

TGFβ activation primes canonical Wnt signaling through the downregulation of AXIN2

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ABSTRACT

Objectives: Aberrant activation of Wnt signaling has been observed in systemic sclerosis (SSc) affected tissues. This study aimed to determine the role of transforming growth factor (TGF) β in driving the increased Wnt signaling, through modulation of AXIN2, a critical regulator of Wnt canonical pathway.

Methods: Canonical Wnt signaling activation was analyzed by TOPFlash TCF/LEF promoter assays. AXIN2 was evaluated *in vitro* by analysis of AXIN2 primary/mature transcripts expression and decay, T β RI blockade, siRNA-mediated TTP-1 depletion and through XAV-939-mediated AXIN2 stabilisation. *In vivo*, Axin2 mRNA and protein expression was determined in skin and lung biopsies from T β RI Δ k-fib transgenic mice and littermate controls.

Results: SSc fibroblasts display increased response to canonical Wnt ligands despite basal levels of Wnt signaling comparable to healthy control (HC) fibroblasts *in vitro*. Notably, we show that SSc fibroblasts express reduced basal expression of AXIN2, which is caused by endogenous TGF β -dependent increase of AXIN2 mRNA decay. Accordingly, we observed that TGF β decreased AXIN2 expression both *in vitro* in HC fibroblasts and *in vivo*, employing T β RI Δ k-fib transgenic mice. Additionally, we demonstrate by AXIN2 loss and gain of function experiments, that the TGF β -induced increased response to Wnt activation characteristic of SSc fibroblasts is dependent on reduced AXIN2 bioavailability.

Conclusions: This study highlights the importance of reduced AXIN2 bioavailability in mediating the increased canonical Wnt response observed in SSc fibroblasts. This novel mechanism extends our understanding of the processes involved in Wnt/ β -catenin-driven pathology and supports the rationale for targeting the TGF β pathway to regulate the aberrant Wnt signaling observed during fibrosis.

INTRODUCTION

Tissue fibroblasts are the key cellular elements of fibrosis, primarily involved in regulating extracellular matrix (ECM) homeostasis. Dysregulated fibroblast activation can result in the excessive synthesis and deposition of collagens and other ECM proteins within tissues leading to organ dysfunction and failure (1,2). Sustained TGF β activity has been shown to be a central mediator of fibroblast activation and can reproduce many of the hallmarks associated with fibrosis both *in vitro* and *in vivo* (3-6). Indeed, TGF β treated fibroblasts display a gene expression profile similar to dermal fibroblasts from diffuse cutaneous systemic sclerosis (dcSSc) patients (7).

The T β RII Δ k-fib transgenic mouse model, characterized by constitutive TGF β signaling through *Col1a2*-mediated fibroblast-specific expression of a kinase deficient TGF β Receptor II gene, replicates key constitutive features of SSc including dermal fibrosis as well as susceptibility to other organ-based complications such as lung fibrosis and pulmonary hypertension (8). This model has proven valuable for the delineation of profibrotic pathways and especially for investigating crosstalk between TGF β and other relevant mediators (9).

Recently, several studies have shown that canonical Wnt signaling is activated during fibrosis in systemic sclerosis (SSc) and in other fibrotic conditions (10-14). Indeed, this activation may have an important role in the initiation/maintenance of fibrosis; however, the biochemical & biophysical mechanisms of this activation are yet to be identified and the relative contribution of crosstalk with TGF β pathway activation is unclear.

Canonical Wnt signaling is dependent on β -catenin bioavailability, which is regulated by the β -catenin destruction complex. This complex is primarily comprised of AXIN, adenomatous polyposis coli, casein kinase-1 and glycogen synthase kinase-3- β , which facilitates the phosphorylation and subsequent ubiquitin-mediated degradation of β -catenin (15). Canonical Wnt agonist activation of this pathway recruits the destruction

complex to the plasma membrane, thereby lowering its capacity to induce β -catenin proteasomal degradation (16). As a result, increased levels of free cytosolic β -catenin can undergo nuclear translocation, leading to the upregulation of canonical Wnt target genes through β -catenin-mediated activation of the TCF/LEF transcription factors.

Specifically, AXIN2 and AXIN1 are essential scaffold proteins for the formation of the β -catenin destruction complex and therefore act as negative regulators of Wnt signaling (17-19). While *AXIN1* is constitutively expressed, *AXIN2* is a direct transcriptional target of canonical Wnt signaling, acting as part of a negative feedback system to control Wnt signaling activation (20,21).

Axin2^(-/-) knockout mice are viable and delivery of *Axin2* cDNA can rescue the *Axin1*^(-/-) lethal phenotype (22,23). Uniquely, heterozygous *AXIN2* germ line mutations in humans can lead to familial tooth agenesis (24). Interestingly, in the chondrocyte lineage TGF β promotes β -catenin accumulation while inhibiting the expression of *Axin1* and *Axin2*, thus providing a context-specific example of the interplay between these pathways (25). Recently, the endogenous Wnt antagonist, DKK-1, has been described to be TGF β responsive with transgenic expression attenuating experimental fibrosis (26). Our study aimed to determine whether increased TGF β signaling could play a role in the observed canonical Wnt signaling hyperactivation of tissue fibroblasts during SSc.

METHODS

Reagents

Adult human dermal fibroblast cultures were obtained from five early dcSSc patients and five healthy controls (HC) (PromoCell GmbH, Germany) (see Supplementary Table 1). Primary cells were immortalised using retrovirus expressing human telomerase (hTERT) to make HC hTERT and SSc hTERT. Briefly, hTERT pBABE (Neo) (kind gift from Prof. M.A. Knowles, University of Leeds, UK) was transfected into Phoenix A cells using

TransIT—293 (Mirus Bio LLC, USA) and virus supernatant containing 8 µg/ml polybrene was added to primary cells and selected with 1000 µg/ml G418 48 hr after transduction. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, UK) containing 10% foetal calf serum (FCS) (Biosera, UK) and incubated at 37°C under 5% CO₂. All experiments conducted with hTERT fibroblasts were validated with primary (not immortalised) fibroblasts. Primary fibroblasts were used between passages 2 and 5. Starvation was performed with 0.5% serum DMEM and stimulations were performed with recombinant human TGFβ (5 ng/ml) (Sigma-Aldrich, UK) or Wnt-3a (100 ng/ml) (R&D Systems, UK). mRNA decay rate was investigated using Actinomycin D (5 µg/ml) (Sigma-Aldrich, UK). Axin stabilization was achieved using the XAV-939 Tankyrase 1/2 Inhibitor (1 µM) (Calbiochem, UK) and TGFβ signaling was inhibited using the SD-208 TβRI Kinase Inhibitor (1 µM) (Sigma-Aldrich, UK).

Study approval

Informed consent was obtained from all patients and studies were granted approval by the Leeds Teaching Hospitals NHS Trust Medical Ethics Committee (LTH REC approval number 10/H1306/88). All scleroderma patients fulfilled the 2013 EULAR/ACR classification criteria for SSc, classified as dcSSc according to LeRoy et al. (27).

TβRIIΔk-fib transgenic mice

The generation of TβRIIΔk-fib transgenic mice has been described previously (8). Constitutive TGFβ signaling is mediated by TβRIIΔk-fib expression and driven by a 6kb pro-*Col1a2* enhancer and minimal promoter, which also directs its expression towards the fibroblast lineage. Dermal and lung tissues were taken from 6-8-week age-matched transgenic (TG) and wild-type (WT) littermates and each experiment was performed on at least 5 mice for each condition. Animals were housed in a clean conventional colony, with

access to food and water *ad libitum*. Strict adherence to institutional guidelines was practiced, and full local ethics committee and Home Office approvals were obtained.

Histology studies

Formalin-fixed skin specimens were embedded in paraffin and sections were cut at 5 μ M. Skin fibrosis was evaluated by dermal thickness between the dermal-epidermal boundary to the dermal-subcutaneous fat boundary and further evaluated by Masson's Trichrome blue staining. Antigen retrieval was performed using 10 mM pH 6.0 sodium citrate and sections were stained with anti-AXIN2 antibody (Sigma-Aldrich, UK) followed by StreptAB-Complex-HRP (Dako Cytomation, Denmark) and visualised with 3,3' diamino-benzidine tetrahydrochloride (DAB; Vector Laboratories). Microscopic analysis was performed using an Olympus BX50 with MicroFire (Optronics) and images captured using Stereo Investigator software at 20X magnification.

Cell lysate preparation

Cells were lysed in RIPA buffer (Sigma-Aldrich, UK), supplemented with complete protease and phosphatase inhibitor cocktails (Roche, Switzerland). Whole cell lysate protein concentration was then quantified using the bicinchoninic acid (BCA) colorimetric protein assay kit (Thermo-Fisher, UK) as per manufacturer's protocol.

Western blotting

Cell lysates were electrophoresed on a 4-12% Bis-Tris gradient gel using the NuPAGE gel system and transferred onto Immobilon-PVDF membrane (EMD Millipore, USA). The membranes were blocked with 5% milk in Tris buffered saline 0.01% Tween 20 (TBST) and incubated with the following antibodies: anti-AXIN2 (NEB, UK), anti-AXIN1 (NEB, UK),

anti- α -SMA (Abcam, UK). Incubation with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody allowed for chemiluminescent detection using enhanced chemiluminescence (ECL) reagent (GE Healthcare, UK). Equal loading of proteins was achieved using the BCA protein assay kit and confirmed by visualization of GAPDH (Abcam, UK).

Quantitative real-time PCR

Total cellular RNA extraction and purification was performed using the RNeasy kit (Qiagen, UK) and cDNA prepared using a SuperScript II first-strand cDNA synthesis kit (Life Technologies, UK) as per manufacturer protocols. Gene expression was quantified by SYBR Green real-time PCR on the ABI Prism 7700 Sequence Detection System (Life Technologies, UK). Specific primers were designed against *AXIN2* (Forward: 5'-CGGGAGCCACACCCTTC-3'; Reverse 5'-TGGACACCTGCCAGTTTCTTT-3'), *ribosomal 18S* (Forward: 5'-GTAACCCGTTGAACCCATT-3'; Reverse 5'-CCAATAATCGGTAGTAGCG-3'), α -SMA (Forward: 5'-TGTATGTGGCTATCCAGGCG-3'; Reverse 5'-AGAGTCCAGCACGATGCCAG-3'), *DKK-1* (Forward: 5'-GACTGTGCCTCAGGATTGTGT-3'; Reverse 5'-CAGATCTTGGACCAGAAGTGTCT-3'). Analysis of the *AXIN2* primary transcript utilised primers spanning intron-exon junctions specifically limiting amplification to unprocessed RNA PT-*AXIN2* (Forward: 5'-TGATGCGCTGACGGATGATT-3'; Reverse 5'-ATCCACTCCCAAGCAAGCC-3'). For mouse *Axin2* expression we employed *Axin2* Forward: 5'-AGCCTAAAGGTCTTATGTGG-3'; Reverse 5'-ATGGAATCGTCCGGTCAGT-3' and ribosomal *18S* primers as shown above. Amplifications were analyzed with SDS v2.3 software (Life technologies, UK) and normalized to ribosomal *18S* using the $\Delta\Delta$ Ct method.

TOPFlash reporter assay

The TOPFlash/FOPFlash luciferase reporter system (gift of Prof. Randall Moon, University of Washington, USA) measured β -catenin driven TCF/LEF transcriptional activation. Fibroblasts were transfected with the TOPFlash construct at a concentration of 1 μ g/ml per well in a 12 well plate using Lipofectamine 2000 transfection reagent (Life Technologies, UK). For transfection efficiency normalization, cells were co-transfected a CMV-Renilla luciferase vector at a concentration of 100 ng/ml. Transfection complexes were removed after 2 hr and cells were stimulated with Wnt-3a in DMEM 0.5% FCS for 24 hr. Cells lysates were prepared and luciferase activity evaluated using the dual luciferase reporter system (Promega, USA) as per manufacturer's protocol. Firefly luciferase activity was normalized to Renilla luciferase activity and expressed as the relative fold change.

Small interfering RNA (siRNA) mediated gene silencing

Cells were seeded to reach a confluence of 70% on the day of transfection in DMEM 10% FCS. On the day of transfection, cell medium was changed to DMEM 5% FCS. Silencer Select, pre-designed, non-targeting (SCR) siRNA (AM4611), *AXIN2* siRNA (S15818), and tristetraprolin (TTP)-1 siRNA (s14978) (Life Technologies, UK) were transfected using NTER transfection reagent (Sigma-Aldrich, UK). Transfection complexes were added to fibroblasts at a final siRNA concentration of 10 nM and removed after 24 hr. Gene silencing was monitored between 24-96 hr post-transfection at RNA and protein level.

Statistical analysis

Statistical analyses were performed using nonparametric Mann-Whitney test for unpaired samples. Experimental data are presented as the mean \pm standard error of the mean (SEM). A P-value ≤ 0.05 was considered statistically significant. Statistical analysis was performed using GraphPad Prism software, version 5.0 (GraphPad Software, Inc., CA, USA).

RESULTS

SSc fibroblasts display an increased canonical Wnt signaling responsiveness.

To measure the activation of canonical Wnt signaling pathway in explanted dermal fibroblasts from SSc and healthy control (HC) fibroblasts, β -catenin-mediated TCF/LEF transcription was quantified by TOPFlash reporter activity. Compared to HC, no increase in basal level TOPFlash activity was observed in SSc fibroblasts. However, following 24 hr treatment with canonical Wnt-3a ligand, SSc fibroblasts displayed a 9.9-fold increase in reporter activity compared to a 4.8-fold increase observed in HC ($P < 0.01$) (Figure 1A). Consistent with these findings, Wnt-3a induced *AXIN2* expression by 5.0-fold in SSc fibroblasts relative to basal levels, compared to the 1.8-fold increase in HC fibroblasts ($P < 0.001$) (Figure 1B). Interestingly, we found that basal *AXIN2* expression was reduced by 52% in SSc fibroblasts (Figure 1B). Since *AXIN2* plays an essential role in regulating the activity of the Wnt signaling pathway, we set out to evaluate the regulatory effects of TGF β on *AXIN2* expression. Stimulation of HC fibroblasts with TGF β for 24 hr reduced the expression of *AXIN2* to 49% at 24 hr ($P < 0.001$), which was analogous to the basal levels of expression observed in SSc fibroblasts, 52% of HC levels (Figure 1C). Additionally, TGF β treatment of SSc fibroblasts further reduced *AXIN2* expression to 20% of HC levels ($P < 0.01$) (Figure 1C). Consistently, *AXIN2* protein expression in HC fibroblasts treated with TGF β was comparable to the level observed in SSc fibroblasts (Figure 1D).

Constitutive TGF β activation leads to tissue fibrosis and reduced Axin2 expression in the T β RII Δ k-fib mouse model of SSc.

The T β RII Δ k-fib mouse model of SSc was utilized to validate the effects of TGF β signaling on *AXIN2* expression *in vivo*. In-line with published data, T β RII Δ k-fib TG mice developed

a significant dermal fibrosis characterized by an increased dermal thickness, collagen deposition, and loss of subcutaneous fat compared to WT controls (Figure 2A) (8, 28). Immunohistochemistry studies determined that dermal tissue from these mice had an overall reduction in the expression of *Axin2*, which was evident in both the epidermal and dermal compartments (Figure 2B-C). Accordingly, in transgenic mice skin *Axin2* mRNA levels were reduced by 65% compared to WT control mice skin ($P < 0.05$) (Supplementary Figure 1A). Furthermore, *Axin2* protein expression was also reduced in lung tissue from these mice, particularly in areas where the normal architecture was lost (Figure 2D).

TGF β priming of fibroblasts reproduces the increased canonical Wnt signaling amplitude observed in SSc fibroblasts through AXIN2 downregulation.

To determine whether the TGF β -mediated decrease in AXIN2 expression could be responsible for the increased sensitivity of fibroblasts to canonical Wnt ligands, 24 hr sequential stimulation experiments were performed. TGF β -primed HC fibroblasts showed a 9.8-fold increase in TOPFlash activation in response to Wnt-3a compared to the 4.8-fold increase in cells not previously treated with TGF β ($P < 0.05$) (Figure 3A). The increased response to Wnt was observable as early as 2 hr following TGF β stimulation and persisted for up to 72 hr (Supplementary Figure 1B). Importantly, fibroblasts treated with TGF β alone did not show any activation of TOPFlash activity (Figure 3A).

Next, we set out to determine the mechanism by which TGF β regulates AXIN2 expression in fibroblasts. TGF β induced a significant and time-dependent reduction in *AXIN2* expression observable within 30 min, which persisted for up to 72 hr ($P < 0.05$) (Figure 3B-C). Similarly, protein expression was decreased within 2 hr and continued up to 72 hr (Figure 3B). In contrast, TGF β treatment had a modest effect on *AXIN1* mRNA and, unlike AXIN2, protein expression remained unchanged (Supplementary Figure 1C).

To determine whether TGF β could regulate *AXIN2* at the transcriptional level, expression of the *AXIN2* primary transcript was evaluated. TGF β induced an increase in primary transcript expression at 10 min and 30 min by 166.9% (P<0.001) and 199.6% (P<0.05), respectively (Figure 3D). At time points \geq 60 min, TGF β reduced the expression of the primary transcript to a maximum of 49.4% (P<0.05) (Figure 3D). As repression on the primary transcript occurred \geq 60 min, Actinomycin D was used to determine the post-transcriptional effects of TGF β upon the mature *AXIN2* transcript. In the presence of TGF β , the half-life of *AXIN2* mRNA decreased from 129 to 27 min across a time period of 30-210 min, which is equal to a 4.7-fold (P<0.01) increase in the rate of mRNA decay (Figure 3E). In the same experimental conditions, SSc fibroblasts showed a basal half-life of 58 min vs 129 min for HC, (2.2-fold faster decay, P<0.05), which was further reduced to 25 min by TGF β stimulation (Figure 3E).

To determine whether these effects were a direct consequence of TGF β receptor activation, the SD-208 T β RI-kinase inhibitor was used to selectively antagonize TGF β signaling. In HC fibroblasts, the TGF β -mediated reduction of *AXIN2* by 64% (P<0.05) was prevented in the presence of SD-208, as expected (Figure 3F). The same effects were observed at protein level (Supplementary Figure 2A), and on *DKK-1* mRNA downregulation and α -SMA upregulation, as expected (Supplementary Figure 2B). More interestingly, a similar pattern was also seen in SSc fibroblasts (Figure 3F). Although *AXIN2* levels in SSc SD208-treated cells were not fully restored to those of HC untreated, we observed a 50% increase in basal *AXIN2* levels (P<0.05) (Figure 3F) confirming that T β RI-kinase endogenous activation is at least in part responsible for the decreased basal levels of *AXIN2* in SSc fibroblasts.

Bioinformatic analysis revealed the presence of several AU-rich elements (ARE) located in the 3'UTR of the *AXIN2* transcript (Supplementary Figure 2C). Recruitment of

TTP-1 to ARE-containing transcripts leads to destabilization and degradation (34-38). Concordantly, siRNA-mediated silencing of *TTP-1* in SSc fibroblasts increased *AXIN2* and *DKK-1* expression relative to non-targeting (SCR) siRNA by 151% ($P<0.05$) and 142% ($P<0.05$) and in HC fibroblasts treated with TGF β by 162.1% ($P<0.01$) and 161.5% ($P<0.01$), respectively (Figure 3G). In line with these data, AXIN2 and DKK-1 protein expression showed a substantial upregulation in siTTP-1 fibroblasts at 48 and 72 hr (Figure 3H).

AXIN2 expression critically regulates the canonical Wnt signaling hyperactivation in TGF β -primed fibroblasts.

TGF β priming reproduced the increased canonical Wnt signaling response in HC fibroblasts at levels equivalent to SSc fibroblasts. Since SSc fibroblasts had a reduction in AXIN2, it was determined whether the TGF β -mediated downregulation of AXIN2 was sufficient to explain this increased activation.

siRNA-mediated silencing of AXIN2 reduced its expression to 50% and similar results were also observed at protein level, at 48 hr and 72 hr, compared to SCR controls (Figure 4A). Wnt-3a stimulation of HC fibroblasts transfected with *AXIN2* siRNA (siAXIN2) showed an increase in TOPFlash activity, which was 5.8-fold higher than Wnt-3a stimulated control cells ($P<0.05$) (Figure 4B).

Following a reciprocal approach, AXIN2 bioavailability was increased by treating fibroblasts with XAV-939, a small molecule inhibitor known to stabilize AXIN by inhibiting its proteasomal degradation (29). As expected, TGF β treated fibroblasts treated with XAV-939 (10.0, 3.0, 1.0 μ M) stabilized AXIN2 in a dose-dependent manner and also prevented the TGF β -induced downregulation of AXIN2 following 48 hr stimulation (Figure 4C, D). Subsequently, the effects of AXIN stabilization on canonical signaling activity were

determined. HC fibroblasts treated with Wnt-3a for 24 hr were used as positive controls of TOPFlash activity (Figure 4E). Co-treatment with incremental concentrations of XAV-939 lead to a dose-dependent suppression of Wnt-3a-induced TOPFlash activation (Figure 4E). Similarly, in TGF β -primed fibroblasts, the Wnt-3a-induced 2.1-fold hyperactivation of TOPFlash activity was completely suppressed in the presence of XAV-939 (Figure 4E).

DISCUSSION

TGF β has an established role in the fibroblast-mediated pathogenesis of tissue fibrosis. Recently, several studies have observed significantly increased Wnt signaling activation in fibroblasts resident in fibrotic tissues, suggesting that the Wnt pathway could be as important as that of TGF β in the pathogenic process (10,11). This study has identified a new mechanism by which TGF β crosstalk mediates hyperactivation of the canonical Wnt/ β -catenin signaling response in fibroblasts, without inducing a direct stimulatory effect.

Although explanted SSc fibroblasts do not display an autonomous increase in canonical Wnt signaling activation, a significant increase in response to canonical agonist treatment is observed when compared to HC. This suggests that canonical signaling hyperactivation *in vivo* is likely to be influenced by the secretion of Wnt agonists from other cell types present in the local microenvironment. Several *in vivo* studies support the role of differential Wnt ligand expression in the increased activation of the canonical Wnt signaling pathway in SSc and models of experimental fibrosis (10,11,30). This study extends these previous observations and indicates the importance of TGF β -responsive AXIN2 expression in the regulation of canonical Wnt signaling pathway hyperactivation.

Consistently, SSc fibroblasts also display a decrease in the basal expression of AXIN2, a critical scaffold protein important in the formation and function of the β -catenin destruction complex (18,19). Supporting the relationship between TGF β signaling and

AXIN2 expression, T β R11 Δ k-fib TG mice, which have constitutively active and fibroblast-specific TGF β signaling, showed reduced Axin2 expression in both fibrotic skin and lung tissue. Indeed, suboptimal β -catenin destruction complex assembly, through the downregulation of the AXIN2 scaffold protein, could explain the increased nuclear β -catenin staining observed in tissue expression studies as well as the amplitude of canonical Wnt signaling evident in SSc fibroblasts (10,11). Complementary to this hypothesis, TGF β induced a quantitatively similar reduction in AXIN2 expression in HC fibroblasts and conferred the enhanced canonical Wnt signaling amplitude observed in SSc fibroblasts. Gene profiling of TGF β responsive genes in dcSSc dermal fibroblasts, limited to an intrinsic SSc gene signature described by Milano et al., showed a significant downregulation of AXIN2 and DKK-1, two important negative regulators of Wnt signaling, despite the overall absence of a distinct Wnt signaling profile (7,32,33). Together these data suggest that a TGF β -primed microenvironment can lead to the increased canonical Wnt/ β -catenin signaling in dermal fibroblasts observed during fibrosis, by its associated repression of key mediators regulating the Wnt signaling pathway. Interestingly, TGF β did not directly induce canonical Wnt activation in dermal fibroblasts and therefore the increased canonical Wnt signaling response *in vivo* is likely to be through secretion of Wnt agonists by other cell types present in the local microenvironment rather than through an autocrine mechanism. These data offer a mechanistic explanation to both our observations on AXIN2 and the data already published by Akhmetshina et al., showing decrease of DKK-1 expression following TGF β stimulation (26). In this sense, TGF β induced mRNA degradation of AXIN2 and DKK-1 can work synergistically in priming and potentiating WNT signaling acting at extracellular level by reduction of a WNT antagonist (reduction in DKK-1) and intercellular level, by decreased activity of GSK complex inhibition.

In this context, our observation of decreased expression of Axin2 in T β RII Δ k mice is rather indicative of a lack of direct Wnt activation in this model and it suggests that the observed increase in Wnt signaling in Scleroderma remains dependent on the presence of Wnt ligands. This is also supported by our *in vitro* data showing lack of TOPFlash activity in cells treated only with TGF β .

Indeed, Wei et al. have observed an increase in Wnt10a ligand in SSc skin and a TGF β independent profibrotic signature in skin from mice with transgenic overexpression of Wnt10a (39). Further studies dissecting the functional effects of physiological or aberrant WNT signaling in this model are warranted to elucidate this cross talk further.

Mechanistically, the TGF β -induced downregulation of AXIN2 in dermal fibroblasts was mediated by an increase in the rate of *AXIN2* mRNA decay, while analysis of the primary transcript indicated involvement in transcriptional repression at later time points. Interestingly, TGF β -mediated repression of AXIN2 in chondrocytes was not similarly affected until 24 hr post-treatment, hence the regulation of AXIN2 by TGF β appears to be context-dependent (25).

The degradation of the majority of eukaryotic mRNAs occurs through poly(A)-tail shortening and is mediated by the recruitment of the RNA-degrading exosome complex (34). TTP-1, a TGF β responsive ARE-binding protein, can facilitate the recruitment of the exosome complex and enhance mRNA decay in transcripts containing 3'UTR ARE motifs, including *TNF*, *c-MYC*, and *CCND1* (34-38). Consistent with the presence of ARE motifs located within the 3'UTR of *AXIN2*, silencing of TTP-1 expression lead to a significant upregulation of both AXIN2 mRNA and protein expression. Likewise, the expression of *DKK-1*, which also harbours 3'UTR ARE motifs, was similarly increased. These data extend upon a previous study linking canonical signaling activation to a reduction in a secreted Wnt signaling regulator, DKK-1, driven by TGF β signaling in lung fibroblasts and the Ad-T β RI model of experimental fibrosis (30). Together, these data suggest that ARE-

mediated mRNA decay of *AXIN2* and *DKK-1*, two regulators of canonical Wnt signaling, might be a common mechanism by which TGF β positively regulates the amplitude of canonical Wnt signaling and contributes to the pathogenic fibrotic response of fibroblasts.

We confirmed our hypothesis with gain and loss of function experiments, where depletion of *AXIN2* in HC fibroblasts increased canonical Wnt signaling and reproduced the SSc fibroblast phenotype. Reciprocally, inhibition of AXIN protein degradation by XAV-939 treatment completely ablated the canonical Wnt signaling hyperactivation conferred by TGF β -priming. *In vivo*, this has proved effective in protecting against experimental fibrosis (29, 40).

Overall, our study highlights the importance of TGF β crosstalk in regulating mediators of the canonical Wnt signaling pathway and shows that it is possible to reproduce the hyper-responsiveness to Wnt stimulation characteristic of SSc fibroblasts through depletion of *AXIN2*. This suggests that the increased canonical Wnt/ β -catenin signaling in fibroblasts, observed during fibrosis, is a consequence of a TGF β -primed microenvironment. This novel mechanism extends our understanding of the processes involved in Wnt/ β -catenin-driven pathology and extends the rationale for developing TGF β targeted treatment to the possibility of regulating the aberrant Wnt activation observed during fibrosis.

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revising it critically for important intellectual content and gave their approval for submission.

REFERENCES

1. Lu P, Takai K, Weaver VM, *et al.* Extracellular Matrix Degradation and Remodeling in Development and Disease. *Cold Spring Harb Perspect Biol* 2011;**3**. doi:10.1101/cshperspect.a005058
2. Cox TR, Eler JT. Remodeling and homeostasis of the extracellular matrix: implications for fibrotic diseases and cancer. *Dis Model Mech* 2011;**4**:165–78. doi:10.1242/dmm.004077
3. Smith EA, LeRoy EC. A possible role for transforming growth factor-beta in systemic sclerosis. *J Invest Dermatol* 1990;**95**:125S – 127S.
4. Leask A, Abraham DJ. TGF- β signalling and the fibrotic response. *FASEB J* 2004;**18**:816–27. doi:10.1096/fj.03-1273rev
5. Varga J, Pasche B. Transforming growth factor-ss as a therapeutic target in systemic sclerosis. *Nat Rev Rheumatol* 2009;**5**:200–6. doi:10.1038/nrrheum.2009.26
6. Sonnylal S, Denton CP, Zheng B, *et al.* Postnatal induction of transforming growth factor beta signalling in fibroblasts of mice recapitulates clinical, histologic, and biochemical features of scleroderma. *Arthritis Rheum* 2007;**56**:334–44. doi:10.1002/art.22328
7. Sargent JL, Milano A, Bhattacharyya S, *et al.* A TGF β -Responsive Gene Signature Is Associated with a Subset of Diffuse Scleroderma with Increased Disease Severity. *J Invest Dermatol* 2009;**130**:694–705. doi:10.1038/jid.2009.318
8. Denton CP, Zheng B, Evans LA, *et al.* Fibroblast-specific Expression of a Kinase-deficient Type II Transforming Growth Factor β (TGF β) Receptor Leads to Paradoxical Activation of TGF β Signalling Pathways with Fibrosis in Transgenic Mice. *J Biol Chem* 2003;**278**:25109–19. doi:10.1074/jbc.M300636200
9. Hoyles RK, Khan K, Shiwen X, *et al.* Fibroblast-specific perturbation of transforming growth factor beta signalling provides insight into potential pathogenic mechanisms of scleroderma-associated lung fibrosis: exaggerated response to alveolar epithelial injury in a novel mouse model. *Arthritis Rheum* 2008;**58**:1175–88. doi:10.1002/art.23379

10. Wei J, Fang F, Lam AP, *et al.* Wnt/ β -catenin signalling is hyperactivated in systemic sclerosis and induces Smad-dependent fibrotic responses in mesenchymal cells. *Arthritis Rheum* 2012;**64**:2734–45. doi:10.1002/art.34424
11. Beyer C, Schramm A, Akhmetshina A, *et al.* β -catenin is a central mediator of pro-fibrotic Wnt signalling in systemic sclerosis. *Ann Rheum Dis* 2012;**71**:761–7. doi:10.1136/annrheumdis-2011-200568
12. Chilosi M, Poletti V, Zamò A, *et al.* Aberrant Wnt/beta-catenin pathway activation in idiopathic pulmonary fibrosis. *Am J Pathol* 2003;**162**:1495–502.
13. Borok Z. Role for alpha3 integrin in EMT and pulmonary fibrosis. *J Clin Invest* 2009;**119**:7–10. doi:10.1172/JCI38084
14. Königshoff M, Kramer M, Balsara N, *et al.* WNT1-inducible signalling protein-1 mediates pulmonary fibrosis in mice and is upregulated in humans with idiopathic pulmonary fibrosis. *J Clin Invest* 2009;**119**:772–87. doi:10.1172/JCI33950
15. Stamos JL, Weis WI. The β -catenin destruction complex. *Cold Spring Harb Perspect Biol* 2013;**5**:a007898. doi:10.1101/cshperspect.a007898
16. Moon RT, Kohn AD, Ferrari GVD, *et al.* WNT and [beta]-catenin signalling: diseases and therapies. *Nat Rev Genet* 2004;**5**:691–701. doi:10.1038/nrg1427
17. Hart MJ, de los Santos R, Albert IN, *et al.* Downregulation of β -catenin by human Axin and its association with the APC tumor suppressor, β -catenin and GSK3 β . *Curr Biol* 1998;**8**:573–81. doi:10.1016/S0960-9822(98)70226-X
18. Liu C, Li Y, Semenov M, *et al.* Control of β -Catenin Phosphorylation/Degradation by a Dual-Kinase Mechanism. *Cell* 2002;**108**:837–47. doi:10.1016/S0092-8674(02)00685-2
19. Chia IV, Costantini F. Mouse axin and axin2/conductin proteins are functionally equivalent in vivo. *Mol Cell Biol* 2005;**25**:4371–6. doi:10.1128/MCB.25.11.4371-4376.2005
20. Jho E, Zhang T, Domon C, *et al.* Wnt/beta-catenin/Tcf signalling induces the transcription of Axin2, a negative regulator of the signalling pathway. *Mol Cell Biol* 2002;**22**:1172–83.
21. Leung JY, Kolligs FT, Wu R, *et al.* Activation of AXIN2 expression by beta-catenin-T cell factor. A feedback repressor pathway regulating Wnt signalling. *J Biol Chem* 2002;**277**:21657–65. doi:10.1074/jbc.M200139200
22. Perry-III WL, Vasicek TJ, Lee JJ, *et al.* Phenotypic and Molecular Analysis of a Transgenic Insertional Allele of the Mouse Fused Locus. *Genetics* 1995;**141**:321–32.

23. Behrens J, Jerchow BA, Würtele M, *et al.* Functional interaction of an axin homolog, conductin, with beta-catenin, APC, and GSK3beta. *Science* 1998;**280**:596–9.
24. Lammi L, Arte S, Somer M, *et al.* Mutations in AXIN2 cause familial tooth agenesis and predispose to colorectal cancer. *Am J Hum Genet* 2004;**74**:1043–50. doi:10.1086/386293
25. Dao DY, Yang X, Chen D, *et al.* Axin1 and Axin2 Are Regulated by TGF- β and Mediate Cross-talk between TGF- β and Wnt Signalling Pathways. *Ann N Y Acad Sci* 2007;**1116**:82–99. doi:10.1196/annals.1402.082
26. Akhmetshina A, Palumbo K, Dees C, *et al.* Activation of canonical Wnt signalling is required for TGF- β -mediated fibrosis. *Nat Commun* 2012;**3**:735. doi:10.1038/ncomms1734
27. LeRoy EC, Medsger TA. Criteria for the classification of early systemic sclerosis. *J Rheumatol* 2001;**28**:1573–6.
28. Derrett-Smith E, Dooley A, Khan, K, Shi-wen X, *et al.* Systemic vasculopathy with altered vasoreactivity in a transgenic mouse model of scleroderma. *Arthritis Res Ther.* 2010;**12**(2):R69.
29. Huang S-MA, Mishina YM, Liu S, *et al.* Tankyrase inhibition stabilizes axin and antagonizes Wnt signalling. *Nature* 2009;**461**:614–20. doi:10.1038/nature08356
30. Bayle J, Fitch J, Jacobsen K, *et al.* Increased expression of Wnt2 and SFRP4 in Tsk mouse skin: role of Wnt signalling in altered dermal fibrillin deposition and systemic sclerosis. *J Invest Dermatol* 2008;**128**:871–81. doi:10.1038/sj.jid.5701101
31. Denton CP, Zheng B, Evans LA, *et al.* Fibroblast-specific Expression of a Kinase-deficient Type II Transforming Growth Factor (TGF) Receptor Leads to Paradoxical Activation of TGF Signalling Pathways with Fibrosis in Transgenic Mice. *J Biol Chem* 2003;**278**:25109–19. doi:10.1074/jbc.M300636200
32. Bayle J, Fitch J, Jacobsen K, *et al.* Increased Expression of Wnt2 and SFRP4 in Tsk Mouse Skin: Role of Wnt Signalling in Altered Dermal Fibrillin Deposition and Systemic Sclerosis. *J Invest Dermatol* 2007;**128**:871–81.
33. Milano A, Pendergrass SA, Sargent JL, *et al.* Molecular subsets in the gene expression signatures of scleroderma skin. *PloS One* 2008;**3**:e2696. doi:10.1371/journal.pone.0002696
34. Houseley J, Tollervey D. The Many Pathways of RNA Degradation. *Cell* 2009;**136**:763–76. doi:10.1016/j.cell.2009.01.019

35. Ogawa K, Chen F, Kim Y-J, *et al.* Transcriptional Regulation of Tristetraprolin by Transforming Growth Factor- in Human T Cells. *J Biol Chem* 2003;**278**:30373–81. doi:10.1074/jbc.M304856200
36. Marderosian M, Sharma A, Funk AP, *et al.* Tristetraprolin regulates Cyclin D1 and c-Myc mRNA stability in response to rapamycin in an Akt-dependent manner via p38 MAPK signalling. *Oncogene* 2006;**25**:6277–90. doi:10.1038/sj.onc.1209645
37. Kang S, Min A, Im S-A, *et al.* TGF- β Suppresses COX-2 Expression by Tristetraprolin-Mediated RNA Destabilization in A549 Human Lung Cancer Cells. *Cancer Res Treat* 2014;**47**:101–9. doi:10.4143/crt.2013.192
38. Blanco FF, Sanduja S, Deane NG, *et al.* Transforming Growth Factor Regulates P-Body Formation through Induction of the mRNA Decay Factor Tristetraprolin. *Mol Cell Biol* 2014;**34**:180–95. doi:10.1128/MCB.01020-13
39. Wei J, Melichian D, Komura K, *et al.* Canonical Wnt Signaling Induces Skin Fibrosis and Subcutaneous Lipotrophy: A Novel Mouse Model for Scleroderma? *Arthritis Rheum.* 2011;**63**(6):1707-17.
40. Beyer C, Reichert H, Akan H, *et al.* Blockade of canonical Wnt signalling ameliorates experimental dermal fibrosis. *Ann Rheum Dis* 2013;**72**:1255–8. doi:10.1136/annrheumdis-2012-202544

Figure 1. SSc fibroblasts have an increased response to canonical Wnt signaling and decreased AXIN2 expression. A) β -catenin-dependent TOPFlash (TCF/LEF) reporter activity was determined in HC and SSc dermal fibroblasts 24 hr after treatment with rhWnt-3a (100 ng/ml) B-C) mRNA expression levels of the canonical Wnt-target *AXIN2*, in HC and SSc dermal fibroblasts 24 hr after treatment with rhWnt-3a (100 ng/ml) (B) or TGF β (5 ng/ml) (C) were quantified by qRT-PCR. Data were normalized to ribosomal 18S RNA and values displayed relative to untreated HC. D) AXIN2 protein expression was determined by Western blot under the same conditions as in figure 1C. Data are shown as mean \pm SEM, n=5; Mann-Whitney test **P<0.01, ****P<0.001.

Figure 2. Constitutive TGF β activation leads to tissue fibrosis and reduced Axin2 expression in the T β RII Δ k-fib mouse model of SSc. A) Dermal fibrosis was evaluated in age-matched WT (left panels) and T β RII Δ k-fib TG mice (right panels) by Masson's trichrome staining (blue staining). B-D) Immunohistochemistry for Axin2 expression (brown staining) in skin (B, C) and lung (D) sections. Insets in B are magnified in C. Sections stained in B-D are matched from mice in A. No staining was detected with an isotype control antibody (data not shown). Scale bars = 100 μ M. Images are representative of 5 independent experiments.

Figure 3. TGF β priming of fibroblasts reproduces the increased canonical Wnt signaling amplitude observed in SSc fibroblasts through post-transcriptional regulation of AXIN2 expression. A) HC fibroblasts incubated in media alone or primed for 24 hr with TGF β (5 ng/ml) prior to sequential Wnt-3a treatment were assayed for TOPFlash activity. B and C) Kinetic analysis of the effect of TGF β treatment on AXIN2 determined by qRT-PCR and Western blot. D) *AXIN2* primary transcript expression, using primers spanning an intron-exon boundary, determined by qRT-PCR. E) Analysis of *AXIN2* stability in HC and SSc fibroblasts following treatment with TGF β and transcriptional inhibitor Actinomycin D (5 μ g/ml), quantified by qRT-PCR and compared to control. F) Evaluation of 48 hr SD-208-mediated T β RI blockade (1 μ M) on *AXIN2*, *DKK-1* and α -*SMA* mRNA by qRT-PCR in HC and SSc fibroblasts. All data is shown relative to HC with no stimulation (100%). G) Effect of TTP-1 knockdown on the TGF β -mediated expression of *AXIN2* and *DKK-1* in HC (with TGF β) and SSc fibroblasts analysed by qRT-PCR. H) Effect of TTP-1 knockdown on the TGF β -mediated expression of *AXIN2*, *DKK-1* and α -*SMA* in HC (with TGF β) quantified by Western blot. Data shown as mean \pm SEM, n=3-8; Mann-Whitney test *P=0.05, **P<0.01, ***P<0.001.

Figure 4. Silencing AXIN2 expression is sufficient to reproduce the canonical Wnt signaling amplitude observed in SSc and TGF β -primed fibroblasts. A) HC fibroblasts were transfected with scrambled (SCR) or AXIN2-specific siRNA (siAXIN2). *AXIN2* mRNA expression

at 24 hr and protein expression at 48-72 hr were quantified by qRT-PCR and Western blot, respectively. B) HC fibroblasts were transfected with the TOPFlash reporter 24 hr prior to siAXIN2 and SCR transfection for 48 hr. Subsequently, cells were treated with or without Wnt-3a for a further 24 hr prior to determination of reporter activity. TOPFlash activity of Wnt3-a-stimulated SCR cells was set to 1. C) HC Fibroblasts were treated with AXIN-stabilizing XAV-939 (1 μ M) for 24 hr and expression of AXIN2 determined by Western blot. D) XAV-939-mediated AXIN2 stability was validated in the presence of TGF β for 24 hr, 1 hr post-XAV-939 treatment, determined by Western blot. E) Fibroblasts were transfected with the TOPFlash reporter for 24 hr, XAV-939 (10, 3, 1 μ M) was added 1 hr prior to treatment with or without TGF β for 24 hr. Fibroblasts were then treated for a further 24 hr with Wnt-3a and luciferase reporter activity assayed. Data shown as mean \pm SEM, n=4-5; Mann-Whitney test *P<0.05, ****P<0.001.