Cytoskeletal protein Flightless I differentially affects TGF-β isoform expression in both *in vitro* and *in vivo* wound models

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ABSTRACT

Flightless I (Flii) is a multifunctional cytoskeletal protein and a negative regulator of wound healing. It affects processes including cellular adhesion, migration and proliferation. These cell processes are also affected by the pro-fibrotic growth factor TGF-β1, which contributes to increased scar formation. Using Flii heterozygous (*Flii*$^{+/-}$), wild-type (WT) and Flii overexpressing (*Flii*$^{Tg/+}$) mice in an incisional model of wound healing, and primary fibroblasts in *in vitro* models of wound healing, we examined whether changes in Flii gene expression could affect specific TGF-β isoform expression and signalling. TGF-β1 levels were increased in Flii overexpressing wounds while TGF-β3 was elevated in Flii-deficient wounds. Wounding fibroblasts *in vitro* led to a translocation of both Flii and TGF-β isoforms from the cytoplasm into the nucleus. Flii not only co-localised with all TGF-β isoforms, but it also associated with the activating protein-1 (AP-1) subunits c-fos and c-jun as well as nuclear Akt. Additionally, siRNA knockdown of Flii gene expression decreased TGF-β1 and Smad 3 and led to an elevation of inhibitory Smad 7, indicating a potential mechanistic role for Flii in TGF-β signalling. We conclude that Flii effects on wound healing could potentially be via its modulatory effects on the TGF-β signalling pathway.

Keywords: Flightless I, TGF-β, cytoskeleton, actin, wounds.

INTRODUCTION

The reorganisation and remodelling of the actin cytoskeleton is important in all aspects of wound repair including lamellipodial crawling of keratinocytes during re-epithelialisation, the migration of fibroblasts across the wound matrix, infiltration of inflammatory cells and wound contraction[1]. The gelsolin family of actin-remodelling proteins play a key role in regulating actin cytoskeletal proteins. Flightless I (Flii) contains a leucine-rich repeat domain, which makes it a unique member of the gelsolin family. This domain allows interactions with other proteins and Flii has been shown to interact

with GTP binding proteins such as ras, cdc42 and rhoA[2], which are involved in the MAPK and cell cycle pathways[3,4]. As well as being an intracellular protein, Flii is also secreted and its extracellular functions include modulation of inflammatory responses and cytokine secretion, both of which may affect wound healing[5,6]. Flii is a negative regulator of wound healing[7,8]. Flii-deficient mice show improved wound healing with increased epithelial migration. In contrast, Flii overexpressing mice have delayed wound healing with gaping wounds, reduced cellular proliferation and migration due to defective focal adhesions[9,10]. Flii is an important developmental protein and it plays an important role in the development and function of the skin's barrier as well as recovery of the intact barrier following skin blistering[11]. While Flii homozygous mice are embryonic lethal, overexpression of Flii results in significantly thinner and more fragile skin and up-regulation of Flii in response to tissue injury or wounding leading to impaired healing and aberrant collagen production[12-16]. Recent studies using Flii neutralising antibodies have identified Flii as a novel mediator of fibrosis and potential anti-scarring target[17].

Three isoforms of transforming growth factor-β, TGF-β1, TGF-β2 and TGF-β3 have been identified as important mediators of cutaneous scarring[18]. TGF-β is an important and essential cytokine that governs a substantial magnitude of cellular processes and activities[19]. The bioactive TGF-β is usually a homodimer made up of two 12.5kDa molecules joined together by a sulfhydryl bond to form a 25kDa molecule[20]. TGF-β signals are transduced through heteromeric complexes of type I and type II serine/threonine kinase receptors. Binding of TGF-β ligand leads to the phosphorylation of type I receptors, which in turn phosphorylate Smad 2/3[19]. This is followed by the formation of a complex with Smad 4 and translocation into the nucleus where they regulate transcription of target genes[21]. Smad 7 inhibits TGF-β signalling by preventing the formation of the Smad 2/Smad 4 complexes which initiate the TGF-β signalling[22]. It interacts with activated TGF-β type I receptor, therefore blocking the association, phosphorylation and activation of Smad 2. Manipulation of TGFβ1 and TGFβ2 isoforms in wound healing have shown to be associated with increased inflammation and scarring[18]. Conversely, exogenous addition of TGF-β3 improves wound healing, suggesting that specific isoforms of TGF-β differentially regulate cellular processes and play an important role in the outcome of wound repair[18].

In this study, using Flii heterozygous and overexpressing mice, we report that manipulation of Flii gene expression differentially affects TGF-β isoforms and also affects TGF-β regulatory proteins c-fos, c-jun and TGF-β signalling proteins, Smads, that regulate and transduce TGF-β signals. This study suggests that Flii may affect wound healing outcomes by modulating the TGF-β signalling pathway.

MATERIALS AND METHODS

Antibodies

Mouse monoclonal anti-Flii antibody (sc-21716), rabbit polyclonal anti-Flii (sc-30046), anti-TGFβ1 (sc-146), anti-c-fos (sc-52), anti-c-jun (sc-45), and anti-Akt (sc-8312) antibodies were obtained from Santa Cruz Biotechnology (CA, USA). Rabbit polyclonal anti-TGFβ2 (AB-12-NA) and goat polyclonal anti-TGF-β3 (AB-244-NA) were obtained from R&D Systems (Minneapolis, USA). Mouse monoclonal anti-Gelsolin (610413) was obtained from BD Biosciences Pharmingen. Mouse monoclonal anti-β-tubulin (T4026) was obtained from Sigma-Aldrich (Sydney, Australia). Biotinylated horse anti-mouse IgG from Vector (Burlingane, CA), Streptavidin Alexa Fluor 555 (S32355) and goat anti-rabbit Alexa Fluor 488 (A11008) from Invitrogen (Oregon, USA) were used in this study.

Animal studies

All animal experiments were approved by the Women's and Children's Health Network (WCHN) Animal Ethics Committee and the Australian National University Animal Ethics Committee following the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, conforming to the Statement on Animal Experimentation by the NHMRC.

Mice

The alleles used were (i) a *Flii* targeted null allele (MGI:2179825: $Flii^{tm1Hdc}$) and (ii) a transgenic strain carrying the human *FLII* gene on a cosmid (MGI:3796829: Tg(FLII)1Hdc)[12,23]. Animals were genotyped as described in (12, 23). Both strains were BALB/c congenic and were maintained by continuous backcross of heterozygous carriers (i.e. $Flii^{+/-}$ or $Flii^{Tg/+}$) to BALB/c animals.

Murine surgery

Flii heterozygous ($Flii^{+/-}$), wild-type (WT) and Flii transgenic ($Flii^{Tg/+}$) mice, 12 weeks old, were anaesthetised using gaseous isofluorane, 5% induction, 2% maintenance and wounded using the same protocol previously described[7]. Briefly, two equidistant, 1 cm full thickness incisions were made through the skin and panniculus carnosus on the flanks of the mice extending 3–4 cm from the base of the skull, 1 cm either side of the spinal column and left to heal by secondary intention. At 3, 7, 14 and 21 days post-wounding, the mice were euthanised using CO_2 asphyxiation and cervical dislocation prior to wound collection. Both left and right wounds were collected and bisected. Half of each wound was fixed in 10% buffered formalin and the other half was snap-frozen in liquid nitrogen. Unwounded skin was also collected from mice from each genotype for controls.

Immunohistochemistry

Histological sections (4 μm) were cut from paraffin-embedded, fixed tissue. Sections were subjected to immunohistochemistry following

antigen retrieval according to the manufacturer's protocols (DAKO Corporation, Botany, Australia). Following blocking in 3% normal horse serum, primary antibodies against TGF-β1 (1:200), TGF-β2 (1:200) or TGF-β3 (1:200) were applied. Species-specific, biotinylated secondary antibodies (1:200) were used and detection was by CY3-conjugated streptavidin (1:200) (Sigma-Aldrich, Sydney, Australia). Fluorescence intensity per unit area was determined using AnalySIS software package (Soft Imaging System GmbH, Munster Germany) and optical fluorescence analysed in the wounds, as previously described[5]. InSpeck Microscope Image Intensity Calibration Kits (Invitrogen, California, USA) were used to define fluorescence intensity levels for constructing calibration curves and evaluating sample brightness. Negative controls included replacing primary antibodies with normal rabbit, mouse, or goat IgG. For verification of staining, non-specific binding was determined by omitting primary or secondary antibodies. All control sections had negligible immunofluorescence.

Cell culture

Human foreskin fibroblasts (HFFs) were donated for this study from a laboratory of A/Prof Pritinder Kaur. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% foetal calf serum (FCS) and antibiotics (100 U penicillin and 100 µg/500 ml streptomycin). Primary fibroblasts were cultured similarly as above except 20% FCS was used. The cultures were incubated at 37°C and 5% CO_2. All cells were serum starved for at least 3 hours prior to experimentation.

Nuclear and cytoplasmic fractionation

Preparations of nuclear and cytoplasmic fractions were done using a nuclear extract kit (Active Motif, California, USA) according to the manufacturer's protocol. Briefly, adherent cells were washed twice with ice-cold PBS containing phosphatase inhibitors, collected by scraping and pelleted by centrifugation. Cytoplasmic fractions were collected by lysing cells in hypotonic buffer followed by adding detergent and nuclei were pelleted by centrifugation. Nuclear pellets were resuspended in complete lysis buffer and the supernatant collected by centrifugation.

Immunoprecipitation

Nuclear and cytoplasmic fractions obtained were pre-cleared using Recombinant Protein G Agarose (Invitrogen, Victoria, Australia). 25 µl of rProtein G was added to 1 ml of lysate and incubated for 10 minutes on a rocking platform at 4°C. The pre-cleared fractions were then divided into two parts of 250 µl for the cytoplasmic fraction and 25 µl for the nuclear fraction. Following this, 5 µl of immunoprecipitation antibody was added to the cytoplasmic fraction and 2 µl of immunoprecipitation antibody was added to the nuclear fraction. The samples were incubated overnight at 4°C on a rocking platform. Then 50 µl and 5 µl of rProtein Agarose were added to the cytoplasmic and nuclear fraction respectively and incubated for a further 2 hours at 4°C on a rocking platform. The fractions were washed 3 times with cold lysis buffer followed by centrifuging at 14,000 g for 10 seconds before discarding of the supernatant. At the final wash, agarose beads were resuspended in 2x SDS Loading Buffer (25 mM Tris pH 6.8, 8% Glycerol, 1% SDS and 0.02% Bromphenol blue) and were mixed thoroughly. The samples were then heated for 3 minutes at 95°C, after which the samples were loaded onto a western gel.

Western blotting

We loaded 20 µg of protein onto gels for all western blot experiments. Protein fractions were electrophoresed on a 4% stacking and 10% separating SDS-PAGE gels at 100 V and then transferred to nitrocellulose by wet transfer (Bio-Rad Laboratories, Regents Park, NSW, Australia) using standard Towbin's buffer with 20% methanol at 100 V for another 1 hour. Membranes were blocked in 5% milk blocking buffer (5% non-fat skimmed milk powder and 0.3% Tween diluted in 1x PBS) for 1 hour and primary antibodies added in blocking buffer and incubated overnight at 4°C. Stringent washes were performed before appropriate secondary antibodies conjugated with horseradish peroxidise were added for 1 hour at room temperature. Washes were then performed before signal detection using Super Signal West Femto Maximum Sensitivity Substrate (Pierce Biotechnology, Rockford, USA) and signal capture using GeneSnap analysis program (Syngene, Maryland, USA). Membranes were stripped and re-probed with β-tubulin (Sigma Aldrich, Sydney, Australia).

siRNA knockdown of Flii gene expression

HFFs were seeded into 6 well tissue culture plates and cultured overnight to achieve 30% to 50% confluence at time of transfection. Sequence of Flii siRNA are as follows: forward: 5'-GCU GGA ACA CUU GUC UGU GTT-3', reverse: 5'-CAC AGA CAA GUG UUC CAG CTT-3'[21]. Scrambled siRNA were used as control. siRNA were transfected into the cells using Lipofectamine 2000 (Invitrogen, Carlsbad, USA). Both siRNA and Lipofectamine 2000 were diluted in Opti-MEM I Reduced Serum Medium (Invitrogen, Carlsbad, USA). Then 250 µl of siRNA (optimised to 1 nM per well) was mixed with 4 µl of Lipofectamine 2000 diluted in 250 µl Opti-MEM and were allowed to complex at room temperature for 20 minutes. We added 500 µl of siRNA:Lipofectamine 2000 complex to each well, mixed and cells incubated for 6 hours before replacing transfection media with DMEM containing 10% FCS only. Cells were incubated for 48 hours prior to gene knockdown assessment.

RT-PCR

Total RNA was extracted from siRNA treated HFFs or primary fibroblasts derived from *Flii*$^{+/-}$, WT, *Flii*$^{Tg/+}$ using TRIzol reagent

Table 1: Primer sequences used in quantitative PCR

Gene	Forward Primer	Reverse Primer
Flii	5'-CCT CCT ACA GCT AGC AGG TTA TCA AC-3'	5'-GCA TGT GCT GGA TAT ATA CCT GGC AG-3'
Gelsolin	5'-CAG ACA GCC CCT GCC AGC ACC C-3'	5'-GAG TTC AGT GCA CCA GCC TTA GGC-3'
Cyclophillin	5'-GGT TGG ATG GCA AGC ATG TG-3'	5'-TGC TGG TCT TGC CAT TCC TG-3'
c-fos	5'-CTA CGA GGC GTC ATC CTC CCG-3'	5'-AGC TCC CTC CGG TTG CGG CAT-3'
c-jun	5'-GAA ACG ACC TTC TAT GAC GAT GCC CTC AA-3'	5'-GAA CCC CTC CTG CTC ATC TGT CAC GTT CTT-3'
TGF-β1	5'-TGG ATA CCA ACT ATT GCT TCA G-'3	5'-GTT GTA GAG GGC AAG GAC C-'3
TGF-β3	5'-AAG CGC ACA GAG CAG AGA TT-'3	5'- AGT GTC AGT GAC ATC GAA AG-'3
Smad 3	5'-GTT GGA CGA GCT GGA GAA GG-3'	5'-TGC TGT GGT TCA TCT GGT GG-3'
Smad 7	5'-GCT CAC GCA CTC GGT GCT CA-3'	5'-CCA GGC TCC AGA AGA AGT TG-3'

(Invitrogen, California, USA) following manufacturer's instructions. Any contaminating genomic DNA was removed using a DNA-free kit (Ambion, Texas, USA). RNA used in each PCR reaction is 50 ng with purity between 1.9 and 2.0 as quantified by the 260:280 ratio using a Beckman Spectrometer. RNA was reversed transcribed to cDNA using reverse transcriptase. cDNA were set up to a final concentration of 1x SYBR Green, 1x Amplitag PCR buffer, 3 mM $MgCl_2$, 5 mM dNTPs, 0.9 µM primers (forward and reverse), 1.25 Units of AmpliTag Gold DNA polymerase in 25 µl of H_2O. The primer sequences are shown in Table 1.

Dual labelling immunocytochemistry

Three x 10^5 cells were plated in a well containing a sterile glass coverslip and placed in six well culture plates until they reached confluency. Monolayer cultures were scratch wounded using a P200 yellow pipette tip and immunocytochemistry performed 30 minutes later. Cells were fixed with cold acetone for 10 seconds, which was then replaced with 1x PBS. Washes were performed after every treatment using 1x PBS. Three per cent Normal Horse Serum (NHS) was used to blocked the cells for 30 minutes at room temperature before incubating with rabbit anti-Flii antibodies (1:200 dilution) overnight at 4°C. Secondary anti-rabbit Alexa Fluor 488 was added at a dilution of 1:1000 for 1 hour in the dark at room temperature. This was then followed by incubating with anti TGF-β1, TGF-β2, TGF-β3, c-fos, c-jun, or Akt antibodies, all at dilution 1:200 for 1 hour at room temperature. The respective biotinylated secondary antibodies were added and coverslips incubated for 1 hour in the dark at room temperature. After this, streptavidin conjugated Alexa Fluor 555 at 1:200 dilution was added for 1 hour in the dark at room temperature. DAPI was added to the cells at 1:1000 dilution before a final wash and then mounted onto a microscope slide using DAKO mounting medium. Negative controls included replacing primary antibodies with species-specific IgG. For verification of staining, non-specific binding was determined by omitting primary or secondary antibodies. All control sections had negligible immunofluorescence. Integrated fluorescence intensity was determined using AnalySIS software package (Soft-Imaging System, Munster, Germany) and images captured using Leica Spectral Confocal Microscope (Leica, San Diego, CA) as previously described[14].

STATISTICAL ANALYSIS

Statistical differences were determined using the Student's *t*-test or an ANOVA. For data not following a normal distribution, the Mann-Whitney U-test was performed. A *p* value of less than 0.05 was considered significant.

RESULTS

Differential effect of Flii on TGF-β1, TGF-β2 and TGF-β3 in incisional wounds

Incisional wounds were created on Flii heterozygous knockout (*Flii*$^{+/}$), WT and Flii overexpressing (*Flii*$^{Tg/+}$) mice. Wounds were collected at 3, 7, 14 and 21 days post-wounding and examined for the expression of the three TGF-β isoforms. TGF-β1, TGF-β2 and TGF-β3 remained unchanged in the unwounded skin of *Flii*$^{+/-}$, WT and *Flii*$^{Tg/+}$ mice (Figure 1A, C, E). Both TGF-β1 and TGF-β2 were significantly elevated in *Flii*$^{Tg/+}$ wounds compared to WT at day 7 post-wounding (75% and 20% increase respectively) and remained significantly elevated at day 14 returning to WT levels at day 21 (Figure 1B, D). In contrast, TGF-β3 was significantly elevated in *Flii*$^{+/-}$ wounds compared to WT at day 7 and day 14 (50% and 30% increase respectively) (Figure 1F). The fluorescent intensity was expressed as a percentage of WT control for each of the TGF-β isoforms (Figure 1G). At day 7 post-wounding, TGF-β3 was significantly elevated in Flii deficient wounds, whereas TGF-β1 was elevated in Flii overexpressing wounds (50% and 70% increase respectively) (Figure 1G).

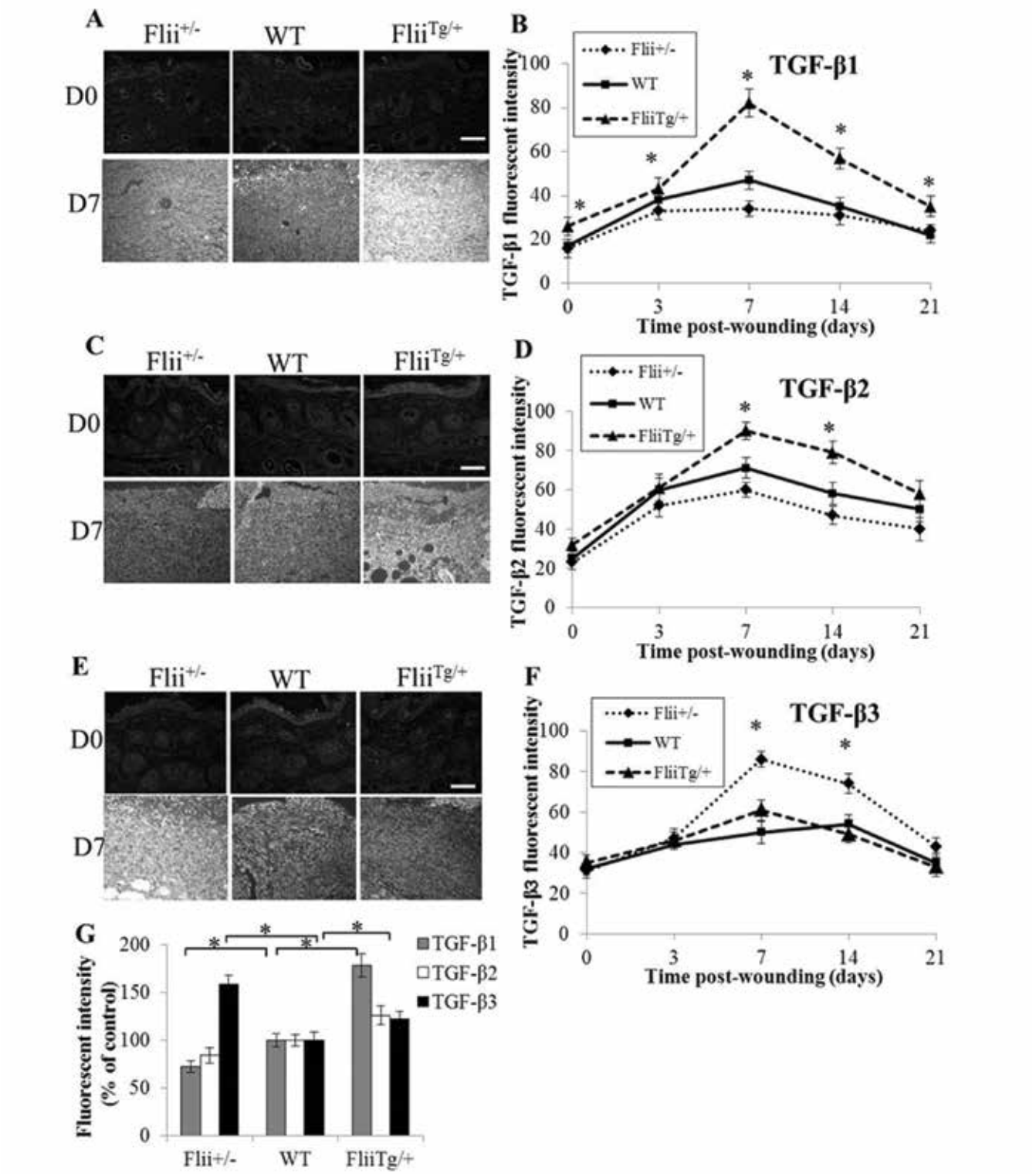

*Figure 1: Transforming growth factor TGF-β1, TGF-β2 and TGF-β3 expression in incisional wounds of Flii+/-, WT and FliiTg/+ mice. (A) Immunohistochemistry for TGF-β1, (C) TGF-β2 and (E) TGF-β3 proteins were performed on wounds of Flii$^{+/-}$, WT and Flii$^{Tg/+}$ mice. Representative images are shown for 0 and 7 day wounds. Unwounded (day 0) skin sections showed negligible TGF-β1, TGF-β2 and TGF-β3 staining. (B) Graph showing TGF-β1, (D) TGF-β2, (F) TGF-β3 fluorescence intensity at 0, 3, 7, 14 and 21 days post-injury. Results represent mean ± S.E.M. n = 6 for each group per time-point. Scale bar = 50 μm in all images. In graphs (B), (D) * denotes significance between WT vs Flii$^{Tg/+}$ and in graph (F) * denotes significance between Flii$^{+/-}$ vs WT and Flii$^{Tg/+}$ ($p < 0.05$). (G) Graph showing TGF-β1, TGF-β2 and TGF-β3 protein expression in Flii$^{+/-}$, WT and Flii$^{Tg/+}$ wounds expressed as a percentage of control (wild-type counterpart). Results represent mean ± S.E.M. n = 6 for each group per time-point. In graph (G) *denotes significance ($p < 0.05$).*

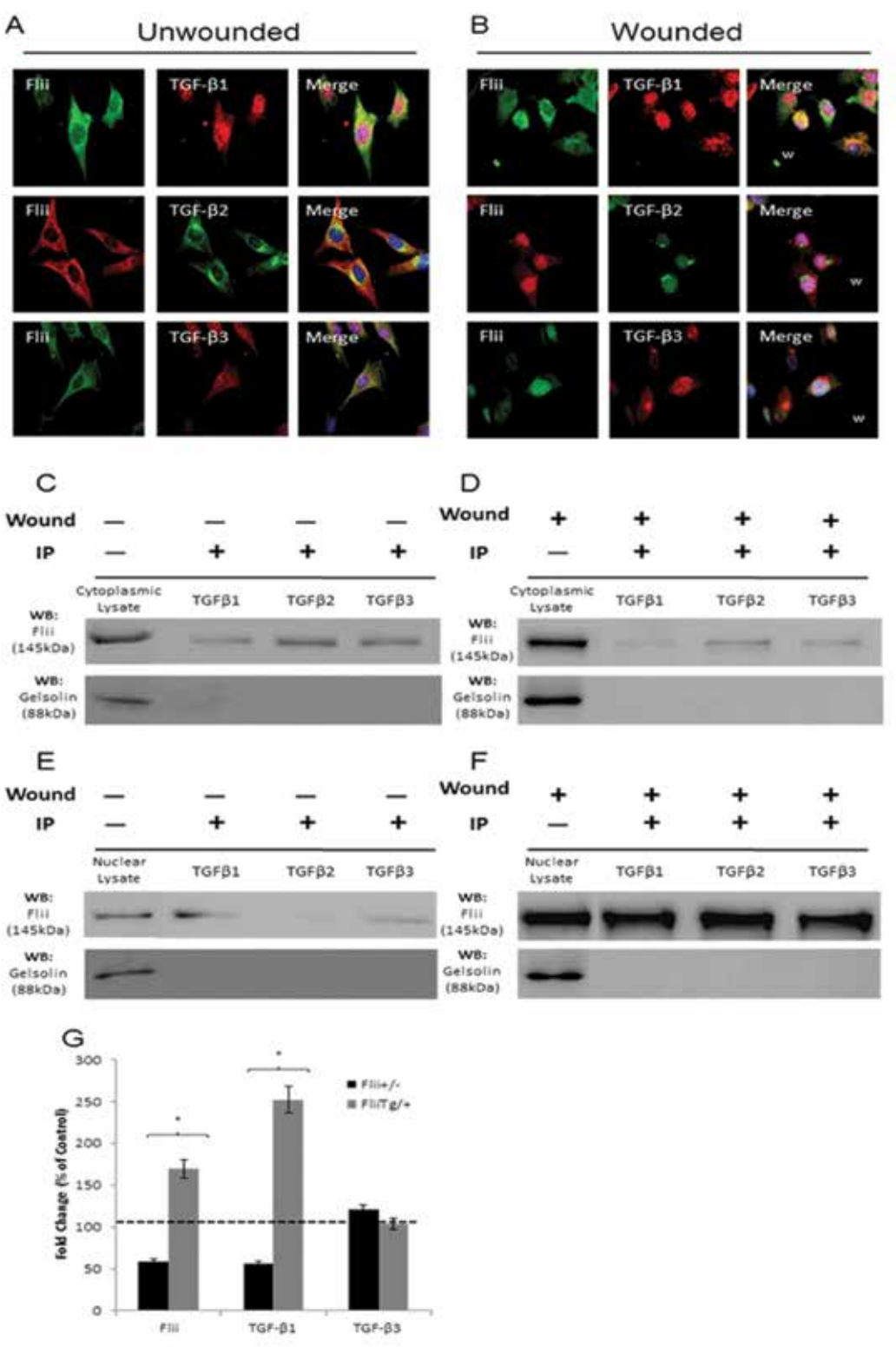

Figure 2: Flii interacts with TGF-β isoforms and translocates from the cytosol into the nucleus in response to wounding.
*(A) Unwounded HFFs immunostained for Flii and the TGF-β isoforms (TGF-β1, TGF-β2 and TGF-β3). (B) Scratch-wounded HFFs immunostained for Flii and the TGF-β isoforms. Co-localisation is represented by yellow in the merged image and pink/white in the nucleus due to the presence of DAPI (blue). Scale bar is 5 μm. (C) Cytoplasmic extracts from wounded and (D) unwounded HFFs immunoprecipitated with TGF-β1, TGF-β2 or TGF-β3 and immunoblotted with Flii antibodies. (E) Nuclear extracts from wounded and (F) unwounded HFFs immunoprecipitated with TGF-β1, TGF-β2 or TGF-β3 and immunoblotted with Flii antibodies. (G) Bar chart representing the fold change in gene expression in primary fibroblasts from $Flii^{+/-}$ and $Flii^{Tg/+}$ mice expressed as a percentage of wild-type fibroblasts. Results represent mean ± S.E.M. n = 6 for each group per time-point. *denotes significance ($p < 0.05$).*

Flii associates with TGF-β isoforms and alters TGFβ-1 gene expression

To test whether Flii and TGF-β isoform expression was altered in response to wounding, scratch-wounded and unwounded monolayers of HFFs were immunostained for Flii and TGF-β1, TGF-β2 and TGF-β3 (Figure 2A, B). Flii and TGF-β staining is either red or green depending on the fluorophore used. DAPI (blue) was also included in the merged image to show the location of the nucleus. Co-localisation of proteins in the cytoplasm is indicated by yellow staining, whereas because of the presence of DAPI in the nucleus, this is seen as pink/white staining. Flii was observed to be present in the cytosol of unwounded cells (Figure 2A). However, 30 minutes post-wounding, Flii was predominantly found in the nucleus and perinuclear region of the fibroblasts (Figure 2B). TGF-β1 and TGF-β3 were present throughout unwounded fibroblast cells including the nucleus and the cytoplasm (Figure 2B). TGF-β2 was mainly found in the cytoplasm of unwounded cells. In response to wounding, all three isoforms translocated into the nucleus (Figure 2B). Flii was observed in the same location as each of the TGF-β isoforms, as represented in the cytoplasm by the yellow colour and in the nucleus by the pink/white colour in the merged images (Figure 2A–B). Although Flii appeared to co-localise with the TGF-β isoforms by immunocytochemistry, immunoprecipitation studies were performed to confirm this. Wounded and unwounded fibroblasts were separated into cytoplasmic (Figure 2C, D) and nuclear (Figure 2E, F) and these were immunoprecipitated with TGF-β1, TGF-β2 and TGF-β3 antibodies. Co-immunoprecipitated proteins from the wounded/unwounded nuclear and cytoplasmic factions were then immunoblotted using Flii antibodies and results confirmed that TGF-β1, TGF-β2, TGF-β3 proteins were predominantly localised with Flii in the nucleus of wounded fibroblasts (Figure 2F). Gelsolin, a member of the same actin-remodelling family of proteins with high homology to Flii was used as a control and showed no localisation with any of the TGF-β isoforms (Figure 2C–F).

Primary fibroblasts derived from *Flii*$^{+/-}$ and *Flii*$^{Tg/+}$ mice were next assessed using RTqPCR to test the effect of differential Flii levels on TGF-β1 and TGF-β3 gene expression. *Flii*$^{+/-}$ and *Flii*$^{Tg/+}$ primary fibroblasts were used to verify Flii transcription levels, and as expected Flii mRNA expression was reduced in *Flii*$^{+/-}$ fibroblasts and increased in *Flii*$^{Tg/+}$ cells (45% decrease and 70% increase respectively) (Figure 2G). Consistent with the *in vivo* protein expression data (Figure 1 B, D, F), our *in vitro* gene studies confirmed that TGF-β1 mRNA was significantly elevated (150% increase) when Flii gene expression was increased. Similarly, TGF-β1 mRNA was significantly reduced (45% decrease) when Flii gene expression was decreased. TGF-β3 mRNA expression levels remained unchanged when Flii gene expression was manipulated (Figure 2G).

Flii co-localises with TGF-β signalling proteins Smad 2/3 and alters Smad 7 gene expression

TGF-β ligands signal via transmembrane receptors, which activate Smad intracellular signal transduction pathways[22]. We have previously shown that TGF-β1 gene expression was reduced by 2.4 fold in Flii siRNA-treated fibroblasts[24]. Given that the TGF-β1 signal is transduced by Smad proteins, we investigated whether Flii might influence Smad expression. Flii was found to co-localise with Smad 2/3 and Smad 7 in both the nucleus and cytoplasm of wounded fibroblasts (Figure 3A). Nuclear and cytoplasmic fractions of wounded and unwounded fibroblasts confirmed that Flii associates with Smad 2/3 (Figure 3B). Smad 7 showed strong cytoplasmic co-localisation with Flii in unwounded and wounded fibroblasts. Increased co-localisation of Smad 7 and Flii was observed in the nucleus of wounded fibroblasts (Figure 3C). When Flii gene expression was reduced by over 80% using siRNA (p = 0.002 vs siRNA control) a significant reduction in Smad 3 gene expression was observed (55% reduction) (Figure 3D); however, Flii siRNA knockdown led to an upregulation of inhibitory Smad 7 (60% increase) (Figure 3D). Gelsolin gene expression was used as a specificity control.

Flii associates with Akt in the nucleus of wounded fibroblasts

TGF-βs act via the classical (Smad-dependent) pathway and also via the non-classical (non-Smad) signalling pathway such as the phosphatidylinositol-3-OH kinase (PI(3)K)–Akt pathway. Importantly, Akt receptors activate non-classical pathways that mediate growth-factor-induced cell proliferation, survival and migration[19]. Given that Flii co-immunoprecipitates with Smad 2/3 and 7, we investigated whether Flii also associates with Akt. Using immunohistochemistry we observed that both Flii and Akt were broadly distributed in the cytoplasm of unwounded cells (Figure 4A). Scratch-wounding resulted in translocation of Flii from the cytoplasm into the nucleus of fibroblasts (Figure 4A). To determine if areas of co-expression indicated interaction between Flii and Akt, nuclear and cytoplasmic fractions of scratch-wounded and unwounded HFFs were immunoprecipitated with Akt antibodies and immunoblotted for Flii and showed that Flii only associates with Akt in wounded nuclear fractions (Figure 4B). These results suggest that Flii may be important in cross-talk between TGF-β and Akt signalling pathways.

Flii associates with c-fos and c-jun

c-fos and c-jun are members of the transcription factor activator protein 1 (AP-1) which regulates TGF-β gene expression[25]. To determine if c-fos and c-jun associated with Flii in response to wounding nuclear and cytoplasmic fractions of scratch-wounded HFFs were immunoprecipitated with c-fos and c-jun antibodies and immunoblotted for Flii. Flii co-immunoprecipitated with both c-fos and c-jun in both cytoplasmic and nuclear fractions (Figure 5A). In addition, gene expression studies revealed that when Flii

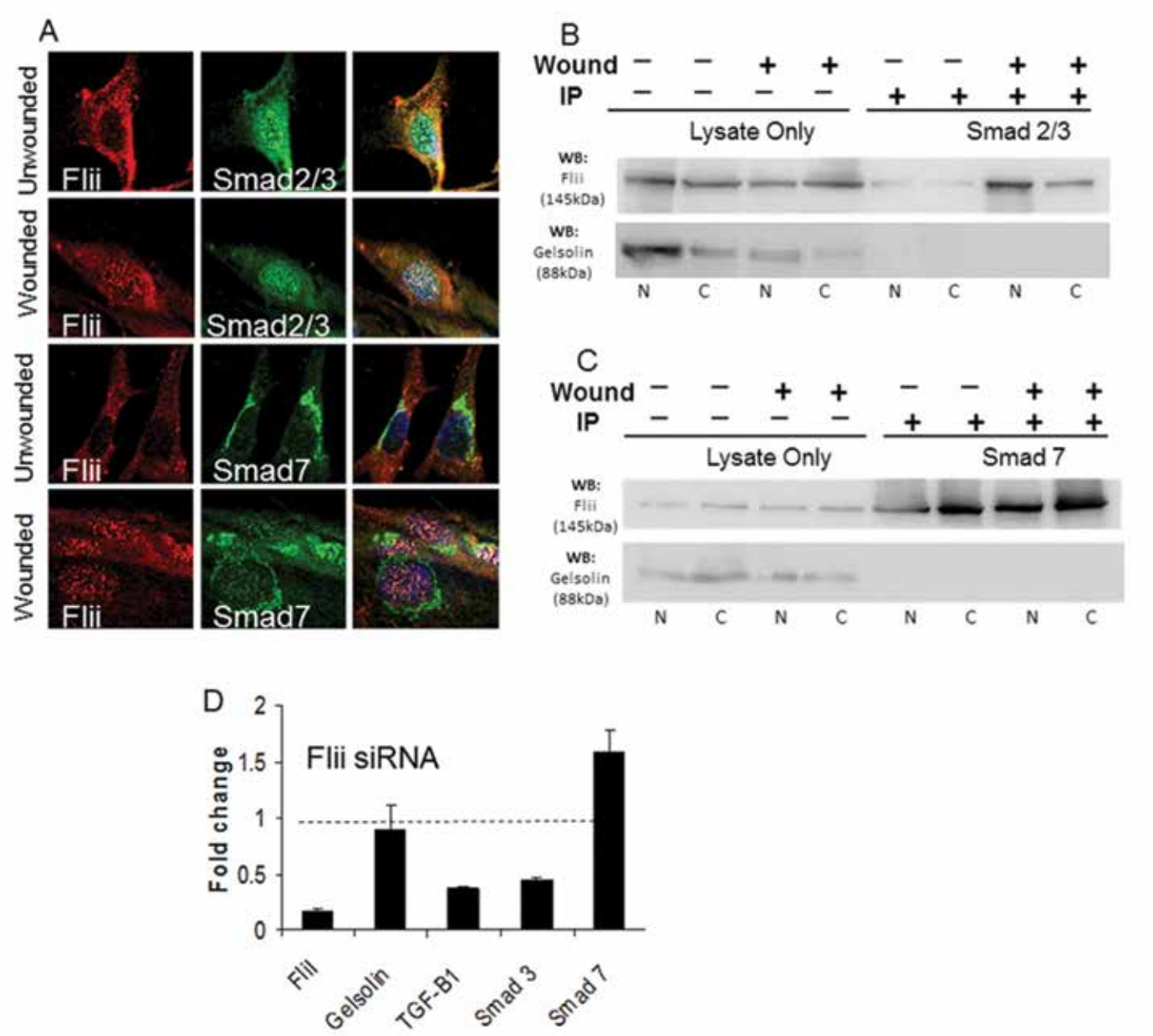

Figure 3: Flii forms a complex with Smad 2/3 and alters Smad 7 gene expression.
(A) Scratch-wounded and unwounded HFFs were immunostained for Flii and the TGF-β signalling proteins Smad 2/3 and 7. Co-localisation is represented by yellow in the merged image and pink/white in the nucleus due to the presence of DAPI (blue). Scale bar is 5 μm. (B) Cell extracts prepared from scratch wounded and unwounded confluent monolayers of HFFs were used in immunoprecipitation experiment. Nuclear and cytoplasmic cell extracts were incubated with antibodies specific for Smad 2/3 (B) and Smad 7 (C) bound to protein A-agarose and precipitates were immunoblotted for Flii. (D) HFFs treated with control or Flii siRNA were analysed for gelsolin, TGF-β1, Smad 3 and Smad 7 gene expression. Flii gene expression was reduced to levels to 22%. Gelsolin gene expression was determined to show specificity of Flii primers. Results represent mean ± S.E.M. n = 3 for each group.

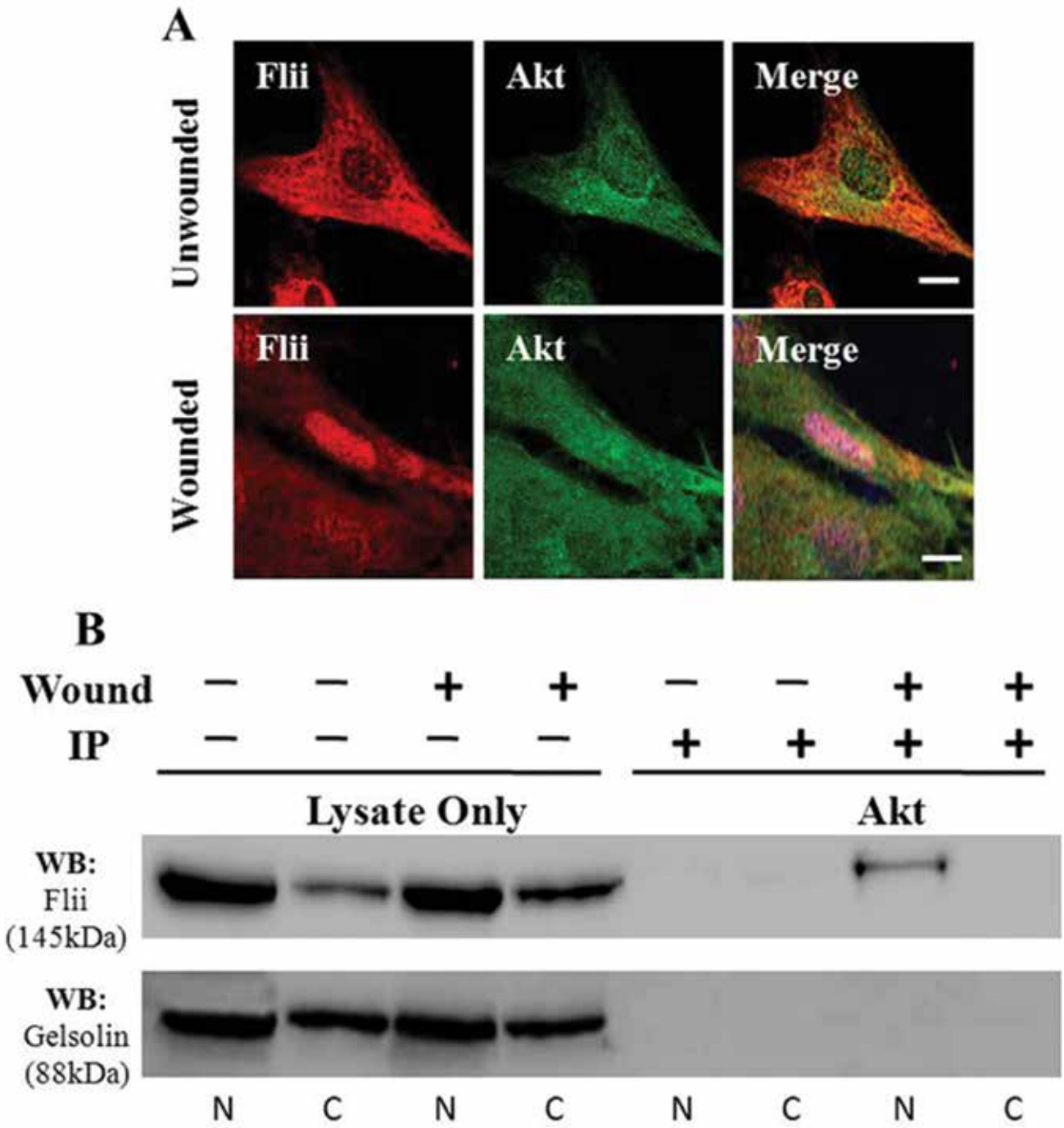

Figure 4: Flii associates with Akt in the nucleus of wounded fibroblasts
(A) HFFs were fixed and stained for Flii and Akt. Flii (red) and Akt (green) staining is shown in (A). Scale bar is 5 μm. (B) Whole cell lysate, nuclear and cytoplasmic extracts prepared from HFFs were incubated with antibodies to Akt and Flii bound to protein A-agarose. Precipitates were analysed by immunoblotting. Flii only co-immunoprecipitated with nuclear Akt in wounded HFFs. n = 3 for each group.

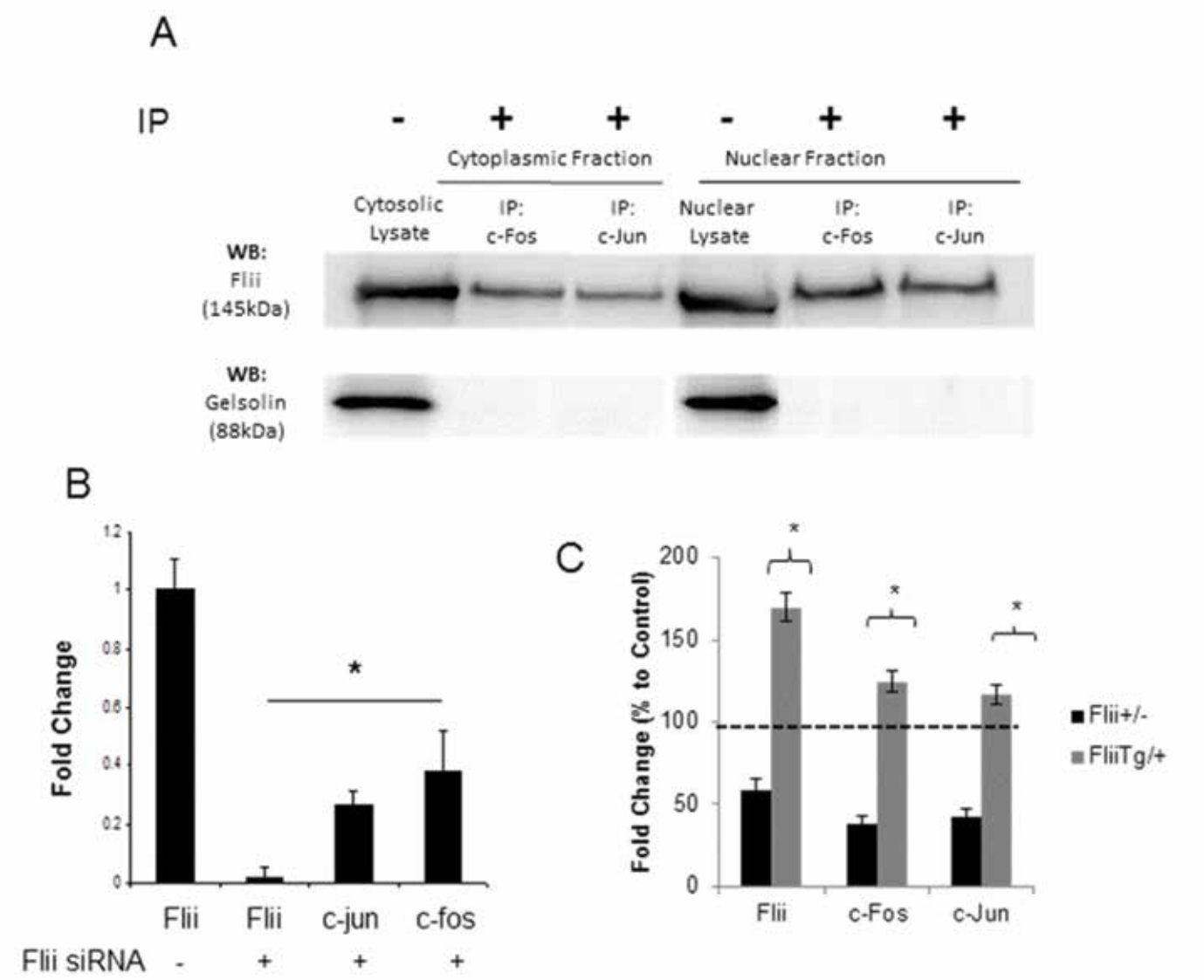

Figure 5: Flii alters c-fos and c-jun mRNA expression.
(A) Nuclear and cytoplasmic fractions of fibroblasts were incubated with antibodies to c-fos, c-jun and Flii bound to protein A-agarose. Precipitates were analysed by immunoblotting for Flii. (B) HFFs were transfected with control or Flii siRNA. Cells depleted of Flii have reduced levels of c-fos and c-jun gene expression, calculated from three replicates of RTqPCR data (, p≤ 0.05). (C) Primary fibroblasts isolated from the skin of mice with reduced Flii gene expression (Flii+/-) and increased Flii gene expression (FliiTg/+) were used in RTqPCR analysis. Cells depleted of Flii have reduced levels of c-fos and c-jun. Results represent mean ± S.E.M. n = 3 for each group. *denotes significance (p < 0.05).*

gene expression was reduced using siRNA, c-fos and c-jun gene expressions were reduced to approximately 55% of their original expression (Figure 5B). *Flii*$^{+/-}$ and *Flii*$^{Tg/+}$ primary fibroblasts were used to investigate c-fos and c-jun gene expression (Figure 5C). c-fos and c-jun mRNA was significantly reduced when *Flii* gene expression was reduced (Figure 5C).

DISCUSSION

Previous studies have described Flii as a negative regulator of wound healing, demonstrating that modulation of Flii activity by either genetic depletion or using neutralising antibodies significantly improves the outcome of wound repair[1,7]. Recent studies have focused on understanding the mechanism behind Flii effects on focal adhesion[9,10] and Flii secretion[26] with subsequent effects of cell migration and cytokine secretion respectively. In addition, studies examining the effect of Flii on healing of partial-thickness burn wounds and blistered skin suggested a possible interplay between Flii and TGF-β isoforms with effects on α-Smooth Muscle Actin expression and collagen contraction respectively[1,15]. This study now shows that Flii not only affects TGF-β expression but that it may also affect specific TGF-β downstream signalling processes through its interaction with members of both the classical and non-classical TGF-β signalling pathways.

Using mice with different levels of *Flii* gene expression and *in vitro* and *in vivo* models of wound healing, we have shown that Flii specifically modulates TGF-β expression. Low TGF-β1, TGF-β2 and high TGF-β3 expression were observed in the wounds of *Flii*$^{+/-}$ mice, which may contribute to the improved healing observed in these mice[7]. In contrast, *Flii*$^{Tg/+}$ wounds had high TGF-β1, TGF-β2 and low TGF-β3 expression and the wounds in these mice had impaired healing[7]. These findings are in agreement with studies which have shown increased levels of α-SMA-positive myofibroblasts and collagen production observed in wounds of *Flii* overexpressing mice[7,15]. The altered TGFβ isoform ratio observed in this study suggests that in addition to Flii effects on actin-remodelling and paxillin phosphorylation which mediates an increased number of focal adhesions and impaired focal adhesion turnover[9,10], Flii also may be involved in TGF-β signalling.

Flii is potentially able to directly regulate TGF-β either at the promoter (via AP-1) or at the protein level. Direct interactions were observed between Flii and all three TGF-β protein isoforms in both cytoplasmic and nuclear cell fractions of wounded cells. Flii has previously been shown to translocate from the cytoplasm to the nucleus in response to wounding[24]. Flii also interacts directly with nuclear receptors and their co-activators, CARM1 and CBP (p300) through GRIP1[8,27]. Other members of the gelsolin family including gelsolin[28] and supervillin[12] have also been identified as nuclear receptor co-activators. The role of nuclear Flii-TGF-β complex remains to be elucidated but it is plausible that Flii-TGF-β complex may act as transcriptional co-activators affecting TGF-β production and pathological fibrotic outcomes of wound healing. Flii intracellular activities include actin remodelling and polymerisation[29], nuclear transcription[30] and protein-protein interaction[31]; however, Flii is not solely an intracellular protein but is secreted through a late endosome/lysosome pathway and is able to bind LPS to dampen cytokine secretion[26], suggesting that it may also have an important effect on inflammation during wound repair. In light of these results demonstrating extracellular Flii function, and results presented in this manuscript, it is tempting to speculate that Flii may also affect the TGF-β signalling pathway via extracellular interactions with TGF-β ligand or TGF-β receptors; however, this is yet to be investigated.

In addition to direct interactions with TGF-β isoforms we show here that Flii also associates with the members of both the classical and non-classical TGF-β signalling pathways. Previous studies have shown the interactions between Smad 2/3 complex with proteins containing the leucine-rich repeat and one such interacting protein, Erbin inhibits TGF-β signalling by physically sequestering Smad 2/3 and preventing transcriptional responses[32]. Considering most interaction between Flii and Smad 2/3 were observed in the nucleus of the wounded cells it is possible that Flii may associate with Smad 2/3 complex to prevent its association with other transcriptional factors preventing induction of target gene expression. Interestingly, reducing *Flii* gene expression led to lower levels of TGF-β1 and Smad 3 gene expression and conversely increased inhibitory Smad 7 gene expression suggesting that cells with reduced Flii levels might be less responsive to TGF-β signals.

Flii effects on the non-classical TGF-β signalling pathway which involves Akt and nuclear and activating proteins-1 (AP-1), c-fos and c-jun showed that Flii specifically interacts with Akt in the nucleus of wounded fibroblasts, suggesting a wound-specific response for Flii and Akt.

A recent study identified that nuclear Akt ensures protein stability by regulating post-translational modifications as well as binding to a protein complex to protect proteolytic degradation[33]. Flii may, therefore, indirectly affect the TGF-β Smad signalling pathway by suppressing the function of Akt to post-translationally phosphorylate Smads, which consequently may affect cellular processes during wound healing including cell survival and the regulation of the cell cycle. In addition, Flii association with AP-1 proteins suggests a possible link between Flii and the TGF-β signalling pathway. Indeed, these results are in agreement with previous findings, suggesting an interplay between Flii and TGF-β1 and Flii interactions with AP-1 proteins in blistered skin[15]. AP-1 proteins bind to the TGF-β promoter to regulate its expression; therefore it is possible that Flii may 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.

In summary, the novel findings of Flii co-localising with multiple proteins involved in TGF-β expression and activity indicated a potential role for Flii in TGF-β responses. Flii may affect wound repair outcomes through its interaction with TGF-β or TGF-β regulatory proteins. Importantly, wounds with decreased Flii expression exhibit a decreased levels of pro-scarring TGF-β1 and high levels of anti-scarring TGF-β3 which may help to explain the observed decreased scarring in porcine pre-clinical models of wound healing[17]. Understanding the cellular mechanism involved in Flii regulation of TGF-β signalling and activity could lead to therapeutic approaches aimed at improving wound healing and reducing fibrosis and pathological scarring.

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CONFLICTS OF INTEREST

The authorial team declares that they have no conflicts of interest.

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