CONCISE REPORT

Inactivation of evenness interrupted (EVI) reduces experimental fibrosis by combined inhibition of canonical and non-canonical Wnt signalling

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ABSTRACT

Objectives Canonical as well as non-canonical Wnt signalling pathways have emerged as core pathways of fibrosis. Their profibrotic effects are mediated via distinct intracellular cascades independently of each other. Thus, inhibition of both pathways may have additive antifibrotic effects. Here, we knocked down evenness interrupted (EVI) to simultaneously target for the first time canonical and non-canonical Wnt signalling in experimental fibrosis.

Methods The antifibrotic effects of siRNA-mediated knockdown of EVI were evaluated in the mouse models of bleomycin-induced skin fibrosis and in fibrosis induced by adenoviral overexpression of a constitutively active TGF- β receptor I (AdTBRI).

Results Knockdown of EVI decreased the release of canonical and non-canonical Wnt ligands by fibroblasts and reduced the activation of canonical and non-canonical Wnt cascades in experimental fibrosis with decreased accumulation of β -catenin and phosphorylated JNK and cJun. Inactivation of EVI exerted potent antifibrotic effects and reduced dermal thickening, myofibroblast differentiation and accumulation of collagen in the mouse models of bleomycin-induced and AdTBRI-induced fibrosis.

Conclusions Inhibition of Wnt secretion by knockdown of EVI inhibits canonical and non-canonical Wnt signalling and effectively reduces experimental fibrosis in different preclinical models. Inhibition of Wnt secretion may thus be an interesting approach for the treatment of fibrosis.

mice.^{3 4 6 10} In addition to canonical Wnt signalling, we and others demonstrated recently that non-canonical Wnt signalling also exerts profibrotic effects.¹¹ The non-canonical Wnt ligand Wnt-5a is overexpressed in SSc and potently stimulates fibroblasts in a TGF- β -dependent manner to release collagen (unpublished data). These findings suggest that both canonical and non-canonical Wnt signalling contribute to fibroblast activation in SSc and might be interesting candidates for targeted therapies. However, approaches for combined inhibition of all Wnt signalling cascades in fibrosis have not been established so far.

Here we evaluated evenness interrupted (EVI) as a potential molecular target for combined and selective inhibition of canonical and non-canonical Wnt signalling. EVI, also known as Wntless or Sprinter, is a multipass transmembrane protein localised in the Golgi and at the cell surface, which is essential for secretion of all canonical and non-canonical Wnt ligands.^{12 13} Although the precise mechanism of action is still under investigation, knockdown of EVI has been shown to block the release of all Wnt proteins in various cell types and organisms. These effects are specific for Wnt proteins, and the secretion of other glycosylated or palmitylated proteins is not affected.¹³

Given the profibrotic effects of canonical and non-canonical Wnt pathways, we hypothesised that the combined inhibition of both pathways by targeting of EVI might be a promising novel antifibrotic approach.

INTRODUCTION

Wnts are a family of evolutionary conserved glycoproteins that play essential roles in developmental processes and adult homeostasis.¹ The intracellular effects of Wnt proteins are mediated by different signalling cascades, which are subcategorised as canonical and non-canonical Wnt signalling pathways. Activation of canonical Wnt signalling relies on stabilisation and nuclear translocation of β -catenin, whereas the effects of non-canonical Wnt are mediated by calcium influx or activation of JNK/Jun signalling independently of β -catenin.² Recent studies characterised canonical Wnt signalling as a core pathway of fibrosis.^{3–10} Canonical Wnt signalling is activated in SSc by overexpression of Wnt-1 and Wnt-10b and decreased expression of endogenous Wnt inhibitors.^{3 5 8} Canonical Wnt signalling stimulates fibroblasts to differentiate into myofibroblasts and is sufficient to induce fibrosis in

MATERIALS AND METHODS**siRNA-mediated knockdown of EVI**

Complexes of siRNA and atelocollagen (Koken, Tokyo, Japan) were prepared as described.^{7 14} The following siRNA duplexes against EVI (Thermo Fisher Scientific, Bonn, Germany) were used: sense, 5'-GUCCAUGGUUCCAGUUUAUUU-3'; antisense, 5'-PAUAAACUGGAACCAUGGACUU-3'. Non-targeting siRNA duplexes served as controls. Atelocollagen/siRNA complexes were injected intracutaneously once weekly, and treatment was started together with the first profibrotic stimulus.

Quantitative real-time PCR

The mRNA levels of EVI were quantified by SYBR green real-time PCR using the following primers:

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mouse—5'-CCGAGGCTGCACTATGAG-3', 5'-GCATGAGGAACTTGAACCTGAAG-3'; human—5'-TGCACCTTCACATCTCCCAAG-3', 5'-TCATAGTAACGGCCCTCATGC-3'.¹⁵

Transfer experiments

Cell culture supernatants from SSc fibroblasts (passages 4–8) transfected either with EVI siRNA or non-targeting siRNA were collected 36 h after transfection. Fibroblasts from healthy volunteers were incubated with undiluted supernatants, and the activation of JNK, Jun and β -catenin signalling was quantified after 1 h and after 12 h using the ImageJ software. Absolute staining intensities were normalised to 4',6-diamidino-2-phenylindole (DAPI) staining. Patient information is provided in online supplementary table S1.

Immunofluorescence staining for β -catenin, p-JNK, p-cJun and vimentin

Formalin-fixed, paraffin-embedded skin sections were stained for anti- β -catenin (Abcam, Cambridge, UK), p-JNK (Abcam, Cambridge, UK), p-cJun (Santa Cruz, Heidelberg, Germany) and vimentin (Abcam, Cambridge, UK). Concentration-matched and species-specific immunoglobulins (Vector Laboratories, Burlingame, California, USA) served as control antibodies. Sections were counterstained with DAPI.^{3 16 17}

Experimental models of dermal fibrosis

Skin fibrosis was induced by injection of bleomycin and by adenoviral overexpression of a constitutively active TGF- β receptor type I mutant (AdTBRI).^{3 5 16} Skin fibrosis was analysed after 3 and 8 weeks, respectively, as described.^{3–7 14–16} The thickness of the subcutis was measured on HE-stained sections at four different sites per mouse. Infiltrating leucocytes were counted on HE-stained sections at ≥ 20 different sites. Staining for pSmad2/3 was performed as described.^{3 18}

Statistics

All data are presented as median with IQR, and differences between the groups were tested by non-parametric Mann–Whitney U test.

RESULTS

Inactivation of EVI inhibits Wnt signalling

siRNA-mediated knockdown of EVI decreased the mRNA levels of EVI by $60 \pm 10\%$ in cultured fibroblasts and by $64 \pm 10\%$ at the end of the injection period in murine skin.

Fibroblasts are the major source of canonical and non-canonical Wnt proteins in SSc. These Wnt proteins activate the releasing fibroblasts as well as neighbouring cells in autocrine and paracrine manners.^{3 6 10} To prove that knockdown of EVI inhibits the secretion of Wnt proteins from fibroblasts, we performed transfer experiments. Cell culture supernatant from fibroblasts transfected with non-targeting siRNA activated

Figure 1 Knockdown of evenness interrupted (EVI) prevents activation of canonical and non-canonical Wnt signalling. Cell culture supernatants of fibroblasts transfected with siRNA against EVI are less effective in inducing nuclear accumulation of β -catenin and phosphorylation of JNK and cJun, demonstrating decreased release of canonical and non-canonical Wnt ligands. Representative images of fibroblasts stained for β -catenin (A), p-JNK (B) and p-cJun (C) are shown at 200-fold magnifications. Left column: staining for β -catenin, p-JNK and p-cJun. Middle column: staining of nuclei with DAPI. Right column: overlay. n.t. siRNA, non-targeting siRNA (n=6 each).

canonical and non-canonical Wnt signalling in fibroblasts with nuclear translocation of β -catenin and accumulation of p-JNK and p-cJun (figure 1A–C). In contrast, the staining intensities of nuclear β -catenin, p-JNK and p-cJun after 1 h were all significantly reduced to 37%, 66% and 16%, respectively, with cell culture supernatant from fibroblasts transfected with EVI siRNA (figure 1A–C). Similar results were obtained after 12 h.

Consistent with an autocrine Wnt stimulation loop in SSc fibroblasts, knockdown of EVI in SSc fibroblasts reduced the mRNA levels of the Wnt target gene axin-2 and of col1a1 (see online supplementary figures S1 and S2).

Canonical and non-canonical Wnt signalling cascades were activated in bleomycin-induced and AdTBRI-induced fibrosis with nuclear accumulation of β -catenin and increased levels of p-JNK and p-cJun as compared to non-fibrotic control mice (see online supplementary figure S2A–C). Injection of EVI siRNA decreased the levels of nuclear β -catenin as well as the levels of p-JNK and p-cJun in bleomycin-challenged and AdTBRI-challenged mice to that of non-fibrotic control mice (see online supplementary figure S3A and S2C).

Inactivation of EVI reduces experimental fibrosis

Inhibition of EVI exerted potent antifibrotic effects and reduced dermal thickening by 56% compared with bleomycin-challenged mice injected with non-targeting siRNA ($p=0.006$) (figure 2A,B). The number of myofibroblasts and the hydroxyproline content were also significantly decreased by 58% and 66%, respectively, upon inactivation of EVI ($p=0.001$ and 0.002 , respectively) (figure 2C,D).

TGF- β exerts its fibrotic effects in part by activating canonical Wnt signalling.³ We thus examined whether inactivation of EVI ameliorates AdTBRI-induced fibrosis. siRNA-mediated knockdown of EVI inhibited dermal thickening in AdTBRI-treated mice by 69% compared with AdTBRI mice injected with non-targeting siRNA ($p=0.02$) (figure 3A,B). Myofibroblast counts and the hydroxyproline content were also reduced by 65% and 48%, respectively ($p=0.008$ and 0.01 , respectively) (figure 3C, D).

Further analyses demonstrated that knockdown of EVI did not alter leucocyte infiltration, reduce subcutaneous atrophy (see online supplementary figure S4A,B) or alter the number of apoptotic cells (see online supplementary figure S5). Despite slight decreases in the levels of pSmad2/3, this trend did not reach statistical significance (see online supplementary figure S6).

DISCUSSION

Several studies highlight the key role of canonical Wnt signalling in the pathogenesis of SSc and related fibrotic diseases.^{3–10} In addition, recent studies demonstrate that non-canonical Wnt signalling is also activated and stimulates the release of collagen via JNK/c-Jun-dependent pathways independently of canonical Wnt signalling.¹¹ Individual inhibition of both pathways demonstrated potent antifibrotic effects in preclinical models of SSc.^{3 5–7} Considering the potent and independent profibrotic effects of canonical and non-canonical Wnt signalling, we hypothesised that combined inhibition of both signalling cascades may exert additive antifibrotic effects. Here, we report the effects of targeting EVI as the first approach for combined inhibition of both pathways. Knockdown of EVI prevented the aberrant activation of canonical and non-canonical Wnt signalling in fibroblasts in vitro and in vivo. Combined inhibition of both Wnt pathways was effective in bleomycin-induced and AdTBRI-induced skin fibrosis as models of early inflammatory and later non-inflammatory stages of SSc,¹⁹ suggesting that such a treatment strategy may be effective in different stages of SSc. Consistent with an autocrine stimulation of fibroblasts by Wnt proteins, knockdown of EVI did not reduce leucocyte infiltration and also decreased the release of collagen from SSc fibroblasts in culture (data not shown). Although inhibitors of EVI itself are not yet available, secretion of Wnt proteins can be targeted pharmaceutically.²⁰ Small molecule inhibitors of the acyltransferase porcupine, which catalyses the palmitoylation of Wnt proteins at conserved Cys77 and Ser20 residues, have been developed very recently. As porcupine is the only enzyme capable of palmitoylating Wnt proteins and as palmitoylation is essentially required for the secretion of the otherwise

Figure 2 Inactivation of evenness interrupted (EVI) inhibits bleomycin-induced dermal fibrosis. siRNA-mediated knockdown of EVI ameliorated bleomycin-induced fibrosis (representative trichrome-stained sections are shown at 100-fold magnification in (A)) with decreased dermal thickening (B), reduced differentiation of resting fibroblasts into myofibroblasts (C) and reduced the hydroxyproline content (D). $n=5$ for each group. n.t. siRNA, non-targeting siRNA.

Figure 3 Knockdown of evenness interrupted (EVI) ameliorates adenoviral overexpression of a constitutively active TGF- β receptor I (AdTBRI)-induced fibrosis. Injection of siRNA against EVI prevents AdTBRI-induced fibrosis (A) and reduces dermal thickening (B), myofibroblast differentiation (C) and hydroxyproline content (D) in mice infected with AdTBRI. Representative trichrome-stained tissue sections are shown at 100-fold magnification. n=5 for each group. n.t. siRNA, non-targeting siRNA.

hydrophilic Wnt proteins, inhibition of porcupine blocks the secretion of all Wnt proteins,²⁰ thereby inhibiting canonical and non-canonical Wnt signalling similar to knockdown of EVI. Based on (1) the effective inhibition of canonical as well as non-canonical Wnt signalling, (2) the potent antifibrotic effects in different mouse models and (3) the availability of novel inhibitors, inhibition of Wnt secretion may be an interesting approach for the treatment of fibrosis in SSc and related disorders. However, further studies with candidate drugs are needed to assess the antifibrotic effects in other organs, to analyse the effects on established fibrosis and to exclude depletion of stem cells and other toxicities upon long-term treatment.

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