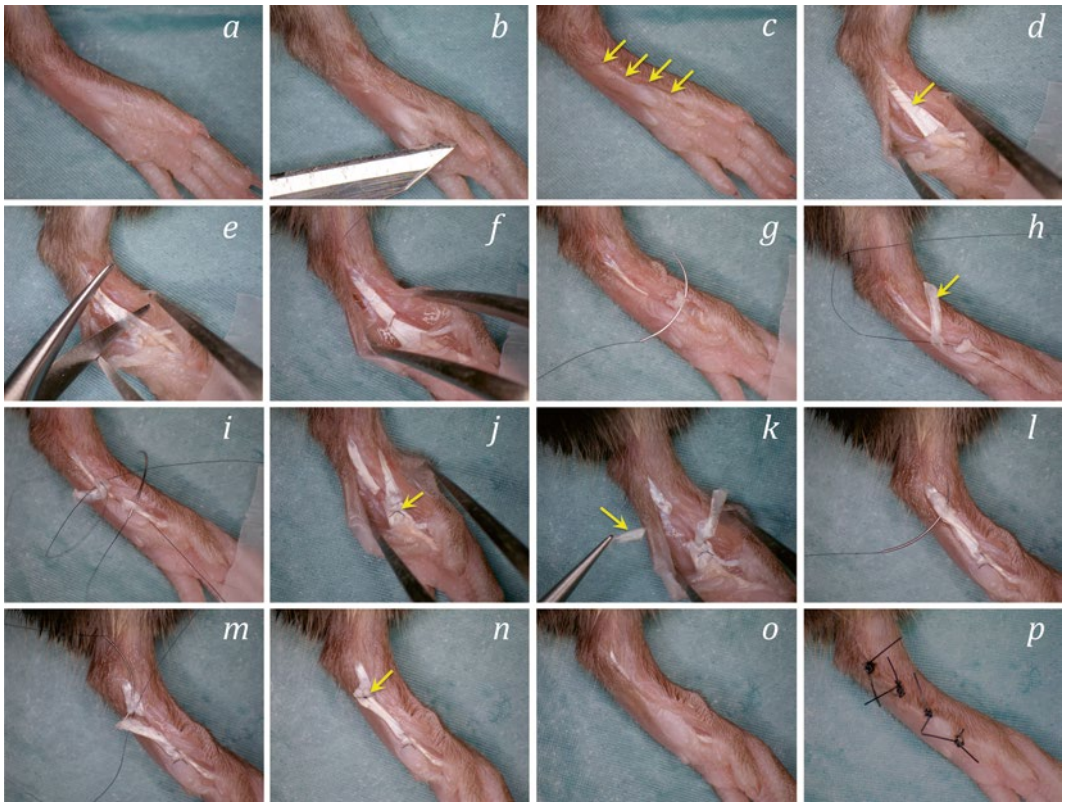

Figure 8: Illustration of the steps of the FDL reconstruction using a live autograft. Adapted from Paper I Figure 2.

Paper II: "Adhesions in a Murine Flexor Tendon Graft Model: Autograft versus Allograft Reconstruction." Hasslund, S., Jacobsen, J., Dadali, T., Basil, P., Vinther, M. Søballe, K., Schwartz, EM., O'Keefe, RJ., Mitten, D., Awad,HA. (2008). Journal of Orthopaedic Research. 26(6), pp.824–33.

Aims and hypothesis: We aimed at establishing a murine model of flexor tendon injury and repair and a functional test of tendon gliding and adhesions. Moreover, in this study we wanted to compare a live autograft to a freeze-dried allograft. We hypothesized that the devitalized tendon allografts, free from live cells, would experience improved digital flexion due to diminished adhesion formation.

Main results:
We established the first murine model of flexor tendon repair and successfully developed a mechanical test of tendon gliding and adhesions in the mouse. The innovative biomechanical flexion test allows calculation of a gliding coefficient based on joint flexion data over a range of applied loads (Figure 9).

We compared live autografts to freeze-dried allografts and found that allografts did not cause increased adhesions compared to autografts. In fact, at 28 days post grafting, the allografts demonstrated a significant improvement in digital flexion (Figure 9). At time points thereafter, a significant improvement in the gliding coefficients for both groups were observed. This improvement was probably caused by a remodeling of the tendon and adhesions, as indicated by the histology (Figure 10). Furthermore, in the grafts that experienced the most adhesions, we observed up-regulation in the levels of GDF-5 and VEGF mRNA, 7 and 20 fold, respectively. This up-regulation was associated with a subsequent resolution of adhesions. In terms of maximum tensile strength, the grafts were found to be similar. Our findings could indicate that freeze-dried allografts might be an attractive alternative to live autografts in flexor tendon reconstruction.

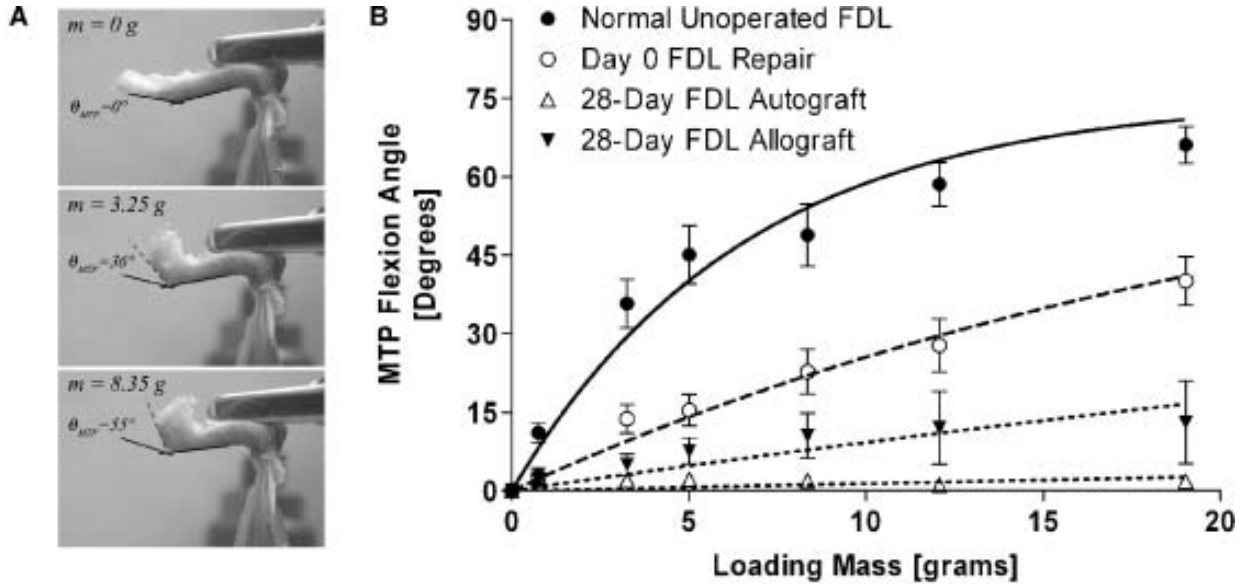

Figure 9: The flexion test. (A) Assessment of the metatarsophalangeal (MTP) joint flexion upon loading of the flexor digitorum longus (FDL) tendon in the direction of the anatomical pull. At each load, a digital picture was taken and the MTP flexion angle was measured relative to the unloaded position. (B) Flexion curves (flexion angles vs. applied loads) of the MTP joint at day 0 and 28 post-grafting. Data points represent measured flexion angles (mean and SEM). Lines represent the best-fit curves based on modeling the data using single-phase exponential association. The gliding coefficient is the rate constant of the rise of the curves. Adapted from Paper II Figure 2.

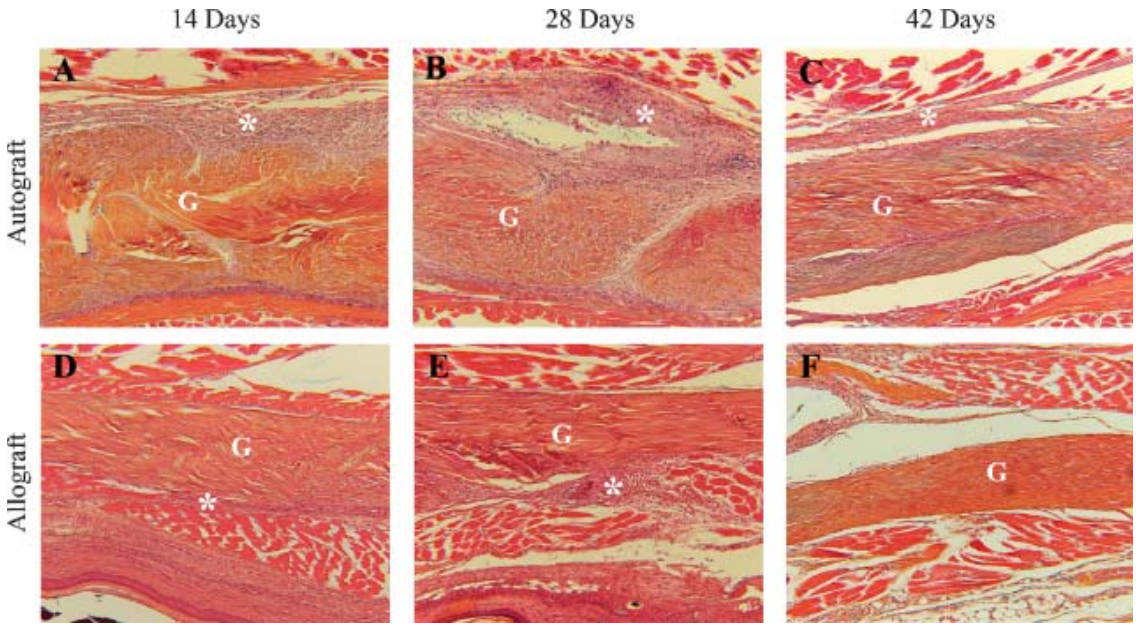

Figure 10: At days 14 and 28, the host junction of both autografted and allografted tendons were surrounded by similar amounts of hypercellular fibrotic scar tissue and appeared enlarged relative to the body of the graft. Remarkable differences in the amount of scar tissue (*) surrounding the middle segment of the graft was observed between days 14 and 28. The autografts (A, B) appear to be encased in this hypercellular tissue, seeming to also invade the graft. In contrast, the allografts (D, E) were much less affected. These differences were less distinct by day 42 (C, F) and the scar tissue appears to have been significantly remodeled in both graft types. Graft tissue is marked G. Sections were stained with Orange G/Alcian Blue (10x). Adapted from Paper II Figure 6.

Paper III: "Freeze-dried Tendon Allografts as Tissue-engineering Scaffolds for GDF-5 Gene Delivery." Basile, P., Dadali, T., Jacobsen, J., Hasslund, S., Vinther, M., Søballe, K., Yasuhiko, N., Hicham Drissi, M., Mitten, D. O'Keefe, R., Schwarz, E., Awad, H. (2008). Molecular therapy. 16(3), pp.466-72.

Aims and hypothesis: We aimed to exploit the hydrophilic capacity of the freeze-dried tendon allograft for loading of recombinant adeno-associated viral (rAAV) vectors for GDF-5 gene delivery. We hypothesized that by inducing an increase in GDF-5 gene expression, the tendon healing could be modified in a way that would lead to reduced adhesions and thereby improved digital flexion.

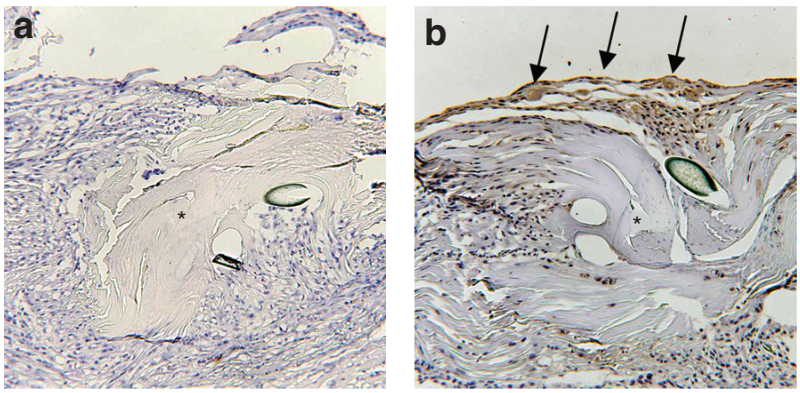

Figure 11: Immunohistochemical sections of (a) the rAAV-*LacZ* and (b) the rAAV-*Gdf5*-loaded FDL allografts at 14 days post grafting. Positive staining of GDF-5 (indicated by the arrows). GDF-5 was presumably synthesized by the transduced host cells surrounding the rAAV-*Gdf5*-treated allograft. Adapted from Paper III Figure 5.

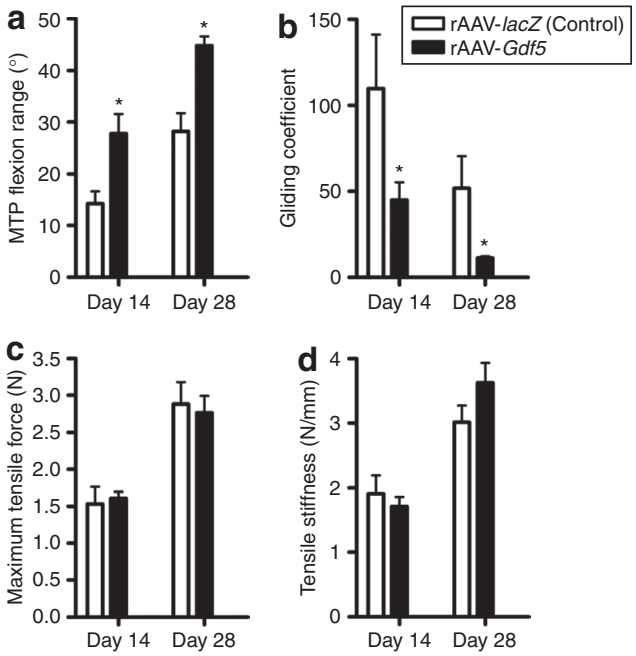

Figure 12: Mice had their FDL tendons reconstructed with freeze-dried allografts loaded with rAAV-*Gdf5* or rAAV-lacZ. Killed and tested at 14 and 28 days after surgery (n = 9). Different biomechanical parameters were determined: (a) the MTP ROM, (b) the gliding coefficient., (c) their maximum tensile force, and (d) their linear tensile stiffness. The data presented are mean values ± SEM. Asterisks indicate significant differences compared to time-matched controls ($P < 0.05$). Adapted from Paper III Figure 4.

Main results: We developed methods to exploit the hydrophilic capacity of the freeze-dried tendon allograft for efficient loading of therapeutic growth factors and gene-delivery vehicles (Figure 11). In this study, we demonstrated that GDF-5 gene delivery via recombinant adeno-associated viral (rAAV) vectors loaded on freeze-dried allografts significantly improved the digital range of motion and the gliding coefficient (Figure 12a and b). The biomechanical properties were maintained but not improved (Figure 12c and d). These results were the first to suggest an anti-fibrotic effect of GDF-5 in the context of tendon healing.

Paper IV: ”Freeze-dried Allografts-Mediated Gene or Protein Delivery of GDF-5 Improves Murine Flexor Tendon Healing.” Hasslund, S., Dadali, T., Vinther, M., Soballe, K., Awad, H. (In review at Journal of Tissue Engenering).

Aims and hypothesis: In this study we aimed to optimize the retention of the rAAV particles as well as the recombinant protein GDF-5 (rmGDF-5) on the freeze-dried tendon allograft. We compared the effects of different dosages of rmGDF-5 and rAAV-*Gdf5* on digital flexion. There are differences in the kinetics of action of protein and viral gene delivery. Therefore, we hypothesized that the anti-fibrotic effect of GDF-5 would vary depending on the method of delivery.

Main results: The optimization studies determined that both the loading time and concentration of the viral vector had dose-dependent effects on their retention of the freeze-dried allograft. Similarly, we found significant incremental effects on the retention of rmGDF-5 when the concentration in the dipping solution was increased.

Functional effects of GDF-5 gene or protein delivery were assessed by the flexion test. The allografts loaded with the lower dose of rAAV-*Gdf5* had significantly improved digital ROM at 14 days post reconstruction. Allografts loaded with the higher dose were not significantly different from controls. Similar results were seen for the rmGDF-5 loaded allografts (Figure 13). Thus it seems that lower doses of GDF-5 suppress adhesion formation more effectively. In regard to the biomechanical parameters, tensile strength and stiffness tended to increase, although the results were not significant (Figure 14).

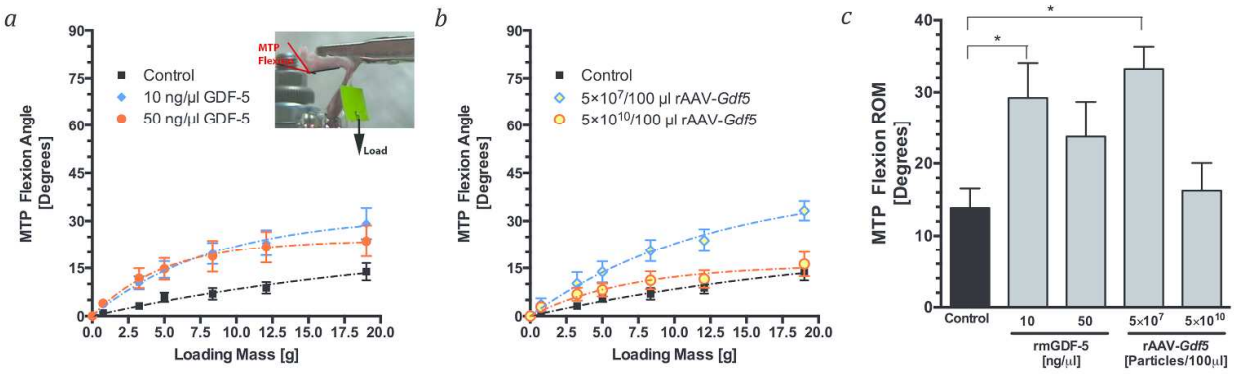

Figure 13: Average MTP joint flexion curves for protein coated allografts (a) and viral coated allografts (b). Digital range of motion (ROM) (c). The control is allografts loaded with rAAV-*LacZ*.. Mean ± SEM (n=8). * p<0.05. Adapted from Paper IV Figure 4.

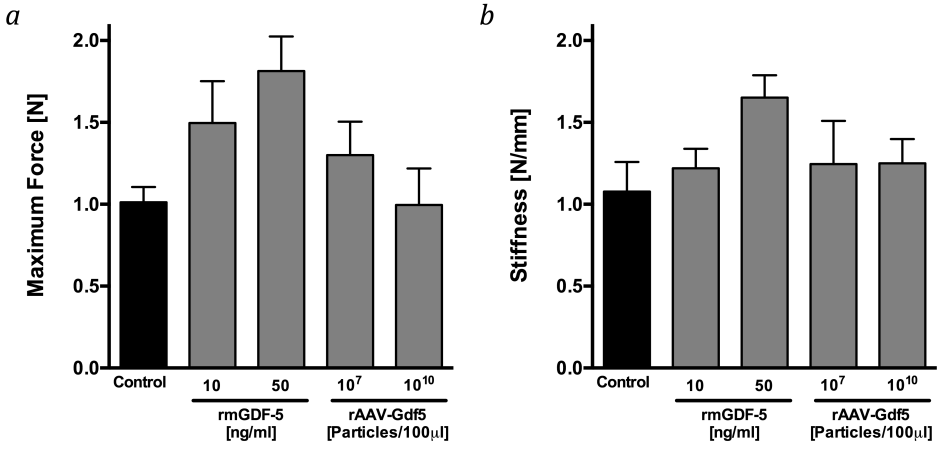

Figure 14: Biomechanical properties measured 14 days post-surgery. FDL tendons are reconstructed with rmGDF-5, rAAV-*gdf5*, or rAAV-*lacZ* (control) loaded allografts, rmGDF-5 loaded allografts, and rAAV-*Gdf5* loaded allografts. Maximum tensile force (strength) (a) and tensile stiffness (b) are presented. Data are presented as mean ± SEM. Adapted from Paper IV Figure 5.

5.0 DISCUSSION AND PERSPECTIVES

The ingenious biomechanical system of the flexor tendons enables us to perform sophisticated finger movements and carry out advanced motoric tasks. It can even be claimed that this ability is one of the prime reasons for the developmental achievements and success of our species. Unfortunately, it is also the complexity of this system that complicates injury to and healing of these structures. Flexor tendon injury and repair is challenged by an imperfect intrinsic healing response leading to the formation of fibrotic adhesions. Despite implementation of modern suture techniques and post-operative motion protocols, rehabilitation following these injuries is highly unpredictable. It is conceivable that further improvement and consistency of the outcome following flexor tendon injuries will warrant manipulation of the biological tendon healing response [35,84,262,263]. Given today's incomplete understanding of the mechanisms contributing to adhesion formation, a need for in vivo screening models for these mechanisms has emerged.

5.1 The murine model of flexor tendon injury and repair

The mouse model is not without limitations, and these have been discussed in detail in Section 3.1 (pp. 30-34) and Paper I. The most important limitation is the inability to perform injury and repair in zone II, since the dimensions of the mouse digits possess insurmountable surgical challenges. A few murine studies of injuries in zone II have been published since we established our model [110,113]. However, in these studies no tendon repair or reconstruction was performed, and no mechanical analysis was carried out. Only one published study has repaired the murine flexor tendons in zone II, but this injury model in fact examined the healing process in vitro rather than in vivo [163]. Although not in zone II, we have succeeded in establishing a reproducible mouse model that permits in vivo healing and adhesion formation and subsequent functional testing of tendon gliding and joint flexion (papers I and II).

5.2 Allografts in flexor tendon repair

Tendon reconstruction by the use of tendon autografts is a common secondary procedure when primary repair is not possible or has failed. Tendon allografts possess several advantages over autografts (outlined in Section 1.1.3 pp. 13–14), but the fear of a less robust healing response and slower biological incorporation has limited their use. The combination of an allograft with a molecular healing enhancer to create a therapeutic allograft could become an attractive clinical alternative to

autografts. We have demonstrated that a freeze-dried allograft can be loaded with a molecular therapeutic (GDF-5) and thereby improve tendon healing (papers III and IV).

Prior to this we investigated the in vivo differences in graft healing by comparing the use of freeze-dried allografts and live autografts for flexor tendon reconstruction. We showed that the biomechanical advantages of autografts were minimal (Paper II). These findings correspond to what has previously been reported [49]. We investigated the graft healing for up to 84 days and found that tensile strength remained less than half the strength of a normal FDL tendon for both auto- and allograft reconstruction (Paper II). As argued in section 3.1.1 (p. 31), a likely reason for the reduced long-term improvement in healing strength was a reduction of the mechanical loading caused by the immobilization procedure. Correspondingly, a lack of mechanical loading has previously been demonstrated to reduce healing strength in Achilles and flexor tendon models [245,264].

Since adhesion formation is a challenging complication to flexor tendon injury and repair, it is indeed relevant to investigate whether allografts alter tendon adhesion formation. We found that freeze-dried allografts did not cause increased adhesion formation compared to live autografts (Paper II), in agreement with the few studies in the literature [49–51,265]. Rather, we observed that allografts experienced decreased fibrotic adhesions and significantly increased tendon gliding (Paper II). Similar findings have recently been reported in a canine model, though the tendon gliding or digit flexion was not evaluated in this study [143].

Several mechanisms could explain the difference in graft adhesion formation. We speculated that autografts and allografts heal by different mechanisms. Live autografts are likely to heal via intrinsic and extrinsic mechanisms, involving graft tenocytes as well as fibroblasts and inflammatory cells from the surrounding tissue. A migration of graft cells into the surrounding tissue and an influx of host cells into the granulation tissue was demonstrated in our murine model, where the flexor tendons of wild type mice were reconstructed by live tendon grafts from LacZ reporter mice [266]. To support this, a recent study has demonstrated that the cells of live tendon grafts are active and significantly contribute to the collagen synthesis [78], whereas freeze-dried acellular allografts must be assumed to lack the contribution from graft cells and will primarily heal by extrinsic mechanisms [78,267]. The source of adhesions can be debated, and in our murine model it appeared that both intrinsic and extrinsic cells contributed to tendon healing as well as adhesion formation [266]. Therefore, we speculate that the lack of cells in the allograft could reduce the degree of adhesion. However, others have proposed that when the balance between intrinsic and extrinsic healing mechanisms is dominated by the extrinsic mechanisms, adhesions are inevitable [5,67,112–114]. If this theory lasts, the lack of adhesion observed in allograft reconstruction in our study could be explained by a contribution of the live cells in the autograft in a manner that induced extrinsic adhesions. Based on this, it can be speculated that the live cells in the autograft produce a signal that will increase ingrowth of adhesions from the surrounding

tissue. The signal produced could be TGF-β, a signaling molecule that has been correlated to increased fibrosis and adhesion formation. Inhibition of TGF-β has led to improved range of motion in our model [171] as well as in other models of flexor tendon healing [166–169]. Therefore, we have performed a gene expression analysis of TGF-β, but we were unable to document a significant TGF-β mRNA response in any of the graft types (Paper II). However, the analyses were only performed on days 14 and 28, which may have been be too late to observe an early TGF-β response [222]. Furthermore, it has been recognized that surface injuries and tissue crushing are correlated to the degree of adhesion [34,59]. This could be explained by a cellular response from the graft. Even the presence of inflammatory cells in the epitenon during tendon healing has been suggested to induce the cells of the epitenon to increase their production of fibronectine, which provides a scaffold for subsequent adhesion formation [115,268].

5.2.1 Perspectives of therapeutic allografts

Allografts are already an approved treatment for tendon injuries, and the American Association of Tissue Banks has developed guidelines to ensure quality and safety. However, the use of allografts in flexor tendon reconstruction has only been sporadically reported [41,42], and potential long-term problems associated with allografts have been a concern. Recently, a follow-up study of allograft flexor tendon reconstruction has been initiated to investigate its long-term effects [269]. Tendon allograft reconstruction was performed in 22 patients, and a preliminary report (7 months–4.5 years) demonstrated functional recovery similar to that of autograft reconstruction and revealed no adverse tissue reaction [269]. However, a clinical recommendation for more widespread use of allografts in flexor tendon reconstruction still lies in the future.

The concept of therapeutic allografts with the ability to improve surgical outcome is intriguing, and a method for graft loading or manipulation would not be limited to flexor tendon allografts, but could be applicable to all tendon allografts. Recently others have demonstrated the potential of therapeutic allografts in animal models. Surface modification of freeze-dried allografts by hyaluronic acid has been documented to decrease flexor tendon adhesion formation [143]. Another approach of allograft manipulation is reseeding decellularized grafts with tenocytes or stem cells [46,270,271]. Neither of these methods has been documented to improve biomechanical properties, but the methods are under continuous development. Bioreactor preconditioning of reseeded allografts has demonstrated promising in vitro results [272,273]. Based on the experimental work in this field, including ours, an increasing interest in the potential of therapeutic allografts for flexor tendon reconstruction has emerged [274].

5.3 The anti-fibrotic potential of GDF-5

Growth and differentiation factor 5 (GDF-5) is a member of the bone morphogenetic protein (BMP) family. GDF-5 has been demonstrated to be involved in tendon development [173,174] and to increase tendon healing strength in animal models [177–180]. In our studies of auto- and allograft tendon reconstruction, we observed an up-regulation of GDF-5 gene expression prior to a resolution of adhesions (Paper II), a finding that has been confirmed in a later study on our model [252]. Furthermore, we have demonstrated that flexor tendon reconstruction using freeze-dried allografts loaded with GDF-5 can improve digit flexion (papers III and IV). To our knowledge, no previous experiments have investigated the effect of GDF-5 on flexor tendon adhesions in vivo. Our findings have been the first to indicate an anti-fibrotic effect of GDF-5. However, the anti-fibrotic mechanisms of GDF-5 are not fully understood. In the following, indirect pathways that can offer a partial explanation will be outlined.

In a microarray study, GDF-5 treatment of tendon fibroblasts was reported to affect the expression profiles of genes involved in cell proliferation, extracellular matrix (ECM) production, and inflammation [275,276]. The down-regulation of pro-inflammatory genes may offer some explanation to the anti-fibrotic effect of GDF-5, since inflammation is suggested to be correlated to the degree of adhesions [115,116,120]. Furthermore, TGF-β inhibition has improved range of motion following flexor tendon surgery in several animal models, including our murine model [166–169,171]. We speculate that the anti-fibrotic role of GDF-5 could be indirect by the inhibition of TGF-β. Indeed, another member of the BMP family, BMP-7, has been demonstrated to antagonize the pro-fibrotic effects of TGF-β in renal fibrosis [277]. Given that GDF-5 and BMP-7 share structural similarity and receptor binding affinity [278], it is plausible that GDF-5 could have a similar antagonistic effect to TGF-β. Another example of an indirect anti-fibrotic effect of GDF-5 could be an increase of vascular endothelial growth factor (VEGF). GDF-5 has been suggested to promote angiogenesis by increasing VEGF gene expression in vitro [279]. This corresponds to our findings of the concomitant increase in GDF-5 and VEGF mRNA expression (Paper II). Hypoxia has been correlated to flexor tendon adhesions [101], thus VEGF could by inducing angiogenesis lead to a reduction of adhesions. Concordantly, in recent studies we have observed VEGF gene therapy to increase digit range of motion in our model (unpublished). However, VEGF is considered to be pro-inflammatory, and inhibition of VEGF has been demonstrated to attenuate lung fibrosis [280,281].

We have investigated different delivery strategies of GDF-5. In this regard, we observed no significant differences between the therapeutic effects of recombinant protein GDF-5 delivery and viral vector mediated GDF-5 gene delivery in vivo (paper IV) and in vitro (paper III). Given the differences in kinetics of protein and viral delivery (papers III and IV), these observations indicate that flexor tendon reconstruction could benefit both from an early GDF-5 supply as well as from sustained delivery. It

would be interesting to investigate the effect of combined protein and viral GDF-5 delivery on flexor tendon adhesion. In addition to the method of delivery, we also investigated the dosage effect. We found no positive dose-response effect, regardless of the type of delivery (Paper IV). On the contrary, the high dosage appeared to circumvent the positive effect of GDF-5. Since one effect of GDF-5 is increased ECM production [275], it could be speculated that a high dosage would lead to excessive collagen formation, causing either more or stronger adhesions. However, the mechanism could depend on the delivery method, and further understanding of the dose-response relation requires additional studies with a wider range of dosages.

As previously mentioned, GDF-5 has improved tendon healing strength in different injury models [177–180]. We observed maintained, although not improved, biomechanical parameters in our initial study (Paper III). As already mentioned, the severing of the proximal muscle could influence its healing strength. The immobilization procedure was changed in our consecutive studies, and we optimized and controlled the loading conditions of the graft (Paper IV). Interestingly, these alterations resulted in a trend toward improved biomechanical parameters compared with the untreated control at 14 days post-surgery (Paper IV). To test tendon healing strength at 14 days post-surgery is probably too early because differences at 14 days could represent the degree of remodeling rather than be a predictive factor of a later increase in tendon healing strength [224]. Studies with extended time series would have to be performed to further investigate the effect of GFD-5 delivery on tensile strength in our model.

5.3.1 Perspectives of GDF-5 in flexor tendon healing

During the last decade, there has been increasing interest in GDF-5 as a potential therapeutic to improve tendon healing. We have identified a mean improvement of digital range of motion in GDF-5-treated animals of approximately 17 degrees with a 95% confidence interval (CI) ranging from approximately 5 to 30 degrees (Paper IV, data from Figure 13). For this type of treatment to be relevant, an improvement of 5 degrees is probably too small to be of clinical relevance. However, 30-degree improvement would indeed be of clinical interest and in fact corresponds to a 40% improvement, comparable to the improvements originally accomplished by the implementation of motion protocols [58]. However, the limitations of our mouse model should be kept in mind, and studies in larger animal models with the option of including early motion protocols need to be performed. Additionally, our data reflect the effect of GDF-5 in a tendon reconstruction model, and the anti-fibrotic potential of GDF-5 could be different in simple tendon repair.

GDF-5 is already being used in several clinical trials. In a running trial, the effect of intradiscal administration of GDF-5 to improve lumbar disc degeneration is being evaluated [282,283]. Completed

clinical trials have demonstrated promising effects of GDF-5 treatment on healing and regeneration of periodontal defects [284]. However, GDF-5 have not been reported in clinical trials for tendon healing,

5.4 Protein versus gene delivery

We have used a freeze-dried tendon allograft to deliver either recombinant protein GDF-5 (rmGDF-5) or viral gene expression vectors encoding GDF-5 (rAAV-*Gdf5*). There are differences in the kinetics of action of protein delivery and gene delivery via viral vectors. The allograft delivery of exogenous rmGDF-5 would lead to an immediate increase in the level of GDF-5 at the repair site. However, the duration of the treatment will depend on the rate of protein release from the graft and will presumably be limited to days. GDF-5 gene delivery via the rAAV vector will be delayed by cell transduction, gene transcription, translation, and protein trafficking. However, once protein expression is established, a continuous delivery will be provided. The duration of the delivery will depend on the transduced cell. We found that rAAV-*Gdf5* delivery provided gene expression from 3 days to at least 21 days following injury and repair in our model (Paper III); this is similar to what has been observed in other models [209]. However, the end point of gene expression needs to be determined in our model.

Despite the differences in kinetics, we did not observe significant differences between the therapeutic effects of protein (rmGDF-5) and gene (rAAV-*Gdf5*) delivery (Paper IV). Nevertheless, we cannot exclude that a difference will develop at a time point exceeding the 14-day time point we evaluated. Furthermore, a single dose of GDF-5 protein treatment has been observed to significantly improve tendon healing strength at 3, but not at 6 weeks post-operatively [181]. A possible explanation could be the limited duration of the treatment, thus we may find that the choice of delivery method can alter the long-term effect of the therapeutic. Concordantly, it has been observed in a chicken flexor tendon injury model that one injection of a recombinant adenoviral vector encoding GDF-7 (BMP-12) improved tendon healing strength after 4 weeks, even though no difference was found after 2 weeks of healing [211]. Since the anti-fibrotic mechanisms of GDF-5 are unknown, it is not possible to predict the optimal time span for GDF-5 therapy, and prolonged time-series studies need to be conducted to properly evaluate the delivery methods.

In our model, protein as well as gene delivery of GDF-5 demonstrated a dose-dependent effect, and the lower dosages appeared to hold a greater anti-fibrotic potential. However, we have not quantified and compared the actual number of GDF-5 molecules delivered by the two methods, and thus we are unfamiliar with the relationship. The low dose of the rAAV-*Gdf5* may result in a higher dose of delivered protein than the high dose of rmGDF-5, or vice versa. The mechanisms of the dose-dependent effect could be different depending on the delivery method. In regard to rmGDF-5

treatment, we speculate that GDF-5 has an optimal range at which the anti-fibrotic pathways are active. Once the range is exceeded, the pathways will be unfavorable, and the anti-fibrotic effect will be reduced. The rAAV-*Gdf5* treatment could also be limited by optimal range for the GDF-5 pathways, but it could also be due to side effects of the viral vector. It has previously been documented that high virus titer could initiate a local inflammatory response in a flexor tendon model [207]. An increased inflammatory response could counteract or confound the effect of GDF-5. However, others have reported that the tissue reaction following rAAV injection is less severe compared with the inflammatory changes already present in injured tendon [285]. Nevertheless, using high viral titer warrants attention when planning to use viral vectors in models of flexor tendon healing.

We have demonstrated that we can control the doses of both recombinant protein and rAAV vectors loaded to the graft (Paper IV). Furthermore, we have documented local delivery of the viral vector to the site of injury (papers III and IV). However, the delivery of recombinant protein has only been indirectly confirmed by the effect on the biomechanical parameters (Paper IV). We have also demonstrated that the loaded dosage of rAAV vectors is correlated to the in vivo gene expression (Paper IV), similarly to what has previously been reported [207]. The biodistribution of the viral treatment needs to be meticulously investigated. Bioluminescence imaging revealed only accumulation of marker gene expression at the site of injury (Paper IV). However, complete exclusion of any off-target gene expression would require investigation by tissue sampling and PCR analysis.

We have demonstrated two strategies for delivery of molecular agents for modification of biological tendon healing. The methods are different in kinetics of action, and the preferred method will depend on the therapeutic and the mechanisms by which it works. Since the kinetics of the methods supplement one another, an interesting possibility might even be to combine the two methods to insure an instant and continuous delivery.

5.4.1 Perspectives of protein vs. viral gene delivery

Delivery of GDF-5 and other members of the BMP family are already in clinical trials [282,283]. The perspectives for allograft delivery of recombinant protein will depend on careful investigation of the biodistribution of the protein in addition to evaluation of potential side effects.

Allograft delivery of viral vectors encoding a therapeutic gene also depends on the biodistribution and side effects of the protein, but on top of that, there are safety issues regarding the vector system. Although rAAV vectors are derived from a nonpathogenic virus and have a minimal risk of genome integration, vector safety is still a concern. No viral vector has yet been accepted for wide clinical use [286]. Tendon injuries are local and nonlethal, and for viral gene therapy to be a relevant clinical alternative, several safety issues need to be addressed. The biodistribution must be accurately

determined. The duration and decay of gene expression needs to be evaluated and potentially controlled. The vector must be optimized to avoid the host immune response and insertional mutagenesis. Nevertheless, flexor tendon healing strength has previously been modified by adenoviral gene transfer of GDF-7 (BMP-12) [211]. Additionally, we have demonstrated the rAAV2 vector to improve digital range of motion following GDF-5 gene transfer (papers III and IV). A concomitant study by Tang and colleagues demonstrated that gene therapy improved flexor tendon healing in regard to both healing strength and adhesion formation using a rAAV2 vector encoding bFGF [186]. Collectively, these studies demonstrate the potential of viral gene therapy as a treatment option for flexor tendon injuries.

6.0 CONCLUSION

We have successfully developed the first murine flexor tendon model that permits in vivo study of simple repair and segmental reconstruction. We have used the model to investigate the differences between autograft and allograft reconstruction. We found that the mechanical advantages of the autograft over the allograft are minimal. We demonstrated how the hydrophilic capacity of the freeze-dried allograft could be exploited to load therapeutic molecules to the graft. Our findings indicate that freeze-dried allografts hold potential to become a valuable, and maybe even preferable, clinical alternative to live autografts.

Furthermore, we investigated the effect of GDF-5, a growth factor involved in tendon development. While GDF-5 has been demonstrated to increase tendon healing strength, we could not confirm this effect in our model. However, we uncovered an anti-fibrotic effect of GDF-5 on flexor tendon healing and demonstrated a significantly improved digital range of motion. The anti-fibrotic effect seemed to be independent of delivery method (protein vs. rAAV). However, we did observe a dose-dependent effect, and a lower dose demonstrated to hold a greater potential. The anti-fibrotic mechanisms of GDF-5 are still unclear, and there is a need for further studies to determine the potential of the anti-fibrotic effect of GDF-5.

Despite the limitations of the murine model, we find that the advantages outweigh the limitations and that the model fulfills its role as a valuable screening tool for new potential modifiers of tendon healing, especially in regard to adhesion formation. The murine model has already demonstrated its value in a number of studies besides those included in this thesis [171,252,266,287]. The model provides a tool to evaluate the molecular, cellular, and biomechanical effects of specific genes and molecules on the tendon healing process.

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APPENDIX

Paper I-IV

Paper I:

"A Mouse Model of Flexor Tendon Repair." Hasslund, S., O'Keefe, R. J., Awad, H. A. Skeletal Development and Repair: Methods and Protocols, Methods in Molecular Biology, vol. 1130, chapter 6. DOI 10.1007/978-1-62703-989-5_6. (Accepted for publication).

Paper II:

"Adhesions in a Murine Flexor Tendon Graft Model: Autograft versus Allograft Reconstruction." Hasslund, S., Jacobsen, J., Dadali, T., Basil, P., Vinther, M. Søballe, K., Schwartz, E. M., O'Keefe, R. J., Mitten, D., Awad, H. A. (2008). Journal of Orthopaedic Research. 26(6), pp. 824–33. DOI 10.1002/jor.20531

Paper III:

"Freeze-dried Tendon Allografts as Tissue-engineering Scaffolds for Gdf5 Gene Delivery." Basile, P., Dadali, T., Jacobsen, J., Hasslund, S., Vinther, M., Søballe, K., Yasuhiko, N., Hicham Drissi, M., Mitten, D., O'Keefe, R., Schwarz, E., Awad, H. (2008). Molecular Therapy. 16(3), pp. 466–72. DOI 10.1038/ sj.mt.6300395.

Paper IV:

"Freeze-dried Allografts-Mediated Gene or Protein Delivery of GDF-5 Improves Murine Flexor Tendon Healing." Hasslund, S., Dadali, T., Vinther, M., Soballe, K., Awad, H. (In review, Journal of Tissue Engineering).

Chapter 6

A Mouse Model of Flexor Tendon Repair

Sys Hasslund, Regis J. O'Keefe, and Hani A. Awad

Abstract

Mouse models offer invaluable cellular and molecular tools for the study of human pathologies including those associated with fibrotic and musculoskeletal diseases. In this methods manuscript, we describe a mouse model of repair and segmental reconstruction of flexor tendons, which in our laboratory has been an invaluable model to study tendon scarring and adhesions. Specifically, we describe in details all the surgical procedures involved, as well as the associated endpoint biomechanical assessments including a novel test of the flexion of the metatarsophalangeal joint as a measure of adhesions, and a standard protocol for biomechanical assessment of the tensile strength of the tendon and repair tissue.

Key words Flexor tendon, Repair, Tendoplasty, Autograft, Allograft, Adhesions, Biomechanics

1 Introduction

Animal models including nonhuman primates [1], canine [2–6], chicken [7–9], rabbit [10–13], and rat [14–16] have been used for decades in studies to identify the cellular processes and factors that lead to scarring and adhesions in flexor tendon repair. Preclinical animal models have also been used to evaluate the effectiveness of therapies that inhibit the formation of adhesions while facilitating the healing of the repair site [17]. These therapies can be loosely classified as either physical or biological treatments. Physical treatments include (1) early controlled passive motion (CPM) rehabilitation protocols [18–20]; (2) optimized surgical and suturing techniques to strengthen the repair while minimizing trauma to the tendon and scar formation [21–23]; and (3) anti-adhesion modification of the graft surface using coatings such as hyaluronic acid or lubricin that serve to reduce the friction during graft gliding [5, 24–26] or serve as physical barriers around the graft to inhibit fibrovascular scar in growth that gives rise to adhesion [27, 28]. In addition to these physical approaches, a number of biologic therapies are currently being empirically investigated including anti-TGF-β1 treatments [29, 30]. For such biologic therapies to

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be clinically successful one must not only demonstrate that they are effective but also understand and control their mechanism of action.

The mouse model offers a powerful toolbox to potentially elucidate the cellular and molecular events involved in scarring because of the availability of genetic models of gain and loss of function. To take advantage of these models, we have developed *the* first murine flexor tendon (flexor digitorum longus or FDL) repair model that permits the in vivo study of simple repair and segmental reconstruction [31–35]. This model provides a means to evaluate the cellular, molecular, and biomechanical effects of specific genes and targets of the healing process. We have developed an innovative biomechanical adhesion test that is highly sensitive and allows calculation of a gliding coefficient at various times following tendon repair or segmental reconstruction [31–35]. The healing process in the murine tendon is completed over the course of 4–6 weeks, with adhesions peaking between 14 and 28 days, making this well-characterized model highly feasible for testing interventions with the potential to improve the functional outcomes [32, 34].

Our work to date has permitted us to delineate several key cellular and molecular features of flexor tendon healing. We first observed that live autografts heal with more extensive scarring and adhesions compared to freeze-dried allografts [32], which suggested that the etiology of adhesions is precipitated intrinsically by the live autograft cells [16]. The mechanism of adhesion formation is likely triggered by inflammation. It has been suggested that the presence of inflammatory cells in synovial sheath and epitenon during tendon healing "induces synovial fibroblasts and epitenon cells to increase their production of fibronectin, which provides a scaffold for subsequent adhesion formation," presumably by secreting growth factors such as TGF-β1 and inflammatory cytokines [16]. Therefore, we postulated that tenocytes in live grafts contribute significantly to the formation of scar tissue in the vicinity of the graft. To test this, we transplanted live FDL grafts from the reporter mouse *Rosa26*$^{LacZ/+}$ in WT recipients and used histological X-gal staining to evaluate the intrinsic contribution of the tendon graft to scarring and adhesions [36]. Consistent with our hypothesis, we observed progressive cellular proliferation and migration as evident by outward flux of β-gal-positive graft epitenon cells contributing to the adhesion tissues that filled and obliterated the gliding space. However, there was also evidence of influx of host cells (β-gal negative) into the gliding space and the graft. Using myeloablated WT mice transplanted with bone marrow from GFP transgenic mice, we have also demonstrated that marrow stem/progenitor cells are mobilized and migrate to the FDL repair site [35]. Altogether, this animal model allowed us to demonstrate that flexor tendon repair

is accomplished both by extrinsic peripheral cells that likely involve synovial fibroblasts, inflammatory cells, and mobilized marrow-derived cells and by intrinsic fibroblasts from the tendon itself. In agreement with published literature implicating TGF-β in a variety of fibrotic pathologies, we have also used this model to confirm the pro-scarring role of TGF-β and demonstrated that TGF-β/Smad3 loss of function in *Smad3*$^{-/-}$ mice leads to improved FDL tendon gliding and MTP joint flexion following surgical repair [33].

Collectively, these studies underscore the unique advantages of the gene deletion and transgenic approaches that the mouse model offers. These studies have also guided our interventional strategies that aim to target the TGF-β1 pathway using factors that antagonize its signaling pathway such as GDF-5 (BMP-14) [31] and using novel gene silencing strategies such as antisense oligonucleotides (ASO) [37], as we have recently demonstrated.

The mouse model is not without limitations, the most important of which is the inability to induce injury and repair in zone II. While mice have zone II-like anatomy [38], only one published study utilized the mouse model of surgical repair of the FDL in a "zone II equivalent," but this injury model in fact examined the repair process of the tendons in vitro rather than in vivo [39], presumably because the dimensions of the mouse digit pose insurmountable surgical challenges. Our approach, described herein, was to create a reproducible in vivo mouse model of flexor tendon repair with functional outcomes, so we decided not to involve zone II. That limitation notwithstanding, we were successful in developing the first functional test of tendon gliding and adhesions in the mouse. While there could be concern about the translational relevance of mouse models, signaling pathways and disease processes are highly conserved across mammalian species, and studies in mice have provided insights about disease and have led to important new therapies in areas including cancers, heart disease, hypertension, diabetes, obesity, osteoporosis, glaucoma, blindness and deafness, and neuropathologies [40] and various skeletal tissue repair processes including tendon repair [39, 41]. Therefore, we believe that the advantages of the mouse model outweigh its limitations.

In this methods chapter, we describe the segmental reconstruction of an FDL tendon gap defect in the murine model using a biologic graft and the relevant biomechanical tests to assess adhesions and healing strength. The methods described herein apply to live grafts from various transgenic strains as well as lyophilized allografts [32]. Simple repair of transverse laceration [34, 35] will not be described in the interest of brevity and to avoid duplicity but can be easily reproduced with guidance from the described protocol.

2 Materials

2.1 Presurgical Prep

1. Hair clipper.
2. Povidone iodine prep solution and 70 % isopropyl alcohol.
3. General surgical disposables such as face masks, sterile drapes, sterile gloves, and sterile gauze.

2.2 Graft Harvest and Surgical Reconstruction

1. Graft donor and recipient mice (*see* **Note 1**).
2. Dissecting microscope with zoom range of 0.7–4.5×. Surgical loupes can also be used if available.
3. Fiber-optic illuminator with dual-obedient goosenecks.
4. Microsurgical instruments, including micro-dissecting, serrated, straight forceps, micro-dissecting spring scissors, micro needle holder, and disposable scalpel #11 with integrated metric ruler on handle.
5. Nonabsorbable Nylon suture (e.g., ETHILON™ 8-0 or 9-0 Black Monofilament, Ethicon).
6. Nonabsorbable silk suture (e.g., Silk Suture 6-0, Ethicon).
7. Fine-tip surgical markers (containing gentian violet ink that is nontoxic, nonsmearing, and nonirritating).

2.3 Anesthetic and Analgesic Drugs

1. Anesthesia drugs: 100 mg/kg Ketamine-HCl and 10 mg/kg xylazine. It is recommended that the drugs be combined together and administered as a single intraperitoneal (IP) injection. The following regimen will produce a surgical level of anesthesia lasting for 15–30 min and sedation of 1–2 h: combine 1.0 ml of 100 mg/ml ketamine-HCl with 1.0 ml of 10 mg/ml xylazine and 8.0 ml 1× PBS. The combined drugs are to be administered at 0.1 ml/10 g body weight via an IP injection using 1 ml syringe with 25G 5/8 in. needle.
2. Analgesic drugs: 0.3 mg/ml Buprenorphine HCl (Buprenex®) and 50 mg/ml flunixin meglumine (Banamine®). It is recommended to administer 0.05 mg/kg Buprenex® subcutaneously once preoperatively and administer 0.5 mg/kg Banamine subcutaneously every 24 h up to 3 days postoperatively.

2.4 Lyophilization Equipment and Supplies (Optional: For Devitalized Allografts)

1. Corning® cryogenic vials, 1.2 ml capacity, or equivalent.
2. Freeze-drying system with appropriate sample racks and flasks (e.g., Labconco FreeZone 1 l Benchtop Freeze Dry System or equivalent).

2.5 Adhesion Testing

1. Support stand with rod and clamps to assemble a customizable adhesion testing apparatus as described in Subheading 3.3.
2. Metric calibration weight set (1–50 g).
3. Digital camera with tripod and remote shutter release.

2.6 Biomechanical Tensile Testing

1. Lab tape.
2. Super glue with high viscosity, gel-like consistency.
3. Gauze pads.
4. Phosphate-buffered saline (PBS, 1×).
5. A uniaxial testing system with an appropriate set of grips for murine soft tissues and tension-calibrated load cell (e.g., Instron 8841 DynaMight™ Servohydraulic Axial Testing System with a 50 N T/C load cell, and custom-made grips with serrated jaws).

3 Methods

Individuals utilizing this protocol should be very aware of your institutional animal welfare policies as well as murine hind limb anatomy (*see* **Notes 2** and **3**).

3.1 Graft Preparation

1. The donor and recipient mouse strains are selected based on the hypothesis tested (*see* **Notes 4** and **5**).
2. Euthanize the donor mice using methods approved by your Institutional Animal Care and Use Committee (IACUC) protocol. If the objective is to use live grafts, the animals should be freshly euthanized just before the reconstruction surgery.
3. Using the clipper, shave the hair from the distal part of the hind limb including the paws. Loose hair can be removed from the plantar surface of the paws by dabbing the area with adhesive tape or moistened gauze.
4. Using a gauze pad or a Q-tip applicator, prep the plantar surface of the hind paw with alternating scrubs of povidone iodine and 70 % isopropanol. Repeat the alternating scrubs three times.
5. Setup a sterile drape, and organize your sterile instruments on a sterile drape.
6. Using sterile gloves and aseptic technique, make an incision over the medial aspect of the plantar surface, starting at the interdigital space between the first and second digit ending at the ankle.
7. At the plantar surface of the calcaneus cut/transect the flexor digitorum brevis and the tendon of musculus flexor digitorum superficialis, and pull both distally.
8. Using the fine-tip surgical marker, draw transverse lines on the FDL tendon defining the 3 mm graft, approximately 2 mm proximal to the branching point (Fig. 1).
9. Using the micro-dissecting spring scissors and straight forceps, cut the 3 mm graft:

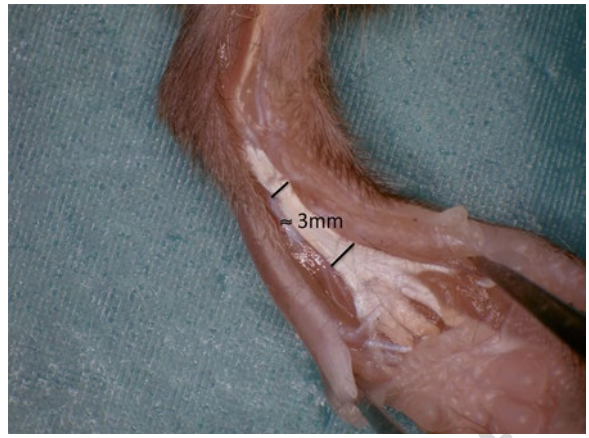

Fig. 1 Photograph of the FDL tendon, showing the region of FDL tendon from which the graft is harvested

(a) If your objective is to use a live graft, transfer the graft to a premarked vial containing sterile PBS. It is recommended that the transplantation surgery be performed immediately after graft harvest without delay to avoid damage to the graft viability.

(b) If your objective is to use a devitalized graft:

- Place the graft in a cryogenic vial containing sterile deionized water.
- When done with graft harvest, freeze the cryogenic vials containing the grafts at −80 °C.
- Once frozen, make sure that the caps of cryogenic vials containing the grafts are loose to ensure that the frozen water in the vessel and tissues can sublimate, and place the vials in the freeze-drying flasks.
- Lyophilize the samples using standard settings overnight. Once lyophilized, the grafts can be stored at −20 or −80 °C.
- Optional: Once lyophilized, the grafts can be loaded with small molecules, gene delivery vectors, or growth factors by doping the grafts in a solution of the molecule of interest at the desired concentration for 2 h.

3.2 FDL Tendon Gap Defect Reconstruction (Tendoplasty)

1. An important aspect of this protocol is to protect the graft from in vivo loading following reconstruction. This can be accomplished by severing the musculotendinous junction (MTJ) [32] during the surgical procedure. Alternatively, the flexor muscles can be transiently paralyzed with BOTOX as described in Subheading 3.3.

2. Anesthetize the animals as described in Subheading 2.3, **item 1**. This should provide a surgical plane of anesthesia that lasts for 15–30 min, which is the average duration of the surgery for a skilled, experienced rodent surgeon.
3. To ensure immediate postoperative pain management buprenorphine should be administered as per Subheading 2.3, **item 2**, preoperatively.
4. Place the animal on a heating pad, and prep the hind leg for sterile surgery as per Subheading 3.1, **steps 2–4**.
5. Affix the foot to the surface of the heating pad by placing sterile tape on the digits, distal to the metatarsophalangeal (MTP) joint.
6. Make an incision over the medial aspect of the plantar surface, from the interdigital space between the first and second digit to the ankle. The incision is continued from the ankle to the knee. If BOTOX is used as per Subheading 3.2, **step 1**a, the incision can be ended at the ankle (Fig. 2a–c). Be careful not to cut any vessels.

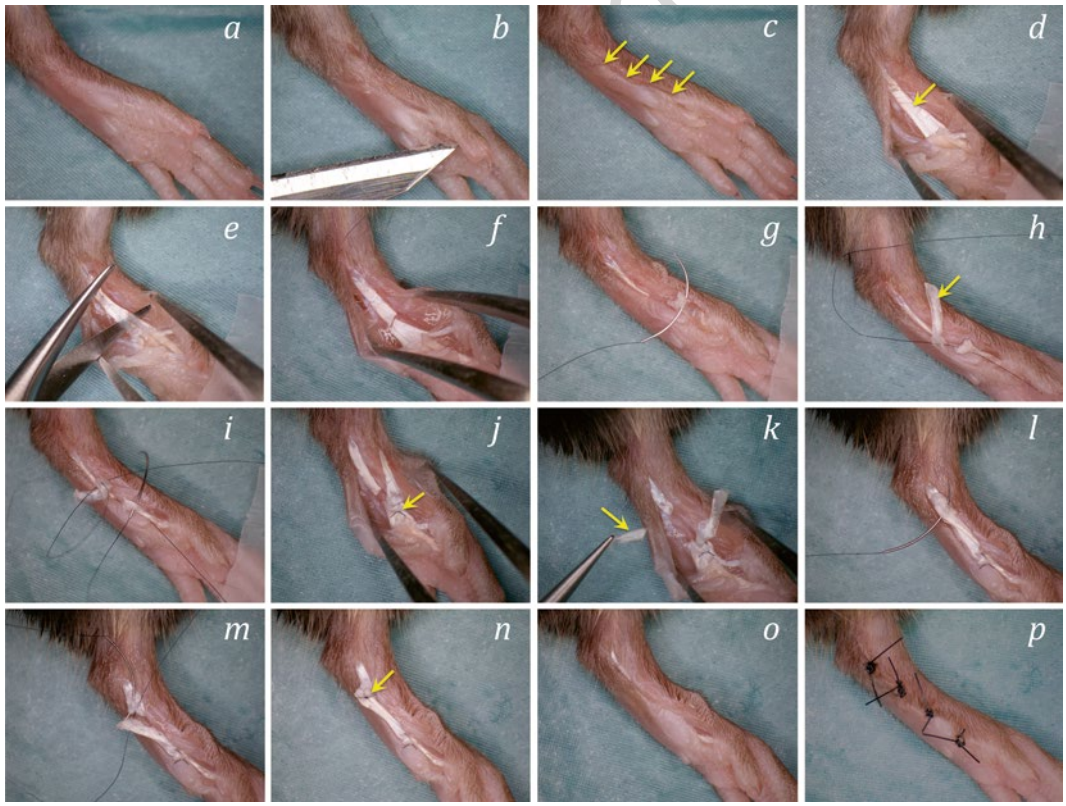

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Fig. 2 Steps of the FDL tendoplasty surgery

7. Below the fascia dissect bluntly to separate the flexor hallucis brevis (FHB) muscle from the flexor digitorum brevis (FDB) muscle. Locate the flexor digitorum tendon below the FDB (Fig. 2d).
8. Transect the tendon in the undivided region 2 mm proximal to the point where it divides and branches to the individual five digits (Fig. 2e, f).
9. Using the micro needle holder and forceps, suture the graft to the distal tendon end using 8-0 nylon suture (Fig. 2g–j). It is important to align the tendon and graft ends. We recommend a horizontal mattress suture pattern. Note: A modified Kessler or other core suture techniques, usually recommended for flexor tendon repair, will cause graft shredding.
10. Transect the proximal end of the tendon, and remove a piece of tendon to create a 2 or a 3 mm gap defect (Fig. 2k).
11. Suture the graft to the proximal tendon end to reconstruct the gap defect, using the same technique applied at the distal end (Fig. 2l–n).
12. Remove the micro retractor, and let the FDB and FHB muscles slide back in place; it might be necessary to actually push them into place (Fig. 2o).
13. If you used BOTOX to unload the tendon, skip this step. If tendon unloading is to be accomplished by MTJ transection rather than BOTOX, locate the tendon muscular junction, and transect the tendon from the muscle using the scalpel.
14. Close the skin 6-0 silk sutures (Fig. 2p).
15. Administer analgesics for pain relief as per Subheading 2.3, **item 2**, or your approved IACUC protocol.
16. Observe the animals carefully for signs of infection, pain, or discomfort. An animal showing severe pain and distress signs or an infection should be immediately humanely euthanized and excluded from the experiment.
17. Remove the skin sutures 7 days postoperatively.
18. At the study endpoint, euthanize the mouse humanely as per your approved IACUC protocol, and harvest the hind limb by disarticulating the tibia from the knee joint. Place in a prelabeled specimen bag and freeze at −20 °C until biomechanical testing of adhesions and tensile strength.

3.3 FDL Tendon Gap Defect Reconstruction (Tendoplasty): Alternative BOTOX Protocol

1. Since severing the MTJ compromises the accrual of biomechanical strength over time, an alternative is to use a BOTOX® (onabotulinumtoxin A) injection [42] into the left hind limb muscle to induce transient paralysis of the flexor muscles 24 h prior to FDL tendoplasty. Previously, Yoneda et al. showed

that a single 30 U/kg intraoperative injection of BOTOX can eliminate as much as 90 % of the in vivo loads on the tendon in the first 3 days but recover as much as 40 % of these in vivo loads over 21 days at a rate of ~2 % per day [42]. Therefore, the BOTOX injection will protect the graft during the early healing phase, but the gradual increase of in vivo loading will likely positively influence the functional (tendon gliding and joint flexion) and biomechanical properties.

2. BOTOX® (Allergan Pharmaceuticals) is supplied as single-use, sterile 100 or 200 U vacuum-dried powder for reconstitution only with sterile, non-preserved 0.9 % sodium chloride (or PBS) prior to injection. Follow the manufacturer's guidelines vis-à-vis reconstituting the BOTOX based on the following considerations: Based on the recommendations of Yoneda [42] and our previous experience (unpublished data), we recommend using an intramuscular (IM) injection of 30 U/kg (or 0.03 U/g). The injection volume should be about 250 μl/kg, which for a 25 g mouse translates to 6.25 μl. The BOTOX should be diluted to a concentration of 120 U/ml (or 0.12 U/μl). It is important to keep the BOTOX on ice or at 2–8 °C until injected. Once reconstituted, the toxin has a limited life even if kept refrigerated (see the product data sheet).
3. Twenty-four hours prior to the tendoplasty surgery, weigh the mouse to determine the BOTOX and anesthesia dose.
4. Anesthetize the mouse as described in Subheading 2.3, **item 1**. Due to the short injection procedure, the mouse can be alternatively anesthetized with isoflurane using a standard anesthesia machine with calibrated vaporizer.
5. Shave the hind leg, and rinse it with ethanol 70 %.
6. Aspirate the desired volume of the reconstituted BOTOX (120 U/ml) based on 0.25 μl/g BW (~6.25 μl for a 25 g mouse) into a 10 μl Hamilton syringe (model 701) using a 32 gauge needle. Insert the needle at the muscle tendon junction, which can be visualized through the skin. The needle should be pointing proximally. Inject the BOTOX in small deposits a few millimeters apart to ensure even distribution of the toxin in the muscle.
7. On the day of surgery (24 h later), verify that the BOTOX-injected hind limbs have lost their ability to grip on the side of their cage when suspended by their tails.
8. Anesthetize the animals as described in Subheading 2.3, **item 1**.
9. From this point forward, follow the subsequent steps described in Subheading 3.2, **steps 2–18**.

3.4 Nondestructive Assessment of Adhesions and the Metatarsophalangeal Joint Flexion

1. It is recommended that the samples be randomly tested, with their identity and treatment blinded to the person performing the test. This is a nondestructive test that will permit subsequent biomechanical testing of the same tendons as described in Subheading 3.5.
2. The testing can be done using a simple-to-build custom apparatus consisting of a support stand, rods, clamps, alligator clips, fishing line, and standard (metric) calibration weights (1–20 N) as depicted schematically in Fig. 3a–c.
3. Thaw the hind limb specimens, and proceed to expose the skin by shaving fur from the medial malleolus to the knee. This can be done with a sharp scalpel. This is done to improve visibility.
4. Make a 10 mm longitudinal skin incision on the posterior side of the tibia to expose the flexor muscle and proximal FDL tendon, taking extra caution not to transect the tendon.

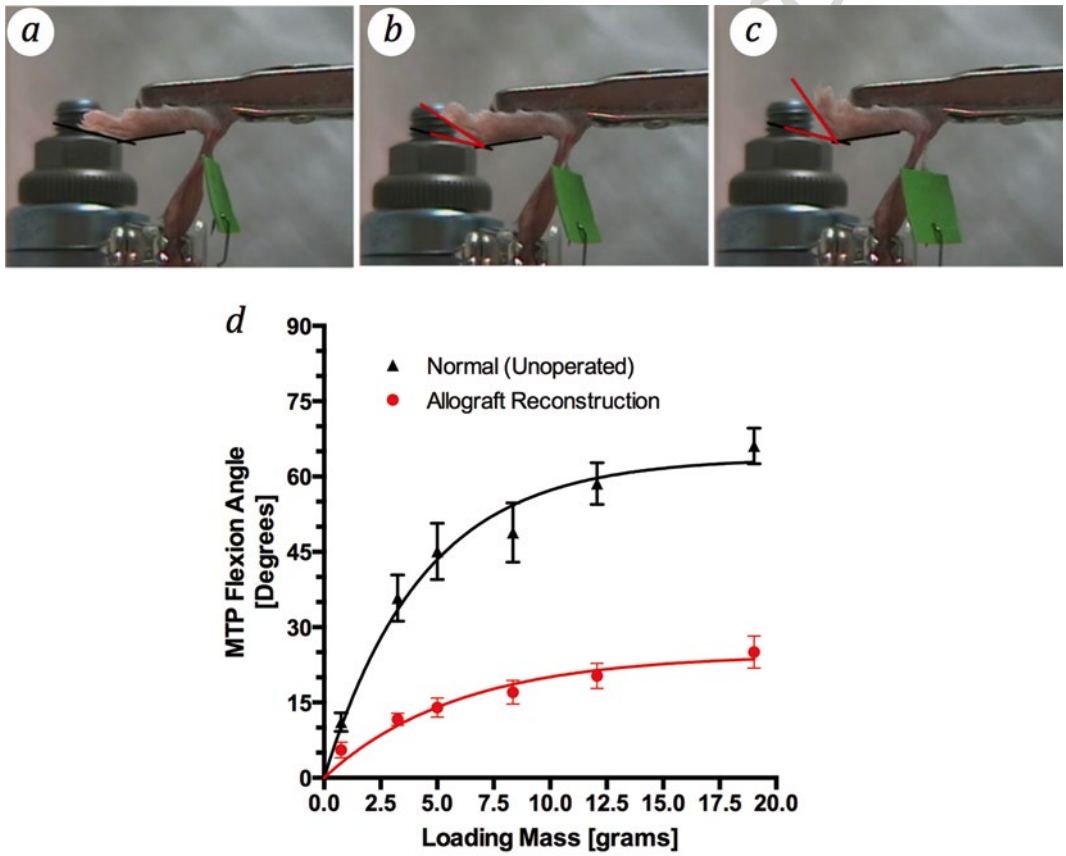

Fig. 3 Assessment of MTP joint flexion upon FDL tendon loading. The proximal FDL tendon is isolated and loaded incrementally using dead weights in the direction of the anatomical pull starting with a neutral unloaded position. At each load a digital picture is taken (**a**–**c**). (**d**) Representative flexion curves (flexion angles versus applied loads) of the MTP joint in normal (unoperated) and allograft reconstructed FDL tendons (day 14 post grafting)

5. Transect the MTJ.
6. Grab the freed proximal end of the tendon with a forceps taking care not to apply any tension and affix a piece of lap tape on the tendon end. Keep the tendon moist with PBS.
7. Mount the hind limb on the testing apparatus upside down by gripping the proximal tibia and foot with clamps. Pass a fishing hook, which has been previously tied to a fishing line, through the taped tendon end (*see* **Note 6**).
8. Set up a digital camera with a remote shutter control on a tripod to take sagittal view images of the mounted foot. Take a digital image to determine the neutral position (zero load) of the MTP joint.
9. Very carefully, load the FDL tendon incrementally from 1 to 20 g in the same anatomical direction as flexor muscle line of force by suspending the standard weights from the line. It is recommended to span that range with 6–7 weights (e.g., 1, 2.5, 5, 7.5, 10, 15, 20 g).
10. Take a digital image with each increment of load to quantify the MTP flexion angle relative to the neutral position (*see* **Note 7**).
11. When the testing is done, the specimens can be frozen and used for biomechanical tensile testing as described in Subheading 3.4.
12. Measure the MTP joint flexion angles from the digital images using ImageJ software (http://rsb.info.nih.gov/ij/) or equivalent software (*see* **Note 8**).
13. Plot the calculated flexion angles versus the applied weights (Fig. 3d). To quantify the resistance to flexion due to adhesions, the flexion data can be fitted using nonlinear regression (e.g., using software such as Prism, GraphPad Software, Inc.) to a single-phase exponential association equation of the form: MTP flexion angle $= \beta \times [1 - \exp(-m/\alpha)]$, where m is the applied load. The curve fit should be constrained to the maximum flexion angle (β) for normal tendons (e.g., in our model, 75° for the maximum applied load of 20 g). The gliding coefficient (α), which regulates the rate of rise of the flexion curve or the ease with which the flexion angle changes with increased loading, is determined by nonlinear regression as a measure of the resistance to MTP joint flexion due to adhesions.

3.5 Biomechanical Testing of Tensile Strength

1. It is recommended that the samples be randomly tested, with their identity and treatment blinded to the person performing the test.
2. Thaw the hind limb specimens, proceed to expose the grafted tendon as described previously, and release it from the tarsal tunnel. Proceed carefully using the dissection microscope to

avoid damaging the tendon. The rest of the foot is left intact (*see* **Note 9**).

3. Prepare a small piece of adhesive lab tape (20 mm × 20 mm) and place (sticky side up) on a gauze pad that has been wetted with PBS.
4. Place a droplet of viscous super glue (~3 mm droplet) on the tape about 1 mm from the distal edge. Using forceps, carefully place sample on the gauze pad and lay the proximal end of the tendon/muscle in the glue droplet on the tape.
5. Add another small droplet of superglue to reinforce adherence of the tendon to the tape. The glue droplet should not smear or diffuse distally into the tendon tissue. Allow the glue to polymerize and harden, but maintain hydration of the tissue by soaking the gauze pad beneath in PBS. Once the glue is hardened, fold the piece of tape over the glued end of the tendon/muscle, aligning the proximal and distal edges of the tape (Fig. 4a).
6. Proceed to carefully mount the specimen onto the testing system. In this chapter, we describe the biomechanical testing setup in our laboratory comprising an Instron 8841 uniaxial testing system (Fig. 4b) and custom-made grips with serrated jaws (inset in Fig. 4b). Alternative testing systems can be used.

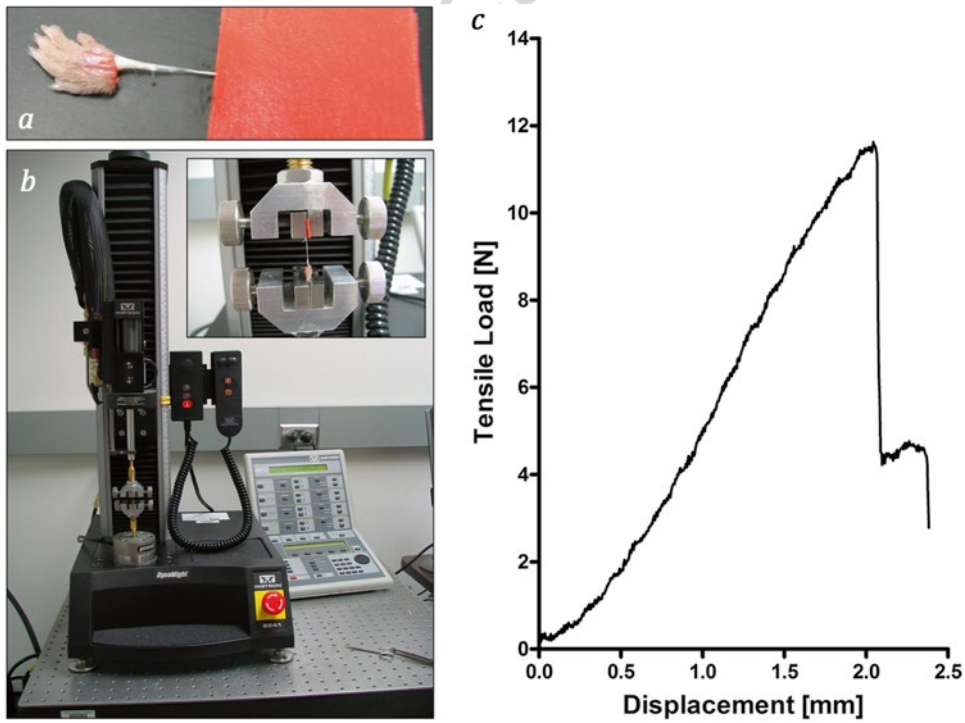

Fig. 4 Biomechanical (tensile) testing of FDL tendon

7. Proceed by first securing the distal bones of the foot in the bottom grips. Once secured, use forceps to slide the taped proximal end of the tendon between the serrated jaws of the top grips, and slowly tighten the grips to securely hold the tendon (inset in Fig. 4b). Make sure to position the actuator such that the tendon is always lax and unloaded during the mounting process. Keep the tendon hydrated by spraying small amounts of PBS during testing (*see* **Note 10**).
8. The mounted tendon is then loaded in tension until failure in displacement control using preset protocols in the instrument software. These protocols typically define parameters such as a preconditioning cyclic regimen (we recommend against using it when testing mouse tendons due to the small forces and displacement that could induce damage), loading rate (e.g., 30 mm/min as per published protocols [32, 43], and an end of test criteria (percentage drop from peak load, e.g., 50 %) as well as the data channels to be logged (make sure to log the displacement and load data), data logging rate (e.g., at a loading rate of 30 mm/min or 0.5 mm/s, the testing will last no more than 6–10 s; therefore, a data sampling rate of 50–100 Hz should generate a reasonably sized data file with enough resolution to capture important events during the test such as yielding and failure), and options to save the data file.
9. Open the data file in data processing software such as a spreadsheet. Some post-processing might be needed to remove data that may have been collected before the tendon was loaded. Plot the force–displacement curves from the data files (Fig. 4c), and determine the following structural properties:
   (a) The maximum tensile force (Newton (N) or equivalent units), which is computed from the peak load in the load–displacement plot.
   (b) The stiffness (N/mm or equivalent units), which is computed from the slope of the linear region of the load–displacement plot.
   (c) The work to failure (N/mm or equivalent units), which is the area under the load–displacement curve up to the peak load.
   (d) The displacement at peak load (mm or equivalent units).
10. At the end of the test, dissect the tendon and identify the mode of failure. Failures in the mid-substance of the tendon/graft are accurate measurements of the repair strength. However, failure near the grips due to stress concentration or slippage tends to underestimate the strength of the tissue or the repair.

4 Notes

1. The choice of the mouse strain or genotype for both donor and recipient is dictated by the hypothesis tested. It is recommended, however, that the size (width and thickness) of the donor and recipient tendons be matched to avoid false measurements of increased or decreased gliding resistance, which in many cases can be accomplished by using age-matched donors.
2. For all animal studies, a protocol defining the scientific rationale and goals for the study, number of animals needed, operative procedure, operative anesthesia, and pre- and postoperative analgesia and care, etc. should be approved by an IACUC.
3. The methods involving graft harvest and surgical reconstruction of the FDL tendon require intimate knowledge of mouse hind limb anatomy. An excellent resource entitled "A Colour Atlas of Anatomy of Small Laboratory Animals, Volume II" by Peter Popesko, Viera Rajtova, and Jindrich Horak (CRC Press; ISBN-10: 0723418233) can be quite useful in familiarizing the reader with the anatomical terminology used herein.
4. If the effect of a certain gene in the graft cells is to be tested, then live grafts could be harvested from mice with a mutation in that gene and the recipients could be phenotypically nonmutant mice (wild type or WT). The choice of the WT and controls is determined based on the genetic background of the mutant mice. If the mutation is generated on a standard inbred background (e.g., C57Bl/6 or other standards), that strain would be appropriate as a control. If a mutation is maintained on a mixed genetic background, WT mice (phenotypically nonmutant mice) from the litter should be used as controls.
5. If the study involves transplanting live or freeze-dried allograft (e.g., for small molecule or growth factor delivery [31]), then the donor and recipient mice should be from unrelated strains (e.g., C57BL/6 and BALB/c or other strains).
6. To standardize the neutral position, at this point the toes should be passively extended by the examiner and allowed to return to the unloaded position.
7. Each increment of weights should be suspended for 30 s before the digital images are taken to avoid creep effects.
8. This should be done by at least two observers blinded to the treatment. To better standardize the measurements, spherical beads may be optionally affixed to the skin to define the edges of the metatarsals and the proximal phalanx, which define the MTP joint flexion angle.

9. From this point forward, the tendon must be kept moist and hydrated, as excessive dryness will have profound effects on the biomechanical properties.
10. While testing in room air and keeping the tissue hydrated is an acceptable protocol, it is preferred to perform these biomechanical tests on the tissue in a physiologic saline bath.

[AU1]

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Adhesions in a Murine Flexor Tendon Graft Model: Autograft versus Allograft Reconstruction

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ABSTRACT: Reconstruction of flexor tendons often results in adhesions that compromise joint flexion. Little is known about the factors involved in the formation of flexor tendon graft adhesions. In this study, we developed and characterized a novel mouse model of flexor digitorum longus (FDL) tendon reconstruction with live autografts or reconstituted freeze-dried allografts. Grafted tendons were evaluated at multiple time points up to 84 days post-reconstruction. To assess the flexion range of the metatarsophalangeal joint, we developed a quantitative outcome measure proportional to the resistance to tendon gliding due to adhesions, which we termed the Gliding Coefficient. At 14 days post-grafting, the Gliding Coefficient was 29- and 26-fold greater than normal FDL tendon for both autografts and allografts, respectively ($p < 0.001$), and subsequently doubled for 28-day autografts. Interestingly, there were no significant differences in maximum tensile force or stiffness between live autograft and freeze-dried allograft repairs over time. Histologically, autograft healing was characterized by extensive remodeling and exuberant scarring around both the ends and the body of the graft, whereas allograft scarring was abundant only near the graft–host junctions. Gene expression of GDF-5 and VEGF were significantly increased in 28-day autografts compared to allografts and to normal tendons. These results suggest that the biomechanical advantages for tendon reconstruction using live autografts over devitalized allografts are minimal. This mouse model can be useful in elucidating the molecular mechanisms in tendon repair and can aid in preliminary screening of molecular treatments of flexor tendon adhesions. J Orthop Res

Keywords: flexor tendon; allograft; autograft; adhesion; biomechanics

INTRODUCTION

Repair of injuries to flexor tendons is complicated by fibrotic adhesions that compromise tendon gliding and limit the range of joint flexion.[1] Adhesions are especially exacerbated in injuries involving flexor digitorum profundus (FDP) and flexor digitorum superficialis (FDS) tendons in Bunnell's "no man's land" or zone II of the hand, which to date remain unsolved clinical problems.[2,3] As an alternative to primary repair, which still represents the standard of care for these injuries,[3] surgeons often use a live tendon autograft especially when primary repair has been neglected or delayed because of infection, or in revision surgery when primary repair had failed.[4]

Unfortunately, flexor tendon grafting procedures also experience post-operative adhesions that limit joint flexion or cause joint contracture. The biological mechanisms of flexor tendon graft repair and adhesion formation are still poorly understood, despite being studied for decades. Adhesions following live autograft reconstruction are thought to arise through intrinsic fibrosis (as a result of suturing or surgical manipulation of the live tendon graft leading to tenocyte necrosis) or through extrinsic fibrosis (whenever the tendon sheath is disrupted leading to synovial and inflammatory cellular influx),[1,5] among other factors.[6–8] On the other hand, flexor tendon reconstruction with allograft tissue has been scarcely reported in the clinical literature, and has been limited to two-stage reconstruction procedures.[9] Few animal studies compared the mechanisms of healing of flexor tendon autografts and allografts. In a canine

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model of flexor tendon reconstruction, freeze-dried allografts have been reported to be tolerated well by the host and to allow flexor tendon function similar to autografts.[10] Others reported that acellular allografts induce minimal adhesion formation in bovine flexor tendons.[11,12] Despite these reports, the biological and biomechanical differences in flexor tendon autograft and allograft healing remain less well studied compared to primary repair, which has been extensively studied.

Based on the scarce evidence from the literature, we hypothesized that an acellular tendon allograft heals without the intrinsic fibrotic adhesions that are normally observed in a live autograft, which experiences excessive scarring. To test this hypothesis and to investigate differences in flexor tendon autograft and allograft repair, we developed a novel mouse model in which we repair a gap defect in the flexor digitorum longus (FDL) tendon of the hind limb with either a live autograft or an acellular, freeze-dried allograft and provide adequate immobilization to induce robust adhesion formation. In this study, we quantitatively examine the autograft and allograft gliding function and biomechanical strength. In addition, using histology and real-time RT-PCR, we examine aspects of cellular and molecular events involved in graft repair and subsequent adhesion formation.

MATERIALS AND METHODS

Processing of Freeze-Dried Allografts

FDL tendon allografts were harvested from donor C57BL/6 mice using aseptic technique and were snap frozen in liquid nitrogen before being placed in a freeze-drying chamber (FreeZone 2.5 Liter Benchtop Freeze Dry System, Labconco Corporation, Kansas City, MO). The tendons were lyophilized for 12 h, after which they were stored at -80°C until the day of surgery. Before grafting, the allografts were reconstituted with sterile saline for 30 min.

Surgical Procedures

Animal studies were approved by the University of Rochester Committee for Animal Resources. Eight-week-old female C57BL/6 mice were randomized into two experimental groups: live autografts and devitalized allografts. The mice were anesthetized with ketamine (60 mg/kg body weight) and Xylazine (4mg/kg body weight) via an intraperitoneal injection. Surgeries were preformed using aseptic technique under a 2× micro dissection magnifying lens. Briefly, a longitudinal plantar incision was made on the left hind foot. The distal FDL tendon of the mouse was isolated and transected on the plantar surface of the metatarsal bones. A 3-mm freeze-dried tendon allograft that has been reconstituted in saline or a freshly harvested live autograft was sutured between the ends of host tendon using an 8-0 nylon suture in a horizontal mattress suture pattern (similar to a modified Kessler technique) (Fig. 1). The tendon was then transected at the proximal musculotendinous junction to temporarily immobilize the flexor mechanism to protect against disruption of the tendon graft early during the repair period and to eliminate early tendon gliding to induce adhesion formation. The skin was closed with 4-0 silk suture. To eliminate favoring the nonoperated limb, the live autografts were harvested from the right limbs. Animals receiving the freeze-dried allografts also had the right FDL tendon transected similar to the autograft donor limb. Mice were sacrificed at 0, 14, 28, 42, 63, and 84 days postsurgery ($n = 9–12$ animals per group per time point) for assessment of the metatarsophalangeal (MTP) joint flexion and biomechanical evaluation. Additional mice were sacrificed at 14 and 28 days for histology ($n = 3$ per group per time point) and for assessment of gene expression by real-time RT-PCR ($n = 3$ per group per time point).

Assessment of Metatarsophalangeal Joint Flexion

To evaluate the range of MTP joint flexion, we developed a novel assay to quantify the resistance to flexion due to grafting and adhesion formation after FDL tendon reconstruction (Fig. 2). Immediately following sacrifice, the lower hind limb was disarticulated from the knee and the proximal FDL tendon along the tibia was released just proximal the tarsal tunnel without disrupting the skin at the ankle or foot. The proximal end of the tendon was then secured between two square pieces of tape using a thin layer of cyanoacrylate as previously described.[13] The lower hind limb was fixed in a custom apparatus where the tibia was rigidly gripped to prevent rotation (Fig. 2A). To standardize the neutral position, the toes were passively extended by the examiner and allowed to return to an unloaded position before a digital image was taken medially to determine the neutral position (zero load) of the MTP joint. The FDL tendon was incrementally loaded in the same anatomical direction as flexor muscle line of force. The loading was accomplished using dead weights (0–19 g) that were statically suspended from a hook and line passing through the proximal FDL tendon/tape composite. The dead weights were suspended for 30 s before the digital pictures were taken to avoid creep effects. With each increment of load, a digital image was taken to quantify the MTP flexion angle relative to the neutral position. The MTP joint flexion angles were measured from the digital images by 2 independent observers (S. H. and J. J.) using ImageJ software (http://rsb.info.nih.gov/ij/) and plotted versus the applied loads (Fig. 2B). Based on the flexion curve of the normal tendon, the flexion data were fitted to a single-phase exponential association equation of the form: $MTP\ Flexion\ Angle = \beta \times [1 - exp(-m/\alpha)]$ ($R^2 = 0.93 \pm 0.07$, $p < 0.05$);

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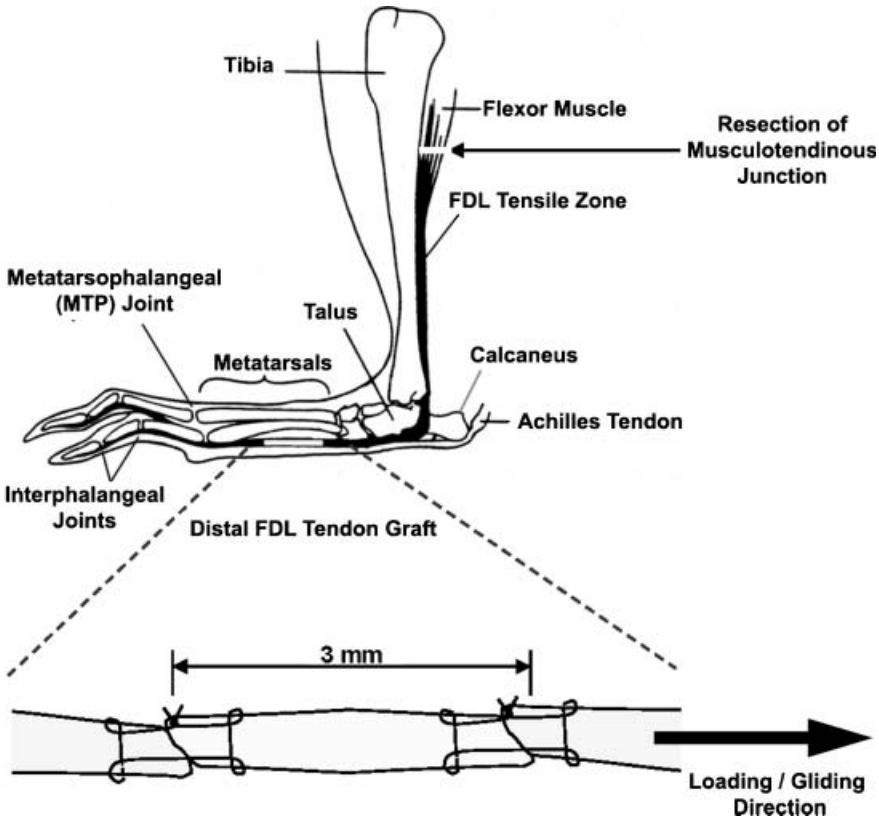

Figure 1. A schematic illustration of the live autograft or freeze-dried allograft reconstruction of the murine distal FDL tendon. The tendon is transected at the proximal musculotendinous junction to temporarily immobilize the flexor mechanism to protect against the disruption of the tendon graft and to stimulate adhesions.

where m is the applied load (Prism GraphPad 3.0, GraphPad Software, Inc., San Diego, CA). The curve fit was constrained to the maximum flexion angle (β) for normal tendons that was determined to be 75° for the maximum applied load of 19 g. The constant α governing the rate of rise of the flexion curve with increased loading was determined by nonlinear regression as a measure of the *resistance* to MTP joint flexion due to impaired gliding and therefore termed the *Gliding Coefficient*.

Biomechanical Testing

Following the adhesion test, the proximal extent of the FDL tendon at the myotendinous junction was identified then freed from surrounding tissue using blunt dissection along the length of the tendon to the tarsal tunnel. The tendon was then released at the tarsal tunnel, with dissection medially along the bone. Once the tendon was freed from the tunnel, the calcaneus was removed, freeing the proximal end of the tendon for direct gripping in the mechanical test as described by Mikic et al. (2001).[13] The distal tendon and graft interfaces were not disrupted or dissected since the mechanical testing involved direct gripping of the distal bones of the foot without disrupting the graft or the branching tendon insertion into the phalanges. The specimens were placed in sterile gauze soaked with saline to maintain adequate tissue hydration. The FDL tendon was then mounted on the Instron 8841 DynaMight™ axial servohydraulic testing system (Instron Corporation, Norwood, MA) using custom grips and tested following published protocols.[13] The tendon was loaded in tension in displacement control at a rate of 30 mm/min until failure. Force-displacement curves were plotted and the maximum tensile force and stiffness were determined. Failure modes were carefully observed and recorded.

Histology

Grafted limbs were harvested at 14 and 28 days post-surgery ($n=3$ autografts and $n=3$ allografts per time point) by disarticulating the intact foot and tibia at the knee joint. Tissues were prepared for histology using routine techniques. Briefly, the harvested lower limb were fixed in 10% neutral buffered formalin with the tibia at 90° relative to the foot, and then decalcified in 10% EDTA at 4°C for 28 days. The decalcified tissues were then dehydrated in a gradient of alcohols and then embedded en bloc in paraffin to preserve the anatomical relationship between the grafted tendon and surrounding tissues. The line of the FDL tendon was marked on the sole of the foot using

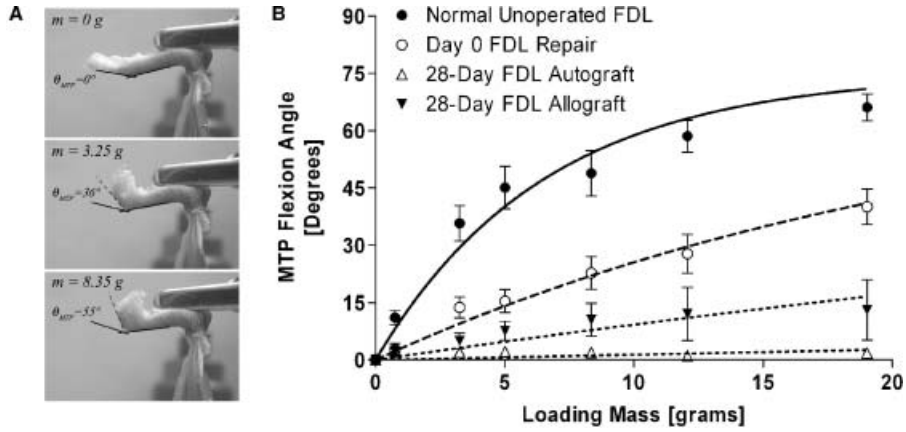

Figure 2. (A) Assessment of MTP joint flexion upon FDL tendon loading. The lower hind limb of the mouse was disarticulated from the knee, and the proximal FDL tendon was isolated and loaded incrementally using dead weights in the direction of the anatomical pull starting with a neutral unloaded position. At each load, a digital picture was taken. Subsequently, the MTP flexion angle was measured relative to the unloaded position. (B) Representative flexion curves (flexion angles vs. applied loads) of the MTP joint in normal (unoperated) and grafted FDL tendons (days 0 and 28 post-grafting). Discrete data points represent measured flexion angles (mean ± SEM). Lines represent best fit curves based on modeling the data using the single-phase exponential association equation $MTP\ Flexion\ Angle = \beta \times [1 - exp(-m/\alpha)]$, where m is the applied mass, β is the maximum flexion angle (75° for normal unoperated FDL tendons), and α is the *Gliding Coefficient*.

India ink with the aid of the reconstruction suture. Serial 3 μm sagittal sections through the FDL tendon plane were then cut, mounted on glass slides, and stained with Orange G and Alcian Blue.

Gene Expression Using Real-Time RT-PCR

RNA isolation and quantitative real-time RT-PCR were performed as briefly described.[14] Grafted tendons from mice sacrificed at 14 and 28 days postsurgery ($n = 3$ autografts and $n = 3$ allografts per time point) and age-matched normal unoperated tendons ($n = 3$) were harvested and immediately frozen in liquid nitrogen. Tendons from either group were pooled and minced by manual homogenization (mortar and pestle) and then flushed through a 22G needle with a syringe for further mechanical breakup of any remaining tissue. Total RNA was isolated using TRIZOL (Invitrogen Corporation, Carlsbad, CA). Single-stranded cDNA was made using a reverse transcription kit (AbGene Inc. USA, Rochester, NY) and used as a template for real-time PCR with SYBR Green PCR Master Mix (AbGene) and gene specific primers (Table 1) in a Rotor-Gene 2000 Real-Time DNA Detection System (Corbett Research, Sydney, Australia). The mean cycle threshold (Ct) values from quadruplicate measurements were used to calculate the gene expression standardized to β-actin expression as an internal control. Gene expression data were normalized and expressed as fold-increase or fold-decrease (mean ± SEM) relative the normal unoperated FDL tendon expression which was normalized to 1.

Statistical Analysis

Data analysis including 2-way Analysis of Variance with Bonferroni post hoc multiple comparisons ($\alpha = 0.05$) and the nonlinear regression analyses were performed using Prism GraphPad 4.0 statistical software. The Gliding Coefficient data were generated by fitting individual tendon flexion curves to the mathematical model using an algorithm in PRISM which utilizes the Marquardt method to minimize the sum of squares of errors between measured and modeled values over a range of α and β values. The goodness of fit was assessed using the R^2 value and by correlating the Gliding Coefficient data to the MTP joint ROM.

Table 1. Primer Sequences for Real-Time RT-PCR

Gene	Forward Primer	Reverse Primer
Tgfb1	5′-CTTTAGGAAGGACCTGGGTT-3′	5′-CAGGAGCGCACAATCATGTT-3′
Gdf5	5′-TCCTTCCTGCTGAAGAAGAACA-3′	5′-TAAAGCTGGTGATGGTGTTGGC-3′
Vegf	5′-TTCAGAGCGGAGAAAGCATT-3′	5′-GAGGAGGCTCCTTCCTGC-3′
Beta-actin	5′-AGATGTGGATCAGCAAGCAG-3′	5′-GCGCAAGTTAGGTTTTGTCA-3′

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RESULTS

Effects of Allograft Processing by Freeze-Drying

To determine the effects of freeze-drying on the mouse FDL tendon tensile biomechanical properties, FDL tendons were harvested from cadaver mice and tested biomechanically in tension either immediately without freezing, after a single −20°C freeze–thaw cycle, after freeze-drying and reconstitution in PBS once, or after freeze-drying and reconstitution in PBS twice ($n = 6$ tendons per group). There were no significant changes in the mechanical properties (failure force, stiffness, and energy to failure) of the once or twice freeze-dried tendons compared to the fresh and fresh-frozen tendons (Fig. 3). When failure modes were examined, 58% of the once or twice freeze-dried tendons failed in the mid-substance whereas 50% of the fresh frozen tendons and 83% of the fresh tendons failed in this manner. The remaining tendons failed either near the insertion to the bone distally or at the proximal tendon-muscle insertion.

Assessment of Metatarsophalangeal Joint Flexion

Both autografts and allografts experienced significant reductions in the range of motion (ROM) of the MTP joint (defined as the flexion angle upon the application of the maximum excursion load of 19 g) (Figs. 2B and 4A). The MTP joint ROM for normal tendons was significantly greater than reconstructed tendons at all time points regardless of the graft type ($p < 0.001$). The ROM for the 14- and 28-day autografts and allografts were reduced compared to the other time points but these differences were not statistically significant. The autografts' MTP joint ROM at 14 and 28 days were only 60% and 40% of the corresponding allografts' ROM, respectively, although these differences were also not statistically significant. These results are consistent with the Gliding Coefficient data. At 14 days post-grafting, the Gliding Coefficient was 29- and 26-fold greater than normal FDL tendon ($n = 8$) for both autografts ($n = 12$) and allografts ($n = 12$), respectively ($p < 0.001$). However, there was no significant difference between autograft and allograft Gliding Coefficients. At 28 days post-grafting, the Gliding Coefficient of the autografts ($n = 9$) was 83-fold ($p < 0.001$) greater that normal tendon ($n = 8$). By contrast, the Gliding Coefficient for freeze-dried allograft tendon ($n = 10$) was increased 16-fold compared to normal tendon, and was 5-fold less than the Gliding Coefficient measured in the autograft tendons ($p < 0.001$). By 42 days and thereafter, the Gliding Coefficients significantly decreased in both groups but remained somewhat higher than normal unoperated FDL tendons (Fig. 4B). Figure 4C shows a strong negative correlation (Spearman's $r = -0.0975$, $p < 0.0001$) between the empirically determined Gliding Coefficient and the measured range of MTP joint flexion, which corroborates the validity of the Gliding Coefficient as quantitative measure of the resistance to joint flexion. Interestingly, simply transplanting a graft and then evaluating the MTP joint flexion immediately after surgery (Day 0, $n = 9$, Fig. 4B) shows that the Gliding Coefficient increases only by twofolds compared to normal FDL tendon, which could be a result of the suture interfering with the gliding of the FDL graft, the enlargement of the graft/host junctures, or skin tightening when the incision was closed.

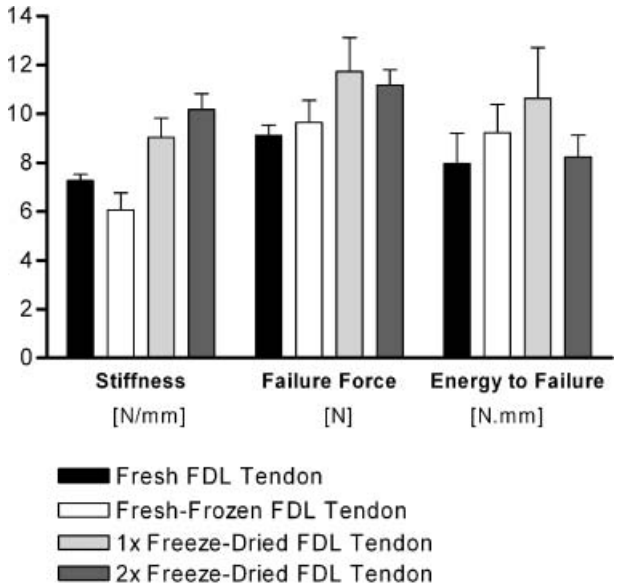

Figure 3. Effects of freeze-drying on the mouse FDL tendon tensile biomechanical properties. FDL tendons were harvested from cadaver mice and tested biomechanically either immediately without freezing (Fresh), after a single −20°C freeze–thaw cycle (Fresh-Frozen), after being freeze-dried and reconstituted in PBS once (1× Freeze-Dried), or after being freeze-dried and reconstituted in PBS twice (2× Freeze-Dried). Data presented as mean ± SEM.

Biomechanical Properties of FDL Tendon Autografts and Allografts

Immediately following the assessment of the MTP joint flexion, the grafted tendons were harvested and tested biomechanically as described. We investigated whether the nondestructive assessment of the MTP joint flexion had any effects on

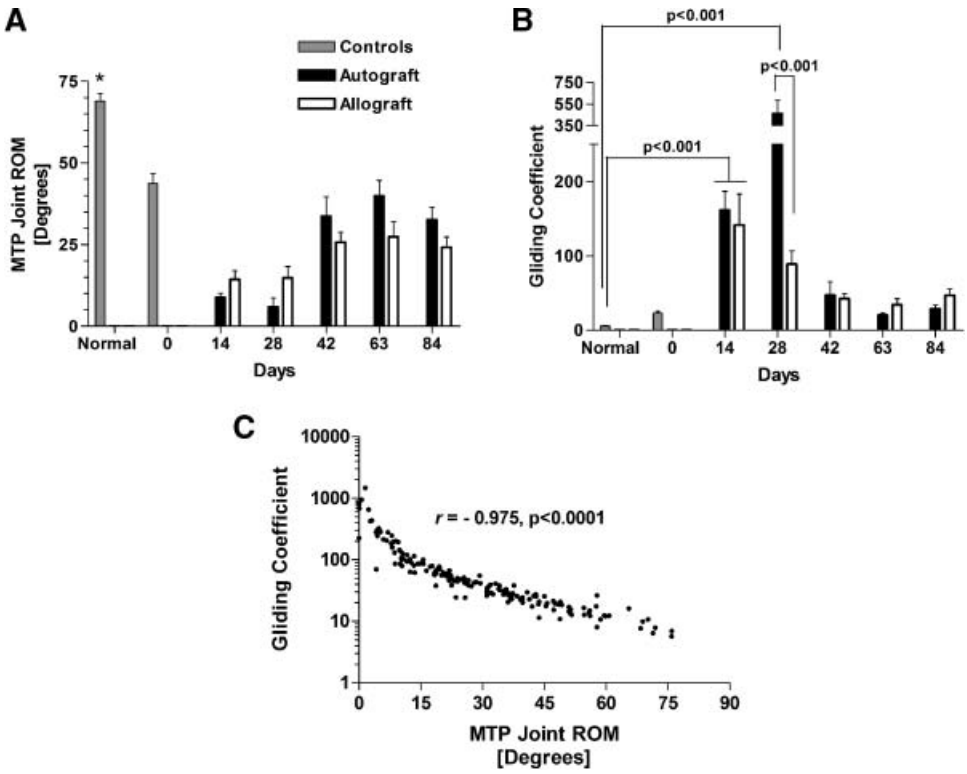

Figure 4. (A) MTP joint flexion ROM and (B) Gliding Coefficients of normal unoperated FDL tendons and FDL tendon autografts and allografts at multiple time points postgrafting (mean ± SEM). Asterisk indicates significant difference between normal and operated tendons ($p < 0.001$). (C) Correlation between the empirically determined Gliding Coefficient and the MTP range of flexion (Spearman's $r = -0.975$, $p < 0.0001$).

the measured tensile biomechanical properties of specimens harvested 28 days post-transplantation. The data in Table 2 demonstrated that the biomechanical properties of fresh autografts or freeze-dried allografts that were tested for MTP joint flexion were not significantly different from specimens that were not tested for MTP joint flexion assessment ($p > 0.05$).

More importantly, there were no significant differences in maximum tensile force or stiffness between live autograft and freeze-dried allograft repairs at any time point up to 84 days post-transplantation (Table 3). While there were mild improvements over time in the tensile strength (as indicated by the maximum tensile force at failure), both autograft and allograft repairs remained less than 50% of the strength of normal FDL tendon. The stiffness for both the autograft and allograft repairs significantly increased over time reaching 75%–90% of the stiffness of normal unoperated FDL tendon. On average, 87% of the autografts failed at the proximal repair site compared to 74% of allografts. The remainder of the tendon grafts failed either at the distal repair site or in the graft itself with the latter being a rare incidence (~3%).

Table 2. Effects of the Nondestructive Assessment of the MTP Joint Flexion on the Tensile Biomechanical Properties of 28-Day FDL Autografts and Allografts[a]

	Maximum Force (N)		Stiffness (N/mm)	
	Autograft	Allograft	Autograft	Allograft
No MTP joint flexion test	1.51 ± 0.23	1.43 ± 0.33	1.33 ± 0.16	1.07 ± 0.18
After MTP joint flexion test	2.19 ± 0.32	2.24 ± 0.31	1.62 ± 0.22	1.58 ± 0.20

[a]Mean ± SEM; $n = 5$ per group.

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Table 3. Tensile Biomechanical Properties of Autografts and Allografts (Mean ± SEM) over Time following FDL Tendon Reconstruction

Normal	Maximum Force (N) 9.71 ± 0.15*		Stiffness (N/mm) 5.14 ± 0.26**	
	Autograft	Allograft	Autograft	Allograft
Day 0	0.80 ± 0.14^{a}	0.45 ± 0.09^{a}	0.36 ± 0.08^{I}	0.24 ± 0.04^{I}
Day 14	$1.54 \pm 0.14^{a,b}$	1.67 ± 0.15^{b}	1.60 ± 0.19^{II}	1.46 ± 0.17^{II}
Day 28	1.79 ± 0.20^{b}	1.83 ± 0.25^{b}	1.45 ± 0.13^{II}	1.33 ± 0.15^{II}
Day 42	3.25 ± 0.26^{c}	2.84 ± 0.21^{c}	3.50 ± 0.30^{III}	3.33 ± 0.19^{III}
Day 63	3.73 ± 0.32^{c}	3.17 ± 0.26^{c}	$3.89 \pm 0.30^{III,VI}$	3.90 ± 0.37^{III}
Day 84	3.56 ± 0.37^{c}	4.17 ± 0.31^{d}	4.60 ± 0.43^{IV}	3.83 ± 0.42^{III}

*$p < 0.001$, compared to autograft and allograft repairs at all time points.
**$p < 0.05$, compared to autograft and allograft repairs at days 0, 14, 28, and 42.
$^{a < b < c < d}$($p < 0.05$).
$^{I < II < III < IV}$($p < 0.05$).

Histology of FDL Tendon Autografts and Allografts

To examine the potential biological mechanisms responsible for the observed reduction in the tendon gliding function at 14 and 28 days post-surgery, and the subsequent restoration of the gliding function at 42 days post-grafting, tendon autografts and allografts were assessed histologically. These analyses revealed that, at 14 and 28 days, the host junctions of both autograft and allograft were surrounded by similar amounts of hypercellular fibrotic scar tissue, and appeared enlarged relative to the body of the graft proper (Fig. 5). However, remarkable differences between autograft and allograft healing were manifested by the amount of fibrotic scar tissue surrounding the middle segments of the grafts (Fig. 6). While autografts were encased by this tissue that appeared to be invading the tendon (Fig. 6A, B), the middle segment of the allografts was largely unaffected by the host and remained mostly acellular at 14 and 28 days post-grafting (Fig. 6D, E). These differences were less profound by day 42 (Fig. 6C, F). These distinct modes of repair are

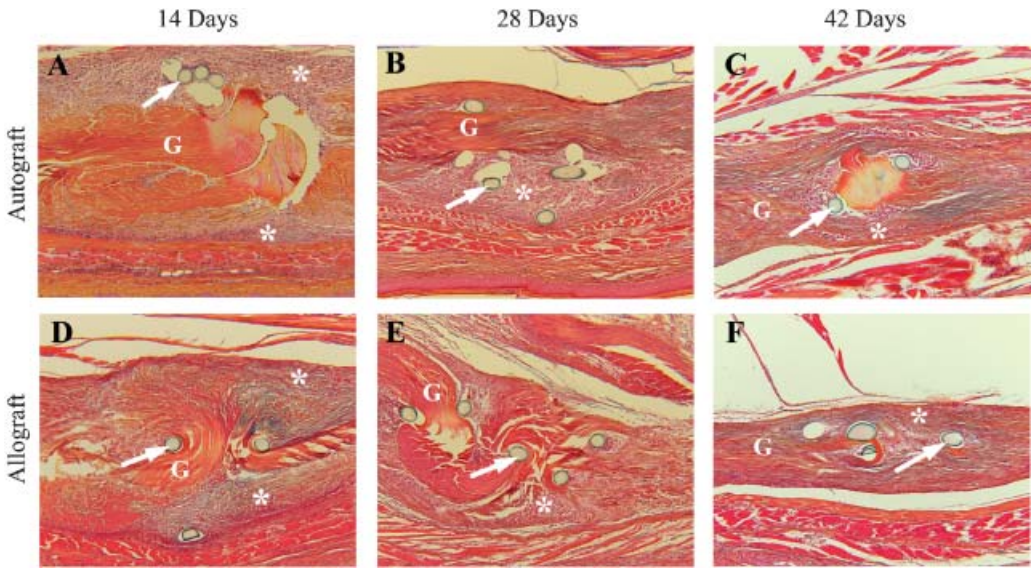

Figure 5. Representative histologic sections of the proximal host–graft junction of the FDL tendon autografts (A–C) and allografts (D–F) at 14, 28, and 42 days postsurgery. Sections were stained with Orange G/Alcian Blue (10×). Of note is that both 14-day and 28-day autograft (A, B) and allograft (D, E) ends adjacent to the suture (arrows) are surrounded by similar amounts of hypercellular fibrotic scar tissue (*) and appear enlarged relative to the body of the graft proper (marked as G). By day 42, the amount of scarring and the enlargement at the graft–host junction are reduced for both autografts (C) and allografts (F).

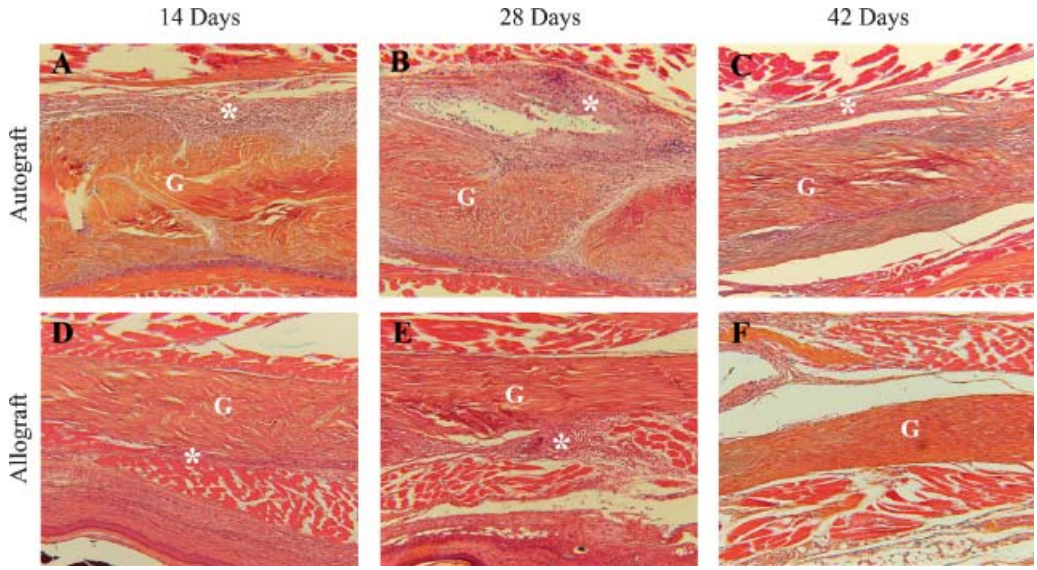

Figure 6. Representative histologic sections of the middle segment of the FDL tendon autografts (A–C) and allografts (D–F) at 14, 28, and 42 days post-surgery. Sections were stained with Orange G/Alcian Blue (10×). Of note are the remarkable differences in the amount of the hypercellular fibrotic scar (*) surrounding 14-day and 28-day autografts (A, B) that appears to be minimal around the acellular allografts (C, D). By 42 days, the scar tissue appears to have significantly remodeled in both autografts (E) and allografts (F). Graft tissue is marked G.

consistent with the differences in the Gliding Coefficients between the grafts at 28 days, and suggest increased adhesions in the autografts at this time, which resolve with subsequent remodeling.

Gene Expression in FDL Tendon Autografts and Allografts

Since it is known that TGF-β1, GDF-5, and VEGF-A are potent growth factors that stimulate vascular invasion, fibrosis and tenocyte differentiation, we assessed their mRNA expression levels in grafted FDL tendons at 14 and 28 days post-surgery, corresponding to the maximum observed reductions in tendon gliding functions. Consistent with the robust intrinsic healing response of the live autografts, *Gdf5*, and *Vegfa* expression levels in autografts were significantly upregulated in 28-day autografts by 7- and 20-fold respectively, compared to normal unoperated tendon ($p < 0.05$), but the *Tgfb1*expression levels were not increased. In contrast, *Tgfb1*, *Gdf5*, and *Vegfa* expression levels were doubled in 28-day allografts compared to normal unoperated tendon controls, although these differences were not significant (Fig. 7).

DISCUSSION

The development of comparative animal models to study the biomechanical and biological factors involved in flexor tendon adhesions is important for advancing our understanding of this debilitating problem and for designing therapeutic and rehabilitation treatment programs. A number of elegant studies in multiple human and animal models have identified passive controlled gliding motion as the most important factor in reducing the risk of adhesion formation.[3,15–18] Other studies have focused on molecular treatment of the flexor tendon injury to provide adhesion-free healing via the delivery of anti-scarring adjuvants that inhibit the effects of TGF-β and bFGF among other factors.[19–23] Despite their promise, these approaches remain experimental and have yet to yield a clinical application,[3] largely because our understanding of the molecular mechanisms involved in the formation of adhesions after flexor tendon injury and grafting remains incomplete.

The novel mouse model of FDL tendon grafts offers a quantitative tool to not only examine the biomechanical aspects of flexor tendon grafts, but also to potentially elucidate the molecular events involved in repair and subsequent adhesion formation via the use of transgenic mouse models of gain and loss of function. However, this model has a number of inherent limitations. The mouse model is admittedly challenging due to the small size of the FDL tendon, which requires microsurgical reconstruction under magnifying lens, however the reproducibility of the data in our study strongly

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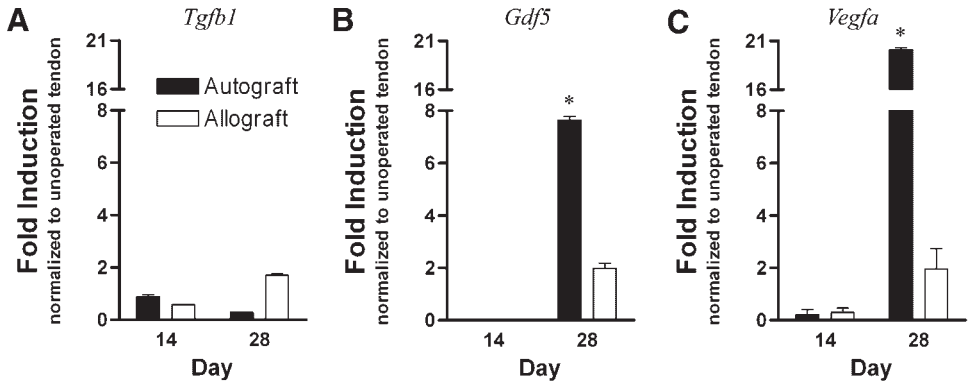

Figure 7. Gene expression of (A) *Tgfb1*, (B) *Gdf5*, and (C) *Vegfa* in FDL tendon autografts and allografts at 14 and 28 days postgrafting. Total RNA was extracted and pooled from three tendon grafts and processed for real-time RT-PCR. Gene expression was standardized with the internal beta-actin control and then normalized by the level of expression in normal unoperated FDL tendon. Data presented as the mean fold induction (over normal unoperated tendon) ± SEM. * $p < 0.05$ versus normal unoperated tendon.

supports the feasibility of this model. In addition, larger animal models (e.g., canine) that more closely resemble the size and anatomy of human flexor tendons allow for testing the effects of passive motion/loading protocols in reducing adhesions,[6,24,25] which we were unable to reproduce in this small animal model for obvious technical reasons. Furthermore, while the mouse FDL tendon graft model does not represent a *true* zone II reconstruction model, we deliberately immobilized the flexor mechanism by severing the proximal FDL tendon insertion in the flexor muscle to abolish early tendon gliding. This resulted in impairment of MTP joint flexion via mechanisms that have the hallmarks of adhesions including histological evidence of fibrotic scar tissue especially around the live autografts similar to those observed in larger animals such as dogs which have been used for years in flexor tendon repair research.

To quantify the effects of adhesions on the biomechanics of the flexor mechanism in our murine FDL tendon model, we developed an innovative Gliding Coefficient as a measure of the resistance to tendon gliding and MTP joint flexion. Since we did not measure the MTP joint flexion angle by another method that would constitute a golden standard against which to assess the accuracy of the measurements, we computed the intra- and inter-observer reproducibility of the joint angle measurements and determined that the average intra- and inter-observer errors were $<1\%$ which provides confidence about the reproducibility of the MTP joint flexion angle measurements. Furthermore, the MTP joint flexion test is nondestructive and allows for subsequent biomechanical testing of the grafts since the maximum applied excursion load of 19 g was about 10% of the failure force of the 14-day grafts which was the earliest healing time point we tested.

As a measure of adhesions, previous studies have reported the digital range of motion upon the application of a single defined load to cause tendon excursion.[21,24] By contrast, the Gliding Coefficient is based on joint flexion data over a range of applied loads that would cause a maximum 75° flexion in a normal unoperated MTP joint. The test offers information about the joint ROM (the plateau) and the resistance to flexion with increased loading (the gliding coefficient). The Gliding Coefficient is similar to the Work of Flexion which measures the resistance to flexion over a range of applied excursions. The work of flexion test is feasible in larger animal models that allow flexion testing under displacement control without the risk of damaging the graft. However, due to the small size of the tendon and low levels of force required to effect flexion, our test was conducted under load control to ensure that we do not induce loading that would be damaging to the tendons. There are other advantages to using the Gliding Coefficient rather than reporting a single joint flexion angle. First, if the "single" flexion angle or ROM is incorrectly reported due to measurement error or due to an error in the determination of the neutral position, it would be difficult to observe this error as an outlier. Instead, by recording and plotting the flexion angle over a range of applied loads, and computing the Gliding Coefficient based on the mathematical model as a "rate" constant for joint flexion under controlled loading, we can easily identify those measurements that deviate from the model and

provide erroneous estimation of the joint function. Since this is a novel measure to assess the resistance to joint flexion under load control, we examined the correlation between the gliding coefficient (GC) and the maximum MTP joint flexion angle range of motion (ROM) and reported a strong negative correlation ($r = -0.97$) which corroborates the GC as a measure of the resistance to joint flexion sensitive to the effects of adhesions and less prone to the effects of errors inherent in measuring a single angle as the ROM.

As hypothesized, at 14 and 28 days post-grafting, both live autografts and reconstituted freeze-dried allografts had significantly greater Gliding Coefficients and hence more adhesions than normal unoperated tendons or time zero repairs. Interestingly, by 42 days post-operatively and thereafter, the Gliding Coefficient was not different than time zero repairs for both autografts and allografts. Histologically, the amount of fibrotic tissue surrounding the 28-day autografts and allografts is markedly reduced by 42 days. There are two possible explanations for these improvements. First, the noted improvement in joint flexion may be a result of the resumption of tendon excursion after the proximal tendon-muscle insertion had been allowed sufficient time to heal and restore the flexor mechanism. This theory is based on the anecdotal observation that the mice more actively used their operated limbs by 28 days. Previous studies have suggested that small flexor tendon excursions following injury may be sufficient for full restoration of the flexion range of motion.[24] Regardless, this feature of our model is different than the clinical experience which suggests that the onset of fibrotic adhesions does not resolve spontaneously and might require meticulous tenolysis surgery.[3] Second, we hypothesized that the marked increase in the expression of *Gdf5* and *Vegfa* mRNA might be involved in the improvements in joint flexion after 28 days. Whether this increased mRNA expression translates into increased GDF5 and VEGF protein synthesis at the repair site after 28 days remains to be verified in future experiments using immunohistochemistry.

In agreement with the limited data in the literature, we found that reconstituted freeze-dried allografts did not cause increased adhesions compared to live autografts. To the contrary, 28-day live autografts experienced a significant fivefold increase in their Gliding Coefficients compared to the processed allografts. Previous studies compared the healing of flexor tendon autografts and freeze-dried allografts implanted in the paws of dogs and reported that: 1) the implanted allografts were tolerated well by the host; and 2) the implanted allografts allowed flexor tendon function similar to that allowed by autografts.[10] Others reported similar observation in bovine flexor tendons suggesting that acellular allografts induced minimal adhesion formation.[11,12] It has been recognized for quite some time that even minor manipulations of a live tendon graft such as the passing of a suture through the tendon induces an "intrinsic" inflammatory stimulation of the resident cells. Since freeze-dried allografts are acellular, the intrinsic inflammatory response is not expected. It is conceivable, therefore, that autograft transplantation may exacerbate the adhesion tissues, presumably resulting from the surgical manipulation of a live graft that might lead to inflammatory stimulation of tenocyte proliferation and migration from the live graft, in addition to other intrinsic and extrinsic factors.[5]

A number of factors may have been responsible for the observation that murine FDL tendon allografts and autografts were similar in terms of their failure tensile properties, but remained significantly weaker than normal unoperated tendons despite modest increases over time. While both grafts initially provided a scaffold to bridge the experimental defect, the two grafts supposedly heal with different mechanisms. Live autografts likely heal via intrinsic and extrinsic mechanisms that involve the graft tenocytes as well as the influx of synovial fibroblasts, precursor cells, and inflammatory cells, respectively.[3,26] As a result, autografts underwent extensive remodeling that negatively affected the rate of accrual of biomechanical strength over time as has been reported for flexor tendon gap defects.[25] By contrast, the acellular allografts can only heal by extrinsic mechanisms. Potenza et al. demonstrated that extrinsic cells from the synovial capsule of the joint populated and contributed to the healing of lacerations within freeze-dried allografts implanted in canine and rabbit knee joints.[27,28] In our model, we observed modest scarring around the mostly acellular middle segment of the allograft at 14 and 28 days that remained isolated resembling a foreign body response. However, at the interface with the host tendon stubs, hypercellular scarring was exuberant in bridging and remodeling the allograft–host juncture, resulting in cellular infiltration into the graft. While the allografts appeared to undergo little remodeling compared to live autografts, the accrual of biomechanical strength was still as slow as live autografts, possibly due to the localization of the repair response to the graft ends. In both groups, however, the abolishment of

tendon gliding and loading due to the deliberate severing of the proximal tendon-muscle insertion is likely the factor that slows return toward normal biomechanical properties. Therefore, clinical interpretations about the biomechanical equivalency of live autograft and freeze-dried allografts from this model should only be made with the limitations of the model in mind.

Admittedly, the clinical utility of freeze-dried (lyophilized) tendon allografts is debatable. There are clinical reports that suggest that freeze-dried allografts are of no significant value in the surgical management of certain indications such as chronic massive rotator cuff tears,[29] and may induce intraarticular reaction when used in ACL reconstruction,[30,31] for example. However, other clinical reports indicate that freeze-dried allografts used for ligament and tendon repairs and arthroscopic reconstruction of ACL deficient knees provide satisfactory clinical results.[32,33] Animal studies including our own data indicate that implanted freeze-dried tendon/ligament allografts are similar in (biomechanical) strength to live tendon/ligament autografts.[10] Furthermore, a freeze-dried animal or human tendon rehydrates easily before surgical implantation without adverse effects on their biomechanical properties as we (Fig. 3) and others have reported.[34]

The patterns of growth factor gene expression have been previously described in flexor tendon healing,[35–37] but not for autograft and allograft flexor tendon reconstruction models. In our study, we evaluated the expression of *Tgfb1*, *Gdf5*, and *Vegfa* transcripts on days 14 and 28 postsurgery, which corresponded to the earliest time point where adhesions were observed. We found a twofold increase in these transcript levels (compared to normal tendon) in 28-day allografts. By contrast, the level of *Tgfb1*mRNA expression was not upregulated in the live autografts at either 14 or 28 days. It is quite possible that the upregulation in *Tgfb1*expression might have been an earlier event in the repair response of autografts and allografts since previous studies suggested that *Tgfb1*mRNA levels are nearly 3.5-fold increased in a rabbit flexor tendon healing model as early as 3 days and remain upregulated through 12 days of healing before returning to normal levels at 24 days.[35] Interestingly, we observed that the level of expression of *Vegfa* was increased by 20-fold in the autografts at 28 days. These observations are somewhat similar to reports that demonstrated that *Vegfa* mRNA levels more than double at 7 and 10 days of healing following canine flexor tendon injury.[36,37] The differences in the levels and temporal kinetics of *Vegfa* upregulation maybe related to the relative size of the graft compared to primary healing. While local and direct GDF-5 protein delivery on collagen sponge implants has been shown to increase the tensile strength of rat Achilles tendon repair tissues,[38] to the best of our knowledge, the effects of this growth factor on flexor tendon adhesion formation are unknown. In our model, we observed that *Gdf5* mRNA levels were sevenfold increased in 28-day autografts, which experienced the highest levels of adhesions. This increase in *Gdf5* transcription was concomitant with the observed increases in VEGF expression. This observation is consistent with recent reports that suggest that GDF-5 promotes angiogenic activity of stromal cells by increasing VEGF gene expression in vitro.[39] How GDF-5 and VEGF might be implicated in the observed adhesions in our murine model of flexor tendon grafts remains to be carefully evaluated.

In conclusion, we developed the first murine model of flexor tendon grafts along with an innovative outcome measure for the quantitative assessment of joint flexion function. Despite its limitations, our model has the potential to enable systematic testing of the cellular and molecular events involved in repair and adhesion formation through the utilization of transgenic mouse models in future studies. Furthermore, the model can potentially aid in rapid and inexpensive screening of novel molecular treatments of flexor tendon adhesions.

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Freeze-dried Tendon Allografts as Tissue-engineering Scaffolds for Gdf5 Gene Delivery

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Tendon reconstruction using grafts often results in adhesions that limit joint flexion. These adhesions are precipitated by inflammation, fibrosis, and the paucity of tendon differentiation signals during healing. In order to study this problem, we developed a mouse model in which the flexor digitorum longus (FDL) tendon is reconstructed using a live autograft or a freeze-dried allograft, and identified growth and differentiation factor 5 (*Gdf5*) as a therapeutic target. In this study we have investigated the potential of rAAV-*Gdf5*-loaded freeze-dried tendon allografts as "therapeutically endowed" tissue-engineering scaffolds to reduce adhesions. In reporter gene studies we have demonstrated that recombinant adeno-associated virus (rAAV)-loaded tendon allografts mediate efficient transduction of adjacent soft tissues, with expression peaking at 7 days. We have also demonstrated that the rAAV-*Gdf5* vector significantly accelerates wound healing in an *in vitro* fibroblast scratch model and, when loaded onto freeze-dried FDL tendon allografts, improves the metatarsophalangeal (MTP) joint flexion to a significantly greater extent than the rAAV-*lacZ* controls do. Collectively, our data demonstrate the feasibility and efficacy of therapeutic tendon allograft processing as a novel paradigm in tissue engineering in order to address difficult clinical problems such as tendon adhesions.

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INTRODUCTION

Successful repair of ruptured flexor tendons, as measured by restoration of digital flexion function, is a great challenge to hand surgeons because the biological cascade of events during healing often causes the tendon proper to adhere indiscriminately to its surrounding tissue.[1] Clinical and experimental observations suggest that formation of adhesions is precipitated by injury to the tendon sheath, surgical manipulation, and immobilization.[2,3] This problem is most challenging with regard to tendon injuries in the "no-man's land" or Zone II. In the past, these were left unrepaired because of the poor prognosis associated with these injuries.[4] As an alternative to primary repair, the transplantation of a tendon graft allows the surgeon to place the graft junctions outside of the confines of the flexor sheath in zone II, where they can be attached distally in Zone I (where no gliding motion takes place) and proximally in Zone III, to the flexor digitorum profundus tendon. However, even simple surgical manipulation of live flexor tendon grafts can result in cellular necrosis and inflammation, leading to adhesion.[4] Therefore, devitalized structures such as freeze-dried tendon allografts or tissue-engineered biomaterial scaffolds are potentially attractive alternatives to live autografts in reconstructing the digital flexor mechanism.

Current tissue engineering strategies using synthetic biomaterial scaffolds have yet to yield clinically usable tendon substitutes. The appeal of these engineered scaffolds is that they can potentially be impregnated with growth factors or genes for targeted and timed release at the site of implantation in order to improve healing. However, many of these "manufactured" scaffolds do not match the mechanical strength of native tissue necessary for the expeditious restoration of function, and they do not remodel in response to daily activity; rather, they break down, producing byproducts that induce inflammation and compromise the repair process.[5] As an alternative, naturally derived materials processed from animal tissue or produced using recombination technology may be better tolerated when implanted. Arguably, the most suitable choice for a naturally derived biomaterial scaffold for tendon tissue engineering would be one that is derived from "allogeneic" tendon tissue. Such scaffolds must meet several functional criteria. As aptly described by Whitlock *et al*., a naturally derived biomaterial scaffold from tendon tissue must be "amenable to host cell-mediated remodeling", "devoid of cellular material to minimize inflammatory potential", and "distinguished by sufficient biomechanical integrity".[5]

The first two authors contributed equally to this work.

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In order to test this concept in a pre-clinical model of tendon adhesion, we recently developed a mouse distal flexor digitorum longus (FDL) tendon-grafting model in which a 3-mm intercalary live autograft or a freeze-dried allograft is implanted.[6] We demonstrated that both autografts and allografts led to significant reductions in the range of motion of the metatarsophalangeal (MTP) joint at 14 and 28 days, which resolved 42 days after surgery. Interestingly, we also observed that the gene expression of the growth and differentiation factor 5 (*Gdf5*) was significantly increased in 28-day grafts, thereby implying that this factor plays a role in the remodeling that leads to the functional improvements observed thereafter. We were intrigued by this observation, because GDF-5 deficiency in mice significantly delays the healing of the Achilles tendon (AT)[7] and adenovirus-mediated *Gdf5* gene expression in the treatment of AT injuries in rats leads to increased strength.[8] On the basis of these findings, and in view of our previous success with recombinant adeno-associated virus (rAAV)-coated cortical bone grafts,[9,10] we hypothesized that the loading of freeze-dried mouse FDL tendon allografts with rAAV expressing *Gdf5* gene would improve the functional properties of the reconstructed tendon and abolish the fibrotic adhesions. On the basis of the data from this study, we report that the remarkable hydrophilic capacity of freeze-dried tendon allografts can indeed be exploited for efficient loading of gene delivery vectors such as rAAV-*Gdf5* to improve the functional outcome of flexor tendon reconstruction.

RESULTS

Processing freeze-dried FDL tendon allografts as gene-delivery scaffolds

We have previously shown that two freeze-drying cycles produce no adverse effects on the mechanical properties of the tendon grafts.[6] Pairs of freeze-dried FDL tendon grafts were reconstituted in a buffer containing 5×10^9 U of rAAV-*lacZ*, and freeze-dried again. Using polymerase chain reaction (PCR) analysis, the rAAV retention efficiency was determined to be ~10% ($2.61 \times 10^8 \pm 1.44 \times 10^8$ genomes per graft, mean value ± SD; see **Supplementary Figure S1**). In order to assess the efficacy of cell transduction *in vitro*, rAAV-*lacZ*-loaded FDL grafts were individually placed in culture wells containing 293 human embryonic kidney cells (**Figure 1a**). X-gal staining showed that large numbers of cells were transduced after a 48-hour incubation with the rAAV-*lacZ*-loaded FDL grafts (**Figure 1b**), whereas a control culture incubated with unloaded FDL grafts was negative for transduction (not shown). Furthermore, the random and attenuated β-galactosidase expression in regions of the wells not proximate to the grafts indicates that the transduction depends on diffusion of the virus after rehydration (**Figure 1c**). In order to assess the transduction efficacy of rAAV-loaded FDL grafts *in vivo*, freeze-dried FDL tendon allografts loaded with rAAV-*lacZ* were implanted in mouse intercalary FDL tendon defects, as previously described.[6] The mice were killed at 7 or 14 days, and the grafted tissues were removed and fixed, paraffin-embedded, and processed for immunohistochemistry with antibodies specific to β-galactosidase. Although the grafts remained mostly acellular, they were surrounded by exuberant hypercellular fibrotic tissue that exhibited intense staining specific for β-galactosidase, thereby suggesting that the host cells in the peripheral tissue were transduced by the rAAV-*lacZ* vector as it slowly diffused out of the implanted graft, with more intense staining on day 7 than on day 14 (**Figure 1d** and **e**).

Kinetics and biodistribution of tendon allograft–mediated gene delivery

We next set out to determine the kinetics and biodistribution of the tendon allograft–mediated gene delivery *in vivo*. FDL

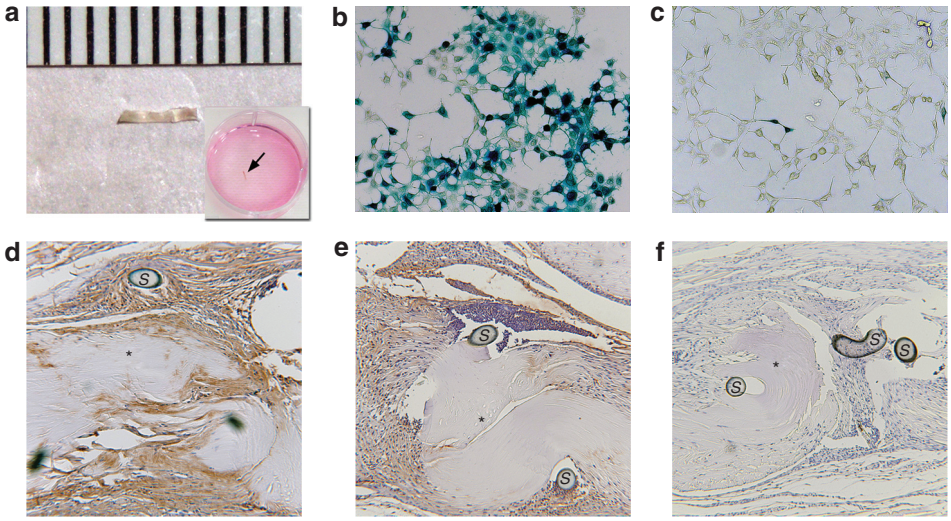

Figure 1 Transduction efficacy of freeze-dried tendon grafts *in vitro* and *in vivo*. (**a**) 3-mm Freeze-dried mouse flexor digitorum longus (FDL) tendon allografts were loaded with 5×10^9 transducing units of rAAV-*lacZ*, and incubated on a confluent monolayer of human embryonic kidney 293 cells for 48 hours (arrow). Representative micrographs of X-gal stained cultures show (**b**) large numbers of LacZ+ cells proximal to the graft, and (**c**) sparse staining in peripheral fields away from the graft. rAAV-*lacZ* loaded FDL allografts were also transplanted into FDL tendon defects of mice ($n = 4$). Representative micrographs of one end of the rAAV-*lacZ* loaded FDL allografts stained with antibodies against β-galactosidase at (**d**) 7 days after transplantation and (**e**) 14 days after transplantation. It is important to note the lack of viable cells and absence of any staining in the freeze-dried allografts (asterisks) that are surrounded by hypercellular and intensely stained fibrotic tissue. (**c**) The specificity of the staining was verified by the absence of non-specific staining in negative controls (**f**, secondary antibody only). *S* indicates remnants of the repair suture. rAAV, recombinant adeno-associated virus.

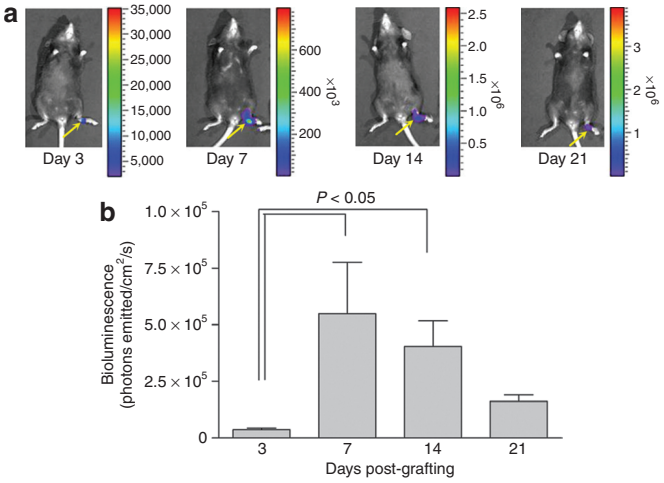

Figure 2 Kinetics and biodistribution of recombinant adeno-associated virus (rAAV)-mediated transduction through the use of processed tendon allografts. (**a**) Temporal bioluminescence images (BLIs) of a representative mouse grafted with a freeze-dried flexor digitorum longus tendon allograft loaded with rAAV-Luc, recorded over 21 days, show the localized biodistribution of rAAV-Luc transduction (heat map-yellow arrows) at the site of allograft implantation in the hind foot. (**b**) Kinetics of *in vivo* rAAV transduction, based on average BLI signal intensity, computed from measurements of total integrated light signal (photons emitted/cm²/s) emitted from a standardized region of interest in a standard 3-minute time interval (mean value ± SEM; $n = 4$).

tendon allografts (3 mm in length) were freeze-dried, reconstituted in a phosphate-buffered saline solution in a vial containing 5×10^9 particles of rAAV-*Luc*, and freeze-dried again pending surgical implantation in FDL tendon defects. In order to assess transduction *in vivo* over time, the mice were imaged on days 3, 7, 14, and 21 after grafting ($n = 4$ mice) using a real-time bioluminescent imaging system. As hypothesized, the only detectable bioluminescent imaging signal was localized to the site of tendon grafting, further supporting the efficacy of targeted gene delivery using processed tendon allografts. Furthermore, the transduction was transient; the bioluminescent imaging signal peaked at day 7 but persisted, albeit at declining levels, up to 21 days after implantation (**Figure 2b**). More sensitive analysis (*e.g.*, PCR) will be needed, however, to determine the reporter gene biodistribution in distant tissues and organs.

Functional verification of rAAV-*Gdf5*

The complementary DNA for *Gdf5* was PCR-amplified and used for creating a plasmid (pAAV-*Gdf5*), which was then used in the production of the rAAV by a helper virus–free method, and purified as previously described.[10] For verifying the specificity of the vector, we performed reverse-transcribed PCR on pAAV-*Gdf5*-transfected human embryonic kidney 293 cells, and demonstrated the predicted 485-base pair PCR product (**Figure 3a**, top).

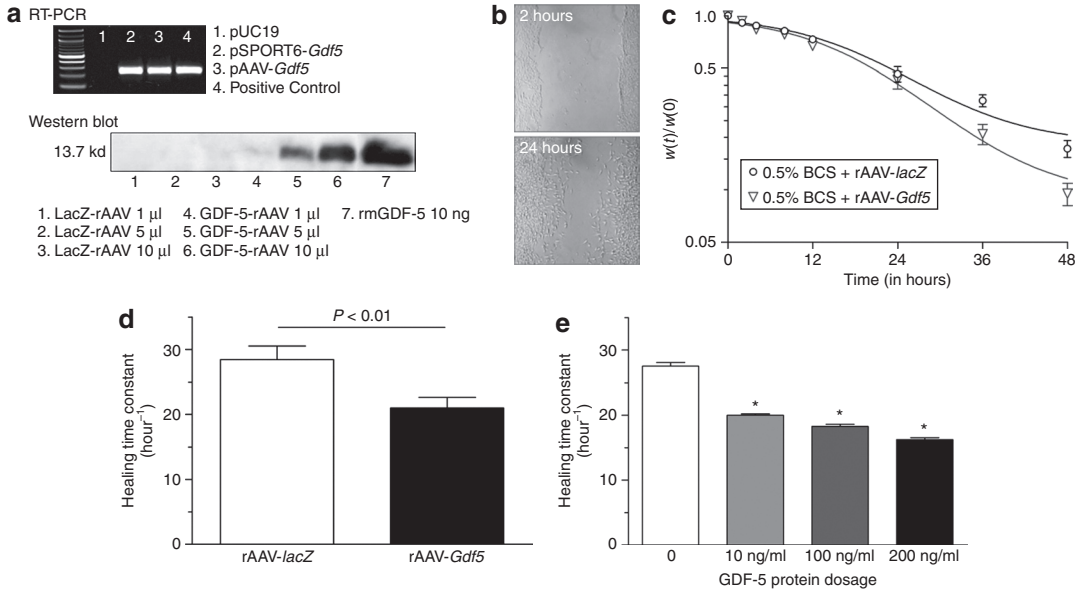

Figure 3 Functional verification of the rAAV-*Gdf5* vector. Human embryonic kidney 293 (HEK293) cells were grown in 6-well plates and transfected with: (1) pUC19, (2) pSPORT6-*Gdf5*, or (3) pAAV-*Gdf5*, and 48 hours later total RNA was harvested from the cells. The messenger RNA was reverse transcribed and used as the template for polymerase chain reaction (PCR) with *Gdf5*-specific primers. (4) The pSPORT-*Gdf5* plasmid was used as template in the positive control. (**a**, top) The ethidium bromide–stained agarose gel shows the predicted 485-base pair PCR product. HEK293 cells were grown in 6-well plates and infected with the indicated amount of rAAV-*lacZ* or rAAV-*Gdf5* (5.0×10^7 particles/ml). After 48 hours in culture, the supernatants were collected and 30 µl was used for Western blotting with GDF-5-specific antibodies. Ten nanograms of recombinant murine GDF-5 was used as a positive control. (**a**, bottom) Autoradiography of the Western blot reveals the predicted 13.7-kd GDF-5 protein. Microwound monolayer assay: (**b**) 80% confluent 3T3 cells were growth-arrested for 24 hours, and then microwounded by passing a pipette tip across the culture well and treated with 0.5% bovine calf serum (BCS) and 5.0×10^7 particles/ml of either rAAV-*lacZ* or rAAV-*Gdf5*. (**c**) The average width of the defect was digitally measured over time and the wound width normalized to the time zero width [$w(t)/w(0)$] versus time was plotted. (**d**) Healing time constants (τ) for the different treatments were computed and plotted as mean values ± SEM. Note that higher τ values indicate slower wound healing rates. (**e**) In a separate experiment, 3T3 cells grown to 80% confluence were microwounded and treated with 0.5% BCS and incremental doses of rmGDF-5. The data presented are mean values ± SEM for the healing time constant (τ) for the different doses of the GDF-5 protein treatments. Asterisks indicate significant differences ($P < 0.01$; $n = 6$ per treatment) compared to untreated controls. GDF-5, growth and differentiation factor 5.

Western blots on culture supernatants from rAAV-*Gdf5*-infected human embryonic kidney 293 cells also demonstrated the predicted 13.7-kd GDF-5 protein (**Figure 3a**, bottom). The effects of rAAV-*Gdf5* gene delivery were evaluated *in vitro* using a standard microwound monolayer assay (**Figure 3b**). These experiments demonstrated that the infection of NIH 3T3 cells with rAAV-*Gdf5* leads to accelerated wound healing when compared with the action of rAAV-*lacZ*-treated controls (**Figure 3c**). We further estimated the healing time constant and found significant differences between the healing rate associated with the rAAV-*Gdf5*-treated wells and that of the controls ($P < 0.05$; **Figure 3d**). It is likely that the effect of rAAV-*Gdf5* in this experiment was masked by the innate ability of the 3T3 cells to proliferate even under serum-deprived, control conditions. Real-time PCR analysis indicated that the accelerated microwound healing rates were attributable to significant early induction of Cyclin D1 and β1-integrin messenger RNA expression, thereby suggesting a synergistic proliferation and migratory effect of rAAV-*Gdf5* (data not shown). In parallel experiments, we treated microwound cultures of 3T3 cells with various concentrations of rmGFDF5 protein, and demonstrated a dose-dependent acceleration of healing with the treatment (**Figure 3e**). Interestingly, the effects of rAAV-*Gdf5* delivery on the microwound healing rate were comparable to the effects of bolus delivery of the GDF-5 protein to these cultures.

Gdf5-targeted Gene delivery for freeze-dried flexor tendon allografts

In order to investigate whether tendon allografts processed as delivery vehicles for therapeutic genes can reduce adhesions and improve the biomechanical properties of the grafted tendons, we performed experiments with FDL tendon allografts loaded with rAAV-*lacZ* (controls) or rAAV-*Gdf5* (treated) in our murine model. MTP flexion tests (See **Supplementary Figure S2**) demonstrated that rAAV-*Gdf5*-loaded allografts were associated with a significantly greater range of joint flexion and a lower gliding coefficient than the *lacZ* control ($P < 0.05$; **Figure 4**) at 14 and 28 days after surgery. The flexion function improved over time between 14 and 28 days after both treatments, but the improvement associated with the rAAV-*Gdf5*-loaded grafts was still significantly greater than that seen in the *lacZ* controls. There were also trends of increasing tensile mechanical properties (maximum force and stiffness) over time, but there were no significant differences between the *Gdf5*- and *lacZ*-treated grafts in this regard. Tendons from mice killed at 14 days after surgery were removed and fixed, paraffin-embedded, and processed for immunohistochemistry with anti-mouse GDF-5 antibody. The data demonstrate positive anti-GDF-5 staining of host cells (arrows) surrounding the grafts loaded with rAAV-*Gdf5*, whereas this is absent in the rAAV-*lacZ*-loaded controls. This finding further validates the efficacy of *Gdf5* gene delivery (**Figure 5**). Next, we histologically examined the implanted allografts at 14 days after surgery (**Figure 6**). Both *Gdf5*-treated and *lacZ*-treated control allografts were surrounded by hypercellular fibrotic tissue at the junction with the host tendon; this could have contributed to impairment of gliding, and consequent reduction in the flexion range of motion (**Figure 6c** and **d**). However, there were marked differences in morphology in the middle segment of the grafts. Whereas the rAAV-*Gdf5*-treated graft was surrounded by organized tissue that resembled neotendon and integrated with the graft (which itself appeared to have been repopulated by cells) (**Figure 6f**), the rAAV-*lacZ* control allograft was mostly acellular and was surrounded by disorganized and hypercellular fibrotic tissue (**Figure 6e**). However, additional assays and immunohistochemistry (for collagen types I and III, for example) are needed in order to confirm these observations.

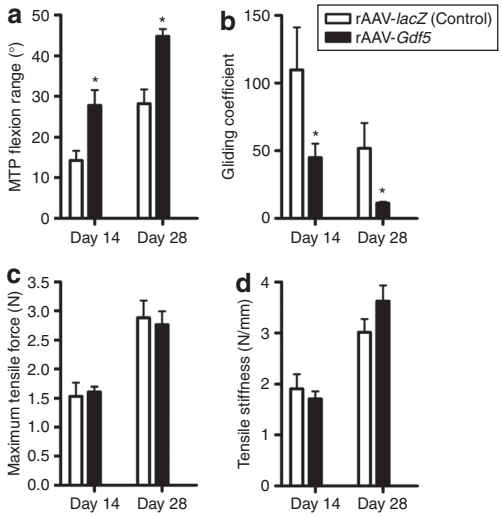

Figure 4 rAAV-*Gdf5* loading of freeze-dried allografts improves the metatarsophalangeal (MTP) flexion range of motion and the gliding function of reconstructed flexor digitorum longus (FDL) tendons while maintaining their biomechanical properties. Mice had their FDL tendons reconstructed with freeze-dried allografts loaded with rAAV-*Gdf5* (treated) or rAAV-*lacZ* (controls) and killed at 14 and 28 days after surgery ($n = 9$ per treatment per time point). The operated hind feet were removed and subjected to the MTP flexion test to determine (**a**) the MTP joint flexion range, and (**b**) the gliding coefficient. The tendons were then isolated and tested biomechanically to determine (**c**) their breaking (maximum) tensile force, and (**d**) their linear tensile stiffness. The data presented are mean values ± SEM. Asterisks indicate significant differences compared to time-matched controls ($P < 0.05$). GDF-5, growth and differentiation factor 5; rAAV, recombinant adeno-associated virus.

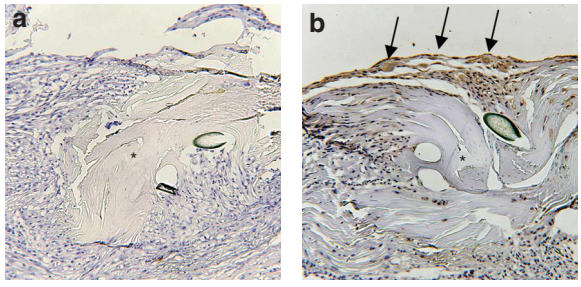

Figure 5 rAAV-*Gdf5* loading of freeze-dried allografts mediates *de novo* GDF-5 protein synthesis by the host cells at the periphery of the implanted allograft. Representative immunohistochemical sections of (**a**) the rAAV-*lacZ*-loaded and (**b**) the rAAV-*Gdf5*-loaded flexor digitorum longus tendon allografts at 14 days after grafting, stained with anti-mouse GDF-5 antibody. It is important to note the matrix-bound GDF-5 (positive staining indicated by arrows), presumably synthesized by the transduced host cells surrounding the rAAV-*Gdf5*-treated allografts (asterisk), that is absent in the rAAV-*lacZ*-treated graft. GDF-5, growth and differentiation factor 5; rAAV, recombinant adeno-associated virus.

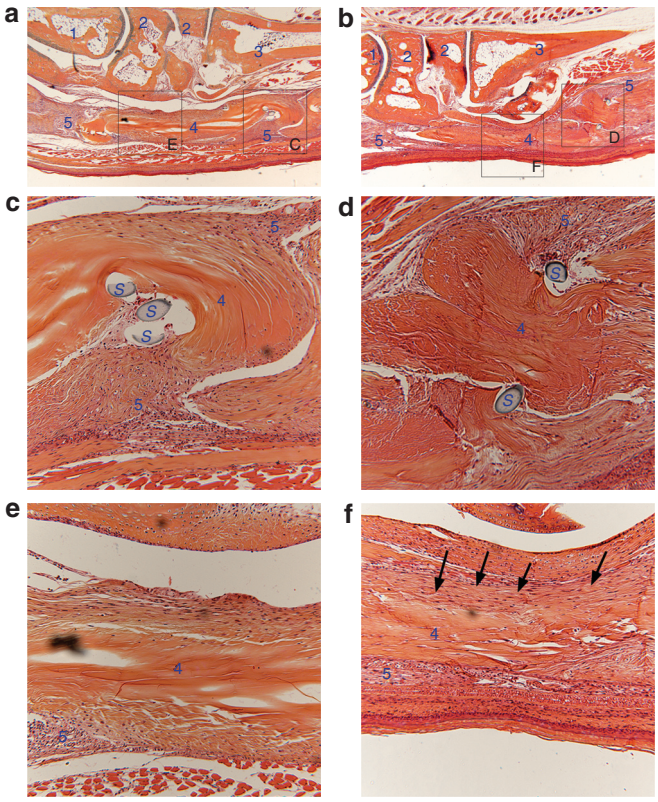

Figure 6 rAAV-*Gdf5* loading of freeze-dried allografts mediates cellular repopulation of the graft and remodeling of the fibrotic scar tissue. Representative histological sections of (**a**,**c**,**e**) the rAAV-*lacZ*-loaded and (**b**,**d**,**f**) rAAV-*Gdf5*-loaded flexor digitorum longus (FDL) tendon allografts at 14 days after grafting, stained with Alcian Blue and Orange G. (**a**,**b**) Micrographs at ×4 show the implanted grafts with their anatomical relationships to the surrounding tissue. Boxed regions shown in the magnified micrographs (×20) depict (**c**,**d**) the distal ends of both grafts, and (**e**,**f**) the middle segment of the grafts. The tissues represented by numbers are: 1) talus, 2) tarsal bones, 3) metatarsal bone, 4) FDL tendon allograft, and 5) fibrotic/inflammatory tissue. *S* indicates remnants of suture. (**f**) Arrows indicate a remodeled tissue that appears to align and integrate with the rAAV-*Gdf5*-loaded allograft, and that also seems to have been repopulated with host cells compared to **e** the mostly acellular rAAV-*lacZ*-loaded allograft. rAAV, recombinant adeno-associated virus.

DISCUSSION

Tendon, ligament, and joint capsular injuries represent 45% of the almost 33 million musculoskeletal injuries that occur each year in the United States, and hand injuries account for 5–10% of annual emergency department visits nationwide.[11] While flexor tendon injuries might represent only a small fraction of these numbers, adhesion formation associated with tendon surgery in general is a much more widespread problem.[12] This problem is not limited to primary repair, but has also been reported in response to tendon reconstruction using autografts or allografts[13] as an inevitable byproduct of the biological cascade of events in tendon healing. It is well established that tendon healing consists of three phases: inflammatory, proliferative, and remodeling. The inflammatory phase involves the recruitment of fibroblasts and macrophages to the site of injury, and phagocytosis of the necrotic tissue. The second phase involves the proliferation of fibroblasts and the formation of a repair scar formed from immature and disorganized collagen matrix. Remodeling then follows in the final phase as the immature collagen fibers in the scar tissue become organized and align with the tendon fibers. While the etiology of adhesion formation has been clinically linked to this last phase,[1] it is hypothesized that adhesions result from post-operative inflammation that extends to surrounding tissue.[14]

Clinical and experimental strategies to abrogate adhesion formation can be categorized as either mechanical or biochemical intervention protocols. Mechanical strategies include post-operative passive motion and rehabilitation protocols early after surgery[3,15]; optimized surgical techniques that involve minimally traumatic manipulation of the tendon, graft, and surrounding tissues[16]; and the use of antiadhesion surface coating of the graft as a physical barrier against adhesion formation.[12,17] Biological and biochemical intervention strategies primarily rely upon growth factor delivery to accelerate the rate of tendon healing and remodeling.[18,19] While a number of growth factors[19–24] could potentially improve the repair of tendons, their effects on tendon adhesion have been left largely unexplored. A rational design for a growth-factor delivery therapy should arguably be based on the natural history of gene expression of growth factors during the different phases of tendon repair, a thorough understanding of the molecular action of these factors, and a sustained delivery mechanism to maximize the therapeutic effects of these factors.

In our mouse FDL tendon–grafting model, we previously observed that that *Gdf5* messenger RNA levels were significantly elevated in 28-day autografts.[6] The increase in *Gdf5* levels was concomitant with the observed increases in *Vegfa* expression, in agreement with findings in other reports.[25] Interestingly, the timing of the peak in *Gdf5* and *Vegfa* gene expression was coincidental with the marked improvements in MTP joint flexion observed thereafter.[6] On the basis of this observation, we hypothesized that GDF-5 plays an important role in the remodeling phase, and that exogenous delivery of paracrine GDF-5 signals will accelerate remodeling and lead to functional improvements in joint flexion. GDF-5 belongs to the category of "cartilage-derived morphogenetic proteins-1" [brachypodism or bone morphogenetic protein-14 (BMP-14)]. The transforming growth factor-β subfamily of proteins which, in addition to GDF-5, includes GDF-6 and GDF-7 (cartilage-derived morphogenetic proteins-2 and -3 or BMP-13 and -12), has been shown to be important for skeletal development in general and for tendon formation and repair in particular.[26,27] The effects of GDF-5 on cell recruitment, migration/adhesion, differentiation, proliferation, and angiogenesis *in vitro* and *in vivo*,[7] as well as its effects on the ultrastructure of the collagen fibrils and the biomechanical properties of normal and repaired tendon tissue have been reported.[7,28] However, the exact mechanism of GDF-5 action in tendon repair has yet to be explored. It has been reported that GDF-5 binds with activin receptor–like kinase 3 and/or activin receptor–like kinase-6 (also termed "BMP type IA" and "BMP type IB" receptors, respectively),[29,30] and this presumably activates the Smad signaling pathway. A recent study demonstrated that constitutive activation and nuclear translocation of Smad8 led to upregulation of the Scelraxis transcription factor and promoted tendon formation in C3H10T1/2 cells (a murine multipotent cell line) which, when implanted on a collagen sponge into a rat AT gap tenotomy model, led to formation of tendon-like tissue.[31]

The hypothesis that GDF-5 might be the paracrine signal that leads to Smad8 activation remains an intriguing possibility despite some preliminary observations that contradict this.[31]

Next we were faced with the decision regarding the selection of the GDF-5 delivery mechanism. The therapeutic window of bolus or topical delivery is not long, because the signaling is almost instantly initiated and short-lived.[32] Alternatively, local transfer of genes that express the relevant healing factors may mediate sustained expression of these factors. The efficacy of various viral vector systems (including retrovirus and adenovirus) in mediating targeted and transient gene transfer in tendon repair has been demonstrated *in vitro* and *in vivo*.[8,33] In a recent study, direct injection of rAAV vectors expressing green fluorescent protein (AdGFP) or BMP-13 (AdBMP-13 or AdGDF-6) into rabbit flexor tendons demonstrated a transient dose-dependent transgene expression up to 12 days *in vivo*.[34] These reports are consistent with our data that demonstrated that rAAV loading of freeze-dried FDL allograft mediates targeted and transient gene expression by host cells at the implant site, with the expression peaking at 7 days but persisting up to 21 days. Notwithstanding the known safety concerns (that are abated by low-dose vector delivery), rAAV-based gene therapy can potentially be a therapeutic option for musculoskeletal tissue (including tendon) reconstruction, in view of the localized and transient expression achieved.

Finally, in the light of our previous findings which suggested that freeze-dried tendon allografts appear to be tolerated well in the host mouse and provide biomechanical scaffolding functions equivalent to those afforded by live autografts,[6] we examined whether tendon allografts can serve as a delivery scaffolds for therapeutic factors, in order to mediate adhesion-free reconstruction of flexor tendon gap defects. Our data indeed show that, despite the modest retention efficiency (~10%), freeze-dried FDL allografts loaded with rAAV-*Gdf5* did transduce local expression of the GDF-5 protein at 14 days, and that this was associated with a significantly improved range of flexion as compared to that achieved by rAAV-*lacZ* controls. While previous studies reported the presence of small foci of bone and fibrocartilage within ectopic tendon/ligament tissue in response to Ad-GDF-6 (or BMP-13) injections in athymic rats,[35] we did not observe such untoward effects in our model.

Interestingly, while we observed beneficial functional effects of rAAV-*Gdf5* on the grafted tendon, we did not observe any significant effects on the biomechanical properties. Earlier reports have suggested that GDF-5-deficient mice displayed a delay in the accrual of biomechanical strength during the initial healing of the AT as compared to control mice.[7] On the other hand, Dines *et al.* (2007), working with a rat model, reported that, at 3 weeks, lacerated ATs that had been repaired with sutures coated with rhGDF-5 showed a greater rate of healing than the repaired tendons in the controls.[36] In both studies, the mechanical properties of the controls, GDF-5 deficient, and GDF-5 augmented tendon repairs were equivalent at later time points. These results suggest that the effects of GDF-5 treatment might be temporally sensitive and dependent on the healing phase. However, our results are not different from those of similar gene therapy–based tendon repair studies. Rickert *et al.* (2005) reported efficacious adenovirus-mediated transfer of *Gdf5* gene by using injections of AdGDF-5 at the site of lacerations in the rat AT, but this was not associated with any significant improvements in mechanical properties.[8] This was in contrast to the previous report by Lou and co-workers (2001) that AdGDF-7 injections at the site of lacerations in chicken flexor digitorum profundus tendon results in delayed but significant onefold improvements in mechanical properties at 6 weeks after treatment.[37] It is therefore possible that other isoforms of the growth and differentiation factor, such as GDF-7, have more potent effects on the mechanical properties of tendon tissue that is undergoing repair. Other possibilities cannot be excluded. The lack of improvement in mechanical properties in the rAAV-Gdf5-treated allografts compared to the controls, as observed by us, may be related to: (i) the dosage used for the treatment (number of rAAV particles transferred), which might have to be optimized in future studies; (ii) the efficiency of rAAV-mediated gene transfer; (iii) the absence of interactions with *in vivo* forces in our model; and (iv) the observation that the transfected host cells resided in the external callus, resulting in remodeling of the fibrotic callus tissue, a reduction in adhesions, and improved gliding function, but not necessarily any remodeling of the graft tissue proper.

On the basis of these findings, we propose a simplified alternative paradigm in tissue engineering, using freeze-dried allograft tissue to deliver cues to the host cells *in situ* to reprogram the repair response. Freeze-dried tendon allografts can provide these delivery functions with a number of desirable characteristics that may be unavailable with synthetic and naturally derived biomaterials. Freeze-dried tendon allografts are biochemically unaltered, because the lyophilization is purely a physical process that leads to dehydration of the tissue. These allografts potentially have an indefinite shelf-life and will likely have less regulatory hurdles to clear *en route* to clinical applications because they can still be classified as "allografts". Furthermore, freeze-dried tendon allografts have biomechanical properties equivalent to fresh or fresh-frozen tendon tissue. Despite being devoid of live cells (which actually confers on them the advantage of not eliciting an immune response leading to graft rejection), they can be readily remodeled and populated by host cells when implanted *in vivo*. Most important, freeze-dried tendon allografts have remarkable native hydrophilic properties that permit efficient reconstitution of the tissue in a physiologic solution containing therapeutic molecules. This concept could be applied to other tendon and ligament models including the anterior cruciate ligament, the AT, and the supraspinatus "rotator cuff" tendon, and could involve loading gene delivery vectors or recombinant or tissue-derived growth factors (see **Supplementary Figure S3**). Future developments could also focus on differential processing (multiple genes and proteins) of composite allograft tissue (bone–tendon–bone) so as to address clinically challenging problems such as soft tissue insertion into bone. Furthermore, such technology can potentially be translatable to other musculoskeletal soft tissue models, including articular cartilage and meniscus tissues.

MATERIALS AND METHODS

rAAV preparation. rAAV-*lacZ* and rAAV-*Luc* stock solutions were purchased, and the single stranded rAAV-Gdf5 vector (serotype 2), which was custom cloned from an existing plasmid (pAAV-Gdf5) containing a cytomegalovirus promoter and the Gdf5 complementary DNA, was purified

and titered at the Gene Therapy Center of the University of North Carolina, Chapel Hill, North Carolina, USA.

Processing of tendon allografts. FDL tendon allografts were aseptically isolated, placed in sterile vials, frozen at −80 °C, and freeze-dried. In order to load the tendon grafts with rAAV vectors, pairs of tendons were soaked in a vial containing 50 µl of phosphate-buffered saline solution containing 5×10^9 U of rAAV (*lacZ*, *Luc*, or *Gdf5*). After allowing the dehydrated grafts to take up the solution for 1 hour, the grafts were snap-frozen and then freeze-dried and stored awaiting experimental use.

Real-time quantitative PCR assessment of rAAV retention in the allografts. rAAV-*lacZ*-loaded FDL tendon grafts were digested in a buffer solution of proteinase K (10 µg/ml Applied Biosystems, Foster City, CA) at 50 °C for 1 hour, and then at 95 °C for 20 minutes to deactivate the enzyme. Samples from a serial dilution of digested virus at standard concentrations of 10^{10}, 10^9, 10^8, 10^7, 10^6, 10^5, and 10^4 U were used for creating a standard curve. Duplicate samples (2 µl) of each standard dilution, along with samples from tendon digests, were added to real-time PCR Master Mix (SYBR Green PCR Master Mix; Applied Biosystems, Foster City, CA) and allowed to react in a Rotor-Gene 2000 Real-Time DNA detection system (Corbett Research, Sydney, Australia) for 40 cycles on a program of 94 °C for 20 seconds, 61.6 °C for 30 seconds, and 72 °C for 30 seconds. The mean cycle threshold (C_t) values were used for calculating the rAAV content and retention efficiency in the tendon samples, on the basis of the standard curve.

Microwound experiments. The microwound assay was performed as previously described by Hocking and Chang.[38,39] Briefly, mouse embryonic fibroblast (NIH3T3; American Type Culture Collection, Manassas, VA) cells were plated and allowed to grow to 80% confluence. The cells were serum-deprived for 24 hours prior to creating wounds. Using a 100-µl pipette tip, wounds were created by scratching the pipette tip across the monolayer, resulting in wounds initially measuring 1.00 mm (±0.20). The cells were then cultured with 0.5% bovine calf serum (American Type Culture Collection, Manassas, VA) and 5.0×10^7 particle U/ml of either rAAV-*lacZ* or rAAV-*Gdf5*. Digital photos of the microwound were taken at 0, 2, 4, 8, 12, 24, 36, and 48 hours. Using a custom Matlab program, the average width of each wound was measured at each time and normalized against the initial wound width ($w(t)/w(0)$). The data were fitted to the equation $w(t)/w(0) = A/(B \cdot \exp(t/\tau) + 1)$ wherein τ represents the "healing time constant" such that wounds that heal faster have a lower healing time constant.

Bioluminescent imaging. In order to demonstrate the efficacy of processed tendon allograft-mediated gene delivery, freeze-dried allografts loaded with rAAV-*Luc* were implanted to reconstruct mouse FDL tendons as described later in this report. Host cells transduced by this virus express the firefly luciferase gene. At each time point following implantation, we injected each mouse with the substrate D-luciferin potassium salt (Xenogen, Cranbury, NJ) which, when cleaved by the transduced luciferase enzyme, emits light that can be captured using a special camera system and software (IVIS 100 Bioluminescent Imaging System, Xenogen, Cranbury, NJ) and the bioluminescence intensity gradients can be represented by a heat map intensity (purple, least intense; red, most intense) computed from measurements of the total integrated light signal (photons emitted/cm^2/s) emitted from a standardized region of interest in a standard time interval (3-minute exposure).

Mouse FDL tendon-grafting surgeries. Animal studies were conducted in compliance with principles and procedures approved by the University of Rochester Committee for Animal Resources. Surgeries were performed using an aseptic technique under a ×2 microdissection magnifying lens. Briefly, a longitudinal plantar incision was made on the left hind foot. The distal FDL tendon of the C57Bl/6 mouse (Jackson Laboratories, Bar Harbor, ME) was isolated and transected on the plantar surface of the metatarsal bones. A 3-mm freeze-dried tendon allograft, that had been previously removed from a C57Bl/6 mouse and loaded with rAAV, was reconstituted in phosphate-buffered saline and sutured between the ends of the host tendon, using an 8-0 nylon suture in a horizontal mattress suture pattern (similar to a modified Kessler technique). The proximal tendon insertion into the flexor muscle was severed to eliminate early gliding, so as to protect the repair during the early phases of healing and to induce adhesion formation. The skin was closed with 4-0 silk suture.

MTP joint flexion test. Immediately after each mouse was killed, the lower hind limbs were disarticulated from the knee and the proximal FDL tendon along the tibia was released just proximal to the tarsal tunnel without disrupting the skin at the ankle or foot. The proximal end of the tendon was secured between two square pieces of tape using a thin layer of cyanoacrylate, as previously described.[36] The lower hind limb was fixed in a custom apparatus, with the tibia rigidly gripped to prevent rotation. In order to standardize the neutral position, the toes of the mouse were passively extended by the examiner and allowed to return to an unloaded position before a digital image was taken medially to determine the neutral position (zero load) of the MTP joint. The FDL tendon was incrementally loaded in the same anatomical direction as the flexor muscle line of force, using dead weights that were statically suspended from a hook and line passing through the proximal FDL tendon/tape composite. The dead weights were suspended for 30 seconds before the digital pictures were taken, so as to avoid "creep" effects. With each increment of load, a digital image was taken to quantify the MTP flexion angle relative to the neutral position. The MTP joint flexion angles were measured from the digital images by two independent observers (P.B. and T.D.) using ImageJ software (*http://rsb.info.nih.gov/ij/*), and plotted against the applied loads. The flexion data were fitted to a single-phase exponential association equation of the form: MTP flexion angle $= \beta \times [1 - \exp(-m/\alpha)]$; where m is the applied load (Prism GraphPad 3.0; GraphPad Software, San Diego, CA). The curve fit was constrained to the maximum flexion angle (β) for normal tendons that was previously determined to be 75° for the maximum applied load. The constant α (gliding coefficient) that governs the rate of rise of the flexion curve with loading was determined by non-linear regression as a measure of the resistance to MTP flexion on account of impaired gliding.

Biomechanical test. Following the MTP flexion test, the proximal end of the FDL tendon was released at the tarsal tunnel, and dissected medially along the bone. Once the tendon was free from the tunnel, the calcaneus was removed, freeing the proximal end of the tendon for direct gripping in the mechanical test, as described elsewhere.[40] The distal bones of the foot were directly gripped in custom grips without disrupting the graft or the branching tendon insertion into the phalanges. The specimen was placed in sterile gauze soaked with saline to maintain adequate tissue hydration until tested. The FDL tendon was then mounted on the Instron 8841 DynaMight axial servohydraulic testing system (Instron, Norwood, MA) and tested using published protocols.[36] The tendon was loaded in tension in displacement control at a rate of 30 mm/minute until failure. Force–displacement data were automatically logged and plotted, and the maximum tensile force and stiffness were determined.

Histology and immunohistochemistry. The grafted limbs were removed by disarticulating the tibia from the knee joint. With the tibia perpendicular to the foot, the FDL tendon was kept in tension by passing a pin through the flexor muscles and the tibia. The tissues were then prepared for histology and analyzed using routine techniques. Briefly, the removed limbs were fixed in 10% neutral-buffered formalin and decalcified in 10% EDTA at 4 °C for 21 days. The decalcified tissues were dehydrated in a gradient of alcohols and then embedded *en bloc* in paraffin. Serial 3-µm sagittal sections through the FDL tendon plane were prepared and stained with Alcian Blue and Orange G. For immunohistochemistry, the rAAV-loaded tendon sections were stained with primary antibodies against β-galactosidase (PAb # GTX26646, GeneTex, San Antonio, TX) or against the murine

GDF-5 (AF853; R&D Systems, Minneapolis, MN). The tissue sections were then treated with appropriate biotin-conjugated secondary antibodies, before being developed with streptavidin-conjugated AEC chromogen (Zymed Laboratories, San Francisco, CA).

Statistical analysis. Data analysis including analysis of variance with Bonferroni post-hoc multiple comparisons ($\alpha = 0.05$) and the non-linear regression analyses to estimate the gliding coefficient from the MTP flexion data were performed using Prism GraphPad 4.0 statistical software.

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SUPPLEMENTARY MATERIAL

Figure S1. Retention efficiency of rAAV on freeze-dried mouse FDL tendon allografts.

Figure S2. Assessment of MTP joint flexion range of motion.

Figure S3. The Concept of the Therapeutically-Endowed Freeze-Dried Tendon Allograft.

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Freeze-dried Allograft-Mediated Gene or Protein Delivery of GDF-5 Reduces Reconstructed Murine Flexor Tendon Adhesions

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Abstract:	Advances in allograft processing open new horizons for clinical adaptation of flexor tendon allografts as delivery scaffolds for antifibrotic therapeutics. Recombinant adeno associated-virus (rAAV) gene delivery of the growth and differentiation factor 5 (GDF-5) has been previously associated with antifibrotic effects in a mouse model of flexor tendoplasty. In this study, we compared the effects of loading freeze-dried allografts with different doses of GDF-5 protein or rAAV-Gdf5 on flexor tendon healing and adhesions. We first optimized the protein and viral loading parameters using RT-PCR, ELISA, and in vivo bioluminescent imaging. We then reconstructed flexor digitorum longus (FDL) tendons of the mouse hindlimb with allografts loaded with low and high doses of recombinant GDF-5 protein and rAAV-Gdf5 and evaluated joint flexion and biomechanical properties of the reconstructed tendon. In vitro optimization studies determined that both the loading time and concentration of the growth factor and viral vector had dose-dependent effects on their retention on the freeze-dried allograft. In vivo data suggest that protein and gene delivery of GDF-5 had equivalent effects on improving joint flexion function, in the range of doses used. However, the lower doses of GDF-5 had more potent effects on suppressing adhesions without adversely affecting the strength of the repair. These findings indicate equivalent antifibrotic effects of GDF-5 gene and protein delivery, but suggest that localized delivery of this potent factor should also carefully consider minimizing the dosage used to eliminate untoward effects, regardless of the delivery mode.

ABSTRACT

Advances in allograft processing open new horizons for clinical adaptation of flexor tendon allografts as delivery scaffolds for antifibrotic therapeutics. Recombinant adeno associated-virus (rAAV) gene delivery of the growth and differentiation factor 5 (GDF-5) has been previously associated with antifibrotic effects in a mouse model of flexor tendoplasty. In this study, we compared the effects of loading freeze-dried allografts with different doses of GDF-5 protein or rAAV-*Gdf5* on flexor tendon healing and adhesions. We first optimized the protein and viral loading parameters using RT-PCR, ELISA, and *in vivo* bioluminescent imaging. We then reconstructed flexor digitorum longus (FDL) tendons of the mouse hindlimb with allografts loaded with low and high doses of recombinant GDF-5 protein and rAAV-*Gdf5* and evaluated joint flexion and biomechanical properties of the reconstructed tendon. *In vitro* optimization studies determined that both the loading time and concentration of the growth factor and viral vector had dose-dependent effects on their retention on the freeze-dried allograft. *In vivo* data suggest that protein and gene delivery of GDF-5 had equivalent effects on improving joint flexion function, in the range of doses used. However, the lower doses of GDF-5 had more potent effects on suppressing adhesions without adversely affecting the strength of the repair. These findings indicate equivalent antifibrotic effects of GDF-5 gene and protein delivery, but suggest that localized delivery of this potent factor should also carefully consider minimizing the dosage used to eliminate untoward effects, regardless of the delivery mode.

INTRODUCTION

Fibrosis and adhesions are frequent complications to flexor tendon injury in the hand.[1] Despite decades of research, an excellent outcome after flexor tendon surgery is still dependent on a skilled and experienced surgeon, a qualified team of occupational therapists, and a very motivated patient. One of the most effective advances in flexor tendon repair is the implementation of early post-operative mobilization, which has become feasible in part due to the development of stronger and more refined suturing techniques.[2] Because of these advances, primary repair outcomes in Zone II injuries are now more successful, and grafts are less frequently used in flexor tendon reconstruction. However, tendon allografts can be the only option in cases of revision surgery and multi-tendon injuries in mutilating scenarios such as combat injuries,[1,3] especially with limitations associated with autografts availability[4,5] and the lack of clinically proven tissue engineered biomaterial scaffolds.[6]

While clinical use of allografts has not been favored in flexor tendoplasty, recent advances in graft processing have enabled novel regenerative applications on the bench and in preclinical models. For example, intrasynovial flexor tendon allografts have been successfully decellularized without affecting the mechanical properties or chemical composition of the tissue[7] and then revitalized by seeding different types of cells (tendon and sheath fibroblasts and stem cells).[8] These studies demonstrate the conceptual feasibility of engineering intrasynovial flexor tendon grafts with epitenon cell layer seeding. However, cell-based tissue engineering approaches still face significant regulatory hurdles before they can become a clinical option.

Alternatively, tendon allografts can be decellularized to minimize the recipient's immune response and can be modified with growth factors to enable their remodeling and incorporation into the host. The growth and differentiation factors (GDFs) 5, 6, and 7 are members of the bone

morphogenetic protein (BMP) family and have been implicated in tendon development and repair.[9-12] It has been previously demonstrated that freeze-dried tendon allografts loaded with recombinant adeno-associated viral (rAAV) for local and transient *Gdf5* gene delivery significantly reduced tendon adhesions and restored the metatarsophalangeal (MTP) joint flexion in mice.[13] Given that these growth factors are morphogens with varied and dose-dependent effects throughout the body that are not limited to tendon biology,[14] we therefore sought to investigate doses that might enhance the repair strength while abating any fibrotic scarring. Considering the differences in kinetics of action of protein (immediate signaling effects) and viral gene delivery (delayed effects that involve transfection, gene expression, protein translation, and signaling), we hypothesized that rAAV-*Gdf5* delivery via freeze-dried tendon allografts will provide a prolonged window of sustained therapeutic effects to improve the tendon biomechanical properties and abolish the fibrotic adhesions. To test this hypothesis, we set out first to optimize the retention of the rAAV particles or the recombinant GDF-5 protein on freeze-dried tendon allograft. We then compared the dose-dependent effects of rAAV-*Gdf5* or GDF-5 protein on the MTP joint flexion and biomechanics of reconstructed mouse FDL tendons.

MATERIALS AND METHODES

Preparation of FDL Tendon Allografts

FDL tendon grafts were aseptically dissected from donors (C57Bl/6 mice) and lyophilized as previously described.[15] The grafts were then digitally imaged to determine their surface area (Image J software, http://rsb.info.nih.gov/ij). The lyophilized tendon grafts were placed in 100µl PBS solution on ice containing rAAV2.5/CMV-*LacZ* (Virus Vector Core Facility, University of North Carolina, Chapel Hill, NC), rAAV2.5/CMV-*Gdf5* (Custom clone previously published (13)), or recombinant murine GDF-5 protein (R&D systems, Minneapolis, MN). After the tendon grafts have been dipped in the rAAV or protein solution for a designated time (as described later), they were lyophilized and stored frozen at -80°C for 1-7 days until analyzed or used for tendon surgeries.

Assessment of rAAV Loading and Retention

To optimize the viral particle loading conditions, several experiments were performed. In the first experiment, the lyophilized grafts were rehydrated in a solution containing rAAV-*LacZ* (5×10^9 particles/100 µl) for 5 minutes to 24 hours. In the second experiment, different concentrations of rAAV-*LacZ* (5×10^7 - 5×10^{10} particles/100 µl) were used to rehydrate the allografts for 120 minutes. To assess the retention of rAAV particles, the processed rAAV-*lacZ* loaded FDL tendon grafts were digested in proteinase K (10µg/ml). Real-time RT-PCR was used to calculate the rAAV content in the tendon samples based on a standard curve in the range of 10^4 - 10^{10} particles/100 µl. Three tendon grafts were loaded and gene expression was measured in triplicate samples for each. The experiments were repeated 3 or 4 times.

Assessment of rmGDF-5 Protein Loading and Retention

To optimize therapeutic protein loading conditions, FDL tendon grafts were processed aseptically by freeze-drying and then dipped in PBS solutions containing rmGDF-5 (10 or 50 ng/µl with 3% bovine serum albumin (BSA) as a carrier protein) for 2 or 24 hours. To assess the retention of the protein, the processed rmGDF-5 loaded FDL tendon grafts were eluted in 120 µl blocking buffer (PBS with 3% BSA and 0.05% Tween-20) for 2 hours on ice, and the eluate was analyzed by Enzyme-linked immunosorbent assay (ELISA).[16] The optical density (OD) for each well was read with a plate reader (Synergy Mx Multi-Mode Reader, BioTek, Winooski, VT) at 450nm wavelength, and calibrated for GDF-5 concentration against a standard curve (10-1000ng/ml), which was included in each ELISA plate. The limit of detection of the assay was 5 ng/ml, and the coefficient of variance for the assay <<1%. Three tendon grafts were loaded and GDF-5 protein retention was assayed in triplicate samples for each. The experiments were repeated 3 or 4 times

Surgical Procedure - FDL Tendon Defect Reconstruction (Tendoplasty)

All animal studies utilized C57Bl/6 mice and were performed in compliance with institutionally-approved animal use and care protocols. Twenty four hours before the tendon reconstruction surgery the left hind limp of the mouse was injected with BOTOX® (Allergan Pharmaceuticals, Irvine, CA) to induce transient paralysis of the flexor muscles in order to protect the reconstructed tendon from rupture upon recovery while allowing controlled, incremental recovery of muscle forces (See Supplemental Material). The next day, aseptic FDL tendoplasty surgeries were preformed as previously described.[13,15] Briefly, the distal FDL tendon of the left hind paw was exposed and transected to create a 3 mm defect at the metatarsals level. A lyophilized allograft loaded with rAAV or recombinant protein was used to reconstruct of the severed tendon using modified horizontal mattress suturing (8-0 nylon suture).

Bioluminescent Imaging (BLI).

To investigate the dose effects on gene delivery kinetics and biodistribution up to 14 days, which corresponds to peak adhesion formation in previous studies,[13] rAAV-*Luc loaded* allografts were implanted in the FDL tendons. Prior to BLI, an intraperitoneal injection of D-luciferin potassium salt (PerkinElmer, Waltham, MA) was administered to each animal. The rAAV induced bioluminescence was then imaged using a 3-minute exposure on the IVIS Spectrum Imaging System (PerkinElmer) and the signal intensity was quantified over a consistent region of interest encompassing the operated foot (n=4 per treatment), as previously described.[13]

Assessment of Metatarsophalangeal (MTP) Joint Flexion

After 14 days of healing the mice (n=8 per treatment) were euthanized, and the hind limbs were dissected below the knee and stored frozen (-20°C) until tested. On the day of testing the proximal FDL tendon was severed from the muscle at the tibia without compromising the healing tissue in the foot. The free tendon end was reinforced using tape and cyanoacrylate. The limb was then inversely suspended in a custom jig where the tibia was secured to prevent sliding and rotation. The flexion angle of the MTP joint under incremental loading was then measured as previously described.[15] The flexion data was used to derive functional parameters including the flexion range of motion (ROM) as previously described.[13,15]

Biomechanical Tensile Testing

Immediately following the assessment of MTP joint flexion, the tendon was released at the tarsal tunnel, and then tested in tension at a rate of 30 mm/min to failure on the Instron 8841 DynaMight™ axial servohydraulic testing system (Instron Corporation, Norwood, MA) as

described.[13,15] The maximum tensile force and stiffness were derived from force-displacement plots.

Statistical analysis

Data analysis included t-tests, analysis of variance (ANOVA) with Newman-Keuls post-hoc multiple comparisons (α=0.05), and non-linear regression to derive the MTP flexion parameters.

RESULTS

Assessment of retention of rAAV particles and protein in tendon grafts in vitro

To optimize the performance of rAAV and rmGDF-5-loaded tendon grafts *in vivo*, we first sought to determine the effects of the concentration and dipping time on graft retention *in vitro.* Not surprisingly, we found significant incremental effects on the retention of rAAV-*LacZ* due to increasing the dipping time (Figure 1*a*). While there were no differences in retention between 5 to 60 minutes, increasing dip-coating time to 120 minutes significantly increased the retention of rAAV particles on the freeze-dried tendon graft compared to 5 and 15 minutes. Increasing the dip-coating time to 24 hours significantly increased the retention of rAAV particles on the freeze-dried tendon graft compared to all other loading times. We also investigated the effects of rAAV concentration in the dipping solution, and observed a dose-dependent improvement in the retention of rAAV particles on the graft (Figure 1*b*) with increased dipping solution concentration.

There were no differences in retention of rmGDF-5 between 2 and 24 hours dipping times (Figure 2*a*). However, there were significant concentration dependent effects on the retention of rmGDF-5 on the graft (Figure 2*b*).

Longitudinal Assessment of rAAV-mediated gene delivery in vivo

To determine the dose effects on the biodistribution and kinetics of reporter gene delivery and transduction *in vivo*, allografts were loaded with 5×10^{7} or 5×10^{10} particles of rAAV-*Luc*, and implanted in FDL tendon defects. BLI was performed on days 3, 7, and 14 after grafting as described. As previously reported,[13] bioluminescence was restricted to the grafted foot (Fig. 3*a*). Furthermore, gene transduction , measured by BLI signal intensity, was dose-dependent, with the

lower dose (5×10^7 particles/100 µl) inducing significantly less intense bioluminescence at days 7 and 14 compared to the higher dose ($p<0.05$, Fig. 3*b*).

Effects of rAAV-*Gdf5* and rmGDF-5 on allograft healing after FDL Tendoplasty

To assess functional effects of GDF-5 gene and protein delivery, MTP flexion and biomechanical tensile testing were performed successively, MTP flexion tests (Fig. 4) demonstrated that the lower dose (5×10^7 particles/100 µl) rAAV-*Gdf5* loaded allografts had significantly improved MTP joint ROM ($p<0.05$; Fig. 4) at 14 days post grafting, while the higher dose (5×10^{10} particles/100 µl) allografts were not significantly different from rAAV-*lacZ* loaded controls. Similarly, the lower dose of rmGDF-5 (10 ng/µl) loaded allografts significantly improved MTP ROM compared to controls ($p<0.05$; Fig. 4) at 14 days post reconstruction, while the higher dose (50 ng/µl) were not significantly different from controls.

The tensile strength and elasticity (maximum force and stiffness, respectively) tended to increase with both doses of rmGDF-5 loaded allografts but not the rAAV-*Gdf5* loaded allografts (Fig. 5), but these differences were not statistically different from the untreated controls.

DISCUSSION

Localized and sustained delivery systems of growth factors to sites of skeletal injury remain a substantial barrier in tissue engineering. A common component of growth factor delivery systems is a biomaterial carrier to provide localization and spatiotemporal regulation of their bioavailabilty after implantation. Biomaterial carriers can be classified in general terms into ECM-mimicking polymer scaffolds or naturally-derived ECM scaffolds.[17] ECM scaffolds such as freeze-dried allografts have a number of desirable characteristics over synthetic polymers in tendon tissue engineering. Tendon allografts have been shown to maintain their biomechanical

when freeze-dried.[15] Their lack of cells and non-self cell surface antigens minimize the foreign body response of the recipient's immune system. They can also be infiltrated by host cells, including fibroblasts, allowing for their incorporation and remodeling *in vivo*.[15,18] Previous studies have demonstrated the feasibility of creating tendon/ligament scaffolds from freeze-dried allografts,[19] and others have demonstrated that decellularized allograft tendon can potentially be combined with donor cells to repair the ACL [20] or flexor tendon.[8] In addition, tendon allografts remain hydrophilic, which enables robust hydration and loading of therapeutics by simply dipping the grafts in an aqueous pharmaceutical solution.[13] In this study, we optimized techniques to use freeze-dried flexor tendon allografts as growth factor and viral gene delivery systems. Both the concentration of the growth factor and titer of the viral vector had a dose-dependent effect on the retention of the therapeutics on the freeze-dried allograft. Maximum retention of GDF-5 protein was achieved within two hours of reconstituting the graft in the therapeutic solution. More importantly, we found no significant differences between the therapeutic effects of the recombinant protein of rAAV-mediated gene delivery of GDF-5 in the range of doses used. This latter observation is consistent with some previous results that demonstrated that low doses of rmGDF-5 and rAAV-*Gdf5* have significant effects on scratch closure rate of monolayer fibroblasts in vitro.[13] The allograft-mediated delivery approach is a clinically compatible procedure, in which a FDA-approved drug or factor can be combined with the allograft at the point-of-care (*e.g.* the operating room). Further, the effects of the protein and the rAAV gene delivery vector were equivalent. However, while rAAV represents a class of gene delivery viral vectors with an acceptable safety profile and is being clinically tested in numerous of FDA approved protocols (http://www.clinicaltrials.gov), there are currently no approved viral vectors for wide clinical use. It is more likely that recombinant forms of the

protein GDF-5 will have a faster route to the clinic, since recombinant BMPs are currently in clinical use.

The effects of various growth factors on tendon healing have been extensively studied.[21] Members of the BMP family, known as growth and differentiation factors (GDFs), have been of particular interest in this area because of their demonstrable induction of tendon phenotype *in vitro* and *in vivo* and their acceleration of tendon healing in preclinical models.[9-12, 16, 22-24] GDF-5 (also called BMP-14) is one of the GDF isoforms whose genetic knockout in mice deregulates tail and Achilles tendon (collagen) ultrastructure leading to inferior biomechanics.[10, 22] It is for these observations that a number of therapeutic and tissue engineering strategies in tendon repair have focused on GDF-5. For example, Rickert et al. demonstrated that coating of surgical suture with GDF-5 accelerates Achilles tendon healing in a rodent model.[11] Yet, the antifibrotic effects of GDF-5 on flexor tendon adhesions have only been recently reported,[13] and confirmed in this current study.

While growth factors often exert potent therapeutic effects, they can also trigger ectopic or untoward responses from targeted or untargeted tissues and cells. For example, factors such as GDFs, are capable of driving ectopic differentiation of stem cells to tendon, cartilage and bone at varying dosages.[12, 25] Indeed, our findings suggest that the lower doses of GDF-5 (delivered either as protein or via rAAV) have more potent effects in suppressing the fibrotic response in tendon healing that leads to adhesions, interestingly without any significant effects the strength or stiffness of the repair tissue. Interestingly, in vivo investigations of tendon repair have previously raised concerns regarding dosage of GDF. For example, ectopic cartilage formation has been reported in preclinical animal models investigating GDF5, especially at higher doses.[26] A time course histological analysis of the stages of the repair response was beyond the scope of

the current work, but will be pursued in future studies to delineate the dose-dependent differences in the biology of tendon healing and the emergences of aberrant tissue differentiation, if any. Nevertheless, our data suggest that localized delivery of GDF-5 delivery must employ a minimal dosage of the growth factor to suppress fibrotic adhesions in our murine model. A more formal investigation of the dose-response effects of GDF-5 should be pursued in future studies.

The antifibrotic mechanism of action of GDF-5 is not understood. A common denominator in the abnormal fibrosis in a number of tissues is TGF- β presumably through inactivation of MMPs.[27-31] We have previously demonstrated that TGF-β/Smad3 loss-of-function in *Smad3*$^{-/-}$ mice leads to improved FDL tendon gliding and MTP joint flexion following surgical repair.[32] Others have shown that specific blockade of TGF-β1/Smad3 signaling is a potent therapeutic intervention against fibrosis.[31] Among the most potent antagonists of TGF-β1 in renal and pulmonary fibrosis, as an example, is BMP-7. In mesangial cells, TGF-β1 increased cell-associated ECM including collagen IV and fibronectin and decreased the level and activity of MMP-2, thereby causing tissue fibrosis.[28] Given that GDF-5 shares similar attributes with BMP-7 in terms of its structure and receptor binding affinity,[33] it is plausible that GDF-5 utilizes a similar mechanism, albeit this has yet to be demonstrated experimentally.

In summary, this study demonstrates that flexor tendon allografts can be manipulated effectively for localized therapeutic delivery, which opens new horizons for clinical utility of flexor tendon allografts, and suggests that localized delivery of potent growth factors, such as GDF-5, should carefully consider minimizing the dosage used to eliminate untoward effects.

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Figure Legends

Figure 1. Retention of rAAV particles on tendon grafts, determined using a quantitative real time PCR assay and primers specific for *LacZ*. Data is presented as mean ± SEM, normalized to the surface area of the graft. Asterisks represent significant differences (* $p<0.05$; ** $p<0.01$).

Figure 2. Retention of recombinant GDF-5 protein in tendon grafts determined using an ELISA assay. Data is presented as mean ± SEM, normalized to the surface area of the graft. Asterisks represent significant differences (** $p<0.01$).

Figure 3. Kinetics and biodistribution of rAAV and allograft mediated gene expression. (*a*) Representative bioluminescence images (BLI) of a representative mouse grafted with a freeze-dried FDL allograft loaded with rAAV-*Luc*. (*b*) In vivo Kinetics of Luc gene expression, based on bioluminescence intensity in a region of interest (ROI) encompassing the foot (mean value ± SEM).

Figure 4. Assessment of MTP joint flexion (inset) following reconstruction with rmGDF-5 or rAAV-*Gdf5* loaded allografts at 14 days post surgery. Average MTP joint flexion curves (*a*,*b*), and maximum MTP flexion range of motion (*c*) of the control (rAAV-*lacZ* loaded) allografts, rmGDF-5 loaded allografts, and rAAV-Gdf5 loaded allografts. Data presented as mean ± SEM Asterisks represent significant differences from control repairs (* $p<0.05$).

Figure 5. Assessment of tensile biomechanical properties of the FDL tendon following reconstruction with rmGDF-5 or rAAV-*Gdf5* loaded allografts at 14 days post surgery. (*a*) Maximum tensile force (strength) and (*b*) tensile stiffness of the FDL tendons reconstructed with

rAAV-lacZ loaded (control) allografts, rmGDF-5 loaded allografts, and rAAV-GDF-5 loaded allografts. Biomechanical properties were measured at 14 days post surgery. Data presented as mean ± SEM.

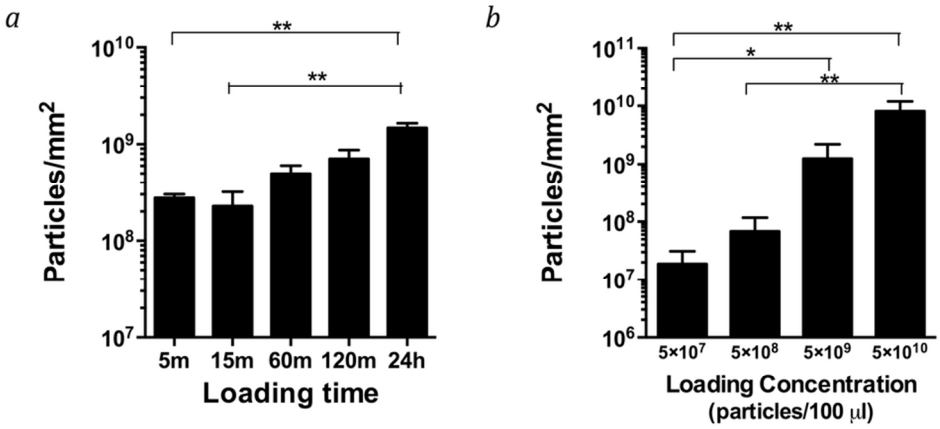

Figure 1. Retention of rAAV particles on tendon grafts, determined using a quantitative real time PCR assay and primers specific for LacZ. Data is presented as mean ± SEM, normalized to the surface area of the graft. Asterisks represent significant differences (* $p<0.05$; ** $p<0.01$).
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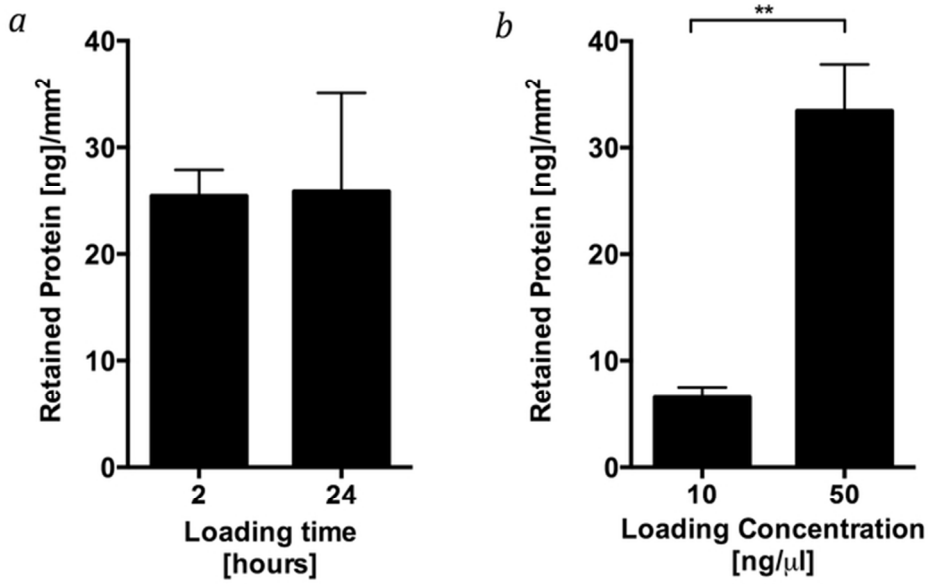

Figure 2. Retention of recombinant GDF-5 protein in tendon grafts determined using an ELISA assay. Data is presented as mean ± SEM, normalized to the surface area of the graft. Asterisks represent significant differences (** $p<0.01$).
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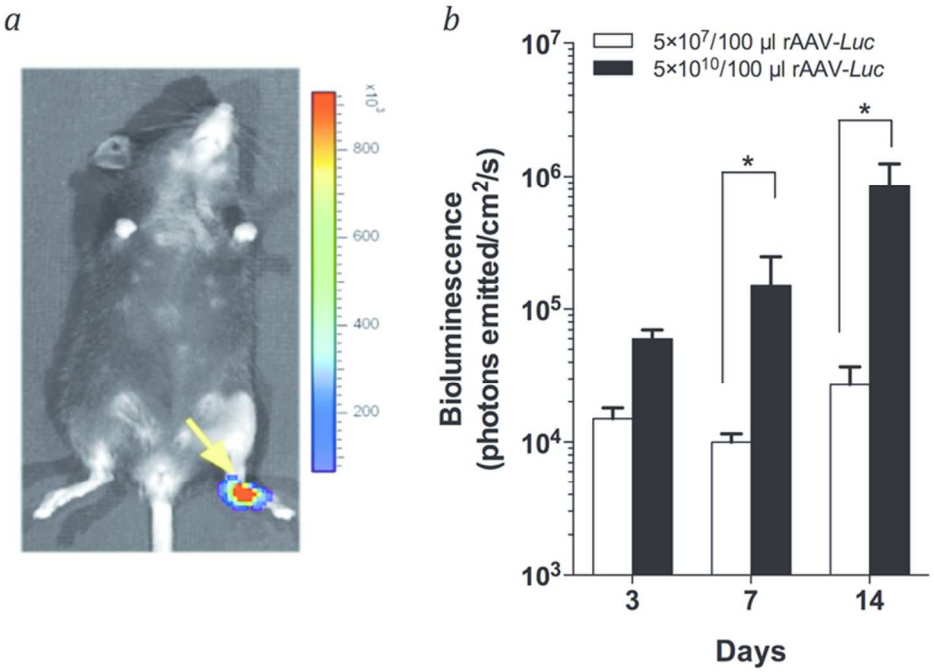

Figure 3. Kinetics and biodistribution of rAAV and allograft mediated gene expression. (a) Representative bioluminescence images (BLI) of a representative mouse grafted with a freeze-dried FDL allograft loaded with rAAV-Luc. (b) In vivo Kinetics of Luc gene expression, based on bioluminescence intensity in a region of interest (ROI) encompassing the foot (mean value ± SEM).
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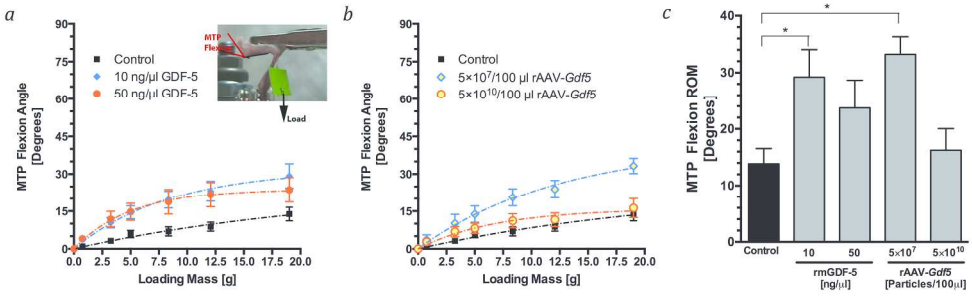

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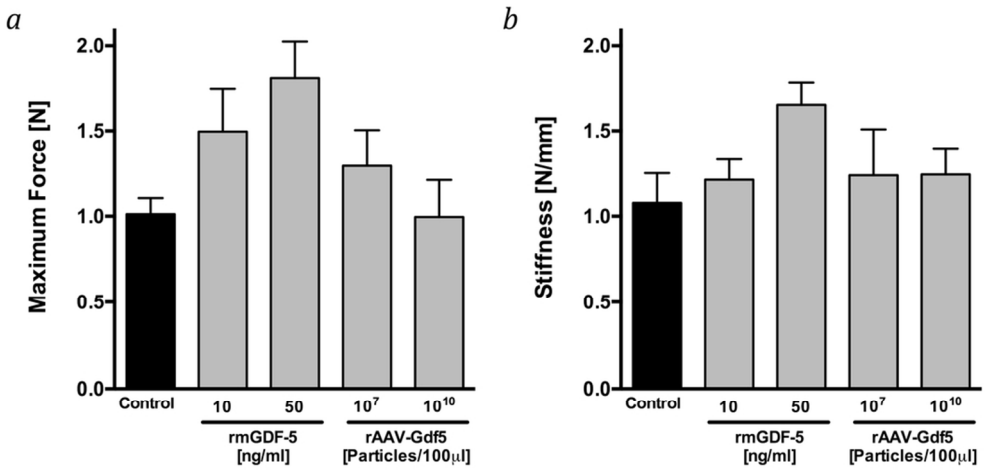
245x73mm (300 x 300 DPI)



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91x46mm (300 x 300 DPI)

Hasslund et al. Supplemental Material

A Protocol for Transient Unloading of the FDL Tendon Using BOTOX

In our previous publications (1-4), our original protocol to protect the graft from in vivo loading involved severing the musculotendinous junction (MTJ). However this additional injury compromises the accrual of biomechanical strength over time. To overcome this limitation, in this study we used an established rodent protocol for BOTOX injection (5) into the treated hind limb muscle to induce transient paralysis of the calf muscles (gastrocnemius and soleus) prior to freeze-dried allograft flexor tendoplasty. Previously, Yoneda et al. showed that a single 30 U/kg intramuscular injection of BOTOX can eliminate as much as 90% of the mouse's paw grip strength (and hence the in vivo load on the tendon) in the first 3 days, but recover as much as 40% of the grip strength over 21 days at a rate of ~2% per day (5).

To protect the graft from rupture, we first performed a feasibility study to adapt the Yoneda rodent protocol (5) for BOTOX-induced paralysis of the mouse's hind limb muscle, and compared this approach with our previous method of MTJ resection. Briefly, 8 week-old female C57BL/6 mice were anesthetized with ketamine (60 mg/kg body weight) and Xylazine (4mg/kg body weight) via an intraperitoneal injection. The left hind legs where shaved and rinsed with 70% ethanol. The BOTOX solution was diluted to a concentration of 120 units/ml and kept on ice until injected. Using a Hamilton syringe (model 701) and a 32 gauge needle, 250 µl/kg of BOTOX was injected into the flexor digitorum longus muscle through the skin. The injection was distributed in small deposits a few millimeters apart to ensure even distribution of the toxin in the muscle.

On the day of surgery, a grip test was performed to document qualitatively whether sufficient muscle paralysis was achieved. In these tests, the mouse was suspended by the tail and allowed

to grip onto a metal stick with the left hind leg. The grip action was qualitatively assessed (No gripping versus gripping). Mice that still had gripping function were not used for surgery.

As we have previously demonstrated (2), transecting the proximal MTJ resulted in elimination of flexor tendon gliding as evident by the hyper-extended paws and failure of the animal to grip on a rod with its operated paw up to 28 days (Fig. S1-*b*) and protected the graft from premature rupture (Fig. S1-*e*). Similarly, a 30 U/kg BOTOX intramuscular injection 1 day prior to surgery eliminated the ability of the mice to grip onto the rod (Fig. S1-*c*), and protected the grafted tendon from premature rupture, which was verified by dissection at 14 and 28 days post surgery (Fig. S1-*f*). Control saline injections resulted in loss of flexor tendon gliding and digital grip (Fig. S1-*a*) due to graft rupture as early as 3 days post-surgery (Fig. S1-*b*). The MTP joint flexion test (which measures the adhesion-related loss of tendon gliding ability) demonstrated that using either method (musculotendinous transection or BOTOX injection) the reconstructed paws had significantly reduced MTP flexion (Fig. S1-*g*) as demonstrated in the decreased MTP joint ROM (Fig. S1-*h*) at 14 and 28 days post tendoplasty, compared to normal unoperated controls.

Thus, our qualitative observations confirm that this BOTOX injection protocol protects the graft from rupture during the early healing phase. More robust gene expression and histological analyses of the healing response in this model along with the grip strength data will be evaluated in future studies to tease out the effects and interactions of innate or delivered growth factors and gradual accrual of *in vivo* loading over time.

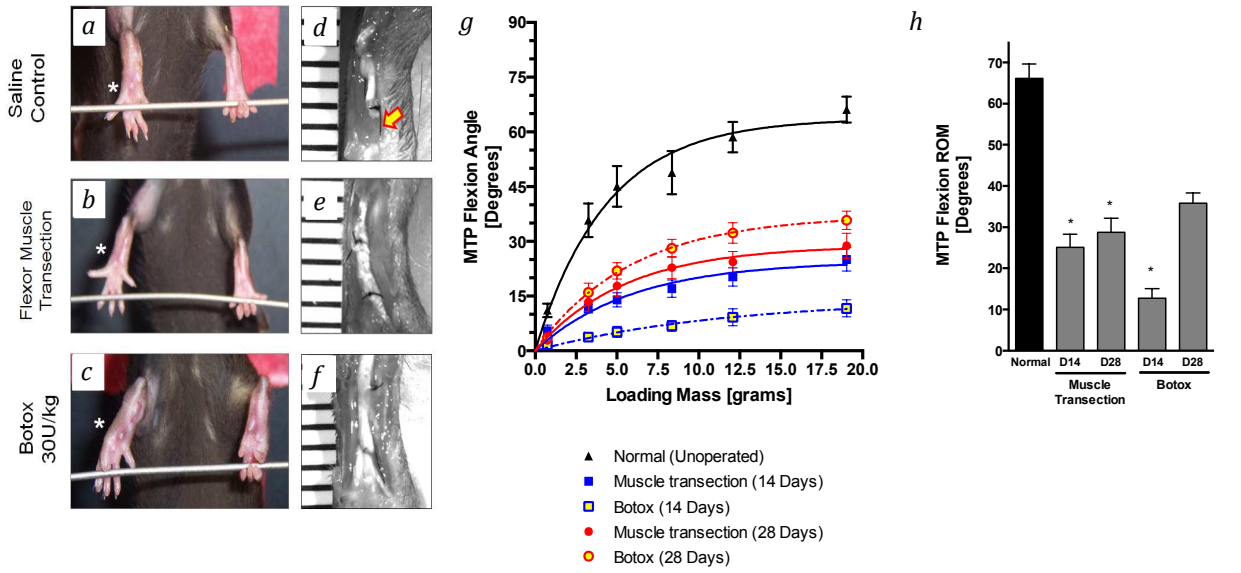

Figure S1. Effects of Botulinum Toxin type A (BOTOX) on digital flexion and grip, allograft integrity, and MTP joint flexion after flexor tendoplasty. Mice either received Saline (*a,d*) or 30 U/kg BOTOX (*c,f*) intramuscular injections or had their proximal musculotendinous junction transected (*b,e*) as described (2) and underwent a flexor tendoplasty a day later. At 14 days, the ability of the mice to grip onto a rod with their operated hind paws (asterisks) was qualitatively assessed (*a-c*) and followed by assessment of the MTP Joint flexion range of motion as a measure of adhesions at 14 days (*g-h*). Data presented as mean ± SEM (n=8). Asterisks represent significant differences from normal unoperated tendon (* $p<0.05$). The paws were then dissected to examine whether the reconstructed tendons remained intact or prematurely ruptured (*d-f*). Arrow in Panel *d* indicates that grafts rupture in the saline treated group.

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