TGFβ activation primes canonical Wnt signaling through the downregulation of AXIN2.

Gillespie J, Ross RL, Corinaldesi C, Esteves F, Derrett-Smith E, McDermott MF, Doody GM, Denton CP, Emery P, Del Galdo F [1]Leeds Institute of Rheumatic and Musculoskeletal Medicine, University of Leeds, Leeds, United Kingdom. [2]NIHR Leeds Musculoskeletal Biomedical Research Unit, Leeds Teaching Hospitals NHS Trust, Leeds, United Kingdom. [3]Centre for Rheumatology and Connective Tissue Diseases, UCL Medical School Royal Free Campus, London, United Kingdom.

Abstract

The activation of the TGF-β and Wnt/β-catenin signaling pathways has been linked to the pathogenesis of tissue fibrosis in systemic sclerosis (SSc) as well as other fibrotic conditions. However, the level of crosstalk between the two pathways is still to be determined. Here we show that dermal fibroblasts from SSc display normal basal Wnt activity and an increased responsiveness to canonical Wnt activation compared to healthy controls (HC). Consistent with these findings, the basal expression of Axin-2, a primary target and regulator of the Wnt signaling pathway was reduced in SSc fibroblasts. TGF-β induced a quantitatively similar *Axin-2* downregulation in HC fibroblasts, which was dependent on TGF-β receptor activation and mediated by increased RNA decay. Furthermore, TβRIIΔ-K transgenic mice, which have a fibroblast specific activation of TGF-β signaling, showed a decreased basal expression of Axin-2 *in-vivo*. Functional studies demonstrated that

TGF-β stimulation enhanced the canonical Wnt response in HC fibroblasts, making it similar to SSc fibroblasts. Mechanistically, gain or loss of Axin-2 bioavailability respectively suppressed or mimicked the effects of TGF-β on canonical Wnt signaling. These studies demonstrate for the first time that activation of the TGF-β pathway sensitizes fibroblasts to canonical Wnt signaling through Axin-2 depletion, and suggest that the increased Wnt/β-catenin signaling during fibrosis can be a consequence of TGF- β activation.

Introduction

Tissue fibroblasts are the key cellular elements of fibrosis, being primarily involved in the turnover of extracellular matrix (ECM) (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 (3, 4, 5, 6). Indeed, TGF-β treated fibroblasts display a gene expression profile similar to the one of fibroblasts from SSc patients (7). Consequently, TGF-β activation has been used to investigate the pathogenesis of tissue fibrosis both *in-vitro* and *in-vivo* (8) (Chris ref).

The TβRIIΔk transgenic mouse model is characterized by a constitutive TGF-β signaling through the fibroblast-specific expression of a kinase deficient TGFβ Receptor II gene, targeted using the specific pro-alpha2(I) collagen (*Col1a2*) gene enhancer and promoter (8). These mice have been shown to develop dermal and pulmonary fibrosis and have shown increased fibroblast activation, myofibroblast number and ECM production (8, 9).

Several studies have shown that Wnt signaling is activated during fibrosis in SSc (10, 11) and other fibrotic (12–14) conditions . Nevertheless, the nature of this activation is yet to be identified and the relative cross talk with TGF-β pathway activation is unclear. Canonical Wnt signaling is mediated by the recruitment of the β-catenin destruction complex to the plasma membrane, which lowers its capacity to induce β-catenin proteasomal degradation (17). As a result, the levels of free cytosolic β-Catenin are increased and can undergo nuclear translocation, leading to the upregulation of Wnt target genes though the activation of LEF/TCF transcription factors.

Axin has been shown to be a critical regulator of Wnt/β-Catenin signaling acting as an essential scaffold protein for the β-catenin destruction complex (18, 19, 20, 21). The two mammalian Axin genes, Axin-1 and Axin-2, are functionally equivalent and both act to negatively regulate Wnt signalling (22). Axin-1 is constitutively expressed, while Axin-2 (also called Conductin or Axil) is a direct transcriptional target of the Wnt pathway, containing TCF binding sites in the promoter and acting as part of a negative feedback system to control Wnt signaling activation (23, 24, 25).

Herein we studied the level of Wnt activation in basal conditions and following ligand engagement in normal and SSc dermal fibroblasts and determined whether TGF-β pathway activation could play a role in Wnt signaling.

Results and Discussion

Recent independent studies have shown increased nuclear β-Catenin localization in fibroblasts resident in SSc skin biopsies in contrast to healthy control tissues [10, 11]. To determine whether explanted fibroblasts from SSc patients displayed an autocrine activation of the Wnt pathway, we analyzed TOPFlash reporter activity, a measure of β-catenin-mediated TCF/LEF transcription. Compared to HC, we observed no increase in the basal level of TOPFlash activity, suggesting that the increased β-catenin nuclear localization observed in tissue fibroblasts *in-vivo* was not a consequence of an increased autocrine or epigenetically stable canonical Wnt activity (Figure 1A). However, treatment of fibroblasts with the canonical Wnt-3a ligand induced an 11.6 fold increase in TOPFlash activity in SSc fibroblasts, significantly higher than the 4.8 fold increase observed in HC fibroblasts ($P<0.01$) (Figure 1A). Consistent with these findings, Wnt-3a-induced *Axin-2* expression by 5.0 fold in SSc fibroblats vs. 1.8 fold in HC fibroblasts ($P<0.001$) . Interestingly, we found that basal *Axin-2* expression was reduced by 52% in SSc fibroblasts (Figure 1B).

These data suggested that SSc fibroblasts have a significantly increased response to canonical Wnt signaling activation. In addition, while Axin-2 is a transcriptional target of Wnt signaling, it also plays an essential role in regulating the activity of the Wnt pathway by recruiting β-catenin and the multi-protein complex that facilitates β-catenin proteosomal degradation. Therefore, we hypothesized that the increased response to canonical activity in SSc fibroblasts could be a result of the decreased basal expression of Axin-2.

As there is strong evidence of a TGF-β-regulated gene expression signature within dcSSc biopsies and in tissue fibroblasts (8, 26), we set out to evaluate the functional effect of TGF-β activity upon Axin-2 expression and consequently Wnt signaling. Stimulation of HC fibroblasts with TGF-β for 24 hours reduced the expression of Axin-2 by 51% ($P<0.001$), which was analogous to the basal levels of expression observed in SSc fibroblasts (52% of HC levels, Figure 1C). Additionally, treatment of SSc fibroblasts with recombinant TGF-β further reduced Axin-2 expression to 20% of HC levels ($P<0.01$). Also at protein level, the amount of Axin-2 expression following TGF- β stimulation was comparable to the amount observed in SSc fibroblasts (Figure 1D).

To validate the effects of TGF-β on Axin-2 expression *in-vivo,* we utilized the TβRIIΔk mouse model. In these mice, a kinase-deficient TβRII gene is selectively expressed in fibroblasts directed by pro-alpha2 collagen promoter and enhancer elements (Figure 1E). The kinase deficient TβRII, once expressed, is responsible for a ligand independent activation of TGF-β which in turn recapitulates the tissue fibrosis characteristic of SSc (8). Twelve weeks old mice, as previously observed, developed a significant increase in dermal thickness, increased collagen deposition and a loss of subcutaneous fat, all indicative of tissue fibrosis (Figure 1F-G). In the same tissue biopsies, immunohistochemistry analysis of dermal Axin-2 expression showed a reduction of the number of Axin-2 positive fibroblasts and therefore decreased overall protein expression (Figure 1H). These data clearly supported our in-vitro findings and further confirmed the functional role of TGF-β signaling on Axin-2 expression.

To determine whether the TGF-β mediated decrease in Axin-2 expression could be responsible for the increase in sensitivity to Wnt canonical ligands, we performed sequential stimulation experiments in which normal fibroblasts were incubated with rhTGF-β for 24hrs and subsequently treated with rhWnt-3a for 24hrs. TGF-β-primed fibroblasts showed an increase in TOPFlash activity 4.0 fold higher than the one observed in fibroblasts treated with Wnt-3a alone ($P<0.005$) (Figure 2A). Importantly, fibroblasts treated with TGF-β alone showed no activation of TOPFlash activity, consistent with what we have observed in SSc fibroblasts. These data indicate that TGF-β does not directly induce canonical Wnt signaling in fibroblasts, however 2 hours TGF-β stimulation was sufficient to induce an enhanced response to canonical Wnt activation similar to the one observed in SSc fibroblasts (Figure s1).

Next, we set out to determine the mechanism by which TGF-β regulates Axin-2 expression in fibroblasts. TGF-β treatment induced a 48% downregulation of *Axin-2* expression ($P<0.01$) within 30mins from activation of the receptor (Figure 2B). This rapid downregulation suggested that the expression of Axin-2 could be modulated at post-transcriptional level. Indeed, actinomycin D experiments showed that the half-life of Axin-2 mRNA was 109 min under control conditions and this was decreased to 26min in the presence TGF-β, which equates to a 419% increase in the rate of mRNA decay (Figure 2C) ($p<0.01$). At protein level, we observed that Axin-2 had a half-life shorter than 2 hours, as determined by cyclohexamide protein stability experiments (Figure 2D). These data indicated that Axin-2 is dynamically regulated and support its role as a rate-limiting factor in Wnt signaling

activation(27),(28). TGF-β treatment suppressed Axin-2 protein expression as soon as 2 hours and by 6 hours the suppression of Axin-2 bioavailability was complete, and comparable with the one induced at 2 hours by cyclohexamide (Figure 2D-E). Further, the suppressive effect of TGF-β on *Axin-2* persisted over a 72hr period at both mRNA (Figure 2F) and protein (Figure 2G) level. To determine whether these effects were a direct effect of TGF-β receptor activation we performed the same experiments in the presence of the TβRI-kinase inhibitor SD-208, which has been shown to selectively antagonize TβRI-induced signaling. In the absence of TβRI activation, TGF-β was unable to suppress Axin-2 expression at both RNA and protein level (Figure 2H-I).

Since activation of the TGF-β pathway has a multitude of intracellular targets, we evaluated the role of TGF-β-induced Axin-2 downregulation upon Wnt signaling by performing gain and loss of function experiments on human dermal fibroblasts. As shown in Figure 3A, transfection of Axin-2 siRNA reduced mRNA expression to 50% compared to scrambled siRNA controls and similar results were also observed at protein level at 72hrs (Figure 3B). Fibroblasts treated with Axin2 siRNA showed an increase in TOPFlash activity, following Wnt-3a stimulation, which was 5.8 fold higher than Wnt3a stimulated control cells ($P<0.05$) (Figure 3C). Hence, the depletion of Axin-2 recapitulated the increased canonical response to Wnt mediated by TGF-β priming.

Following a complementary approach, we set out to increase the bioavailability of Axin-2 by treating dermal fibroblasts with a small molecule inhibitor XAV939, which

has been shown to stabilize Axin protein by protecting it from proteasomal degradation (29).

As expected, XAV939 stabilized Axin-2 in a dose-dependent manner (Figure 3D) and also prevented the TGF-β-induced downregulation of Axin-2 following 72 hour stimulation (Figure 3E). Indeed, these effects were apparent as early as 24 hours and persisted up to 72hrs (Figure s2).

In the presence of XAV939, TGF-β primed fibroblasts showed an increase in TOPFlash activity following Wnt-3a stimulation equivalent to fibroblasts treated with Wnt-3a alone ($P<0.01$) (Figure 3F). These data clearly indicated that the effects of TGF-β on Wnt canonical signaling were mediated by a decreased Axin-2 bioavailability. Altogether, these gain and loss of function studies suggest that Axin-2 downregulation is both sufficient and necessary for the TGF-β-induced enhancement of the fibroblast response to canonical Wnt signaling activation.

In conclusion, we identify a new mechanism by which TGF-β primes dermal fibroblasts to increase their responsiveness to canonical Wnt activation without inducing a direct stimulatory effect. Further, we demonstrate that the Wnt priming is caused by the downregulation of Axin-2, a negative regulator of canonical Wnt signaling. Therefore targeting of TGF-β pathway may be of therapeutic benefit in resolving the aberrant Wnt/ β-catenin signaling observed during fibrosis.

Methods

Detailed methods are described in the supplemental file. Briefly, dermal fibroblasts from 3 early diffuse cutaneous dcSSC patients and 5 healthy controls (HC) were

stimulated with recombinant human (rh)TGF-β (5ng/ml) and/or rhWnt-3a (100ng/ml) (SIGMA). TGF-β signaling was inhibited using SD-208 (1μM) (Sigma Aldrich, UK) mRNA levels were quantified by SYBRGreen (life Technologies, UK) qRT-PCR and protein expression was measured by western blotting or immunohistochemistry.

mRNA stability was investigated using actimomycin D (ug/ml), protein half-life with cyclohexamide (ug/ml). For the gain and loss of function studies, Axin-2 protein expression was stabilized using XAV939 (1uM) (Calbiochem, UK) and commercially validated Silencer Select siRNAs (Life Technologies) were transfected using Qiafect transfection reagent (Qiagen, UK). Both TOPFlash (TCF/LEF) luciferase (30) and Renilla firefly luciferase reporters were transfected with lipofectamine (life Technologies). SSc data were normalized to HC untreated samples for comparison. *In-vivo* expression studies were performed on TβRIIΔk transgenic mice.

Study approval

Informed consent was obtained from all patients, and studies were granted approval by the Leeds Teaching Hospitals NHS Trust Medical Ethics Committee. All scleroderma patients fulfilled the American College of Rheumatology (ACR) classification criteria for SSc, classified as dcSSc according to skin involvement evaluated by the modified Rodnan total skin score (mRSS).

Statistical analysis

Statistical analyses were performed, using nonparametric Mann-Whitney U tests for unpaired samples. Experimental data are presented as the mean ± SEM. A P-value

<0.05 was considered statistically significant. Statistical analysis was performed using GraphPad Prism software, version 5.0 (San Diego, CA, USA).

Conflict of interest:

Acknowledgments

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Address correspondence to: Dr Francesco Del Galdo, Institute of Rheumatic and Musculoskeletal Medicine, and Leeds Musculoskeletal Biomedical Research Unit, University of Leeds, Leeds lS9 7TF, UK.

References

1. Lu P, Takai K, Weaver VM, Werb Z. Extracellular Matrix Degradation and Remodeling in Development and Disease [Internet]. *Cold Spring Harb Perspect Biol* 2011;3(12). doi:10.1101/cshperspect.a005058

2. Cox TR, Erler JT. Remodeling and homeostasis of the extracellular matrix: implications for fibrotic diseases and cancer. *Dis Model Mech* 2011;4(2):165–178.

3. Smith EA, LeRoy EC. A possible role for transforming growth factor-beta in systemic sclerosis. *J. Invest. Dermatol.* 1990;95(6 Suppl):125S–127S.

4. Leask A, Abraham DJ. TGF-β signaling and the fibrotic response. *FASEB J* 2004;18(7):816–827.

5. Wynn T. Cellular and molecular mechanisms of fibrosis. *J Pathol* 2008;214(2):199–210.

6. Varga J, Pasche B. Transforming growth factor-ss as a therapeutic target in systemic sclerosis. *Nat Rev Rheumatol* 2009;5(4):200–206.

7. Sargent JL et al. A TGFβ-Responsive Gene Signature Is Associated with a Subset of Diffuse Scleroderma with Increased Disease Severity. *Journal of Investigative Dermatology* 2009;130(3):694–705.

8. Denton CP et al. Fibroblast-specific Expression of a Kinase-deficient Type II Transforming Growth Factor β (TGFβ) Receptor Leads to Paradoxical Activation of TGFβ Signaling Pathways with Fibrosis in Transgenic Mice. *J. Biol. Chem.* 2003;278(27):25109–25119.

9. Sonnylal S et al. Postnatal induction of transforming growth factor beta signaling in fibroblasts of mice recapitulates clinical, histologic, and biochemical features of scleroderma. *Arthritis Rheum.* 2007;56(1):334–344.

10. Wei J et al. Wnt/β-catenin signaling is hyperactivated in systemic sclerosis and induces Smad-dependent fibrotic responses in mesenchymal cells. *Arthritis Rheum.* 2012;64(8):2734–2745.

11. Beyer C et al. β-catenin is a central mediator of pro-fibrotic Wnt signaling in systemic sclerosis. *Ann Rheum Dis* 2012;71(5):761–767.

12. Chilosi M et al. Aberrant Wnt/beta-catenin pathway activation in idiopathic pulmonary fibrosis. *Am. J. Pathol.* 2003;162(5):1495–1502.

13. Borok Z. Role for alpha3 integrin in EMT and pulmonary fibrosis. *J. Clin. Invest.* 2009;119(1):7–10.

14. Königshoff M et al. WNT1-inducible signaling protein-1 mediates pulmonary fibrosis in mice and is upregulated in humans with idiopathic pulmonary fibrosis. *J. Clin. Invest.* 2009;119(4):772–787.

15. Karagiannis GS et al. Cancer-Associated Fibroblasts Drive the Progression of Metastasis through both Paracrine and Mechanical Pressure on Cancer Tissue. *Mol Cancer Res* 2012;10(11):1403–1418.

16. Anastas JN, Moon RT. WNT signalling pathways as therapeutic targets in cancer. *Nat Rev Cancer* 2013;13(1):11–26.

17. Moon RT, Kohn AD, Ferrari GVD, Kaykas A. WNT and [beta]-catenin signalling: diseases and therapies. *Nat Rev Genet* 2004;5(9):691–701.

18. Hart MJ, de los Santos R, Albert IN, Rubinfeld B, Polakis P. Downregulation of β-catenin by human Axin and its association with the APC tumor suppressor, β-catenin and GSK3β. *Current Biology* 1998;8(10):573–581.

19. Salic A, Lee E, Mayer L, Kirschner MW. Control of β-Catenin Stability: Reconstitution of the Cytoplasmic Steps of the Wnt Pathway in Xenopus Egg Extracts. *Molecular Cell* 2000;5(3):523–532.

20. Liu C et al. Control of β-Catenin Phosphorylation/Degradation by a Dual-Kinase Mechanism. *Cell* 2002;108(6):837–847.

21. Sakanaka C, Weiss JB, Williams LT. Bridging of β-catenin and glycogen synthase kinase-3β by Axin and inhibition of β-catenin-mediated transcription. *PNAS* 1998;95(6):3020–3023.

22. Chia IV, Costantini F. Mouse axin and axin2/conductin proteins are functionally equivalent in vivo. *Mol. Cell. Biol.* 2005;25(11):4371–4376.

23. Jho E et al. Wnt/beta-catenin/Tcf signaling induces the transcription of Axin2, a negative regulator of the signaling pathway. *Mol. Cell. Biol.* 2002;22(4):1172–1183.

24. Mutational spectrum of β-catenin, AXIN1, and AXIN2 in hepatocellular carcinomas and hepatoblastomas [Internet]. , *Published online: 09 July 2002; | doi:10.1038/sj.onc.1205591* 2002;21(31). doi:10.1038/sj.onc.1205591

25. Leung JY et al. Activation of AXIN2 expression by beta-catenin-T cell factor. A feedback repressor pathway regulating Wnt signaling. *J. Biol. Chem.* 2002;277(24):21657–21665.

26. Sargent JL et al. A TGFβ-Responsive Gene Signature Is Associated with a Subset of Diffuse Scleroderma with Increased Disease Severity. *J Invest Dermatol* 2009;130(3):694–705.

27. Tan CW et al. Wnt Signalling Pathway Parameters for Mammalian Cells. *PLoS ONE* 2012;7(2):e31882.

28. Krüger R, Heinrich R. Model reduction and analysis of robustness for the Wnt/beta-catenin signal transduction pathway. *Genome Inform* 2004;15(1):138–148.

29. Huang S-MA et al. Tankyrase inhibition stabilizes axin and antagonizes Wnt signalling. *Nature* 2009;461(7264):614–620.

30. Veeman MT, Slusarski DC, Kaykas A, Louie SH, Moon RT. Zebrafish prickle, a modulator of noncanonical Wnt/Fz signaling, regulates gastrulation movements. *Curr. Biol.* 2003;13(8):680–685.

Fig 1. SSc fibroblasts have an increased response to canonical Wnt signaling and low Axin-2 expression. A) Human dermal fibroblasts from HC and SSc patients were transfected with TOPFlash (TCF/LEF) reporter (1ug) and treated with rhWnt-3a (100ng/ml) for 24hrsTOPFlash activity of HC fibroblast was set to one, all other values were calculated as multiple thereof B) qRT-PCR data from the same experimental conditions in A. Bars represent mean of three different experiments, mRNA levels were normalised for Ribosomal RNA input . Axin levels of HC fibroblasts in basal condition were normalised to 100 , all other values expressed as multiple thereof. C) HC and SSc fibroblasts

were stimluated with TGF-β (5ng/ml) for 24hrs and assayed for Axin-2 mRNA expression by RT-PCR as in B). D) Western Blots for Axin-2 a-SMA as positive control and GAPDH as housekeeping in unstimulated SSc fibroblasts and in HC fibroblasts stimulated or not with TGF-β for 72hrs. E) Schematic representation of the TβRII kinase-deficient (TβRIIΔk) transgene construct, regulated by a fragment of the *COL1A2* enhancer (–19.5 to –13.5 kb) fused with a minimal *COL1A2* promoter that also directs expression to the fibroblast lineage. F-G) Dermal fibrosis resulting from constitutively active TβRII within fibroblasts. Biopsies were taken from age-matched TG and WT littermates. Skin sections were stained with Masson's trichrome, isotype control. H) Immunohistochemistry for Axin-2 in the same skin biopsies from F and G. Axin-2 positive cells stained in Brown (red arrow heads). Original Magnification 400X.

Fig 2. TGF-β-mediated downregulation of Axin-2 primes Wnt signaling in fibroblasts. A) HC fibroblasts were transfected with the TOPFlash reporter, then they were treated with either Wnt-3a or TGF-β for 24 h, or pretreatef with TGF-b for 72hrs and then Wnt-3a for 24hrs before reporter activity was evaluated. TOPFlash activity of WNT3a stimulated cells was set to 1 and all other values calculated as multiple thereof B) q-RT PCR fro Axin2 in HC fibroblasts treated with TGF-β for 30-120min. Axin-2 levels, normalised by ribosomial RNA in basal condition were set to 100 , all other values were expressed as multiple thereof. C) HC Fibroblasts were treated or not with TGF-β for 10-120min in the presence of actinomycin D (100ug/ml) . Axin-2 mRNA was quantified by qRT-PCR. Values and error bars represent mean and standard error of three different experiments. D) HC fibroblasts were treated with TGF-β for 2-6hr and protein lysates were assayed by western blot for Axin-2 and GAPDH. E) Cycloheximide (100ug/ml) was added to HC fibroblasts for 2-6hrs, protein lysates were extracted and assayed by western blot. F) Fibroblasts were treated with TGF-β for 24-72hr and mRNA quantified by RT-PCR. G) Western Blots for Axin-1, Axin 2 and GAPDH in HC fibrobalsts following TGF-β treatment for 48-72hrs. H-I) HC Fibroblasts were treated with SD-208 (1uM) for 1hr prior to 24hrs TGF-β treatment. mRNA levels of Axin-2 and SMA were quantified by rt-

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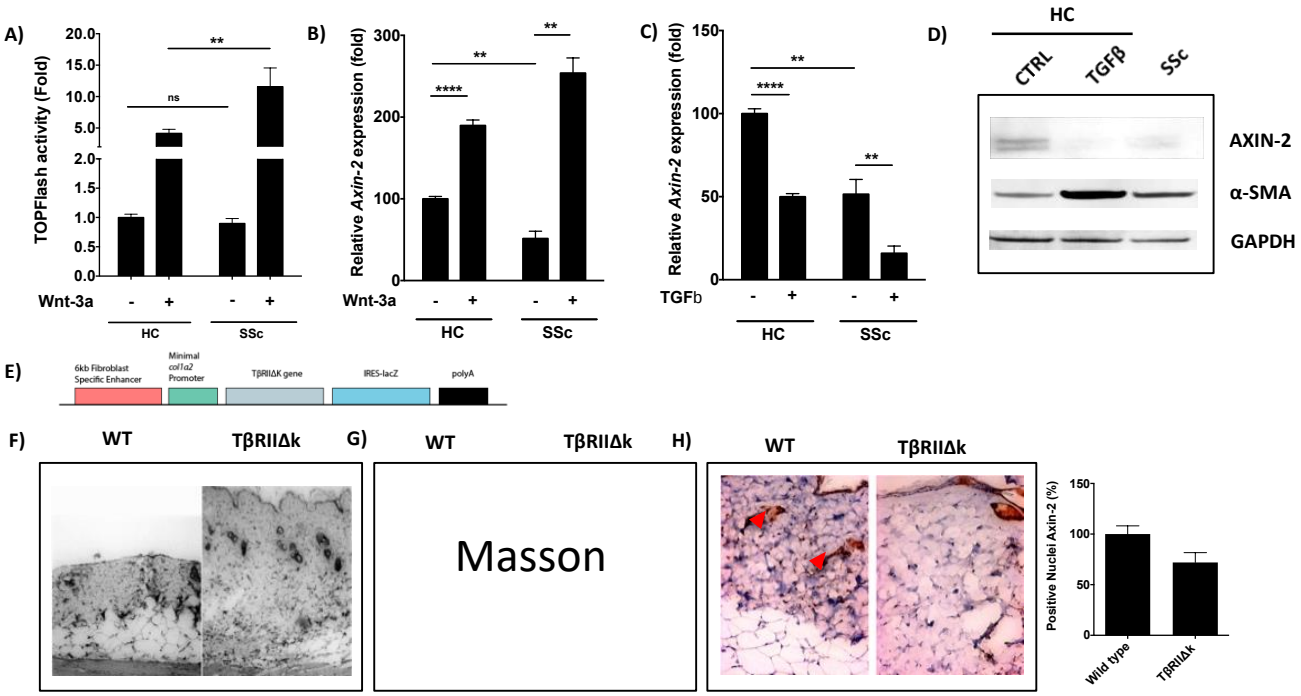
Fig 3. Axin-2 downregulation is sufficient and necessary to prime Wnt signaling following TGF-β stimulation. A-B) HC fibroblasts were transfected with scrabbled (Scr) or Axin-2 specific siRNA. mRNA levels for axin-2 were quantified by RT-PCR at 24 hrs (A). B) Western Blots for Axin-1 Axin-2 and GAPDH following 72hrs siRNA treatment. C) Fibroblasts were transfected with TOPFlash reporter 24hrs prior to transfection with Axin-2 and Scr siRNA for 72hrs. Subsequently, cells were treated with or without Wnt-3a for a further 24hrs and reporter activity was measured. TOPFlash activity of Scrabbled cells wtimluated with Wnt3-a was set to one, other values were calculated as multiple thereof. D-E) HC Fibroblasts were treated with XAV939 (1uM) 1hr before being stimulated or not with TGF-β for 24hrs. Lysates were prepared and assayed by western blot. E) Fibroblasts were transfected with the TOPFlash reporter for 24hrs. Then XAV939 was added 1hr before treatment with or without TGF-β for 72Hrs.. Fibroblasts were then treated for a further 24hrs with Wnt-3a before TOPFlash Reporter activity was evaluated.



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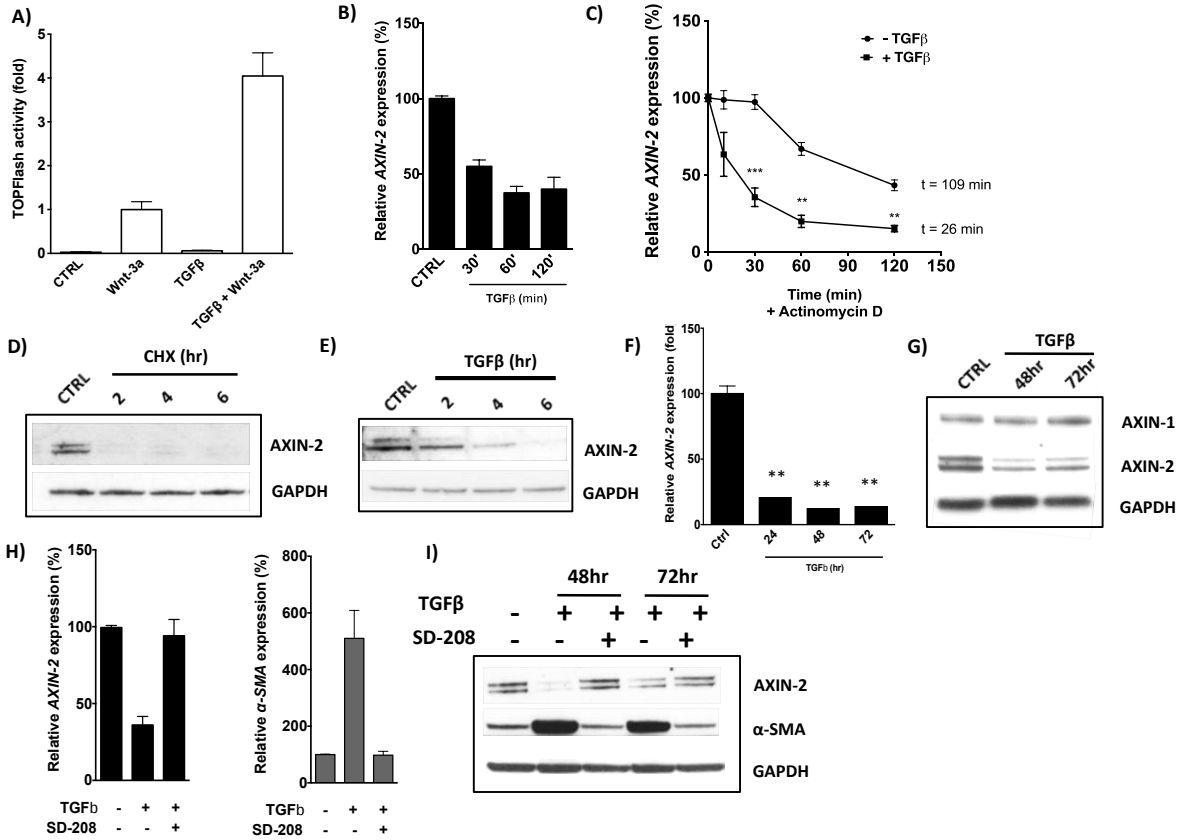

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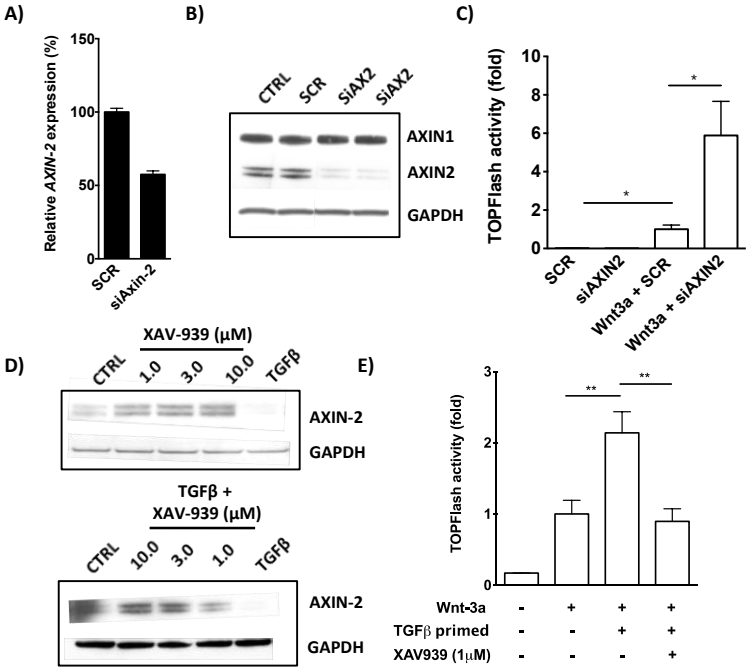

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