

PATHOBIOLOGY IN FOCUS

Canonical Wnt signaling in systemic sclerosis

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Fibroblastic disorders are characterized by abundant accumulation of extracellular matrix proteins such as collagen in a variety of organs, which results in structural changes and dysfunction of the affected organ. Thus fibrotic diseases are characterized by a high morbidity and mortality and also lead to major socioeconomic costs. Systemic sclerosis (SSc) is a prototypic multi-systemic fibroblastic disease, which affects the skin and a variety of internal organs, including the lungs, heart and gastrointestinal tract. Targeted antifibrotic therapies are not yet available for clinical use in SSc. In recent years, canonical Wnt signaling has been profoundly characterized as an important mediator of sustained fibroblast activation in fibrotic diseases. In the present review, we will summarize current research on the canonical Wnt signaling pathway in SSc and discuss translational implications and potential limitations of prolonged Wnt inhibition.

Laboratory Investigation (2016) **96**, 151–155; doi:10.1038/labinvest.2015.154; published online 11 January 2016

Fibroblastic disorders account for up to 45% of deaths in Europe and Northern America. Moreover, fibrotic tissue responses cause major socio-economic costs that are estimated to be 10 billions of dollars per year in the United States of America alone.¹ Although key pathways of tissue fibrosis have been identified, this knowledge has not been translated to the clinic and targeted antifibrotic therapies are not yet available for most fibrotic diseases. Fibrosis may arise upon defined triggers in certain cases, but in many cases, the initiating stimulus remains elusive (so-called 'idiopathic' fibroblastic diseases). Activated fibroblasts are key players in fibroblastic diseases. A variety of different cells may differentiate into myofibroblasts, acquire a contractile phenotype with expression of alpha-smooth-muscle actin (*α*-SMA) and release abundant amounts of extracellular matrix proteins, such as collagen.² In physiological tissue responses, the myofibroblast differentiation is tightly controlled and the repair responses are terminated upon completion of the repair.¹ However, in fibrotic diseases, persistent myofibroblast activation leads to massive deposition of extracellular matrix proteins. The excessive accumulation of collagen disturbs the architecture of the affected tissue and results in functional impairment. SSc is an idiopathic systemic fibroblastic disorder that affects the skin and various internal organs. Among the autoimmune rheumatological diseases, SSc is marked by the highest case-related morbidity, and the 10-year survival of patients with a diffuse disease is only as low as 60–70%.³ Early stages of SSc are characterized by alterations of the small vessels with apoptosis of endothelial cells and the

formation of perivascular, inflammatory infiltrates, while later stages are dominated by progressive accumulation of extracellular matrix.^{2,4} Although several key mediators that trigger profibrotic responses such as TGF- β or PDGF have been identified, the failure to effectively terminate wound-healing responses and the mechanisms leading to the persistent myofibroblast activation in SSc and other fibrotic diseases are only partially understood.⁵

Leroy⁶ demonstrated already in the 1970s that SSc fibroblasts retain their activated phenotype *in vitro* in the absence of exogenous profibrotic stimuli. Ongoing research has been investigating mechanisms that lead to the sustained activation of fibroblasts. Several mechanisms such as the activation of paracrine and autocrine loops along with epigenetic changes may contribute to the endogenous activation of profibrotic pathways.^{2,4} In recent years, morphogen pathways, including Wnt, Hedgehog and Notch, have gained considerable attention as key mediators of fibroblast activation in fibrotic diseases.^{7–15} Here we review the key role of canonical Wnt signaling in fibrosis, exemplified in SSc as a prototypic fibroblastic disorder. Although we will focus on SSc in this review, evidence from other fibrotic diseases confirms the important pathophysiological role of Wnt signaling.

WNT SIGNALING PATHWAYS

Wnt proteins are lipid-modified secreted glycoproteins that have important roles in mammalian embryonic development and in adult tissue homeostasis. The lipid modification is an essential step for the secretion of Wnt proteins. Nineteen

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Received 10 October 2015; revised 16 November 2015; accepted 23 November 2015

Wnt ligands have been described in mammals that regulate cell signaling through a variety of ligand–receptor interactions.^{15–17} A variety of 10 different Frizzled (Fzd) receptors have been described. These receptors may not only be able to compensate for each other but also enable highly context- and tissue-specific regulation. Wnt receptors transduce their intracellular signaling by canonical, β -catenin-dependent pathways and several so-called non-canonical pathways, which are not directly dependent on β -catenin.¹⁷ The canonical Wnt pathway is activated by binding of Wnt ligands to a coreceptor complex, including Fzd and low-density lipoprotein-related protein receptor proteins 5 or 6 (LRP5/6). The activated receptor complex recruits Dishevelled (Dsh) and Axin to the cell membrane and consequently mediates the destabilization of the β -catenin destruction complex, a cytosolic protein complex that promotes the proteasomal degradation of β -catenin by phosphorylation.¹⁶ In addition to Axin, the β -catenin destruction complex comprises adenomatous polyposis coli (APC), glycogen-synthase-kinase 3 (GSK-3 β) and casein kinase I alpha (CK1 α). APC and Axin primarily function as scaffolding proteins within this complex and GSK-3 β and CK1 α subsequently phosphorylate β -catenin and prime it for ubiquitination and degradation. Upon inhibition of the destruction complex, β -catenin is stabilized, translocates to the nucleus and interacts with members of the T-cell factor/lymphoid enhancer-binding factors (TCF/Lef) transcription factors and several co-factors to modulate the activation of target genes.¹⁷

The activation of this core pathway can be regulated at several levels. Binding of Wnt ligands to their coreceptors is modified by secreted Wnt antagonists, including secreted frizzled-related proteins (SFRPs), Dickkopf (DKK) proteins or Wnt inhibitory factor (WIF).^{18,19} SFRPs and WIF prevent the interaction of Wnt ligands with the Fzd receptor by directly binding to Wnt proteins. In addition, SFRPs also dimerize with Fzd and with each other and can inhibit or enhance Wnt signaling in a context-specific manner. In SSc, increased levels of SFRPs were associated with inhibition of canonical Wnt signaling.²⁰ DKK proteins interfere with the formation of the Fzd-LRP5/6 coreceptor complex by binding to LRP.²¹ R-spondins are extracellular Wnt agonists, which enhance Wnt signaling by reducing the degradation of Fzd and LRP.²²

Tankyrases are members of the family of poly (ADP-ribose) polymerases (PARPs) and mediate the degradation of Axin via poly-ADP-ribosylation resulting in the destabilization of the β -catenin destruction complex and stabilization of β -catenin.²³ Finally, the transcriptional activity of the β -catenin/TCF complex is modified by cofactor binding, eg, by the coactivator p300.¹⁵

In addition to canonical Wnt signaling, several non-canonical Wnt signaling pathways, including the Wnt/Calcium pathway and the Planar-Cell polarity pathway, have been described.¹⁶ In contrast to the canonical Wnt signaling pathway, which is relatively well characterized, the

non-canonical-cascades are less well understood. The focus of this article is to review the role of canonical Wnt signaling in SSc; a more detailed review of the Wnt signaling cascades can be found elsewhere.

ACTIVATION OF CANONICAL WNT SIGNALING IN FIBROTIC DISEASES

In recent years, evidence has been accumulating that the canonical Wnt signaling pathway has a central role in fibrosis and activation of canonical Wnt signaling has been described in various fibrosing disorders, including pulmonary, renal and liver fibrosis.^{7,11,12} In fibroblasts from SSc patients, the increased expression of the Wnt proteins Wnt1 and Wnt10b along with the decreased expression of endogenous Wnt antagonists such as DKK1, SFRP1 and WIF1 have been observed.^{7,20,24} The nuclear accumulation of β -catenin occurs in fibrotic skin from SSc patients compared with healthy controls.^{7,8} Consistently, classical targets of canonical Wnt signaling are elevated in skin samples from SSc patients.^{7,20} Similar results were also observed in other fibrotic tissues, such as non-alcoholic liver fibrosis and idiopathic pulmonary fibrosis.^{7,25,26} Of note, the increased expression of central canonical Wnt pathway components is also reflected in murine models of SSc, such as the model of bleomycin-induced dermal fibrosis and in tight-skin (tsk-1) mice.^{7,8}

ACTIVATION OF CANONICAL WNT SIGNALING INDUCES FIBROSIS

The aberrant activation of canonical Wnt signaling potently activates fibroblasts *in vitro* and *in vivo*. *In vitro*, treatment with recombinant Wnt proteins such as Wnt1 and Wnt3a activates fibroblasts, induces the differentiation of resting fibroblasts into myofibroblasts and increases the release of extracellular matrix.⁷

In vivo, the overexpression of several key mediators of the canonical Wnt pathway induces fibrosis: the transgenic overexpression of Wnt10b in subcutaneous adipocytes under a FABP4 promoter resulted in a massive generalized dermal fibrosis with first fibrotic changes at the age of 3 weeks and progressive increase of dermal thickening, hydroxyproline accumulation and myofibroblast counts.⁷ Of note, the profibrotic effects of Wnt10b overexpression were remarkably pronounced.

In another mouse model with an inducible, fibroblast-specific overexpression of a stabilized mutant of β -catenin, dermal fibrosis developed rapidly within 2 weeks after induction and was progressive over time.⁸ Similar results were also described in a mouse model characterized by conditional overexpression of a stabilized β -catenin-mutant selectively in fibroblasts of the murine ventral dermis by Hamburg and Atit.²⁷ In addition, Hamburg-Shields *et al*²⁸ recently published that the conditional overexpression of β -catenin in dermal fibroblasts is associated with the increased expression of extracellular matrix genes *in vivo*. Moreover, in another approach, pharmacological activation

of canonical Wnt signaling by pharmacological inhibition of GSK-3 β exacerbated skin fibrosis.¹⁰

ACTIVATION OF CANONICAL WNT SIGNALING BY TGF- β

Canonical Wnt signaling closely interacts with TGF- β signaling, a key pathway of fibrosis.⁷ TGF- β activates canonical Wnt signaling *in vitro* and *in vivo*. Stimulation of cultured fibroblasts with recombinant TGF- β 1 induced the nuclear accumulation of β -catenin and the activation of TCF/Lef-responsive elements in reporter assays.⁷ *In vivo*, the activation of TGF- β signaling in the skin by overexpression of a constitutively active TGF- β receptor type I (TBRIact) induced nuclear accumulation of β -catenin and increased the transcription of target genes in the skin. Treatment with SD-208, a selective small-molecule inhibitor of the TGF- β receptor I, significantly ameliorated activation of canonical Wnt signaling in experimental models of fibrosis.

TGF- β REGULATES CANONICAL WNT SIGNALING ON THE LEVEL OF ITS ENDOGENOUS INHIBITORS

TGF- β may regulate canonical Wnt signaling by modulating the expression of its endogenous inhibitors.^{7,20} Treatment with recombinant TGF- β 1 inhibited the expression of DKK1 at the transcriptional level in cultured dermal fibroblasts. Moreover, reduced expression of DKK1 was found in the TBRIact mouse model. Moreover, the stimulatory effects of TGF- β on canonical Wnt signaling were abrogated by recombinant DKK1.⁷ Consistently, transgenic overexpression of DKK1 prevented the TBRIact-induced activation of canonical Wnt signaling, thus demonstrating that TGF- β -induced downregulation of DKK1 significantly contributes to the activation of canonical Wnt signaling in experimental fibrosis.

Furthermore, transgenic overexpression of DKK1 significantly reduced dermal thickening, hydroxyproline content and differentiation of myofibroblasts in TBRIact-induced fibrosis. Inhibition of TGF- β -dependent activation by DKK1 has also been demonstrated in pericytes in kidney fibrosis.²⁹ These studies highlight that TGF- β -induced activation of canonical Wnt signaling is required for complete penetrance of TGF- β -induced fibrosis.⁷

To analyze the downstream effectors that mediate the TGF- β -induced downregulation of DKK1, knockdown experiments with siRNA and pharmacological inhibitors were performed. The results suggested that TGF- β -induced downregulation of DKK1 is mediated via mitogen-activated kinase p38 and is independent of SMAD3/4 signaling and also the non-canonical TGF- β cascades JNK, Rac and Rock.⁷ In addition, recent data could show that TGF- β downregulates SFRP and DKK1 via promoter hypermethylation-induced transcriptional silencing.²⁰ These results suggest that TGF- β regulates DKK1 expression at several levels.^{7,20}

In addition, a study of Wei *et al*³⁰ demonstrated that Wnt3a can also activate TGF- β signaling via autocrine, Smad-dependent loops. These results suggest that canonical Wnt signaling and TGF- β signaling might be mutually

reinforcing each other to persistently drive myofibroblast activation in SSc.³⁰

INHIBITION OF CANONICAL WNT SIGNALING AMELIORATES EXPERIMENTAL FIBROSIS

Thus several independent studies from different groups demonstrate that Wnt signaling is activated in SSc and can promote fibroblast activation *in vitro* and *in vivo*, thus providing a strong rational to target canonical Wnt signaling in SSc.^{7,8,27} Indeed, genetic inactivation of several different effector molecules within the canonical Wnt signaling pathway ameliorated fibrosis: postnatally induced, fibroblast-specific deletion of β -catenin prevented the development of bleomycin-induced skin fibrosis, a model of the early, inflammatory stages of SSc.⁸ Moreover, the transgenic overexpression of DKK1 ameliorated fibrosis in different complementary fibrosis models, including bleomycin-induced dermal fibrosis, tsk-1 mice and TBRIact-induced fibrosis.⁷ Knockdown of evenness-interrupted, a transmembrane protein at the Golgi and the cell surface required for secretion of Wnt ligands, inhibited the activation of fibroblasts *in vitro* and reduced experimental fibrosis *in vivo*.³¹

Taken together, inhibition of canonical Wnt signaling by genetic approaches targeting different levels of Wnt signaling, including ligand secretion, interaction of ligands with their receptors or intracellular signaling, exerts antifibrotic effects in complementary murine models.

TRANSLATIONAL IMPLICATIONS

Given the potent and consistent antifibrotic effects of inactivation of canonical Wnt signaling in genetic proof-of-concept studies, canonical Wnt signaling might offer potential for targeted therapies for fibrotic diseases. For decades, canonical Wnt signaling has been considered 'undruggable' owing to the lack of typical pharmacological targets in the core pathway. However, in recent years, several pharmacological approaches have been developed that potently and selectively inhibit canonical Wnt signaling. Some of them have already successfully completed early clinical trials.³²

PKF118-310 and ICG-001 are small-molecule inhibitors that interfere with the interaction of β -catenin with TCF and CBP, respectively. In experimental fibrosis models, treatment with PKF118-310 or ICG-001 prevented the development of bleomycin- and TBRIact-induced dermal fibrosis.³³ Moreover, PKF118-310 or ICG-001 also exerted antifibrotic effects in therapeutic settings with preestablished bleomycin-induced dermal fibrosis.³³ Consistently, Henderson *et al*³⁴ described antifibrotic effects of ICG-001 in bleomycin-induced lung fibrosis. Moreover, antifibrotic effects were described in the ureter-obstruction model of renal interstitial fibrosis.³⁵ Of interest, PRI-724, another inhibitor of the CBP- β -catenin interaction has recently been evaluated in a phase I clinical trial as a 7-day treatment course in patients with solid tumors with an acceptable toxicity profile and further phase I-II studies are in progress.³²

Tankyrases (TNKS 1 and 2) mediate the proteasomal degradation of Axin by poly-ADP-ribosylation. The degradation of Axin results in the destabilization of the β -catenin destruction complex, nuclear accumulation of β -catenin and activation of canonical Wnt target genes. Treatment with XAV939, a selective tankyrase inhibitor, reduced the activation of cultured fibroblasts and prevented bleomycin- and TBR1act-induced skin fibrosis.³⁶

Another approach to pharmacologically target Wnt pathway components in fibrotic diseases evolved from the observation that endogenous Wnt antagonists DKK1 and SFRP1 are downregulated in fibroblasts from SSc patients by promoter hypermethylation.²⁰ 5-Aza-2'-deoxytidine (5'-aza), an inhibitor of DNA methyltransferases, inhibited hypermethylation-induced epigenetic silencing of DKK1 and SFRP1 resulting in inhibition of canonical Wnt signaling *in vitro* and *in vivo* and exerted antifibrotic effects.²⁰ Although inhibitors of DNA methyltransferases are not specific and modulate a variety of other pathways besides canonical Wnt signaling, they do have a high translational potential. First inhibitors of DNA methyltransferases have already been approved for the treatment of hematological malignancies and would be available for clinical use in fibrotic diseases as well while other pharmacological Wnt pathway modulators are still in earlier stages of clinical testing.

POTENTIAL LIMITATIONS OF PROLONGED WNT INHIBITION

Concerns of long-term inhibition of canonical Wnt signaling in chronic diseases are particularly related to its role in stem cell maintenance. Canonical Wnt signaling is regulating proliferation and also differentiation of adult stem cells and its inhibition may thus result in toxicity, in particular in tissues with high cellular turnover, such as the intestine. Indeed, genetic manipulation of Wnt signaling by constitutive overexpression of DKK1 selectively in the epithelial layer in the mouse intestine resulted in a disorganized mucosa structure in the homozygous transgenic F2 generation.³⁷ Similar results were observed 10 days after i.v. administration of an adenoviral DKK1-expressing construct in adult SCID mice.³⁸ In contrast to the findings in these genetic models, pharmacological inhibition of canonical Wnt signaling in mice was rather well tolerated without obvious clinical evidence for significant gastrointestinal toxicity. This discrepancy is likely due to a less complete suppression of canonical Wnt signaling with the pharmacological approaches as compared with the genetic models. Although the remaining Wnt activity might be sufficient to limit toxicity in those relatively short mouse models, treatment of human fibrotic diseases would require chronic treatment for years and could thus lead to long-term complications by slowly progressive rarefaction of stem cells upon chronic Wnt inhibition. A potential approach to minimize those potential complications might be combination therapies.³⁹

CONCLUSIONS

Multiple lines of evidence highlight the central role of canonical Wnt signaling for the persistent myofibroblast activation in fibrotic diseases. Increased activation of canonical Wnt signaling has been shown on several levels in skin samples from SSc patients and in other fibrosing disorders. Furthermore, the pathological activation of canonical Wnt signaling is reflected in experimental models of SSc. Forced activation of canonical Wnt signaling stimulated myofibroblast differentiation and collagen release from cultured fibroblasts and induced fibrosis in mice. In contrast, inhibition of canonical Wnt signaling by several different approaches and at different levels ameliorated fibrosis in complementary mouse models of SSc. Thus canonical Wnt signaling might be a potential target for antifibrotic therapies. Several pharmacological inhibitors of key components of the canonical Wnt pathway have demonstrated antifibrotic effects in preclinical studies in well-tolerated doses and some of those molecules showed promising results in first clinical trials. A major concern that needs to be carefully considered, in particular for long-term applications in chronic diseases, is toxicity related to stem cell depletion. Further studies are required to assess potential adverse effects of long-term treatment with candidate inhibitors and to limit potential toxicity.

DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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