Increasing the level of cytoskeletal protein Flightless I reduces adhesion formation in a murine digital flexor tendon model

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Abstract

Background: Surgical repair of tendons is common, but function is often limited due to the formation of flexor tendon adhesions which reduce the mobility and use of the affected digit and hand. The severity of adhesion formation is dependent on numerous cellular processes many of which involve the actin cytoskeleton. Flightless I (Flii) is a highly conserved cytoskeletal protein, which has previously been identified as a potential target for improved healing of tendon injuries. Using human in vitro cell studies in conjunction with a murine model of partial laceration of the digital flexor tendon, we investigated the effect of modulating Flii levels on tenocyte function and formation of adhesions.

Methods: Human tenocyte proliferation and migration was determined using WST-1 and scratch wound assays following Flii knockdown by siRNA in vitro. Additionally, mice with normal and increased levels of Flii were subjected to a partial laceration of the digital flexor tendon in conjunction with a full tenotomy to immobilise the paw. Resulting adhesions were assessed using histology and immunohistochemistry for collagen I, III, TGF-β1 and -β3.

Results: Flii knockdown significantly reduced human tenocyte proliferation and migration in vitro. Increasing the expression of Flii significantly reduced digital tendon adhesion formation in vivo which was confirmed through significantly smaller adhesion scores based on collagen fibre orientation, thickness, proximity to other fibres and crimping. Reduced adhesion formation was accompanied with significantly decreased deposition of type I collagen and increased expression of TGF-β1 in vivo.

Conclusions: These findings suggest that increasing the level of Flii in an injured tendon may be beneficial for decreasing tendon adhesion formation.

Keywords: Flii, Flightless I, Tenocyte, Tendon, Adhesions
Background

Tendon repair is a tightly orchestrated cascade of cellular events that results in the restoration of tendon continuity [1, 2]. The function of the restored tendon is highly dependent on the size and severity of adhesions that form during the healing process as a consequence of the significant inflammatory response that occurs after injury [3]. Formation of adhesions leads to the flexor tendons being bound to each other and surrounding structures hence preventing normal gliding actions [4–6]. For a tendon to heal effectively with its function intact, there must be a re-establishment of strength and gliding functions with minimal adhesion formation [7]. In order for this to occur, a delicate balance must exist between intrinsic and extrinsic repair processes, allowing migration and proliferation of cells to the site of injury to allow effective tendon repair [7–10]. The actin cytoskeleton is important in facilitating the cellular migratory and proliferative processes and proteins that regulate the actin cytoskeleton are integral to the repair process [11–13].

Flightless I (Flii) is an actin remodelling protein, which has been well defined as a negative regulator of cutaneous wound healing [14–16]. Mice with decreased Flii expression (Flii<sup>−/−</sup>) show improved wound repair and enhanced re-epithelialization, whilst mice overexpressing Flii (Flii<sup>Tg/Tg</sup>) have impaired repair responses and delayed recovery of the intact skin barrier post injury [14–17]. In contrast to its role in dermal wound repair, Flii has also been shown to positively influence the regeneration of hair follicles [18], and digit regeneration has been observed in Flii<sup>Tg/Tg</sup> mice following severe proximal amputation of murine claws [19]. Recent studies have further shown that explanted tendons from Flii overexpressing mice have significantly elevated tenocyte outgrowth compared to Flii<sup>−/−</sup> mice [20]. In addition, there is a large body of evidence showing the importance of Flii in cellular adhesion and matrix remodelling [21–23] as well as tissue inflammation during wound healing and in inflammatory skin conditions [24–28], consequently, here, we investigated the effect of altering Flii expression in vivo on tendon adhesion formation. A murine model of tendon adhesion was used in these studies is well established [29] and mice limbs and paws are frequently utilised due to the structural and biological similarity to the human hand. In this study, the effect of increased expression of Flii on tendon adhesion formation was investigated using a murine tendon injury model. Histological and immunohistochemical analysis of the tendons and the resulting adhesions was performed. The findings suggest that increasing Flii levels may be beneficial for reducing tendon adhesion formation.

Methods

siRNA knockdown of Flii

Flii siRNA transfection was undertaken following optimised experiments described previously [30]. Briefly, human fibroblasts and tenocytes were seeded in triplicate at 30-50% confluency on collagen-coated 24 well plates containing 500 μL DMEM and 20% FCS without antibiotics. Transfection efficiency was optimised using Block-iT fluorescent oligo in the presence of Lipofectamine 2000. Next, cells were seeded on collagen-coated plates for 24 h before Flii and scrambled siRNA were transfected into the cells using Lipofectamine 2000. siRNA was diluted in Opti-MEM I reduced serum media to a final concentration of 60 nM and then incubated 20 min at room temperature with Lipofectamine 2000 to form siRNA: Lipofectamine complex. A total of 500 μl was added to each culture well and incubated with cells for 6 h before replacing transfection media with 10% FCS-supplemented DMEM. Cells were incubated for 48-72 h for gene knockdown assessment, and protein was collected for standard Western blotting experiment demonstrating reduction in Flii expression following previously described protocols [15] (results demonstrated in Fig. 1a). Following optimisation of the protocol, siRNA-treated human tenocytes and fibroblasts were used in proliferation and migration assays described below. No treatment control cells were labelled as control. The oligo nucleotides used in this study are as follows:

Flii siRNA: sense 5′ → GCUGGAACACUUGU CUGUGTT → 3′
(GenePharma, Suzhou, China); antisense 5′ → CACAGACAAGUGUUCCAGCTT → 3′
(GenePharma, Suzhou, China); scrambled siRNA sense 5′ → UUCGCCAAGUGUCUCCAGTT → 3′ (Gene-Pharma), antisense 5′ → ACGUGACACGUGC CGGA ATT → 3′
(GenePharma, Suzhou, China); Block-iT (Invitrogen, Carlsbad, USA).
Proliferation assay
Human tenocytes and fibroblasts were used in a WST-1 metabolic proliferation assay, and transfected with Flii siRNA following established protocols [15]. Cells were cultured until confluent in a 37 °C, 5% CO2 incubator before seeding into 96-well plates at a density of 4 × 10^4 cells/well. After 24 h, the media was replaced with serum-free DMEM and incubated for 6 h to synchronize the cell cycle, and proliferation was assessed according to manufacturer’s protocols (Roche Applied Science, Munich, Germany). Briefly, 10 μL of WST-1 reagent was added to the cells and left at 37 °C for 30 min. The presence of the formazan product was quantified using a dual absorbance of 450 nm and 600 nm using a plate reader.

Migration assay
Human tenocytes and fibroblasts were used in a scratch wound assay and transfected with Flii siRNA following established protocols [15]. Cells were plated into 96-well plates and allowed to reach confluence before being wounded using a Woundmaker® (Essen Bioscience, Michigan, USA) which creates a uniform wound of 700-800 μm in each well of the 96 well ImageLock® plate. Cells were placed into an Incucyte®TM (Essen Bioscience, Michigan, USA) at 37 °C and 5% CO2 where images were automatically taken every 3 h for 24 h. The resulting images were analysed using Image Pro Plus 7.1 to determine the effect of Flii siRNA on cell migration.

Murine surgery
All experiments and maintenance of mice were conducted according to Australian Standards for Animal Care under protocols approved by the Women’s and Children’s Health Network Animal Ethics Committee (WCHN) and carried out in accordance with the Australian code of practice for the care and use of animals for scientific purpose (AE952/9/2016).

Flii^{+/+} mice generation
All mouse strains were congenic on the Balb/c background and Balb/c littermates were used as wild-type (WT) control animals. Transgenic Flii overexpressing mice (strain name: (Tg1FLII) 2Hdc) were generated as described previously [15] by incorporating a 17.8-Kb fragment of a human cosmid clone that spans the entire FLII locus, with animals homozygous for the transgene. These animals carry two copies of the mouse Flii allele designated Flii^{+/+} [31]. This transgenic strain was backcrossed to Balb/c animals for 10 generations before being intercrossed and homozygous animals were identified by PCR. Following this, the colony was maintained by crossing animals which were identified as homozygous for the transgene. These animals carry two copies of the mouse Flii gene and two additional copies of the human Flii gene (Flii^{+/+}) results in elevated levels of Flii protein in various tissues [15, 32]. Flii^{+/+} mice are viable and reproduce normally with an average litter size of 7.0 [32].

Digital tendon adhesion model
Digital tendon adhesion surgery was performed on WT and Flii^{+/+} mice following an established model [29]. Humane endpoints were at 3, 7, 14, 21 and 28 days post-surgery (n = 6 per group/timepoint). Each mouse received a 50% partial laceration of the digital flexor tendon to the third and fourth digit of both the right and left hind paw (Fig. 1a). Using a Nikon Trinocular dissecting microscope, the digital flexor tendon was exposed through a transverse skin incision and a standardised partial laceration was performed between the A1 and A3 pulley over the proximal phalanx. The skin was closed over the wound with a single 7/0 silk suture. Following the partial laceration of the digital flexor tendons, a further skin incision was made distal to the ankle joint of the left hind limb and a proximal tenotomy was performed of the deep and superficial flexor tendons. The incision was closed with two single 7/0 sutures. The tourniquet was removed, and mild pressure applied to restore blood flow in the foot. The mice were then given a single injection of Temgesic (buprenorphine 0.03 mg/kg) for analgesia. Upon recovery, the mice were visually inspected to ensure mobilisation of the right hindlimb and complete immobilisation of the left hindlimb.

Digit processing
Upon completion of the prescribed endpoint, mice were humanely euthanized and both hind feet were cleaned with 70% ethanol and removed intact, proximal to the ankle, placed into a 6-well plate and covered with 10% buffered formalin overnight. The bone was decalcified in 5% EDTA in 1× PBS for 5 days on a plate shaker. The EDTA was washed out on the fifth day in 3 changes of PBS × 2 min. Feet were placed in 70% ETOH and processed by dehydration in graduated alcohol washes (70% for 2 h, 80% for 1 h, 90% for 1 h, 95% for 1 h and 100% for 3 h). The tissue was then cleared in xylene for 3 h, followed by paraffin wax infiltration under pressure for 4 h at 62 °C. Upon completion of processing, the appropriate toes were dissected from the feet and embedded in paraffin wax [29].

Histology
Paraffin-embedded samples were sectioned (4 μm) for histological assessment and stained with haematoxylin and eosin (H&E) or Masson’s trichrome staining as described previously [33, 34]. Digits were cut transversely for 400 μm to reach the tendon, following this, 4 μm
sections were cut and visualised to ensure consistent sectioning. The epidermis was included to aid orientation, and care was taken to include the damaged tendon area. In some orientations, the tendon may appear to be completely lacerated in the histological sections; however, as demonstrated in Fig. 2, tendons were partially lacerated, and this visual phenomenon may be due to structure of the tendon (i.e. large and small tendon bundles) or orientation of the sample.

Immunohistochemistry
Immunohistochemical experiments were undertaken on all digits collected from Balb/c WT and Flii\textsuperscript{Tβ/β} mice at days 0, 3, 7, 14, 21 and 28 post-surgery. Following antigen retrieval, 3% normal goat serum diluted in PBS was used for blocking for 30 min. Primary antibodies were used at 2 μg/ml and included mouse α-Flii (Santa Cruz sc-21716), rabbit α-Collagen I (Rockland 600-401-103), rabbit α-Collagen III (Rockland 600-403-105), mouse α-TGFβ1 (Santa Cruz sc-52893) and rabbit α-TGFβ3 (Santa Cruz sc-83). Species-specific Alexa Fluor 488, 568 or 633-conjugated secondary antibodies (1:400, Invitrogen, Carlsbad, USA) were diluted in PBS and applied for detection. Nuclear counterstain 4,6-diamidino-2-phenylindole (DAPI) was applied last. The slides were mounted in Dako Fluorescent Mounting Medium (DAKO Corporation, Sydney, Australia) and viewed using an Olympus Epifluorescent microscope.

Image analysis
H&E staining of the tendons were analysed microscopically. Adhesion size was determined by measuring the total size of the adhesion around the tendon using the Image Pro-Plus 7.0 programme (Media Cybernetics Inc, Rockville, USA). Any adhesion formation seen at 4 × magnification was deemed as positive for adhesion formation. Masson’s trichrome stained slides were analysed for collagen formation. Briefly, a macro was created using Image Pro-Plus 7.0 which calculated the number of blue/green (collagen) pixels vs red (muscle/connective tissue) pixels within the tendon/adhesion. As tendon adhesions are made up of 99% collagen type I, this was used as a confirmation of adhesion formation. The adhesions were scored out of a maximum of 10 points for collagen fibre orientation, thickness, proximity to other fibres and crimping. Zero points would indicate an adhesion that had the same collagen structure as a normal tendon. Immunohistochemical samples were analysed by determining fluorescence intensity using the AnalySIS software (Soft-Imaging System GmbH, Munster, Germany). For verification purposes, negative control sections were included in each experiment which excluded staining with the primary antibody or secondary antibody. All negative control sections had negligible immunofluorescence

Statistical analysis
Data was analysed using the Student’s t test to compare between two groups or an ordinary two-way ANOVA with Tukey’s multiple comparison test when comparisons between more than 2 groups were required. A p value of < 0.05 was considered significant.

Results
Attenuation of Flii using siRNA decreases human tenocyte proliferation and migration
To investigate if human tenocytes and fibroblasts respond equivalently to Flii, siRNA was used to decrease gene expression and the resulting effects on cell proliferation and migration were determined. Flii expression was successfully decreased in both cell types following siRNA treatment (Fig. 1a). Reducing Flii expression in human tenocytes significantly decreased cell proliferation (p = 0.0007) (Fig. 1b). The opposite effect was observed in human fibroblasts with significantly increased proliferation observed following Flii knockdown through siRNA transfection (p = 0.0001) (Fig. 1c). Reducing Flii in human tenocytes significantly delayed migration, shown using the in vitro scratch wound assay (Fig. 1d-e), but improved fibroblast migration was observed (Fig. 1d-e). The impairment in human tenocyte migration following siRNA treatment (Fig. 1d-e) was evident at all time points measured (p = 0.0004, 0.009, 0.0001, 0.0002 and 0.001 respectively).

Increasing Flii levels decreases digital flexor tendon adhesions in vivo
Considering that reducing Flii expression showed impaired properties of human tenocytes, we proceeded to investigate the effect of Flii over-expression using a murine model of digital tendon adhesion formation. Mice with normal and increased levels of Flii were subjected to a partial laceration of the digital flexor tendon in the third and fourth digit of both hind paws in conjunction with a full tenotomy to immobilise the paw (Fig. 2a-f). Tendon rupture is extremely uncommon in this model and was not encountered in the mice. To ensure reproducibility of the 50% partial laceration, the divided tendons were stained with H&E and analysed (Fig. 2g). Partial lacerations were performed in both genotypes, and no difference in fibre division was detected between mice. The partial lacerations were found to have an average of 53% ± 4.3% fibre division (Fig. 2h), which was reproducible and robust as a standardised model. Immediately following the partial laceration, the skin was closed over the injury using a single 7/0 silk suture
(Fig. 2d). A proximal tenotomy was also performed in the left limb alone, distal to the ankle joint to confirm the superficial and deep tendons could be accessed and divided (Fig. 2e, f). This caused complete immobilisation of the digits of the affected limb but did not affect the mouse’s ability to move around. The effect of the proximal tenotomy on tendon adhesion formation was assessed at day 21 where it was observed that 54% ± 6.2 of the right digits with mobilisation had formed adhesions when compared with 82% ± 11.7 of the left digits with immobilisation ($p = 0.046$) (Fig. 2i-l). Adhesions were significantly larger ($p = 0.001$) at day 21 in mice with immobilised digits 34697.83 $\mu$M2 ± 863.3 when compared with mobilised digits 18654.44 $\mu$M2 ± 570.3 (Fig. 2l) and these were used for analysis in the rest of the study.

$Flii^{Tg/Tg}$ mice have impaired cutaneous healing but significantly smaller digital tendon adhesions than WT mice

The effect of Flii expression on digital tendon adhesion formation in immobilised paws was investigated using H&E stained sections of mice hind-paw digital tendons injured with a 50% laceration and harvested at days 3, 7, 14, 21 or 28 using 12-week-old female WT or $Flii^{Tg/Tg}$. The injury to the skin that occurred to access the tendon and sheath was also assessed and revealed that $Flii^{Tg/Tg}$ mice had significantly slower re-epithelialisation than WT mice ($p = 0.011$) (Fig. 3a-f) and a significantly thickened epidermis on days 3 and 7 ($p = 0.045$ and $p = 0.049$ respectively) (Fig. 3f). Digital tendon adhesions in WT and $Flii^{Tg/Tg}$ mice were measured at days 3, 7, 14, 21 and 28 post 50% laceration injury. Adhesions were recognised as a fibrotic mass between the tendon and
surrounding structures with architecture visually different to the dermal and tendon structures (Fig. 4a–j). FliiTg/Tg mice formed significantly smaller adhesions than WT mice on days 7, 14, 21 and 28 post-injury ($p = 0.0003, 0.0006, 0.0007$ and $0.0003$ respectively) (Fig. 4k).

Flii is upregulated in FliiTg/Tg tendon adhesions

The expression of Flii was assessed using immunohistochemistry and found to be significantly upregulated in the adhesions of FliiTg/Tg mice at days 7, 14, 21 and 28 post 50% laceration injury compared with WT mice ($p = 0.0003, 0.0006, 0.0007$ and $0.0003$ respectively) (Fig. 4k).

FliiTg/Tg mice have slower, more organised collagen deposition and reduced adhesion score

Masson’s trichrome staining was used to differentiate between connective tissues such as collagen and cytoplasm. A digital macro was used to quantify red vs. green staining at days 21 and 28 post-injury. It was determined that FliiTg/Tg mice had significantly lower collagen deposition in the healing tendon than WT mice ($p = 0.048$ and $0.044$ respectively) (Fig. 6a–e). Adhesions in FliiTg/Tg mice had a reduced adhesion score with significantly more organised collagen fibres than those of WT mice ($p = 0.05$) (Fig. 6f). FliiTg/Tg adhesions were more consistent with the organisation seen in the uninjured tendon, with fibres following a crimped wavy path and appearing similar in structure to normal tendons.

FliiTg/Tg mice adhesions commence remodelling process slower than WT mice adhesions

Immunohistochemical staining allowed the investigation of levels of collagen type I and III (Coll I and III respectively) in the established adhesions at days 21 and 28 (Fig. 7). Whilst unwounded tendons and established adhesions are composed primarily of Coll I, Coll III is secreted by tenocytes in the early stages of the healing
process [35]. Comparing the levels of these two collagens allowed the investigation of the rate of the healing process and the severity of adhesion formation. WT mice had significantly increased Coll I in the adhesions at days 21 and 28 compared with the FliiTg/Tg mice (p = 0.044 and 0.032 respectively) (Fig. 7a-i). FliiTg/Tg mice still had low levels of Coll III present in the adhesion at days 21 and 28 whereas WT mice had no Coll III detectable (Fig. 7j).

FliiTg/Tg mice adhesions express higher levels of TGFβ1 and lower levels of TGFβ3 than WT mice

TGFβs are a family of important growth factors known to be active in all the phases of tendon healing. TGFβ isoforms are involved in a variety of healing processes including collagen production and intrinsic/extrinsic cell migration. Flii has been shown to regulate TGFβ expression [36], and increased Flii levels have been shown to upregulate the pro-fibrotic TGFβ1 isoform and downregulate the anti-scarring TGFβ3 isoform in cutaneous wounds [25, 36]. Here, the expression of TGFβ1 and TGFβ3 in the adhesions and surrounding dermis in WT and FliiTg/Tg mice was investigated using immunohistochemistry. TGFβ1 expression in tendon adhesions was significantly higher in FliiTg/Tg mice than WT mice, at days 14, 21 and 28 (p = 0.048, 0.044 and 0.039 respectively) with expression in both genotypes peaking at day 21 post injury (Supplementary Figure 1 and Fig. 8a). TGFβ1 expression in the surrounding dermis was also significantly elevated in FliiTg/Tg mice at days 3 and 7.
(p = 0.01 and 0.046 respectively) when compared with WT mice, with expression peaking at days 3 and 7 post injury respectively (Fig. 8b). In contrast, TGFβ3 expression was significantly elevated in both the adhesion at days 14, 21 and 28 (p = 0.033, 0.009 and 0.018 respectively) (Fig. 8c) and the dermis in WT mice at day 3 (p = 0.044) when compared with Flii^+/− mice (Fig. 8d). Both genotypes had peak expression of TGFβ3 in the adhesion at day 21 and in the surrounding dermis at day 3 post injury (Fig. 8c-d).

Discussion

Tendon injuries require a complex re-organisation of the actin cytoskeleton in order to facilitate essential processes for repair including migration and proliferation whilst minimising tendon adhesions which hinder repair and functionality. Tenocytes make up the majority of the cellular content in tendons and are integral in coordinating the healing process following injury including release of signalling molecules to recruit a variety of growth factors, cytokines and inflammatory cells to the injury site in order to facilitate successful healing [37]. We have previously shown the effect of differential Flii gene expression on murine tenocyte function in vitro [20]. In this study, we investigated the effect of modulating Flii levels on human tenocyte function and formation of tendon adhesions using a murine model of a partial laceration of the digital flexor tendon.

Flii belongs to a family of actin remodelling proteins critical in modulating cellular responses during development, tissue healing and regeneration [16, 17, 21, 38]. Previous studies have shown that Flii is expressed in murine tenocytes and its expression is upregulated in response to wounding using in-vitro models [20]. In order to ascertain the role of Flii in tendon adhesion formation, we first investigated the effects of reducing Flii expression on human tenocyte function and fibroblast proliferation and migration properties in vitro. In agreement with previous studies [15], we found that reducing Flii using siRNA resulted in improved human fibroblast proliferation and migration properties in vitro. This however contrasted with human tenocytes where reduction of Flii leads to negative cellular
responses including reduced tenocyte proliferation and migration.

Digital tendon adhesions form as the result of a large inflammatory response following injury and result in the tendon being bound to surrounding structures, preventing normal gliding function and significantly reducing the mobility and function of the affected digit and the hand [39–41]. To determine the role of Flii in adhesion formation, a murine model of digital tendon adhesion formation was used with mice with either normal or increased expression of Flii [32]. Overexpression of Flii resulted in significantly smaller tendon adhesions when compared with WT mice, over a period of 28 days suggesting that Flii is important in regulating tendon adhesion formation. The model involves penetrating the tendon sheath, allowing the influx of extrinsic cells from the surrounding tissue which results in the formation of an adhesion. With FliiTg/Tg mice showing significantly smaller tendon adhesion size, this suggests that Flii may affect adhesion formation through regulation of extrinsic cell migration to the wound site. Interestingly, an upregulation of Flii expression appears to be positively correlated with decreased adhesion formation which is in contrast to previous research in cutaneous repair which has indicated that Flii negatively regulates cutaneous healing [14, 17, 32, 42]. This may be due to a tissue-specific effect, as previous research has shown that Flii can positively regulate healing in mammals in areas, which have retained a regenerative phenotype including the digit tip [19]. In order to access the tendon, an incision was made through the epidermal and dermal layers of the digit. The cutaneous wound notably had impaired healing in the FliiTg/Tg mice compared with WT mice, with slower re-epithelialisation and a significantly thickened epidermis. This shows that the positive effect of Flii seen on adhesion formation was not due to a positional

**Fig. 5** Adhesions in FliiTg/Tg mice have upregulated Flii levels. Representative images immunostained for Flii expression in WT (a-e) and FliiTg/Tg (f-j) mice from days 3, 7, 14, 21 and 28 post-injury. Flii expression is detected as red fluorescence and DAPI staining detected as blue fluorescence. t, tendon; d, dermis; dotted line, adhesion area. Magnification × 10. Scale bar = 200 μM and refers to all images. (k) Graphical representation of Flii expression in the tendon adhesions of WT and FliiTg/Tg mice at 7, 14, 21 and 28 days post 50% partial laceration injury. No adhesions were detected at day 3 post injury. n = 6. Data represented as mean ± SEM. *p ≤ 0.05
effect, as the skin in the same area retained its negative healing outcomes with increased Flii levels and suggests a cell-specific response to Flii results in the positive response of smaller adhesions in Flii

Immunohistochemical staining of Flii showed a significant increase in Flii expression in the tendon adhesions and surrounding dermis of FliiTg/Tg mice, suggesting that any improvements seen in the formation of adhesions were likely due to increased Flii levels. Negligible staining for Flii was observed in intact tendons likely due to their being mainly composed of collagen type I protein. Flii may be expressed in tenocytes within the tendon, but these cells are found sporadically throughout the tendon and would be hard to detect in significant numbers in vivo cross sections.

Collagen type I is the main component of tendon adhesions and cutaneous scarring [43, 44]. Flii

![Figure 6](image)

**Fig. 6** FliiTg/Tg mice have slower, more organised collagen deposition within the adhesion and significantly decreased adhesion score. (a-d) Representative images of tendon adhesions in WT (a-b) and FliiTg/Tg (c-d) mice at days 21 and 28 post-injury, stained with Masson’s trichrome. Dotted line represents adhesion area; t, tendon. Magnification × 20, scale bar = 200 μM. (e) Graph showing significantly decreased collagen deposition in the healing tendons of FliiTg/Tg mice compared with WT at day 21 and 28. (f) Graph showing decreased adhesion score indicative of collagen fibres appearing more similar to those of unwounded tendons. n = 6. Data represented as mean ± SEM. *p ≤ 0.05
is also vital in determining the physiological outcome of the resulting scar, with slower more organised collagen type I production resulting in a better healing outcome [15]. Flii/Tg mice had less collagen type I than WT mice in the tendon adhesions. Moreover, these collagen fibres in Flii/Tg mice appeared closer to that of unwounded tendons. This suggests that during tendon repair, increased Flii levels may cause a more controlled cellular response, leading to slower, more organised collagen type I deposition. The mechanism by which Flii regulates collagen type I deposition is still unknown, but it has been suggested that it may modulate transcription of genes including TGFβ1 in wound healing [15].

Flii has been shown to play a critical role in TGFβ1/SMAD-mediated transcription of collagen which is important for fibrosis and wound repair [25]. Through its role in interacting with the SWI/SNF complex, Flii recruits the TGFβ-responsive element (TRE) in a TGFβ-dependent manner, facilitating TGFβ-induced chromatin accessibility to target genes including the COLIA2 gene promoter [45]. Flii is also critical for the recruitment of Brahma-related gene 1 (BRG1), a core ATPase associated with SMAD2 and SMAD3, to the COLIA2 promoter region leading to collagen production [45]. In response to wound healing, Flii translocates from the cytoplasm to the nucleus where it forms a transcription complex with activating proteins-1 (AP-1) c-fos and c-jun which bind to the TGF-β promoter to regulate its expression [25]. Flii may therefore function as a nuclear receptor co-activator through its direct interaction with c-fos and c-jun and/or other co-activator complexes may modulate TGF-β gene expression. Flii has further been shown to regulate TGF-β1/SMAD signalling indirectly by suppressing the function of Akt in the nucleus of wounded fibroblasts which is involved in the post-translational phosphorylation of SMADs [36]. TGFβ1 is upregulated shortly after tendon injury, specifically in the tendon sheath and the epitelen [46, 47].
often leads to increased production of collagen type I subsequently resulting in excessive, disordered collagen type I production, leading to adhesions and fibrosis [48]. Flii modulates TGFβ expression, with decreased levels of TGFβ1 and increased levels of TGFβ3 detected in cutaneous healing studies in Flii+/− mice [36]. Although FliiTg/Tg mice adhesions had significantly higher TGFβ1 levels early in the healing process, these peaked at day 3 and dropped off significantly by day 14. In comparison, WT mice adhesions do not see a peak in TGFβ1 levels until day 7, and these levels remain relatively high across the course of healing. This delayed expression of TGFβ1 may help to explain why the formation of adhesions occur earlier with significantly higher collagen type I levels in WT vs FliiTg/Tg mice adhesions.

Conclusions
In summary, this study has shown that overexpression of Flii leads to smaller tendon adhesions with collagen organisation more closely resembling unwounded tendons. If translated into the human situation this could potentially lead to improved mobility and function of an injured digit or hand. Whilst the mechanism for tendon adhesion formation remains to be elucidated, these studies support further investigations aimed at determining if Flii could be a potential target for a therapeutic intervention for decreasing human tendon adhesion formation.

Supplementary information
Supplementary information accompanies this paper at https://doi.org/10.1186/s13018-020-01889-y.

Additional file 1: Figure S1. TGFβ1 and TGFβ3 expression in WT mice at days 3, 7, 14, 21 and 28 post injury. TGFβ1 and TGFβ3 expression in WT and FliiTg/Tg mice at days 3, 7, 14, 21 and 28 post injury. Representative images of TGBβ1 expression (A-E), and TGFβ3 expression (F-J) in WT mice from days 3-28 post partial laceration injury. (K-O) are composite images of the two stains. Representative images of TGBβ1 expression (A1-E1), and TGFβ3 expression (F1-J1) in FliiTg/Tg mice from days 3-28 post partial laceration injury. (K1-O1) are composite images of the two stains. TGFβ1 is represented by red staining, TGFβ3 represented by gold staining and DAPI by blue staining. t = tendon. d = dermis. Dotted line represents tendon adhesion area. Magnification x 10. Scale bar = 200 μM.

Abbreviations
AP-1: Activating protein-1; Col: Collagen; DAPI: 4',6-diamidino-2-phenylindole; EDTA: Ethylenediaminetetraacetic acid; ETOH: Ethanol;
Fli1: Flightless; H&E: Haematoxylin and eosin; PBS: Phosphate buffered saline; TGF-β: Transforming growth factor beta; WT: Wild type

Authors’ contributions
AJC, JEJ, PJ A and ZK conceived all experiments. JEJ performed all experiments and analysis with the assistance of ZK. All authors contributed to manuscript writing and have approved the final submitted and published versions.

Funding
This work was supported by an Australian Postgraduate Award scholarship to JEJ. ZK is supported by a Foundation Fellowship from the University of South Australia. AJC is supported by NHMRC Senior Research Fellowship GNT1102617.

Availability of data and materials
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate
All experiments and maintenance of mice were conducted according to Australian Standards for Animal Care under protocols approved by the Women’s and Children’s Health Network Animal Ethics Committee (WCHN) and carried out in accordance with the Australian code of practice for the care and use of animals for scientific purpose (AE952/9/2016).

Consent for publication
Not applicable

Competing interests
The authors declare they have no competing interests.

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Received: 2 June 2020 Accepted: 13 August 2020
Published online: 27 August 2020

References
1. Legrand A, Kaufman Y, Long C, Fox PM. Molecular biology of flexor tendon healing in relation to reduction of tendon adhesions. J Hand Surg [Am]. 2017;42(9):222–6.
2. Gelberman RH, Manske PR. Factors influencing flexor tendon adhesions. Hand Clin. 1985;1:135–42.
3. Martin P. Wound healing—aiming for perfect skin regeneration. Science. 1997;276(5309):75–81.
4. Miller JA, Ferguson RL, Powers DL, Burns JW, Shalaby SW. Efficacy of hyaluronic acid/nonsteroidal anti-inflammatory drug systems in preventing postsurgical tendon adhesions. J Biomed Mater Res. 1997;38(1):25–33.
5. Sammer DM, Chung KC. Advances in the healing of flexor tendon injuries. Wound Repair Regen. 2014;22(6):S125–9.
6. Ding B, Wang X, Yao M. Photothermal tissue bonding technique for improving healing of hand tendon injury. Surg Innov. 2019;26(2):153–61.
7. Manske P. Flexor tendon healing. The Journal of Hand Surgery: British & European Volume. 1988;13(3):237–45.
8. Chen Z, Rui Y, Xu Y, Zhang Q, Sun Z, Zhou J, et al. Effect of tendon hydrogel on healing of tendon injury. Exp Ther Med. 2018;15(6):515–9.
9. Gulec A, Turk Y, Aydin BK, Ercokac OF, Safali S, Ugurluoglu C. Effect of curcumin on tendon healing: an experimental study in a rat model of Achilles tendon injury. Int Orthop. 2018;42(8):1905–10.
10. Long C, Wang Z, Legrand A, Chattopadhaya A, Chang J, Fox PM. Tendon tissue engineering: mechanism and effects of human tenocyte coculture with adipose-derived stem cells. J Hand Surg [Am]. 2018;43(2):183 e1–9.
11. dos Remedios CG, Chhabra D, Kelic M, Dedova IV, Tsubakihara M, Berry DA, et al. Actin binding proteins: regulation of cytoskeletal microfilaments. Physiol Rev. 2003;83(2):433–73.
12. Hall A. Rho GTPases and the actin cytoskeleton. Science. 1998;279(5350):509–14.
13. Hall A, Nobes CD. Rho GTPases: molecular switches that control the organization and dynamics of the actin cytoskeleton. Philos Trans R Soc Lond Ser B Biol Sci. 2000;355(1399):965–70.
14. Adams DH, Ruzehaji N, Strudwick XL, Greenwood JE, Campbell HD, Arkell R, et al. Attenuation of Flightless I, an actin-remodelling protein, improves burn injury repair via modulation of transforming growth factor (TGF)-beta1 and TGF-beta3. Br J Dermatol. 2009;161(2):326–36.
15. Cowin AJ, Adams DH, Strudwick XL, Chan H, Hooper JA, Sander GR, et al. Flightless I deficiency enhances wound repair by increasing cell migration and proliferation. J Pathol. 2007;201(5):572–81.
16. Kopecki Z, Cowin AJ. Flightless I, an actin-remodelling protein and an important negative regulator of wound repair. Int J Biochem Cell Biol. 2008;40(8):1415–9.
17. Kopecki Z, Yang GN, Arkell RM, Jackson JE, Melville E, Iwata H, et al. Flightless I over-expression impairs skin barrier development, function and recovery following skin blistering. J Pathol. 2014;232(3):541–52.
18. Waters JM, Lindo JE, Arkell RM, Cowin AJ. Regeneration of hair follicles is modulated by Flightless I (Flii) in a rodent vivisera model. J Invest Dermatol. 2011;131:838–47.
19. Strudwick XL, Waters JM, Cowin AJ. Flightless I expression enhances murine claw regeneration following digit amputation. J Invest Dermatol. 2017;137(1):228–36.
20. Jackson JE, Kopecki Z, Anderson PJ, Cowin AJ. In vitro analysis of the effect of Flightless I on murine tenocyte cellular functions. J Orthop Surg Res. 2020; In Press.
21. Kopecki Z, Arkell R, Powell BC, Cowin AJ. Flightless I regulates hemidesmosome formation and integrin-mediated cellular adhesion and migration during wound repair. J Invest Dermatol. 2009;129(8):2031–45.
22. Kopecki Z, O’Neill GM, Arkell R, Cowin AJ. Regulation of focal adhesions by Flightless I involves inhibition of paxillin phosphorylation via a Rac1-dependent pathway. J Invest Dermatol. 2011;131(7):1450–9.
23. Arora PD, Nakajima K, Nanda A, Plaha A, Wilde A, Sacks DB, et al. Flightless anchors IQGAP1 and R-Ras to mediate cell extension formation and matrix remodeling. Mol Biol Cell. 2020;mbcE19100554.
24. Chong HT, Yang GN, Sidhu S, Ibbotson J, Kopecki Z, Cowin AJ. Reducing Flightless I expression decreases severity of psoriasis in an imiquimod-induced murine model of psoriasisform dermatitis. Br J Dermatol. 2017;176(3):705–12.
25. Kopecki Z, Arkell RM, Strudwick XL, Hirose M, Ludwig RJ, Kern JS, et al. Overexpression of the Flii gene increases dermal-epidermal blistering in an autoimmune ComI mouse model of epidermolysis bullosa acquisita. J Invest Dermatol. 2011;223(5):401–13.
26. Kopecki Z, Stevens NE, Chong HT, Yang GN, Cowin AJ. Flightless I alters the inflammatory response and autoantibody profile in an OVA-induced atopic dermatitis skin-like disease. Front Immunol. 2018;9:1833.
27. Wang T, Chung TH, Ronni T, Gu S, Du YC, Cai H, et al. Flightless I homolog negatively modulates the TLR pathway. J Immunol. 2006;176(3):1355–62.
28. Ruzehaji N, Kopecki Z, Melville E, Appleby SL, Bonder CS, Arkell RM, et al. Attenuation of Flightless I improves wound healing and enhances angiogenesis in a murine model of type 1 diabetes. Diabetologia. 2014;57(2):402–12.
29. Wong KS, Lui YH, Kapacee Z, Kadler KE, Ferguson MW, McGrouther DA. The cellular biology of flexor tendon adhesion formation: an old problem in a new paradigm. Am J Pathol. 2009;175(5):1938–51.
30. Martens PJ, Ly M, Adams DH, Penzkover KR, Strudwick X, Cowin AJ, et al. In vivo delivery of functional Flightless 1 sRNA using layer-by-layer polymer surface modification. J Biomater Appl. 2015;30(3):257–68.
31. Jackson JE, Yang GN, Jackson JE, Melville EL, Galley MP, Murrell DF, et al. Cytoskeletal protein Flightless I inhibits apoptosis, enhances tumor cell invasion and promotes cutaneous squamous cell carcinoma progression. Oncotarget. 2015;6(34):36426–40.
32. Thomsen N, Chappell A, Ali RG, Jones T, Adams DH, Matthaei KI, et al. Mouse strains for the ubiquitous or conditional overexpression of the Flii gene. Genesis. 2011;49(8):681–8.
33. Jackson JE, Kopecki Z, Adams DH, Cowin AJ. Flii neutralizing antibodies improve wound healing in porcine preclinical studies. Wound Repair Regen. 2012;20(4):523–36.
34. Kopecki Z, Luchetti MM, Adams DH, Strudwick X, Mantamadiotis T, Stopppacciaro A, et al. Collagen loss and impaired wound healing is associated with c-Myb deficiency. J Pathol. 2007;211(3):351–61.
35. Sharma P, Maffulli N. Basic biology of tendon injury and healing. The Surgeon: journal of the Royal Colleges of Surgeons of Edinburgh and Ireland. 2005;3(5):309–16.

36. Chan H, Kopecki Z, Waters J, Powell B, Arkell R, Cowin A. Cytoskeletal protein flightless-I differentially affects TGF-beta isoform expression in both in vitro and in vivo wound models. Wound Practice & Research: Journal of the Australian Wound Management Association. 2014;22(3):169.

37. Andarawis-Puri N, Flatow EL, Slosovsky LJ. Tendon basic science: development, repair, regeneration, and healing. J Orthop Res. 2015;33(6):780–4.

38. Yang GN, Strudwick X, Border C, Kopecki Z, Cowin AJ. Effect of Flightless I expression on epidermal stem cell niche during wound repair. Advances in Wound Care. 2019;Online Ahead of Print.

39. Khanna A, Friel M, Gougoulias N, Longo UG, Maffulli N. Prevention of adhesions in surgery of the flexor tendons of the hand: what is the evidence? Br Med Bull. 2009;90:885–109.

40. Matthews P, Richards H. Factors in the adherence of flexor tendon after repair: an experimental study in the rabbit. J Bone Joint Surg (Br). 1976;58(2):230–6.

41. Mortensen HM, Skov O, Jensen PE. Early motion of the ankle after operative treatment of a rupture of the Achilles tendon. A prospective, randomized clinical and radiographic study. J Bone Joint Surg Am. 1999;81(7):983–90.

42. Cameron AM, Turner CT, Adams DH, Jackson JE, Melville E, Arkell RM, et al. Flightless I is a key regulator of the fibroproliferative process in hypertrophic scarring and a target for a novel antiscarring therapy. Br J Dermatol. 2016;174(4):786–94.

43. Liu X, Wu H, Byrne M, Krane S, Jaenisch R. Type III collagen is crucial for collagen I fibrillogenesis and for normal cardiovascular development. Proc Natl Acad Sci U S A. 1997;94(5):1852–6.

44. Dale PD, Sherratt JA, Maini PK. A mathematical model for collagen fibre formation during foetal and adult dermal wound healing. Proceedings Biological sciences / The Royal Society. 1996;263(1370):653–60.

45. Lim MS, Jeong KW. Role of flightless-I (Drosophila) homolog in the transcriptional activation of type I collagen gene mediated by transforming growth factor beta. Biochem Biophys Res Commun. 2014;454(3):393–8.

46. Chang J, Most D, Stelnicki E, Siebert JW, Longaker MT, Hui K, et al. Gene expression of transforming growth factor beta-1 in the rabbit zone II flexor tendon wound healing. evidence for dual mechanisms of repair. Plast Reconstr Surg. 1997;100(4):937–44.

47. Chang J, Thunder R, Most D, Longaker MT, Lineaweaver WC. Studies in flexor tendon wound healing: neutralizing antibody to TGF-beta 1 increases postoperative range of motion. Plast Reconstr Surg. 2000;105(1):148–55.

48. Chan KM, Fu SC, Wong YP, Hui WC, Cheuk YC, Wong MWN. Expression of transforming growth factor β isoforms and their roles in tendon healing. Wound Repair Regen. 2008;16(3):399–407.

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Author/s:
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Title:
Increasing the level of cytoskeletal protein Flightless I reduces adhesion formation in a murine digital flexor tendon model.

Date:
2020-08-27

Citation:
Jackson, J. E., Kopecki, Z., Anderson, P. J. & Cowin, A. J. (2020). Increasing the level of cytoskeletal protein Flightless I reduces adhesion formation in a murine digital flexor tendon model.. J Orthop Surg Res, 15 (1), pp.362-. https://doi.org/10.1186/s13018-020-01889-y.

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