

Article

Tsk2/+ Mice Demonstrate Increased TWEAK/FN14 and TGF- β Pathways During Early Dermal Fibrosis

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Abstract

Background/Objectives: Our prior work demonstrated that a non-synonymous mutation in procollagen type III (*Col3a1*) at residue 33 (C33S) was responsible for the skin fibrosis seen in the Tsk2/+ mouse. We previously showed increased expression of the TWEAK receptor/fibroblast growth factor-inducible 14 (FN14) in Tsk2/+ skin and systemic sclerosis (SSc) skin; however, the role of TWEAK in Tsk2/+ had not been assessed. **Methods:** Primary dermal fibroblasts from Tsk2/+ and WT mice were examined for differential gene expression at different ages. Wild-type and C33S-mutated COL3A1-expressing plasmids were transfected into *Col3a1*KO fibroblasts and treated with FN14 or TGF- β inhibitors. **Results:** Tsk2/+ mice had increased FN14 protein compared to their respective non-diseased counterparts and had increased mRNA expression of a panel of transcripts associated with a fibrotic signature (*Icam1*, *Irak2*, and *Tweak*). COL3A1-KO cells transfected with a COL3A1^{Tsk2}-expressing plasmid displayed significantly higher levels of this fibrotic signature compared to COL3A1^{WT}-expressing plasmid. FN14 and TGFBR1 inhibitors significantly reduced the fibrotic signature compared to untreated Tsk2/+ cells. **Conclusions:** The upregulation of FN14 in Tsk2/+ mouse skin and primary dermal fibroblasts suggests that this pathway may be crucial to fibrosis development. Inhibition of both FN14 and TGFBR1 reduces the fibrotic signature in both *Col3a1*Tsk2 transfections, suggesting a similarity between the Tsk2/+ model and SSc and confirming that the Tsk2/+ mouse model is suitable for studying the initiating events of SSc fibrosis.

Keywords: Tsk2/+ mouse; fibrosis; FN14; TWEAK; model for scleroderma; TGF- β signature

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1. Introduction

Systemic sclerosis (SSc) is a complex autoimmune disease of unknown etiology. The disease manifests itself with fibrosis of the skin and internal organs [1]. SSc is classified into two major subsets: the milder, limited cutaneous SSc (lcSSc), with primarily skin involvement early in disease progression and late organ involvement, and the more aggressive diffuse cutaneous SSc (dcSSc), with early skin and organ involvement. More recently, analysis of gene expression profiles in the skin of patients with lcSSc and dcSSc has identified additional subsets within these broad categories [2–4]. Pathologically, SSc is characterized by three key processes: widespread small vessel vasculopathy, immune activation/inflammation, and progressive fibrosis driven by persistent fibroblast activation [5–7].

Animal models have been used to understand the pathogenesis of SSc; however, no model is perfect. Unlike the chemically induced models, such as the hypochlorous model [8,9] and the bleomycin model [10,11], the tight skin 2 (Tsk2/+) mouse is a genetic model of SSc that spontaneously recapitulates several essential characteristics of the human disease, including the loss of subdermal adipose tissue and an increase in elastic fibers in the skin [12]. The mouse closely resembles the diffuse proliferative subset of SSc, displaying a prominent TGF- β signature [13,14]. Tsk2/+ mice are born with the ‘tight skin’ phenotype and can be identified as early as 3 weeks of age, and skin fibrosis develops by 10 weeks of age [12]. The causative mutation in Tsk2/+ mice is a cysteine-to-serine substitution at residue 33 in the N-terminal peptide region (PIIINP) of the alpha procollagen III gene (*Col3a1*) [13]. This mutation is autonomous, as transfections with the plasmid encoding *Col3a1* containing the C33S mutation into COL3A1-KO fibroblasts induce type I collagen (COL1A1) expression [13].

Tumor necrosis factor superfamily member 12 (TNFSF12), also known as tumor necrosis factor-like weak inducer of apoptosis (TWEAK), and its receptor, fibroblast growth factor-inducible molecule 14 (FN14), have garnered recent interest for their roles in fibrotic disease. TWEAK is expressed in two forms: a membrane-bound and a soluble form (CD163) [15]. The receptor for TWEAK, FN14, is a type 1 transmembrane protein with a TNF receptor-associated factor binding site [16–19]. Exposure of cardiac fibroblasts to ectopic TWEAK increases cell proliferation, NF- κ B, COL1A1, and MMP9 [20–22]. In human gingival fibroblasts, TWEAK expression upregulates FN14, VCAM, IL-6, and ICAM [23]. Studies have shown that this axis may be an essential player in fibrosis [24–26], although one study did not concur [27]. TGF- β 1 was reported to induce FN14 [28] and TWEAK induced TGF- β 1 expression [29]. In support of this observation, we found increased *Fn14* gene expression and FN14-positive cells in the dermis of 4-week-old female Tsk2/+ mice [13,14]. We also found that patients with SSc had increased FN14 gene expression and protein levels in the skin [13,14]. Serum TWEAK levels are elevated in both dcSSc and lcSSc subsets [30], but, intriguingly, low bronchoalveolar lavage TWEAK levels correlated with pulmonary fibrosis [30]. Peripheral blood mononuclear cells may not be the primary source of TWEAK in SSc [31]. The authors proposed that TWEAK enhanced CCL5, as higher CCL5 levels were observed in patients without pulmonary fibrosis. Kowal-Bielecka et al. found increased soluble TWEAK/CD163 ratios and proposed that they may be protective against digital ulcers but may be associated with more severe disease in SSc [32]. More recently, Lu et al. showed that TWEAK and FN14 were elevated in SSc skin and that FN14 antagonism reduced bleomycin-induced skin fibrosis in mice [24]. Considering the known role of TWEAK/FN14 in activating fibroblasts, its reported induction by TGF- β [28], and our observations of increased FN14 expression in the dermis of both SSc patients and Tsk2/+ mice [13,14] we further investigated the function of TWEAK/FN14 in the Tsk2/+ skin. This axis represents a critical yet underexplored component of the fibrotic cascade in SSc.

The skin of Tsk2/+ mice mimics some essential features of a TGF- β 1 gene signature, including increased TGF- β 1 protein at specific ages [12,14]. The mouse closely resembles the diffuse proliferative subset of SSc, displaying a prominent TGF- β signature [14]. TGF- β is widely regarded as the master regulator of fibrosis in SSc, driving fibroblast differentiation and activation. These observations suggest that the FN14/TWEAK pathway may contribute to the establishment of skin fibrosis in Tsk2/+, matching some features of the diffuse proliferative subset of SSc [14]. Building on our earlier data, we examined the role of TWEAK and its receptor, FN14, in mediating Tsk2/+ dermal fibrosis and further characterized the suitability of the Tsk2/+ mouse as a model for SSc skin fibrosis.

Therefore, the objective of this study was two-fold: first, to definitively test the causal role of the TWEAK/FN14 axis in the spontaneous skin fibrosis of the mouse by using genetic or pharmacological antagonism; and second, to further characterize how the TWEAK/FN14 pathway contributes to the broader pro-fibrotic gene signature, particularly in relation to TGF- β signaling, thereby assessing the utility of the model. We demonstrate that TWEAK and FN14 are elevated in the dermis of Tsk2/+ mice, and the Tsk2/+ mutation specifically upregulates COL1A1, which p38 MAPK mediates, confirming the critical involvement of this pathway in SSc-like spontaneous fibrosis.

2. Materials and Methods

All studies and procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Drexel University, Protocol Number 20460, and conducted in accordance with recommendations in the “Guide for the Care and Use of Laboratory Animals” [33]. Primary dermal fibroblasts were isolated from the skin of 1-day-old neonatal COL3A1-KO mice as previously described [13].

2.1. Nanostring Analyses

Tsk2/+ (n = 5) and WT (n = 6) primary dermal fibroblasts (200,000 cells) were plated in 6 cm dishes and cultured for 48 h in complete Dulbecco’s Modified Eagle’s Medium supplemented with 10% FBS, 50 U/mL penicillin, and 50 mg/mL streptomycin. The fibroblasts were released from the dish with 0.25% trypsin, washed, and RNA isolated with the RNeasy kit (Qiagen, Redwood City, CA, USA). The purified RNA was shipped to Nanostring Technologies (Seattle, WA, USA) for analysis using the Immunology Gene Array Panel. Analyses of the results were performed with the nSolver Analysis Software v2.0.

2.2. RNA Isolation and Quantitative PCR

RNA was isolated from total skin or from primary Tsk2/+ and WT fibroblast cell lines at different ages (2, 4, 8, and 17+ weeks). The skin was homogenized in Trizol, then a phase extraction using chloroform and centrifugation was performed. The aqueous phase was collected, and ethanol was added to 1.5 times the collected volume. RNA was then extracted using the RNA Isolation kit (Clontech, Mountain View, CA, USA). The cDNA was synthesized according to the manufacturer’s recommendations using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Waltham, MA, USA). The relative quantities of all transcripts were measured using the SYBR green reagent using an HT7900 instrument (Applied Biosystems) and normalized to β -actin.

The selection of 2, 4, 8, and 17+ weeks for gene expression analysis in the Tsk2/+ mouse captures critical transition points from the initiation of molecular stress to the establishment of chronic dermal fibrosis. These time points align with distinct physiological and signaling milestones in the model. At 2 weeks, this is the earliest detectable phase of the disease, and dermal thickening is minimal. At four weeks, the Tsk2/+ phenotype becomes histologically undeniable, and the skin is physically thicker. By eight weeks, the mouse has physically moved out of its rapid growth phase and into a state of established fibrosis, and mice at 17 weeks and beyond provide insight into the long-term stability of the phenotype and its corresponding gene expression.

2.3. Transient Transfections

COL3A1-KO fibroblasts cultured in complete DMEM were transfected with *Col3a1*^{Tsk2/+} or *Col3a1*^{WT} plasmids or empty vector (mock) as described previously [34]. Transfection efficiency was determined as described by Long et al. [13]. A subset of the transfection experiments included 1.25 μ M SM16 (Sigma Aldrich, St. Louis, MO, USA) or 1.25 μ L/mL DMSO (control). An additional subset of transfections included 100 ng/mL FN14 block-

ing antibody or 100 ng/mL MOPC21 isotype IgG antibody (control). The FN14 blocking antibody and MOPC21 were a kind gift from Biogen Idec (Cambridge, MA, USA). Gene expression was analyzed by qRT-PCR in the transfected fibroblasts, normalized to β 2-microglobulin (B2M), and calculated using the $\Delta\Delta C_t$ method. The data were then graphed as the fold change relative to mock for Col3a1Tsk2/+ and Col3a1WT.

2.4. Histology

Paraffin-embedded skin sections derived from Tsk2/+ and WT mice were deparaffinized, and antigens were unmasked with 10 mM citrate buffer at 100 °C for 30 min, allowed to cool in PBS, and blocked with 5% goat serum (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Rabbit anti-FN14 antibody (Abcam, Waltham, MA, USA, Cat. #ab109365) diluted 1:100 in PBS was applied to the sections overnight at 4 °C. Slides were washed three times with PBS to remove unbound antibody, then incubated with Cy3-conjugated goat anti-rabbit IgG (diluted 1:200) (Jackson ImmunoResearch Laboratories, Cat. #111-165-003) for 40 min at room temp. Slides were washed in PBS as described, mounted with DAPI (Life Technologies, Frederick, MD, USA), and viewed. Positive FN14 cells were quantified at 400 $\times$ magnification with a Nikon Eclipse 80i microscope. The data comprises at least 3 mice per age and genotype, with 6 to 9 randomly selected images quantified per mouse.

2.5. Fibroblast Proliferation Assay

COL3A1-KO fibroblasts were seeded at a density of 10,000 cells per well in 96-well plates (in quintuplicate), and cells were allowed to adhere overnight. The cells were washed gently with PBS, and 100 ng/mL isotype control antibody (MOPC21), 100 ng/mL FN14 antibody, 1.25 μ L DMSO, or 1.25 μ M SM16 were added to the cells in complete DMEM. Cells at the zero time point were assayed immediately with the MTT reagent, and at 24, 48, and 72 h. The optical densities of the wells were determined at 570 nm and 690 nm using a Sunrise TECAN plate reader (Morrisville, NC, USA) and graphed as fold increases relative to day 0.

2.6. Western Blotting

After the transient transfections, cells were lysed in NP40, and 30 μ g of whole-cell lysate was separated on a 10% polyacrylamide gel and then transferred to a PVDF membrane (ThermoFisher Scientific, Waltham, MA, USA). The membrane was blocked in Tris-buffered saline containing 5% skim milk, and the primary antibody was incubated on the membrane overnight. Primary antibodies used were phosphorylated p38 (Cell Signaling, Danvers, MA, USA, Cat # 9211S), total p-38 (Cell Signaling, Cat # 9212S), COL1A1 (Cell Signaling, Cat # 39952S), pro-COL3A1 (Santa Cruz Biotechnology, Dallas, TX, USA, Cat # sc-166316), COL3A1 (ThermoFisher Scientific, Cat # PA5-27828), and diluted 1:100 before use. HRP-conjugated Secondary antibodies employed in these analyses were diluted 1:2000 (Jackson ImmunoResearch, West Grove, PA, USA) and detected using Supersignal West Pico Chemiluminescent Substrate according to the manufacturer's directions (Thermo Fisher Scientific, Rockford, IL, USA).

2.7. FN14-KO X Tsk2/+ Breeding and Analyses

Tsk2/+ mice were bred to FN14-KO mice (Biogen, Cambridge, MA, USA), resulting in Tsk2/+.FN14-KO heterozygotes. Female Tsk2/+.FN14-KO heterozygotes were then backcrossed to FN14-KO males to create Tsk2/+.FN14-KO mice. The Tsk2/+.FN14-KO mice were assessed for tight skin beginning at 3 weeks of age and for fibrosis development at 20 weeks of age.

2.8. Statistical Analyses

Statistical significance was calculated using GraphPad Prism 10, with Student's *t*-test or one-way ANOVA for multiple comparisons. A *p*-value < 0.05 was considered to be significant.

3. Results

3.1. *Tsk2*^{+/+} Neonates Have Increased Procollagen 3 in the Dermis

While investigating the timeline of fibrosis development in *Tsk2*^{+/+} mice, we found that fibrosis is detectable at about 2 weeks of age [12]. Our prior study also demonstrated that COL3A1 is elevated in the dermis at 4 weeks of age and continues to increase in the reticular fibers with age [13]. However, to establish these parameters, there must be a continuum of abnormal collagen expression in the *Tsk2*^{+/+} dermis that begins early in life. COL3A1 is the dominant collagen expressed in the skin of fetuses and rapidly declines after pups are born [35]. When we investigated pro-COL3A1 in neonatal dermis, we found elevated pro-COL3A1 levels, but similar mature COL3A1 levels, suggesting that abnormalities in *Col3a1* expression are well established before they become physically detectable in the skin (Figure 1). This finding implies that there is early active fibrogenesis in neonatal *Tsk2*^{+/+} mice, at a time when the skin has not yet accumulated enough collagen to be tight.

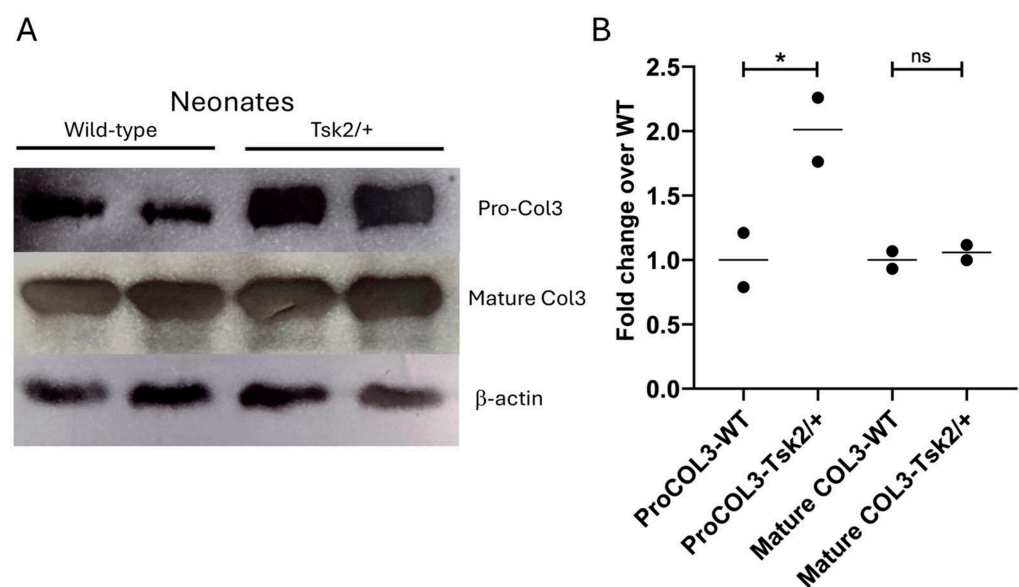


Figure 1. Increased pro-COL3A1 in *Tsk2*^{+/+} neonatal skin. (A) Western blotting analyses of WT and *Tsk2*^{+/+} neonatal skin for pro-COL3A1 and mature COL3A1, demonstrating dysregulated collagen processing prior to the detection of dermal fibrosis. β-actin was used as a loading control. (B) Densitometric quantification of proCOL3 and mature Col3 normalized to β-actin. Statistical analyses: one-way ANOVA with Tukey's multiple comparisons test; * *p* < 0.05, ns = not significant.

3.2. *Tsk2*^{+/+} Skin and Skin-Derived Fibroblasts Show Increased Components of the Profibrotic Signaling Pathway

Our previous data showed increased *Tweek* gene expression at 4 weeks of age in the skin of female mice [14]; however, male mice were not analyzed at that time. In a separate study, we confirmed that female mice had an increase in FN14-positive cells at 4 weeks of age (Figure 2A). In male mice, we observed two cycles of increased FN14-positive cells at 2 and 8 weeks of age (Figure 2B). This observation was confirmed by immunofluorescence staining for FN14-positive cells in male *Tsk2*^{+/+} skin at 2 weeks of age (Figure 2C) and at 4 weeks of age in female skin (Figure 2D).

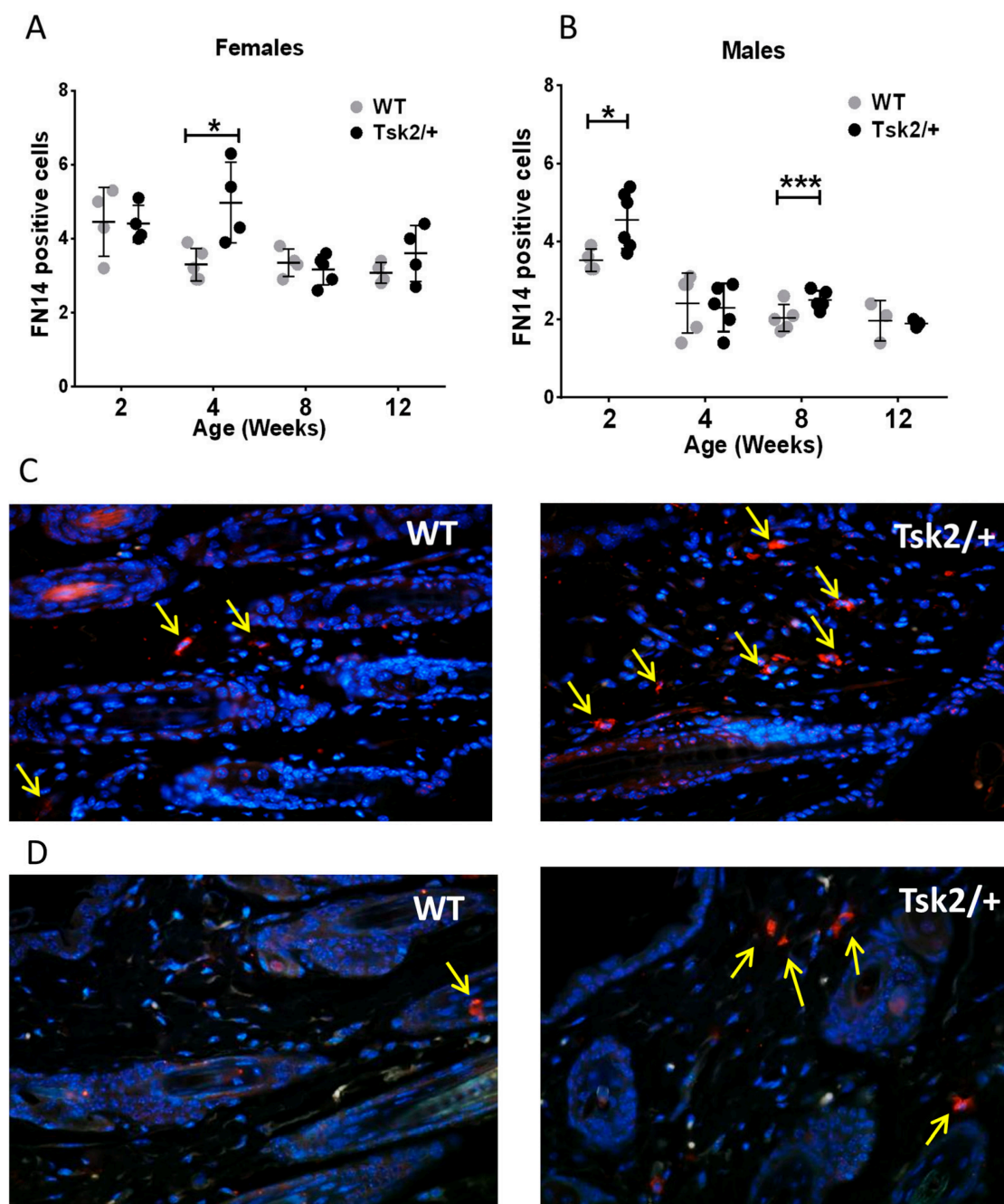


Figure 2. Increased FN14 is elevated at 4 weeks in females and 2 and 8 weeks in male Tsk2/+ mice. Histological quantification of FN14 at different ages of (A) female and (B) male Tsk2/+ mice revealed distinct cycling frequencies. (C) Representative images of FN14+ cells in the skin of mice at 2 weeks of age in males, and (D) the skin of female mice at 4 weeks of age. Magnification of images was at 400 $\times$. FN14 positive cells were stained red, and the nuclei of the cells were counterstained blue with DAPI. * $p < 0.05$. *** $p < 0.001$. N = 3–6 per age and sex.

These data suggest that the TWEAK/FN14 pathway may mediate the fibrotic signature in Tsk2/+ mouse skin at specific time points. Intriguingly, the increased expression of FN14-positive cells correlated with the increased expression of other fibrotic-related genes seen at 4 weeks of age in females and at 2 and 10 weeks of age in males (Table 1) when compared to WT mice of the same age and sex.

Table 1. Fibrosis-related gene expression in Tsk2/+ mouse skin at different ages.

Gene	Female Mice				Male Mice			
	2 wk	4 wk	10 wk	17+ wk	2 wk	4 wk	10 wk	17+ wk
<i>Col1a1</i>	0.42	5.79	0.81	1.17	1.12	0.71	1.81	0.54
<i>Col3a1</i>	0.44	7.48	0.91	1.5	1.22	0.34	1.91	0.81
<i>Col5a1</i>	0.67	4.89	0.85	0.87	1.7	0.28	2.23	0.59
<i>Fbn1</i>	0.68	2.69	0.98	1.84	1.62	2.57	3.42	0.7
<i>Eln</i>	0.42	12.62	0.35	2.25	2.69	1.47	0.44	0.95
<i>Fbln2</i>	0.7	2.57	1.28	2.19	1.79	1.23	2.29	0.77
<i>Fbln5</i>	1.05	1.72	1.82	1.22	1.42	0.98	1.64	0.8
<i>Acta2</i>	1.37	5.28	0.82	2.18	3.04	1.44	1.33	0.52
<i>Comp</i>	0.86	3.25	0.89	0.92	1.73	1.42	1.38	0.85
<i>Sparc</i>	0.51	4.92	0.82	0.51	1.28	1	2.31	0.71
<i>Ctgf</i>	0.58	1.88	0.62	1.39	1.38	0.47	1.33	0.83
<i>Ccn3</i>	0.57	12.01	1.18	ND	1.13	1.7	5.94	ND

Numbers represent the magnitude of change in the Tsk2/+ skin compared to WT mouse skin at the same age. RNA was extracted from skin, cDNA was synthesized, and qRT-PCR was used to measure the expression level for each transcript and normalized to β -actin expression. *Col1a1* = Type I Collagen; *Col3a1* = Type III Collagen; *Col5a1* = Type V Collagen; *Fbn1* = Fibrillin 1; *Eln* = Elastin; *Fbln2* = Fibulin 2; *Fbln5* = Fibulin 5; *Acta* = Alpha Smooth Muscle Actin; *Comp* = Cartridge Oligo Matrix Protein; *Sparc* = Secreted Protein Acidic and Rich in Cysteine; *Ctgf* = Connective Tissue Growth Factor; *Ccn3* = Cellular Communication Network Factor 3. An unpaired Student's *t*-test calculated significant increases or decreases in Tsk2/+ vs. WT skin. The values in blue bold show statistically significant changes ($p < 0.05$) in expression, whereas the values in black bold show a trend for significance (p values between 0.05 and 0.07). ND = not done. N = 4–6 mice per strain and sex at each age.

3.3. Tsk2/+ Mice Have an Increased Pro-Inflammatory Gene Signature in Their Dermis

To better understand signaling in Tsk2/+ skin, we used the Nanostring Immunology Panel to assess the expression of 800 inflammatory genes. In this panel we found 10 genes to be significantly elevated over wild-type mice; including *Irak2* (1.34 fold, $p = 0.005$), *Zeb1* (1.31 fold, $p = 0.012$), *Traf4* (1.33 fold, $p = 0.02$), *Xbp1* (1.17 fold, $p = 0.02$), *Tnfsf12/Tweak* (1.32 fold, $p = 0.02$), *Tgfb3* (1.37 fold, $p = 0.02$), *Cul9* (1.51 fold, $p = 0.03$), *Cd164* (1.12 fold, $p = 0.04$), *Icam1* (1.72 fold, $p = 0.05$), and *Pou2f2* (1.11 fold, $p = 0.05$). *Ilf3* (0.81 fold, $p = 0.015$) and *Litaf* (0.89 fold, $p = 0.04$) were significantly decreased (Figure 3). We also noted a trend for increased expression for *Crlf2* (1.10 fold, $p = 0.06$), *Igf2r* (1.31 fold, $p = 0.06$), and *H2-dma* (1.26 fold, $p = 0.07$). The gene expression data from the Nanostring panel can be found in the (Supplementary Data).

3.4. The C33S Mutation in PIIINP of COL3A1 Promotes Fibrosis in Tsk2/+ Fibroblasts

Our previously published data identified the C33S mutation to be the likely key driver of collagen deposition in the dermis of Tsk2/+ mice. Tsk2/+ skin displays a partial TGF β signature, suggesting that the C33S mutation drives fibrosis via this mechanism [13]. Therefore, we further examined the role of the Tsk2/+ mutation by transfecting COL3A1-KO fibroblasts with a plasmid constructed to carry the wild-type *Col3a1* (*Col3a1*^{WT}) gene or the Tsk2/+ mutation (*Col3a1*^{Tsk2/+}) gene. Fibrosis-related genes tested included *Col1a1*, α -*Sma*, *Icam1*, *Irak2*, *Mfap4*, *Tweak*, and *Fn14*, which were measured by qRT-PCR (Figure 4). The rationale for including *Mfap4* in this panel is that it is emerging as a critical biomarker that bridges ECM assembly and cellular activation. It is heavily involved in the proper formation of elastic fibers [36], but when dysregulated, it drives pathological remodeling [37]. As Tsk2/+ has increased elastic fibers in the skin [38], we included this gene.

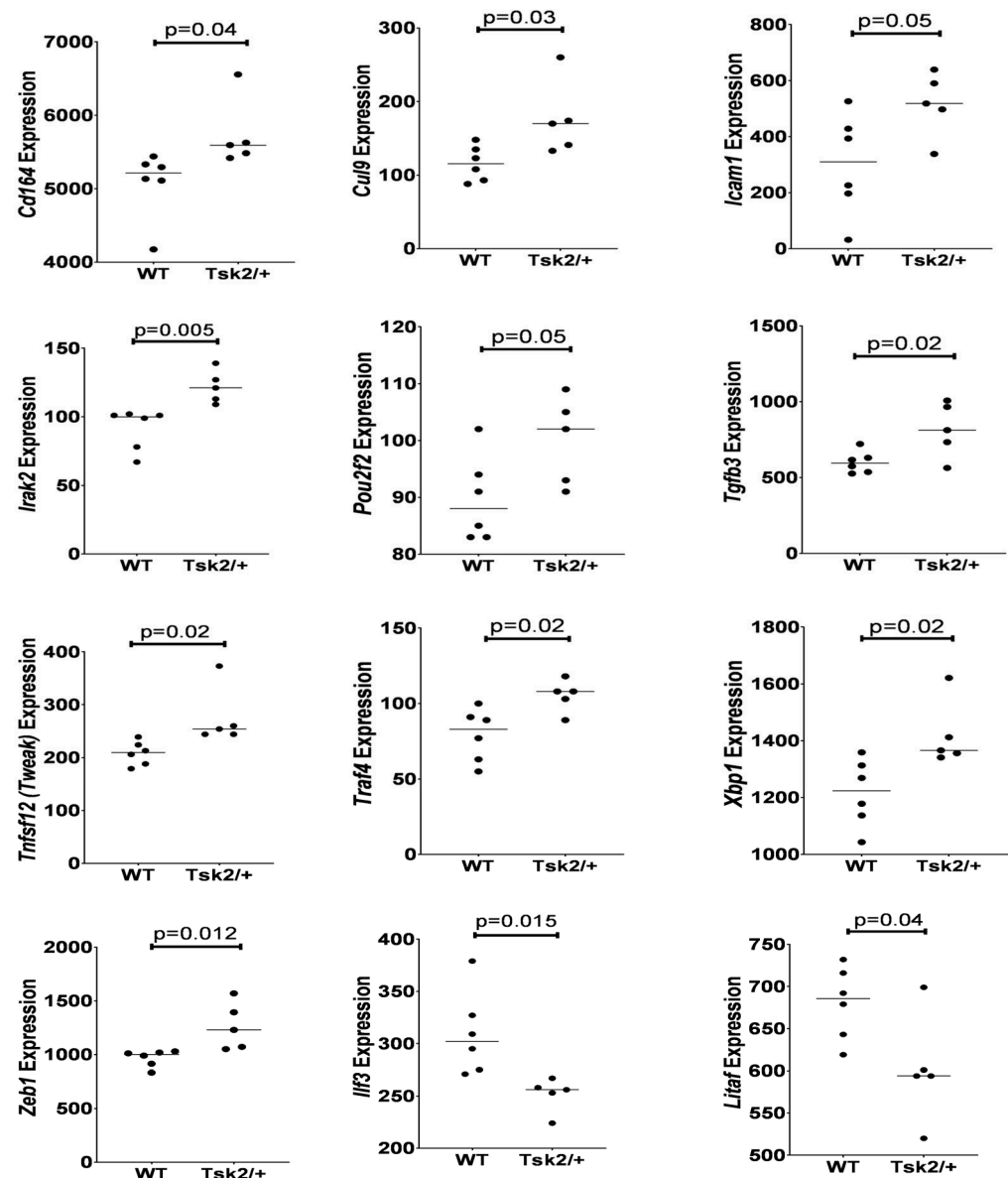


Figure 3. Increased expression of inflammatory genes in the *Tsk2*^{+/+} mouse skin. Expression was assessed using the Nanostring Immunology Panel, and data were analyzed with nSolver Analysis Software (Nanostring). *Cd164* = Cluster designation molecule 164/Endolyn; *Cul9* = Cullin 9; *Icam1* = Intercellular adhesion molecule-1; *Ilf3* = Interleukin enhancer binding factor 3/NP90; *Irak2* = Interleukin 1 receptor associated kinase-2; *Litaf* = Lipopolysaccharide-induced TNF factor; *Pou2f2* = POU class 2 homeobox 2; *Tgfb3* = Transforming growth factor beta-3; *Tnfsf12* = TNF superfamily member-12 (also called Tweak, TNF-related WEAK inducer of apoptosis); *Traf4* = TNF receptor associated factor-4; *Xbp1* = X-box binding protein-1; *Zeb1* = Zinc finger E-box binding homeobox-1.

Our prior studies demonstrated that the plasmids were transfected at equal efficiency [13]. However, to ensure that the *Tsk2*^{+/+} mutation did not directly alter the expression of the *Col3a1* gene in the plasmid constructs, we measured their expression in the COL3A1-KO-transfected cells and found no difference between COL3A1^{Tsk2/+} vs. COL3A1^{WT} (Figure 5A). This gave us confidence that any observed changes in gene expression are due solely to the PIINP mutation. We show in Figure 5B that COL3A1-KO fibroblasts transfected with the *Col3a1*^{WT} construct induced the expression of *Col1a1* by 1.27-fold over the empty vector (mock control) ($p < 0.001$), and the *Col3a1*^{Tsk2/+} construct induced *Col1a1* gene expression by 1.66-fold more over mock control ($p < 0.0001$). The difference between the *Col3a1*^{Tsk2/+} transfected cells vs. the *Col3a1*^{WT} transfected cells

was 1.38-fold ($p < 0.001$) (Figure 5B). Immunoblotting confirmed elevated COL1A1 protein in the culture media of the transfected cells, with greater levels in COL3A1-KO fibroblasts transfected with *Col3a1*^{Tsk2/+} plasmid than in those with the control plasmid or the *Col3a1*^{WT} plasmid (Figure 5C,D). These results suggest that the C33S mutation in *Col3a1* specifically drives *Col1a1* expression.

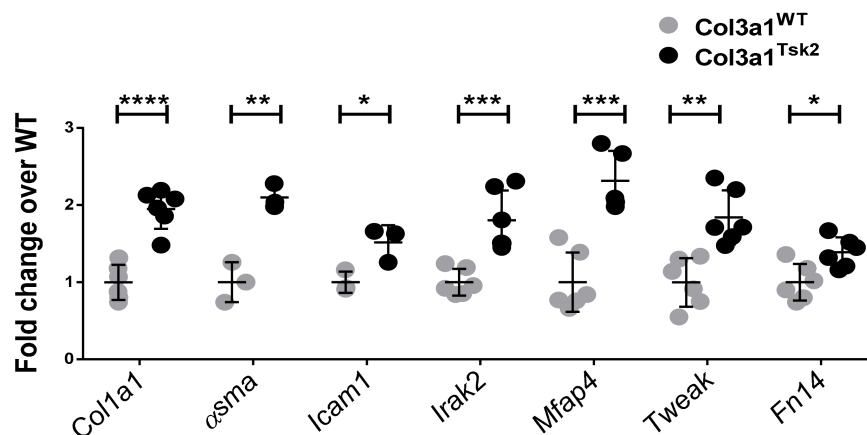


Figure 4. The PIIINP mutation in *Col3a1* specifically drives the expression of fibrotic genes in mouse skin fibroblasts. COL3A1-KO dermal fibroblasts were transfected with plasmids carrying either the wild-type *Col3a1* (*Col3a1*^{WT}) gene or the Tsk2/+ mutation (*Col3a1*^{Tsk2/+}) gene ($n = 3-6$). RNA was harvested after 48 h and measured for the transcripts of interest by quantitative PCR (qPCR). Col1a1 = type 1 collagen; αsma = alpha smooth muscle actin; Icam1 = Intercellular adhesion molecule-1; Irak2 = Interleukin 1 receptor associated kinase-2; Mfap4 = Microfibril associated protein-4; Tweak = TNF-related WEAK inducer of apoptosis (also called Tnfsf12 = TNF superfamily member-12); FN14 = FGF inducible-14 (Tweak receptor). Statistics: one-way ANOVA with Tukey's multiple comparisons test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

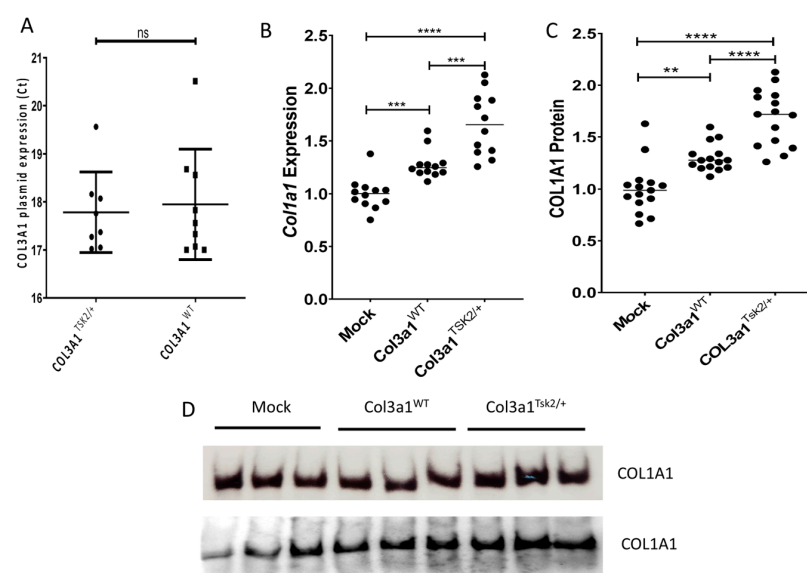


Figure 5. Transfection of COL3A1-KO fibroblasts with the *Col3a1*^{Tsk2/+} plasmid increases *Col1a1* gene expression and COL1A1 protein in the culture media. COL3A1-KO fibroblasts were transfected with *Col3a1*^{WT} or *Col3a1*^{Tsk2/+}. Mock transfections using an empty plasmid with the same backbone as the test plasmids were also used (mock). (A) Expression of *Col3a1* ($n = 8-9$) and the (B) Expression of the *Col1a1* gene 48 h after transfection ($n = 12$), (C) Densitometric quantification of COL1A1 protein secreted into the media at 48 h after the transfection ($n = 15$), (D) Representative immunoblots for COL1A1 secreted into the media from two independent experiments. Statistics: A and B, unpaired t -test; C and D, one-way ANOVA. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ns = no significant difference.

3.5. Increased *Tsk2*^{+/+} Dermal Fibrosis Gene Transcripts Are Dependent on TGF β RI Receptor and FN14 Receptor Signaling

To further evaluate the contribution of TGF- β signaling to the *Tsk2*^{+/+} gene profile, we transfected COL3A1-KO fibroblasts with *Col3a1*^{WT} or *Col3a1*^{Tsk2/+} plasmids and blocked the TGFBR1 with the ALK5 small molecule inhibitor, SM16. SM16 was dissolved in DMSO, and an equivalent volume of DMSO served as the vehicle control. We found that SM16 abolished the *Tsk2*^{+/+} fibrotic gene signature, except for *Fn14*, which remained significantly elevated (Figure 6A). For the *Mfap4* gene, there was a trend for increased expression with the *Col3a1*^{Tsk2/+} plasmid ($p = 0.07$), which was abolished with SM16. Viability was not affected by SM16 or the DMSO vehicle (Figure 6B). Overall, these findings confirm that TGF- β signaling is involved in *Tsk2*^{+/+} dermal fibrosis.

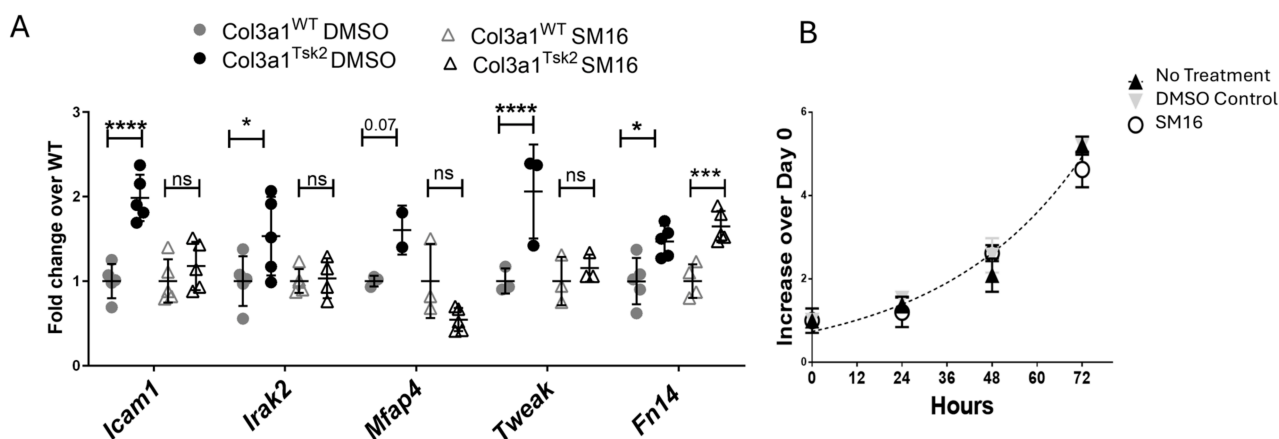


Figure 6. Inhibition of the TGF β receptor abrogates increased gene expression caused by the C33S mutation in the PIIINP region of the *Col3a1* gene. COL3A1-KO fibroblasts were transfected with *Col3a1*^{WT} or the *Col3a1*^{Tsk2/+} plasmid, then 1.25 μ M SM16 or 1.25 μ L/mL DMSO (control) was added to the cells. Cells were harvested after 48 h, RNA was extracted, and gene expression was measured by qPCR. (A) Gene expression profile ($n = 3$ –5), (B) MTT assay for cell viability, ($n = 4$ –9). Statistical analyses: 2-way ANOVA with Tukey’s multiple comparisons test; * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$, ns = no significant difference.

To further evaluate the *Tsk2*^{+/+} signature, we assessed whether TWEAK/FN14 was involved in *Tsk2*^{+/+} fibrosis. We transfected COL3A1-KO fibroblasts with plasmid constructs and then blocked FN14 signaling with an antibody specific to FN14. MOPC21 served as the isotype control for the FN14 antibody. We found that blocking FN14 signaling abolished the increased expression of all evaluated genes (Figure 7A). Cell viability remained unaffected by either the FN14 blocking antibody or the MOPC21 isotype control (Figure 7B). Overall, these observations suggest that the FN14 receptor is also involved in *Tsk2*^{+/+} dermal fibrosis.

3.6. In Vivo Deletion of FN14 Does Not Ameliorate *Tsk2*^{+/+} Fibrosis

Tsk2^{+/+}.FN14-KO mice still had tight skin detectable in the interscapular region by 3 weeks of age and persisting throughout their lifespans. We evaluated the contribution of FN14 to the traditional hallmarks of the *Tsk2*^{+/+} mouse and measured mouse weights at 20 weeks of age, and footpad thickness according to Long et al. [12], and found that the deletion of FN14 did not alter the fact that the *Tsk2*^{+/+} mice were still smaller and had thicker footpads compared to their wild-type counterparts (Figure 8).

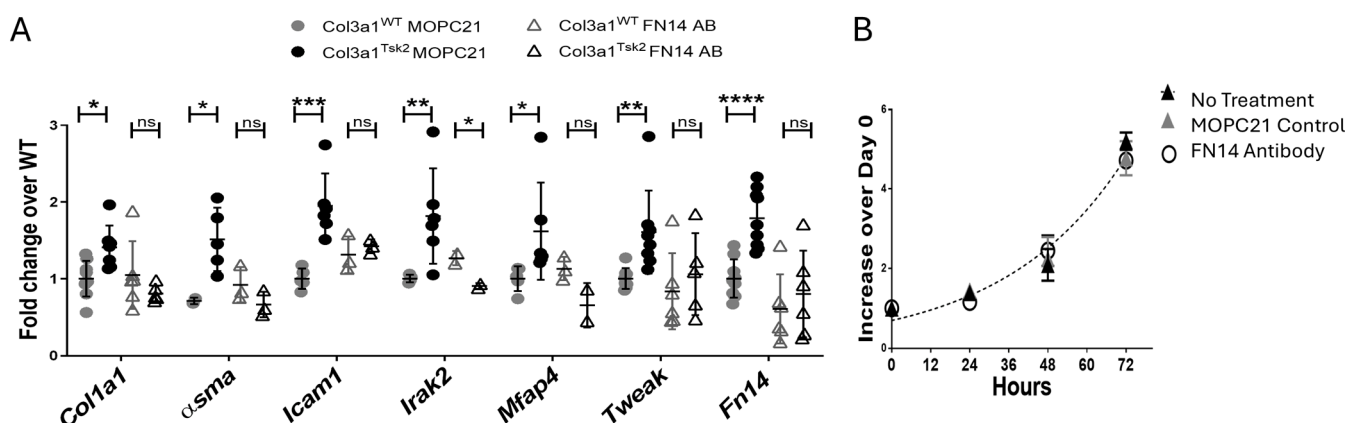


Figure 7. Inhibition of the FN14 receptor abrogates increased gene expression caused by the C33S mutation in the PIINP region of the *Col3a1* gene. COL3A1-KO fibroblasts were transfected with Col3a1^{WT} or the Col3a1^{Tsk2/+} plasmid, then 100 ng/mL FN14 blocking antibody or 100 ng/mL MOPC21 isotype IgG antibody (control) was added to the cells. Cells were harvested after 48 h, RNA was extracted, and gene expression was measured by qPCR. (A) Gene expression profile (n = 4–9), (B) MTT assay for cell viability, (n = 4–9). Statistical analyses: 2-way ANOVA with Tukey's multiple comparisons test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ns = not significant.

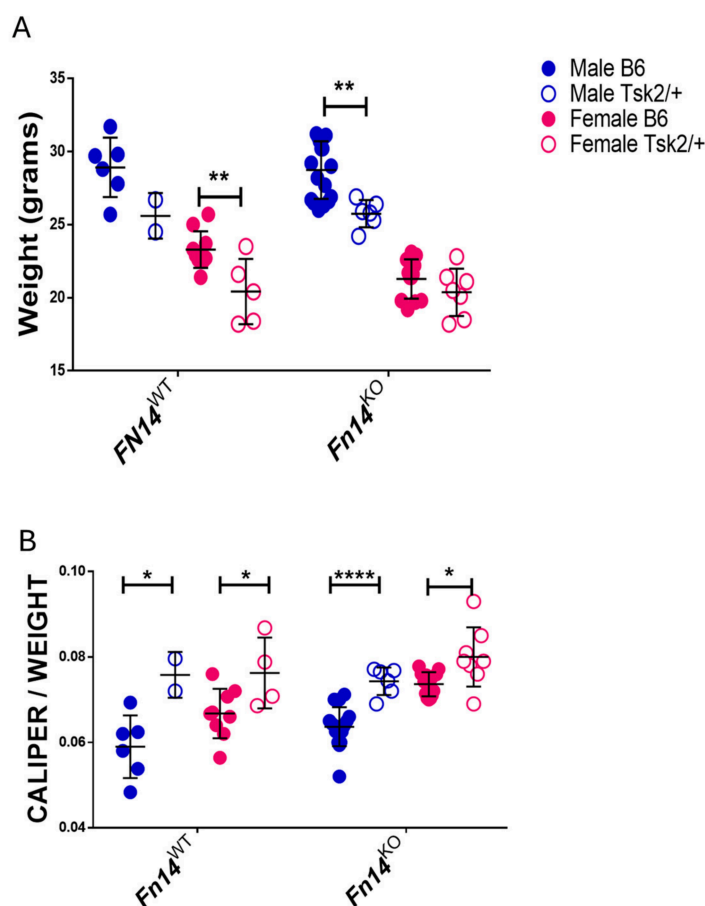


Figure 8. Tsk2.FN14-KO mice display the hallmarks of traditional tight skin, small size, and increased footpad thickness. (A) Male and female mice were weighed and correlated to the presence or absence of FN14; (B) footpad thickness was measured and graphed as caliper width/weight (n = 2–10). Statistical analyses: Two-tailed unpaired Student's *t*-test. * $p < 0.05$, ** $p < 0.01$, and **** $p < 0.0001$.

3.7. The *Tsk2*^{+/+} Mutation Increases the Phosphorylation of p38 MAPK

It has previously been shown that p38 MAPK can act as a stress kinase or a growth kinase, depending on the initiating signal from the ECM [39]. TGF- β is a key cytokine that activates the p38 MAPK pathway; therefore, we determined whether p38 MAPK signaling contributes to *Tsk2*^{+/+} dermal fibrosis. COL3A1-KO fibroblasts transfected with the *Col3a1*^{Tsk2/+} plasmid showed increased phosphorylated p38 when compared to COL3A1-KO fibroblasts transfected with the *Col3a1*^{WT} plasmid. This shows that phosphorylated p38 may be an essential signaling molecule driving fibrosis in the *Tsk2*^{+/+} dermis (Figure 9A,B). When p38 was inhibited with SB202190, we observed reduced COL1A1 protein in the culture medium (Figure 9C,D), further defining the role of p38 in *Tsk2*^{+/+} dermal fibrosis.

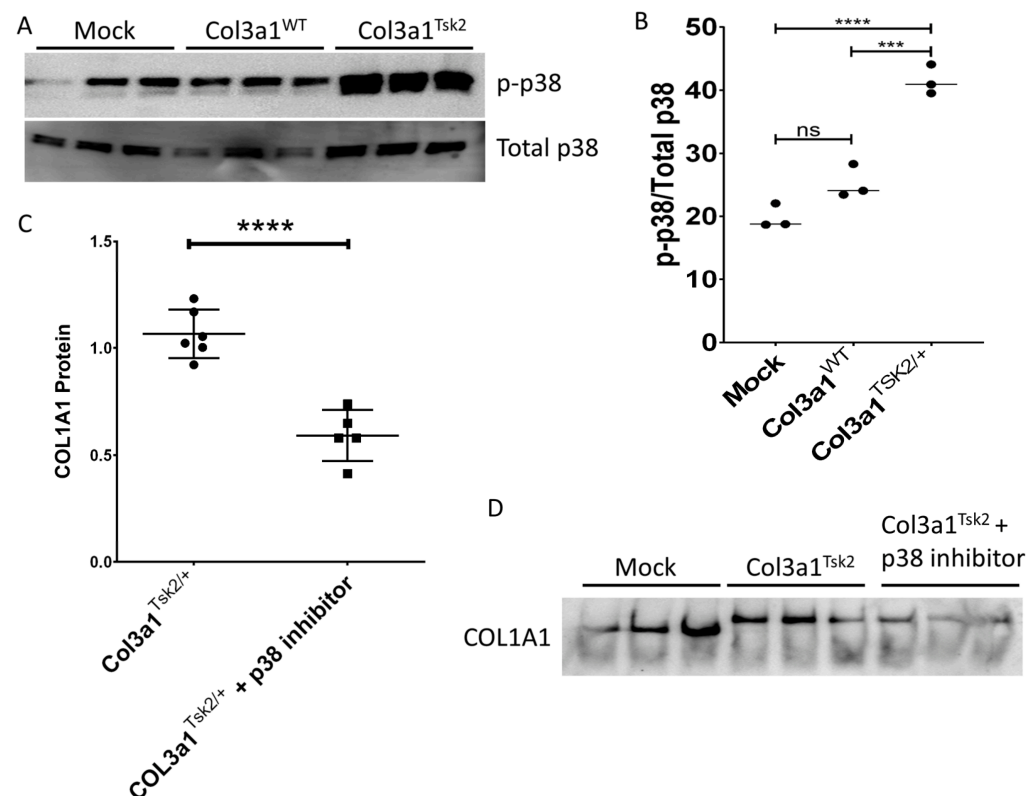


Figure 9. The increased COL1A1 protein is dependent on phosphorylated p38. (A) Representative immunoblots of phosphorylated p-38 and total p-38 in mock, Col3a1^{WT}, and Col3a1^{Tsk2/+} transfected COL3A1-KO fibroblasts (n = 3). (B) Densitometric quantification and determination of the amount of phosphorylated p38 in the samples. (C) Inhibition of p-38 with SB202190 reduced COL1A1 secreted into the culture media (n = 5–6). (D) A representative immunoblot for COL1A1 in the media. Statistical analyses: (A) One-way ANOVA, (B) unpaired *t*-test, (C) paired *t*-test; *** *p* < 0.001, **** *p* < 0.0001, ns = not significant.

4. Discussion

The *Tsk2*^{+/+} mouse has long been recognized as a genetic model for SSc, recapitulating key features such as spontaneous skin fibrosis, loss of hypodermal adipose tissue, and a distinct TGF- β signature [12–14]. However, the specific molecular mechanisms linking the causative C33S mutation in *Col3a1* to this complex phenotype have remained incompletely understood. In this study, we definitively demonstrate that the *Tsk2*^{+/+} mutation autonomously drives a profibrotic program through two distinct yet converging signaling axes: the TGF- β and TWEAK/FN14 pathways. Furthermore, we provide the first evidence that TWEAK/FN14 signaling is not merely a bystander pathway but a critical, neces-

sary driver of fibrosis in this model, as its blockade abrogates the fibrotic gene signature in fibroblasts.

A pivotal finding of this study is the presence of elevated procollagen III in the dermis of Tsk2/+ neonates, prior to the onset of palpable skin tightness or histological fibrosis, which typically manifests around 2 weeks of age [12]. This accumulation of procollagen III, alongside normal levels of mature collagen III (Figure 1), suggests a fundamental defect in collagen processing or trafficking started by the C33S mutation. This may induce cellular stress states, such as the unfolded protein response, early in development. This hypothesis is supported by our Nanostring data, which show elevated Xbp1, a transcription factor centrally involved in the ER stress response [40]. The continuum of abnormality suggests that the Tsk2/+ mouse is an ideal model for studying the *initiating* molecular events of SSc, rather than just the end-stage fibrotic scarring.

Our transfection data demonstrate that the C33S mutation is cell autonomous. Introducing the *Col3a1*^{Tsk2/+} plasmid into COL3A1-KO fibroblasts was sufficient to upregulate a broad panel of fibrotic markers, such as *Col1a1*, *αSma*, and *Icam1*, and signaling mediators, including *Irak2* and *Fn14*, independent of the systemic immune system. This experimental design allowed us to dissect the signaling requirements for this phenotype. While it is well established that TGF-β is a master regulator of SSc fibrosis [14], our results using the ALK5 inhibitor SM16 reveal a more nuanced hierarchy in the Tsk2/+ mouse. SM16 successfully reduced the expression of extracellular matrix genes (*Col1a1* and *Mfap4*) and inflammatory markers (*Icam1*), confirming the dependence on TGF-β signaling. However, SM16 did not significantly reduce Fn14 expression. This contrasts with previous reports on other cell types, suggesting TGF-β induces FN14 [28]. In Tsk2/+ fibroblasts, this shows that Fn14 upregulation may be driven by mechanisms upstream of, or in parallel to, canonical TGF-β signaling, potentially via the ER stress/unfolded protein response pathway, as indicated by the *Xbp1* data.

Crucially, blockade of the FN14 receptor using a neutralizing antibody completely ablated the Tsk2/+ fibrotic gene signature, including *Col1a1*. This places the TWEAK/FN14 axis as a central bottleneck in the pathology of this model. If TGF-β inhibition does not reduce FN14, but FN14 inhibition blocks the fibrotic output, it implies that the TWEAK/FN14 axis is essential for the perpetuation of the TGF-β-driven fibrotic response, or that it acts as a requisite co-signal. This mirrors findings in human SSc, where TWEAK/FN14 levels correlate with disease severity and fibrotic progression in the skin [24,32]. The upregulation of *Tweak* and *Fn14* observed in the dermis of Tsk2/+ mice, which exhibit sex-specific cycling peaks (Figure 2), further underscores the dynamic nature of this pathway during disease progression. The cyclical nature of *FN14* expression, particularly in males, may explain variability often seen in static time-point analyses and suggests that therapeutic timing targeting this axis is critical.

We also identified *Irak2* and phosphorylated p38 MAPK as key intracellular effectors in this cascade. *Irak2* was significantly upregulated in Tsk2/+ skin and in transfected fibroblasts. IRAK2 is known to activate p38 MAPK downstream of Toll-like receptors and IL-1 receptors [41]. Our data show that the Tsk2/+ mutation leads to hyperphosphorylation of p38, and pharmacological inhibition of p38 significantly reduced COL1A1 secretion. This further links the initial stress of mutant collagen (potentially acting as a DAMP or inducing ER stress) to the inflammatory activation of fibroblasts via an IRAK2-p38 axis, culminating in excessive matrix deposition.

Finally, the immune profiling of Tsk2/+ skin revealed a specific pro-inflammatory and pro-fibrotic transcriptional landscape. The upregulation of *Zeb1*, a driver of epithelial-to-mesenchymal transition [42], further aligns with the proliferative, invasive phenotype of SSc fibroblasts. Concurrently, the downregulation of negative regulators such as *Ilf3* and

Litaf suggests a loss of homeostatic inhibitors in fibrosis. One of the key functions of *Ilf3* (NP90) is to suppress the senescence-associated secretory phenotype, and it is significantly reduced in senescent fibroblasts [43]. This suggests that, as it decreases in *Tsk2/+* skin, the tissue is failing to suppress the pathological secretory state. *Litaf* is a complex transcription factor, and its reduced expression has been found in hypertrophic cardiac fibroblasts, linking low levels of this transcription factor to an activated, fibrotic cellular state [44]. However, this signaling may be organ-dependent or cell-specific, as increased expression was associated with liver fibrosis [45]. Its downregulation in the *Tsk2/+* mouse parallels this finding and supports a pro-fibrotic environment, as observed in cardiac fibroblasts.

Intriguingly, the observation that *Tsk2/+* skin is profibrotic while their lungs remain disease-free is a paradox but can be rationalized by the TWEAK/FN14 pathway. We have identified the TWEAK/FN14 axis as one pathway leading to skin fibrosis, whereas it may play an opposing role in the lung. In the dermis, the TWEAK/FN14 axis, in parallel with TGF- β , acts as a pro-fibrotic driver, and the blockade of either receptor in the *Tsk2/+* fibroblasts completely ablated the fibrotic signature. However, studies in SSc show increased TWEAK/FN14 in skin but an inverse correlation between circulating TWEAK levels and pulmonary fibrosis severity [13,14,24,30]. The C33S mutation in COL3A1 in the *Tsk2/+* mouse specifically affects the PIIINP segment, which is a key regulator of collagen fibrillogenesis and growth-factor tethering. Dermal tissue is uniquely dependent on the proper COL1A1-to-COL3A1 ratio during development [35]. Any dysregulation of collagen processing in the *Tsk2/+* neonate may result in failure to sequester TGF- β in the skin, leading to localized fibrosis. In contrast, the lung utilizes a different matrix composition to maintain TGF- β latency. The presence of other redundant protein tethers, such as Latent TGF-Binding Protein-1 and -4, in the pulmonary matrix might sequester excess TGF- β [46], thereby neutralizing the effect of the mutant COL3A1 in that tissue.

The paradoxical failure of the *in vivo* studies in which FN14 was deleted to resolve dermal fibrosis in the *Tsk2/+* mouse, compared with the efficacy of acute *in vitro* blockade, highlights a critical distinction between specific pathway inhibition and genetic deletion in a cell-autonomous model. While FN14 serves as one amplifier of the fibrotic program, its absence from conception likely allows for significant developmental compensation of other pro-fibrotic pathways. In the *Tsk2/+* neonate, the *Col3a1* C33S mutation acts as an internal initiator, triggering chronic ER stress and the XBP1s-mediated unfolded protein response independently of surface receptor activity. This intrinsic danger signal is further reinforced by the upregulation of IRAK2 and a robust TGF- β gene signature, both of which converge on the p38 MAPK bottleneck. Consequently, in a chronic *in vivo* environment, the loss of FN14 is likely bypassed by these redundant pro-fibrotic signals. We propose that while the TWEAK/FN14 axis remains a viable therapeutic target for interrupting established signaling, the initiation of *Tsk2/+* fibrosis is driven by a multi-nodal network in which the structural mutation in procollagen III provides a constant stimulus that exceeds the need for a single receptor. Combination targeting of the TGF- β receptor and FN14 may confer greater efficacy in *Tsk2/+* mice and may suggest better success in controlling fibrosis in SSc.

Future studies are needed to delineate further the functional role of the C33S mutation in the *Tsk2/+* mouse. Furthermore, it is crucial to determine the upstream driver of FN14 expression and define whether it is driven by ER stress or another mechanism. A central paradox identified in these studies is that although TGF- β inhibition (by SM16) reduced the fibrotic signature, it did not reduce *Fn14* expression. These studies, using inducible or fibroblast-specific conditional knockouts, would be instrumental in bypassing developmental rewiring to confirm the role of FN14 in adult fibrotic maintenance. Elevated *Xbp1* indicates accumulation of misfolded or mutant procollagen III within the cell, which

could act as an intracellular danger signal, triggering the unfolded protein response and further driving inflammation and fibrosis. In *Tsk2*/+, it will be essential to determine whether the mutant procollagen III acts as a danger-associated molecular pattern. We have investigated the role of NLRP3 in *Tsk2*/+ and found that its deletion does not affect the phenotype [38]. However, the mutant COL3A1 could be activating another inflammasome, thereby driving inflammation and fibrosis. Elevated *Irak2*, an intermediary in TLR signaling, and hyperphosphorylated p38 MAPK (Figure 9) further suggest that the mutant collagen might not only be a structural defect but also act as a danger signal. While the *Tsk2*/+ mouse serves as a robust model for the cell-autonomous initiation of dermal thickening, it fails to recapitulate the spontaneous pulmonary fibrosis characteristic of human SSc. Investigating the tissue-specific expression of growth factor tethers, such as LTBP and fibrillin, in the *Tsk2*/+ lung may elucidate mechanisms of pulmonary resilience. Further studies are needed to investigate other signal transduction pathways in the mouse to better understand those that are altered by the C33S mutation. Elucidation of other signaling pathways in the mouse may help to uncover novel therapeutic targets for the treatment of SSc. One interesting observation from these studies is the distinct temporal expression of FN14 between male (biphasic peaks at 2 and 8 weeks) and female (peak at 4 weeks) mice. SSc shows a strong female predilection in humans, but the *Tsk2*/+ model shows fibrosis in both sexes. Expanding longitudinal studies to include high-resolution spatial transcriptomics could help determine how these temporal spikes coordinate the recruitment of inflammatory cells and the subsequent activation of the p38 MAPK bottleneck across distinct stages of disease progression. Understanding why the timing differs between male and female mice could reveal a hormonal regulation of the TWEAK/FN14 axis.

5. Conclusions

In conclusion, this study characterizes the *Tsk2*/+ mouse as a model of early-stage SSc pathophysiology, driven by a *Col3a1* mutation that triggers a specific inflammatory and fibrotic cascade. We demonstrate that while TGF- β is a major driver, the TWEAK/FN14 axis is an indispensable component of this pathology. The inability of TGF- β inhibition to downregulate *Fn14*, combined with the efficacy of FN14 blockade in preventing fibrosis, highlights FN14 as a promising, distinct therapeutic target.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/rheumato6020013/s1>, Table S1: Complete list of Immunology Gene Panel (Nanostring); File S1: Uncropped Westerns in the Figures.

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