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BASIC SCIENCE

Transforming growth factor-β-dependent Wnt secretion controls myofibroblast formation and myocardial fibrosis progression in experimental autoimmune myocarditis

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Aims Myocardial fibrosis critically contributes to cardiac dysfunction in inflammatory dilated cardiomyopathy (iDCM). Activation of transforming growth factor-β (TGF-β) signalling is a key-step in promoting tissue remodelling and fibrosis in iDCM. Downstream mechanisms controlling these processes, remain elusive.

Methods and results Experimental autoimmune myocarditis (EAM) was induced in BALB/c mice with heart-specific antigen and adjuvant. Using heart-inflammatory precursors, as well as mouse and human cardiac fibroblasts, we demonstrated rapid secretion of Wnt proteins and activation of Wnt/β-catenin pathway in response to TGF-β signalling. Inactivation of extracellular Wnt with secreted Frizzled-related protein 2 (sFRP2) or inhibition of Wnt secretion with Wnt-C59 prevented TGF-β-mediated transformation of inflammatory precursors and cardiac fibroblasts into pathogenic myofibroblasts. Inhibition of T-cell factor (TCF)/β-catenin-mediated transcription with ICG-001 or genetic loss of β-catenin also prevented TGF-β-induced myofibroblasts formation. Furthermore, blocking of Smad-independent TGF-β-activated kinase 1 (TAK1) pathway completely abrogated TGF-β-induced Wnt secretion. Activation of Wnt pathway in the absence of TGF-β, however, failed to transform precursors into myofibroblasts. The critical role of Wnt axis for cardiac fibrosis in iDCM is also supported by elevated Wnt-1/Wnt-5a levels in human samples from hearts with myocarditis. Accordingly, and as an *in vivo* proof of principle, inhibition of Wnt secretion or TCF/β-catenin-mediated transcription abrogated the development of post-inflammatory fibrosis in EAM.

Conclusion We identified TAK1-mediated rapid Wnt protein secretion as a novel downstream key mechanism of TGF-β-mediated myofibroblast differentiation and myocardial fibrosis progression in human and mouse myocarditis. Thus, pharmacological targeting of Wnts might represent a promising therapeutic approach against iDCM in the future.

Keywords Experimental autoimmune myocarditis • CD133 inflammatory progenitor • Cardiac fibroblasts • Myofibroblast • TGF-β signalling • Wnt-TAK1 signalling

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Translational perspective

A growing body of evidence suggests that myocardial fibrosis developing through the excessive accumulation of pathogenic myofibroblasts is a direct cause of impaired cardiac function in inflammatory heart diseases. Using the mouse model of inflammatory dilated cardiomyopathy, we describe a novel transforming growth factor-β (TGF-β)-dependent molecular mechanism controlling progression of post-inflammatory fibrogenesis. We describe how profibrotic cytokine TGF-β through activation of TGF-β-activated kinase 1 (TAK1) induces secretion of Wnt proteins, which activate the canonical Wnt pathway causing formation of pathogenic myofibroblasts. Our results indicate that pharmacological targeting of extracellular Wnts or the canonical Wnt pathway might represent a promising therapeutic option preventing accumulation of new pathogenic myofibroblasts. Such anti-fibrotic therapy in inflammatory cardiomyopathy patients potentially could prevent disease progression. Some researchers consider cardiac remodelling as a dynamic process with a substantial turnover of pathogenic cells. Therefore, inhibition of their *de novo* formation could eventually lead to resolution of the established fibrotic tissue and consequently to improvement of heart function in some patients. Furthermore, targeting of Wnts or Wnt pathway represents an attractive alternative for anti-TGF-β treatment by offering a broader range of available pharmacological compounds with well-defined function.

Introduction

Inflammatory dilated cardiomyopathy (iDCM) is a distinct entity among the specific heart muscle diseases and is defined as cardiac dysfunction due to myocardial inflammation. Following acute myocarditis, patients may develop progressive heart failure in up to 30% of cases with survival rate <40% after 10 years.[1,2] Dilation of heart chambers, impaired systolic function, and myocardial fibrosis are hallmarks of the typical end-stage heart failure phenotype in iDCM.[1,2] Progressive pathological remodelling and excessive accumulation of myofibroblasts following cardiac inflammation is a major cause of heart dysfunction in iDCM.[3–5] Myofibroblasts produce fibrillar collagen, mainly type I, which decreases cardiac compliance and impairs both systolic and diastolic heart function, as evidenced by echocardiography or magnetic resonance.[5–8] So far, little is known about cellular and molecular mechanisms regulating transition of acute myocarditis into iDCM and end-stage heart failure phenotype.

Experimental autoimmune myocarditis (EAM) represents a rodent model of iDCM. Immunization of susceptible animals with myosin heavy chain-α (αMyHC) peptide together with a strong adjuvant results in $CD4^+$ T-cell-mediated myocarditis. Inflammation peaks 14–21 Days after the first immunization. Later on, inflammation largely resolves, but some animals develop the typical end-stage heart failure phenotype, including ventricular dilation and myocardial fibrosis.[4,5,9]

In the EAM, transforming growth factor-β (TGF-β) signalling controls the transition from myocarditis to post-inflammatory fibrosis phenotype.[3] We recently demonstrated that multipotent $CD133^+$ progenitors infiltrating the heart during myocarditis convert into collagen-producing myofibroblasts when exposed to TGF-β1. In fact, inflammatory $CD133^+$ cells represent the major cellular source of post-inflammatory myofibroblasts in EAM.[3,4]

Transforming growth factor-β signalling plays a crucial role in embryonic development, tissue homeostasis, and pathogenesis of a variety of diseases. Active TGF-β binds to the transmembrane TGF-β type II receptor, which in turn recruits and activates the TGF-β type I receptor initiating canonical Smad signalling by phosphorylation of Smad2 and Smad3 proteins.[7,10] Stimulated TGF-β type I receptor also activates a number of Smad-independent signalling cascades distinctly from transcription. Polyubiquitination and autophosphorylation of TGF-β-activated kinase 1 (TAK1) represents an example of non-canonical pathway activated by TGF-β1.[11]

Wnt signalling represents another important molecular pathway regulating developmental processes and tissue pathogenesis. The family of Wnt ligands consists of highly evolutionarily conserved glycoproteins, which after binding to receptor complexes can trigger various outputs including β-catenin-dependent (canonical) or independent (non-canonical) responses.[12] In canonical response, binding of Wnt ligands to Frizzled/Low Density Lipoprotein Receptor-related Proteins receptor complexes initiates a series of molecular events resulting in cytoplasmic stabilization of β-catenin, which is subsequently translocated to the nucleus. In the nucleus, β-catenin complexes with the TCF (T-cell factor)/LEF (lymphoid enhancer factor) transcription factors to initiate the transcription of Wnt target genes.[13] Natural regulation of Wnt pathway occurs predominantly at the level of extracellularly secreted inhibitors. The family of secreted Frizzled-related proteins (sFRP) represents one of the most important natural inhibitors of Wnt signalling. sFRP bind directly to Wnt proteins and prevent their interactions with Frizzled receptors or directly bind to Frizzled receptors.[14] Interplay between TGF-β and Wnt pathways has been recently postulated,[15] but nothing is known about the role of Wnts in inflammatory heart failure.

Methods

Balb/c mice were injected subcutaneously with 150 μg of αMyHC (Ac-RSLKLMATLFSTYASADR-OH; Caslo) peptide emulsified 1:1 with Complete Freund's Adjuvant (CFA, Difco) on Days 0 and 7. sFRP2 (200 μg/kg, R&D Systems), Wnt-C59 (2.5 mg/kg, R&D Systems), or ICG-001 (500 μg/kg, Selleckchem) was intravenously injected every second day between Days 17–29 of EAM. Control mice received solvent only. Animal experiments were approved by local authorities. Full methods are available in the supplementary material online, Methods.

Results

Blocking Wnt signalling by secreted Frizzled-related protein 2 prevents transforming growth factor-β1-induced transformation of heart-inflammatory progenitors and cardiac fibroblasts into pathogenic myofibroblasts

In EAM, TGF-β signalling critically controls the accumulation of pathological myofibroblasts in the post-inflammatory heart.[3] On

the cellular level, this process mainly involves TGF-β-mediated transformation of heart-infiltrating CD133$^+$ progenitors into alpha smooth muscle actin (αSMA)$^+$/collagen I$^+$/fibronectin$^+$ pathological myofibroblasts.[3,4] To identify molecular pathways involved in this process, we compared gene expression profiles of inflammatory CD133$^+$ cells isolated from hearts during the inflammatory phase at Day 21 of EAM, expanded and stimulated without (control) and with TGF-β1 for 24 hours. The analysis resulted in differential expression of 485 genes with identified function. Gene ontology analysis of differentially expressed genes suggested that TGF-β1 activates Wnt signalling pathway in differentiating CD133$^+$ cells (Supplementary material online, *Figure S1*). Quantitative analysis confirmed up-regulation of common endogenous Wnt target genes *Axin2*,[16] *Tcf7*,[17] *Ephb3*,[18] but also *Wnt1*, *Wnt5a*, *Wnt10b*, and *Wnt11* and down-regulation of *Sfrp2* transcripts in TGF-β1-treated cells (Supplementary material online, *Figure S1*).

To address whether Wnt signalling was essential for transformation of precursor cells into myofibroblasts, we differentiated inflammatory CD133$^+$ cells and cardiac fibroblasts (alternative source of pathogenic cardiac myofibroblasts) with TGF-β1 in the presence or absence of physiological Wnt inhibitor sFRP2, which sequesters Wnt ligands and thus prevents their association to receptor complexes. Addition of sFRP2 to TGF-β1-treated cells inhibited Wnt target gene expression (*Figure 1A* and *B*), nearly completely prevented formation of fibronectin$^+$/αSMA$^+$ myofibroblasts, and inhibited TGF-β-induced contraction (*Figure 1C* and *D*). Accordingly, we found that the expression of genes characteristic for myofibroblasts including *Acta*, *Fn1*, *Col1a1*, and *Tnc* and involved in tissue remodelling such as *Mmp8*, *Mmp9*, *Timp2*, and *Timp3* were impaired in TGF-β1-treated cells cultured in the presence of sFRP2 (*Figure 1E*).

Transforming growth factor-β triggers Wnt secretion and activation of the canonical Wnt pathway

Gene expression analysis showed that addition of sFRP2 prevented expression of the common Wnt target genes in response to TGF-β1 treatment. These findings prompted us to measure secreted Wnt proteins. In the canonical response, Wnts activate TCF/LEF transcription factor complex through β-catenin. We measured TCF/LEF activity in SuperTopFlash (STF)-reporter cells (expressing firefly luciferase under the control of TCF/LEF-responsive element) treated with supernatant of cultured CD133$^+$ cells and mouse or human cardiac fibroblasts stimulated with TGF-β1. Supernatant collected from each cell type stimulated with TGF-β1 for 1- or 24-h-induced β-catenin/TCF/LEF-responsive gene expression. Addition of sFRP2 to the reporter cells completely abolished this response (*Figure 2A*).

Extracellular Wnts can activate canonical Wnt pathway in target cells. Accordingly, TGF-β1 increased the pool of the active form of β-catenin and the total β-catenin cellular content in CD133$^+$ cells (*Figure 2B*) and induced nuclear accumulation of β-catenin 1–2 h after treatment (*Figure 2C*). β-catenin response to TGF-β1 was completely prevented by addition of sFRP2 (*Figure 2B* and *C*). Furthermore, we observed that TGF-β1 induced transcription of *Wnts* as early as 1 h after treatment (Supplementary material online, *Figure S2*). Wnt protein secretion can be blocked with Porcupine inhibitor Wnt-C59. Addition of Wnt-C59 to TGF-β1-treated cells abrogated TGF-β1-induced expression of Wnt target genes and myofibroblast-specific genes (*Figure 3A* and *B*) and prevented formation of αSMA$^+$ myofibroblasts (*Figure 3C*).

T-cell factor/β-catenin-mediated transcriptional activation is crucial for transforming growth factor-β1-mediated myocardial fibrosis

To address whether TGF-β1-induced fibrosis required activation of the canonical Wnt signalling pathway, we used tankyrase inhibitor JW55 (triggering β-catenin cytoplasmic degradation and thus preventing its nuclear translocation and transcriptional activity)[19] as well as FH-535[20] and ICG-001[21] (both suppressing TCF/β-catenin-mediated transcription) in differentiation studies. All inhibitors not only prevented transcription of Wnt target genes in TGF-β1-challenged cells (*Figure 3D*), but also effectively reduced the conversion of CD133$^+$ cells and cardiac fibroblasts into myofibroblasts (*Figure 3E*, Supplementary material online, *Figures S3 and S4*). In hearts of mice with EAM, we found elevated levels of β-catenin (Supplementary material online, *Figure S5*). Using β-catenin$^{flox/flox}$ cardiac fibroblasts, we demonstrated that genetic loss of β-catenin resulted in impaired expression of myofibroblast-specific genes and reduced contractile properties of TGF-β1-treated cells (*Figure 3F*, Supplementary material online, *Figure S6*).

Transforming growth factor-β-activated kinase 1 mediates transforming growth factor-β1-induced Wnt secretion

Transforming growth factor-β1 activates the canonical Smad pathway and a number of Smad-independent signalling cascades. In CD133$^+$ cells, we observed phosphorylation of Smad2 in response to TGF-β1 (Supplementary material online, *Figure S7A*). On the other hand, TGF-β triggers TAK1 phosphorylation in our model (Supplementary material online, *Figure S7B*). To address which pathway triggers Wnt secretion, we used two selective inhibitors specifically blocking the canonical Smad (SB-431542) or Smad-independent TAK1 (5Z-7-oxo-zeaenol) pathway. Addition of 5Z-7-oxo-zeaenol, but not SB-431542, to TGF-β1-treated cells prevented the nuclear translocation of β-catenin and abrogated expression of Wnt target genes (*Figure 4A* and *B*). Next, we used supernatant of cells stimulated with TGF-β1 in the presence or absence of these two inhibitors to measure TCF/LEF activity in the reporter cells. Our results clearly showed that blockade of TAK1, but not of the Smad pathway, prevented accumulation of Wnts in supernatant in response to TGF-β1 (*Figure 4C*). Importantly, none of these inhibitors affected externally induced Wnt-3a-mediated TCF/LEF response of the reporter cells (*Figure 4D*). Our results showed that 5Z-7-oxo-zeaenol also effectively prevented TGF-β1-induced transcription of *Wnts* (Supplementary material online, *Figure S7C*).

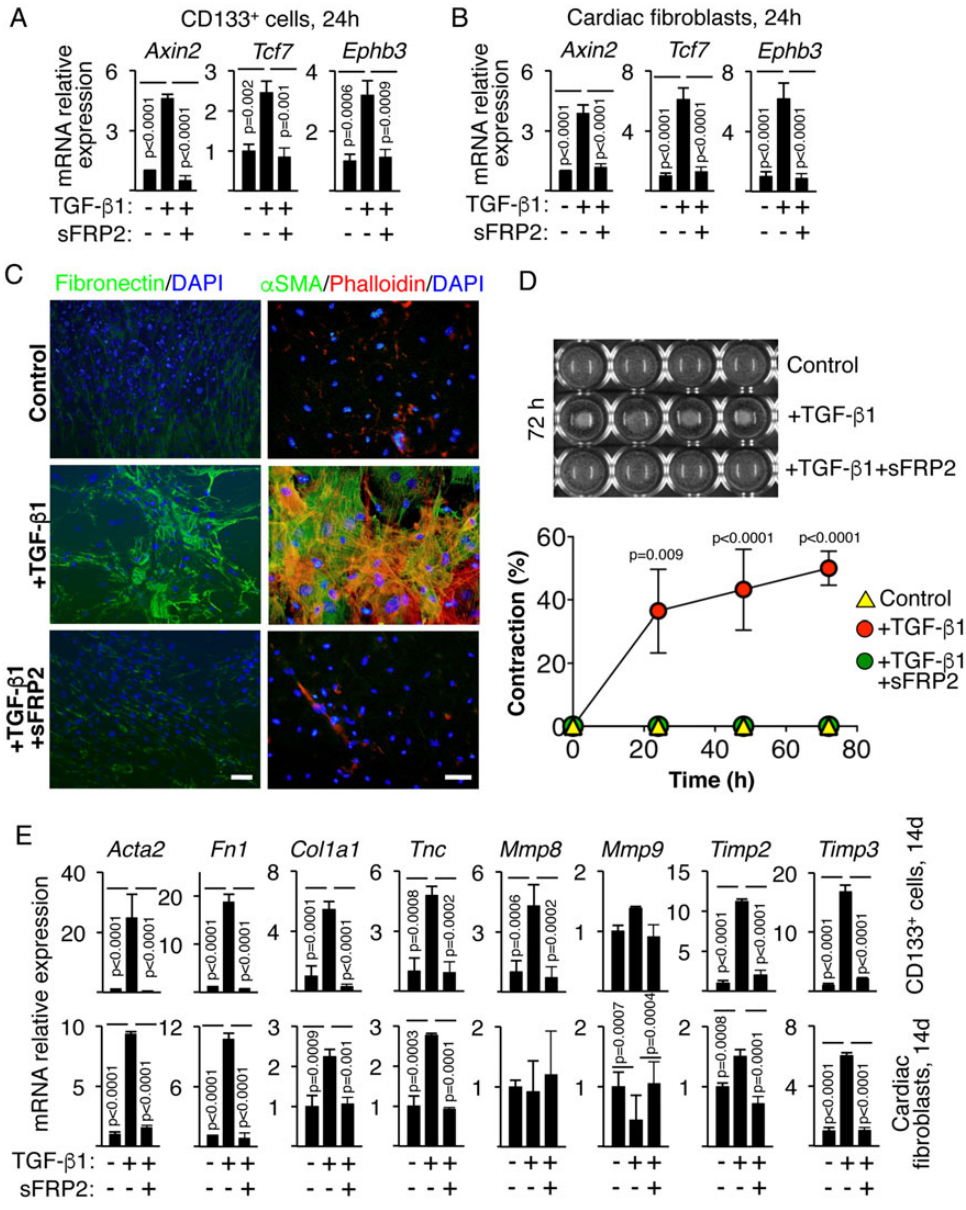

Figure 1 Secreted Frizzled-related protein 2 prevents transforming growth factor-β-induced formation of myofibroblasts from heart-inflammatory progenitors and cardiac fibroblasts. Heart-inflammatory CD133$^+$ cells expanded from myocarditis-positive hearts or cardiac fibroblasts were treated without or with transforming growth factor-β1 or transforming growth factor-β1 + secreted Frizzled-related protein 2. (*A* and *B*) Relative mRNA levels of the common Wnt target genes in CD133$^+$ cells (*A*, $n = 8$) and in cardiac fibroblasts (*B*, $n = 8$) 24 h after stimulation. Representative immunofluorescence of fibronectin, αSMA, and stress fibres (Phalloidin) 14 Days at the indicated condition are shown in (*C*). 4′,6-diamidino-2-phenylindole (blue) visualizes nuclei. Bar = 20 μm. Data are representative for 3 experiments. (*D*) Representative image and quantification of the contraction activity, $n = 5$. (*E*) Relative expression of myofibroblast-specific genes in CD133$^+$ cells (top, $n = 8$) and in cardiac fibroblasts (bottom, $n = 8$) 14 Days after stimulation. *P* values computed using one-way ANOVA and the Bonferroni post-hoc test.

Co-activation of transforming growth factor-β1 and canonical Wnt signalling pathways is required for myofibroblast differentiation

Next, we asked whether Wnts in the absence of TGF-β1 could trigger myofibroblast differentiation. Wnt-1/Wnt-5a cocktail, Wnt-3a-conditioned medium, or GSK3 inhibitor—CHIR, all potently induced TCF/β-catenin-mediated transcription (*Figure 5A*). Treatment with Wnt-1/5a, Wnt-3a, or CHIR led to nuclear accumulation of β-catenin (*Figure 5B*) and induced expression of the common Wnt downstream target genes in CD133$^+$ cells (*Figure 5C*) and cardiac fibroblasts (not shown). However, we observed only a modest up-regulation of myofibroblast-specific

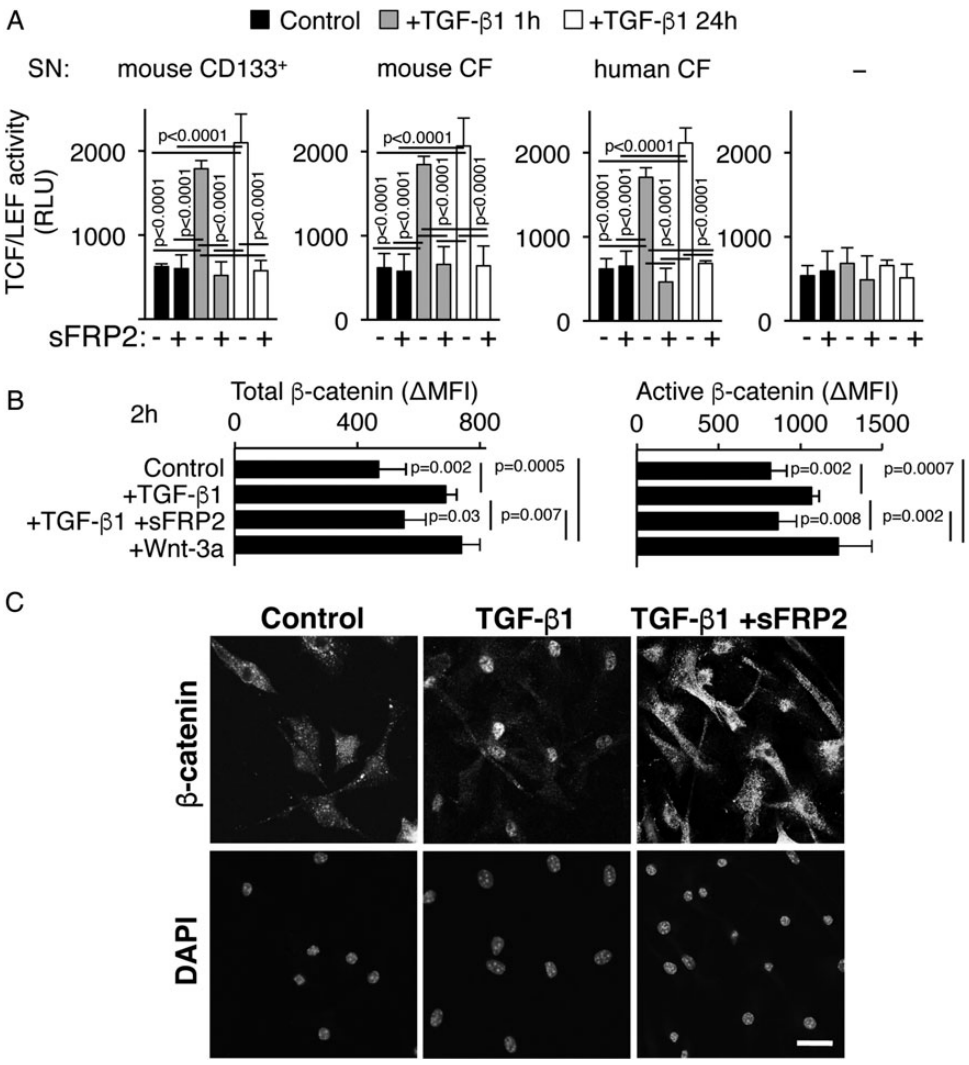

Figure 2 Transforming growth factor-β induces Wnt secretion and activation of the canonical Wnt pathway. (*A*) Heart-inflammatory CD133$^+$ cells, mouse or human cardiac fibroblasts (CF) were cultured without (control, black) or with transforming growth factor-β1 for 1 h (grey) or 24 h (white). Amount of Wnt ligands secreted into supernatants was measured as the activity of T-cell factor/lymphoid enhancer factor-luciferase reporter expressed in STF-reporter cell line. Graphs represent activity of T-cell factor/lymphoid enhancer factor promoter determined as relative luciferase units of the reporter cells cultured in the indicated supernatant in the presence or absence of secreted Frizzled-related protein 2. Culture medium (−) was used as a control. Data are representative for three experiments. *P*-values computed using one-way ANOVA and the Bonferroni *post hoc* test: (*B*) Quantification of total β-catenin and active β-catenin using flow cytometry analysis in CD133$^+$ cells treated for 2 h as indicated. Graphs show differences of median fluorescence intensity (Δ MFI) for cells stained with and without primary antibodies. $n = 5$. *P*-values computed using one-way ANOVA and the Bonferroni *post hoc* test. Data are representative for three experiments. (*C*) Localization of β-catenin (top) in CD133$^+$ cells stimulated for 2 h with transforming growth factor-β1 in the presence or absence of secreted Frizzled-related protein 2 as indicated. 4′,6-diamidino-2-phenylindole (bottom) visualizes nuclei. Bar = 20 μm. Data are representative for four experiments.

genes and inefficient formation of αSMA$^+$ myofibroblasts (*Figure 5D*). Inhibition of Wnt secretion with Wnt-C59 suppressed TGF-β1-mediated myofibroblast differentiation. However, addition of CHIR (that induces the canonical Wnt pathway) to TGF-β1/Wnt-C59-treated cells induced nuclear accumulation of β-catenin and effectively promoted myofibroblast differentiation (*Figure 5E–H*).

Wnt secretion is essential for development of post-inflammatory fibrosis and ventricular dysfunction in experimental autoimmune myocarditis

Next, we asked how the expression of Wnt ligands is regulated in the heart during EAM progression. We found that inflammatory

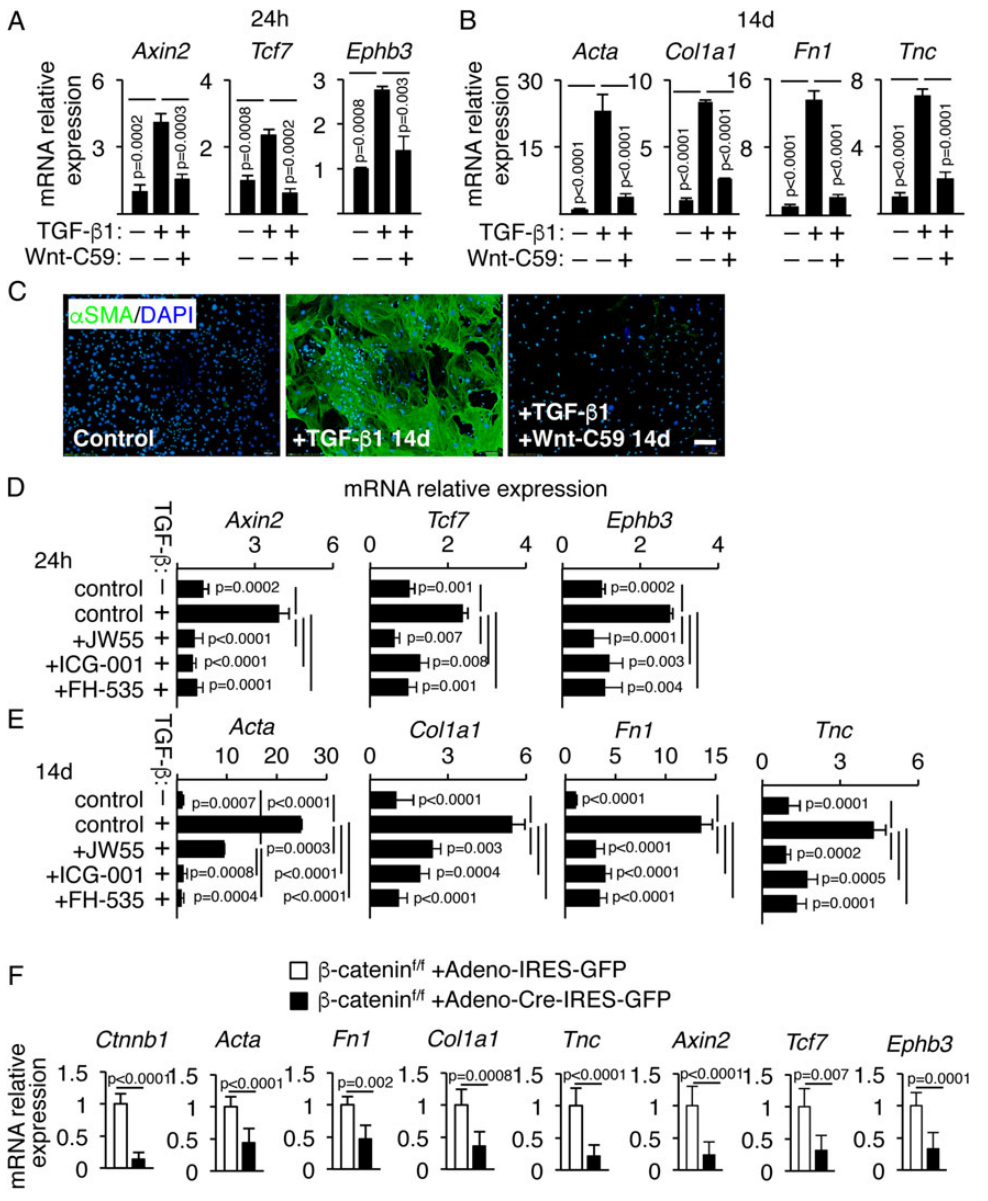

Figure 3 Wnt secretion and T-cell factor/β-catenin-mediated transcriptional activation are essential for transforming growth factor-β-mediated myocardial fibrosis. (*A*–*C*) Heart-inflammatory CD133^{+} cells were stimulated without or with transforming growth factor-β1 in the presence or absence of Wnt secretion blocker Wnt-C59. Shown are relative mRNA levels of the common Wnt target genes 24 h after stimulation (*A*, $n = 6$) and myofibroblast-specific genes (*B*, $n = 6$) and representative immunofluorescences of αSMA (*C*, green) both 14 Days after stimulation. 4′,6-diamidino-2-phenylindole (blue) visualizes nuclei. Bar = 20 μm. *P*-values computed using one-way ANOVA and the Bonferroni *post hoc* test. (*D* and *E*) CD133^{+} cells were treated without (control) or with transforming growth factor-β1 in the absence or presence of the activator of β-catenin degradation JW-55 (10 μM), the inhibitors of T-cell factor/β-catenin-mediated transcription FH-535 (10 ng/mL), or ICG-001 (10 μM), respectively. Shown are relative mRNA levels of the common Wnt target genes (*D*, $n = 4–8$) 24 h after stimulation and myofibroblast-specific genes (*E*, $n = 4–8$) 12 Days after stimulation. *P*-values computed using one-way ANOVA and the Bonferroni *post hoc* test. (*F*) β-Catenin$^{flox/flox}$ cardiac fibroblasts were infected by Adeno-Cre-IRES-GFP or by control particles (Adeno-IRES-GFP) and cultured for 10 Days in the presence of transforming growth factor-β1. Shown are relative mRNA levels of β-catenin (*Ctnnb1*), myofibroblast-specific genes, and Wnt target genes ($n = 5$). *P*-values computed using Student's *t*-test.

cells infiltrating the myocardium of αMyHC/CFA-immunized mice (d21) represented potent producers of Wnt-1 and Wnt-5a. In the post-inflammatory heart (d40), elevated Wnt-1 and Wnt-5a levels were maintained in fibrotic regions including adjacent cardiomyocytes (*Figure 6A*). Importantly, we found also high levels of Wnt-1 and Wnt-5a in human heart samples with active myocarditis, which was in contrast to unaffected cardiac tissue (*Figure 7*).

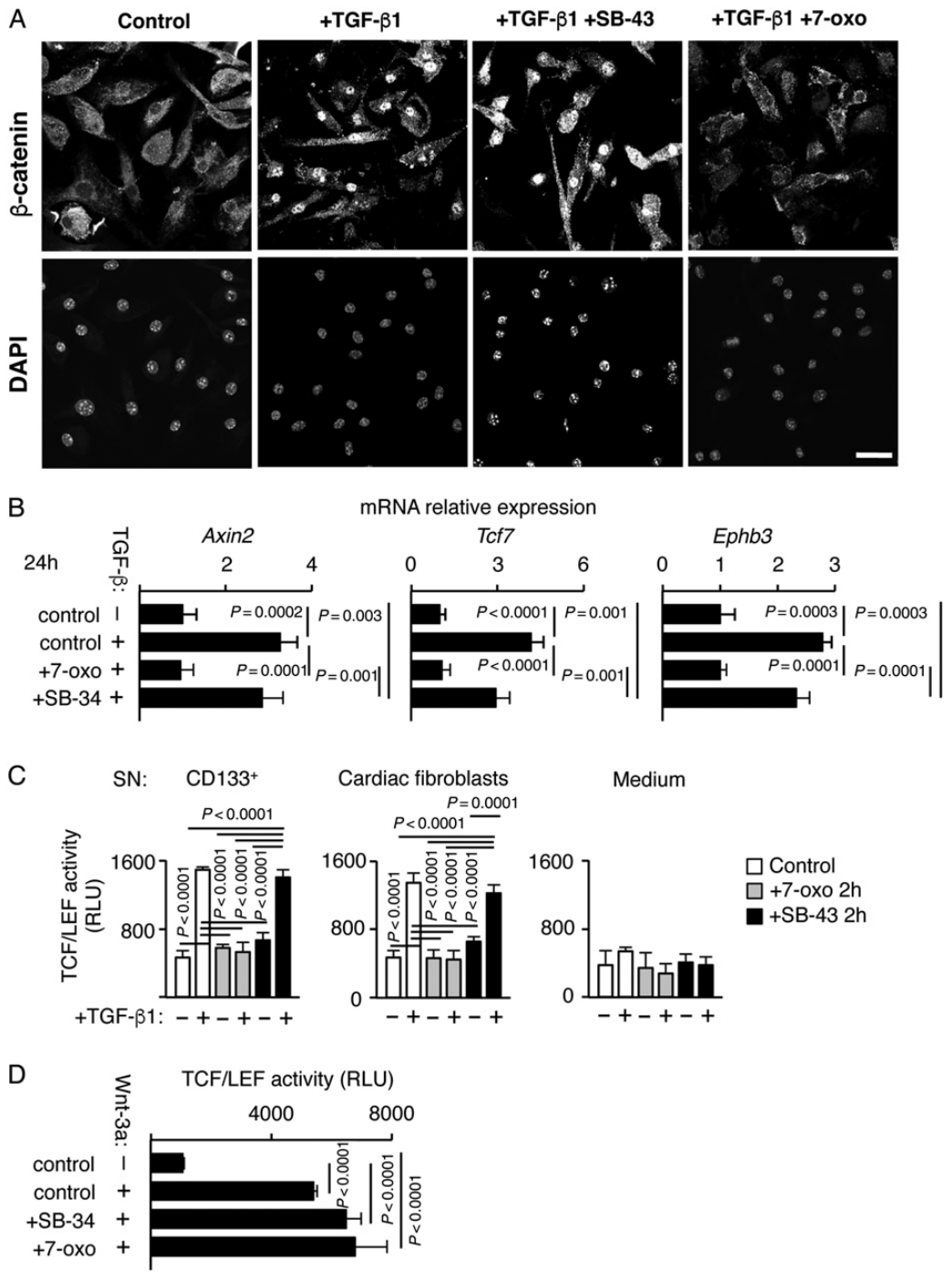

Figure 4 Transforming growth factor-β-induced Wnt secretion requires transforming growth factor-β-activated kinase 1 activation. (*A* and *B*) $CD133^+$ cells were stimulated with transforming growth factor-β alone or in the presence of Smad signalling blocker SB-431542 (SB-43) or transforming growth factor-β-activated kinase 1 inhibitor 5Z-7-oxo-zeaenol (7-oxo) as indicated. Localizations of β-catenin (top) 2 h after treatment are shown in (*A*). 4′,6-diamidino-2-phenylindole visualizes nuclei. Bar = 20 μm. Data are representative for three experiments. Relative mRNA levels of the common Wnt target genes 24 h after stimulation are shown in (*B*). $n = 6$. *P*-values computed using one-way ANOVA and the Bonferroni *post hoc* test. (*C*) Supernatant of $CD133^+$ cells or cardiac fibroblasts cultured without (control, white) or with transforming growth factor-β alone or in the presence of 7-oxo (grey) or SB-43 (black) for 2 h was used to stimulate cells expressing T-cell factor/lymphoid enhancer factor-luciferase reporter. Culture medium (medium) was used as a control. Graphs represent activity of T-cell factor/lymphoid enhancer factor promoter determined as luciferase activity in the reporter cells. Data are representative for three experiments. $n = 3–4$. *P*-values computed using one-way ANOVA and the Bonferroni *post hoc* test. (*D*) T-cell factor/lymphoid enhancer factor promoter activity of reporter cells treated without or with Wnt-3a-conditioned medium in the presence or absence SB-43 or 7-oxo as indicated. $n = 6$.

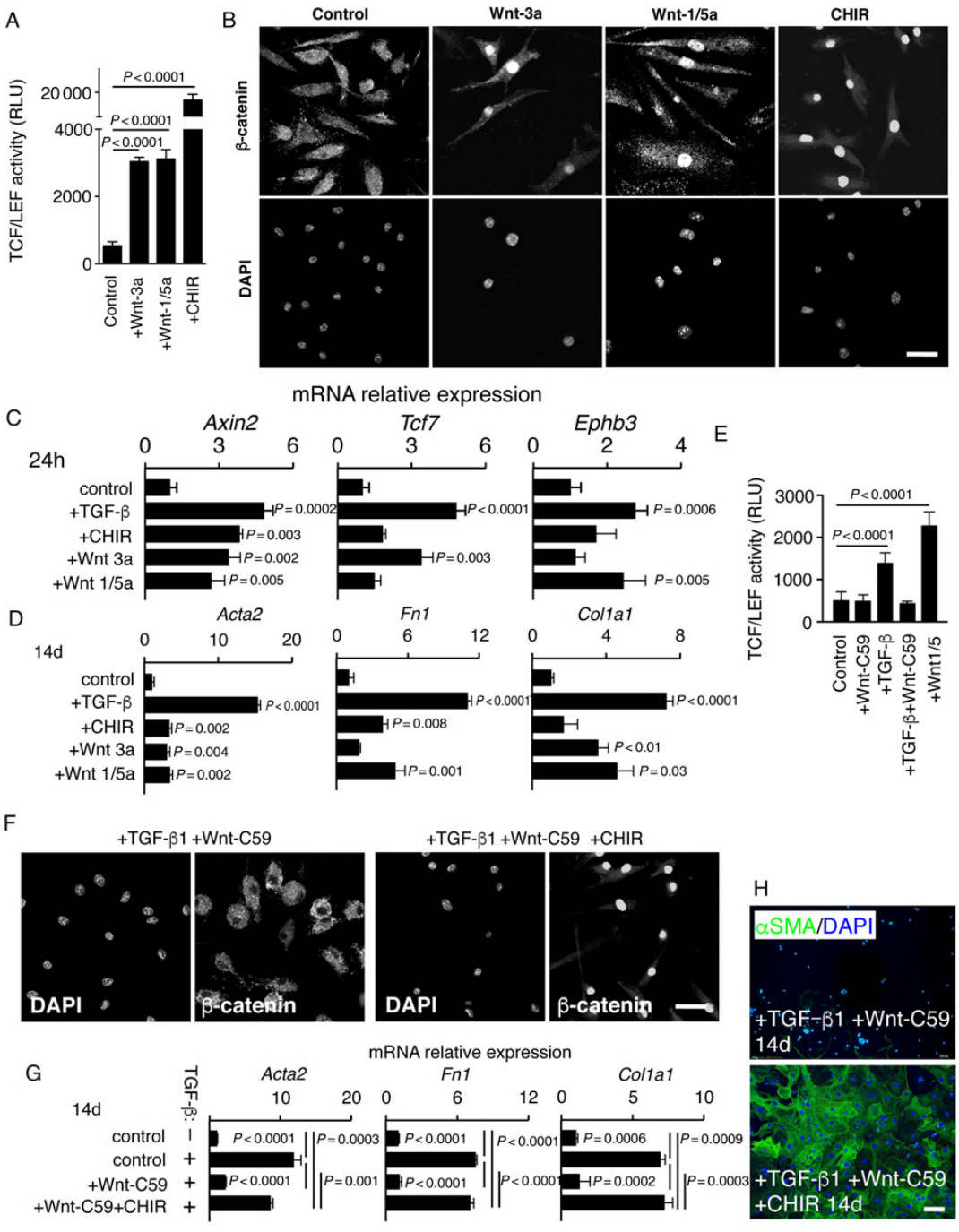

Figure 5 Activation of Wnt pathway in the absence of transforming growth factor-β1 fails to induce myofibroblast differentiation. (*A*) Activity of T-cell factor/lymphoid enhancer factor-luciferase reporter in reporter cells stimulated without or with GSK3 inhibitor—CHIR, Wnt-3a-conditioned medium, or Wnt-1/5-a recombinant proteins as indicated ($n = 4$). *P*-values computed using one-way ANOVA and the Bonferroni *post hoc* test. (*B*) Shown are localizations of β-catenin (top) 2 h after the indicated treatment of $CD133^+$ cells. 4′,6-diamidino-2-phenylindole (bottom) visualizes nuclei. Bar = 20 μm. (*C* and *D*) Analyses of Wnt-targeted genes expression (*C*, 24 h, $n = 6$), and myofibroblast-specific gene expression (*D*, 14 Days, $n = 6$) of heart-inflammatory $CD133^+$ cells treated as indicated. *P*-values vs. control, computed using one-way ANOVA and the Bonferroni *post hoc* test. (*E*) Activity of T-cell factor/lymphoid enhancer factor-luciferase reporter in reporter cells stimulated without or with transforming growth factor-β, Wnt-C59, or Wnt-1/5-a recombinant proteins as indicated ($n = 4$). *P*-values computed using one-way ANOVA and the Bonferroni *post hoc* test. (*F*–*H*) $CD133^+$ cells were treated with transforming growth factor-β1 and Wnt-C59 in the absence or presence of CHIR as indicated. (*F*) Representative localizations of β-catenin 2 h after indicated treatment. (*G*) Myofibroblast-specific genes expression ($n = 6$). (*H*) Immunofluorescences of αSMA 14 Days after stimulation induction. Data are representative for three experiments. Bars = 20 μm. *P*-values computed using the one-way ANOVA and the Bonferroni *post hoc* test.

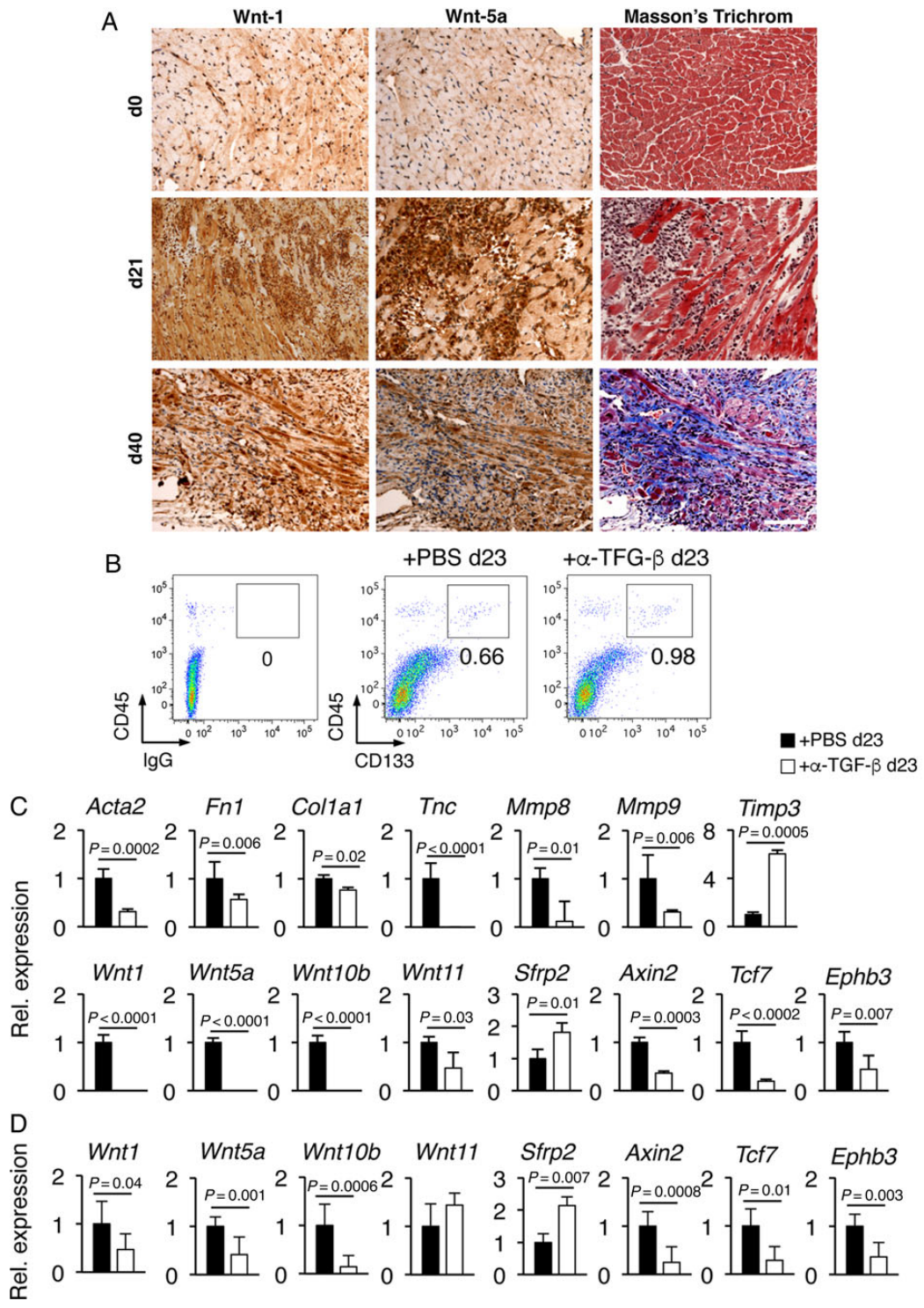

Figure 6 Transforming growth factor-β-mediated gene expression of heart-infiltrating progenitors *in vivo*. (*A*) BALB/c mice were immunized at Days 0 and 7 with αMyHC/CFA, and hearts were harvested at Days 0 (healthy), 21 (myocarditis), and 40 (fibrosis). Representative immunohistochemistry of heart sections using anti-Wnt-1, and -Wnt-5a antibodies and Masson's trichrome staining (tissue fibrosis, blue) at the indicated stages of EAM. Data are representative for four hearts. Bar = 100 μm. (*B–D*) BALB/c mice were immunized at Days 0 and 7 with αMyHC/CFA and injected either with PBS or anti-transforming growth factor-β neutralizing antibody between Days 17–22. (*B*) Representative flow cytometry analyses of heart-infiltrating ($CD45^+$) $CD133^+$ progenitors at Day 23 of EAM in both groups. Numbers indicate percentage of positive cells in the adjacent gates. Relative gene expression levels of FACSorted heart-infiltrating $CD45^+CD133^+$ cells (*C*) or of the whole hearts (*D*) from both groups. n.d, not detected, $n = 5$. *P*-values were computed using the two-tailed Student's *t*-test.

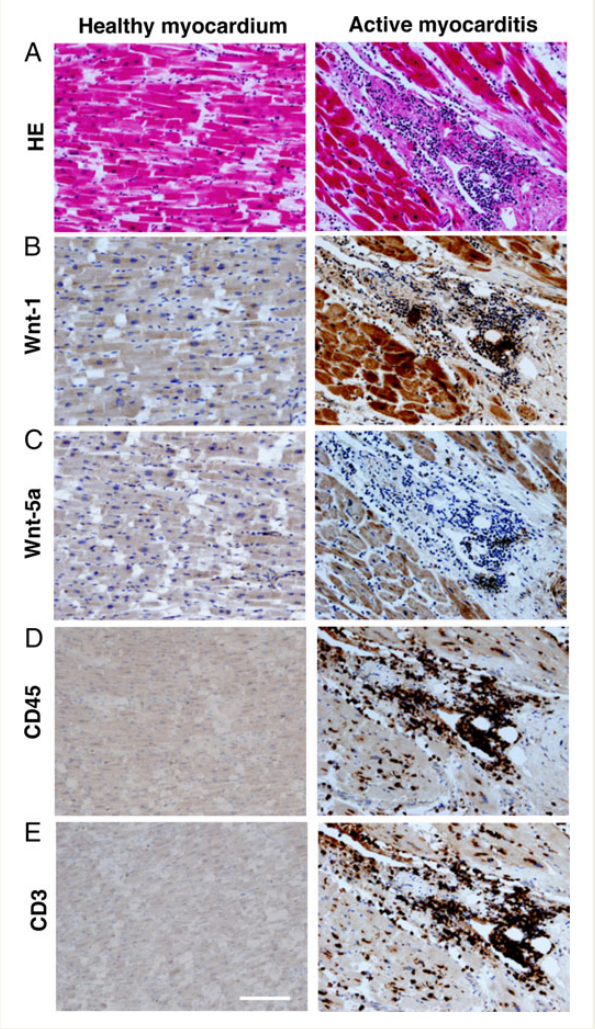

Figure 7 Wnt levels are up-regulated in the myocardium of patients with active myocarditis. Representative haematoxylin and eosin staining (*A*) and immunohistochemistry with anti-Wnt-1 (*B*), -Wnt-5a (*C*), -CD45 (*D*), and -CD3 (*E*) antibodies of sections of human unaffected hearts (left) and of human hearts with active myocarditis (right). Bar = 100 μm.

Injection of anti-TGF-β-blocking antibody during acute myocarditis between Days 17–22 into αMyHC/CFA-immunized mice prevents cardiac fibrosis in EAM.[3] Here, we showed that anti-TGF-β treatment suppressed expression of not only myofibroblast-specific genes, but also of *Wnts* and Wnt target genes in the whole myocardium, and in inflammatory $CD133^+$ cells, respectively (*Figure 6B–D*).

To address the relevance of Wnt pathway in EAM, we treated αMyHC/CFA-immunized mice with Wnt-C59, ICG-001, or sFRP2 between Days 17–29. In all treatments, mice were nearly completely protected from post-inflammatory fibrosis in EAM (*Figure 8A–D*). Cardiac fibrosis promotes ventricular dysfunction.[4] Accordingly, in αMyHC/CFA-immunized mice treated with sFRP2, the blunted cardiac fibrosis was associated with preserved left ventricular dimensions and improved systolic function as assessed by transthoracic echocardiography at Day 60 after disease induction (*Figure 8E*).

Discussion

We previously demonstrated that TGF-β signalling controls development of myocardial fibrosis in EAM.[3,4] Here, we further elucidated the underlying mechanisms and identified Wnt secretion followed by activation of the canonical Wnt cascade as a key downstream molecular pathway of TGF-β-mediated cardiac fibrogenesis. We observed this mechanism not only in heart inflammatory cells but also in mouse and human cardiac fibroblasts suggesting a common mechanism. We demonstrated that Wnt secretion during tissue remodelling is indeed crucial for the development of cardiac fibrosis in EAM model. Activation of β-catenin in response to TGF-β has been observed also in skin[15] and pulmonary fibroblasts.[22] Noteworthy, several lines of evidence suggest that the canonical Wnt can, in turn, positively regulate TGF-β signalling.[23]

Transforming growth factor-β signalling activates canonical Smad signalling and also a number of Smad-independent signalling cascades. Using 5Z-7-oxo-zeaenol, we found that inhibition of TAK1 pathway prevented TGF-β-mediated Wnt secretion. In fibrotic processes, TAK1-dependent TGF-β signalling has been mainly implicated in the activation of MAPK- and JNK-dependent pathways.[11] Interestingly, MAPK and JNK kinases are activated in non-canonical pathways triggered by Wnts. Moreover, recent findings demonstrated that TAK1 activation led to Wnt-dependent transcription.[24] Our results might imply that Wnts represent extracellular mediators of TAK1-dependent activation of its downstream signalling pathways.

Although the role of Wnts in iDCM has not been addressed so far, several reports pointed to an important role of Wnt pathway in pathophysiology of ischaemic heart disease. In rodent models of myocardial infarction, sFRPs were reported to reduce fibrosis and improve cardiac function.[25,26] Furthermore, blockade of Frizzled receptors with the specific antagonist improved post-infarction cardiac function and reduced collagen content.[27] Recent findings showed that activation of the canonical Wnt pathway by inhibiting GSK3β in cardiac fibroblasts promoted fibrogenesis in ischaemic hearts.[28] In contrast to ischaemic heart disorders, inflammatory cells represent a major cellular compartment in myocarditis. Our results clearly demonstrated that Wnt signalling is essential for myofibroblast formation from both, cardiac fibroblasts and heart-inflammatory progenitors ($CD133^+$ cells). It seems that irrespective of the cellular targets, the same downstream molecular mechanisms of TGF-β are involved in cardiac fibrogenesis.

Frizzled receptor stimulation with Wnt ligands can trigger gene transcription independently of β-catenin by activating planar cell polarity pathway and Wnt/Ca^{2+} pathway. Wnt-C59 inhibits Wnt secretion and sFRP2 scavenges extracellular Wnts, thus both eliminate bioavailable Wnt ligands from interaction with Frizzled receptors. Although both inhibitors blocked TGF-β-mediated differentiation, it remained unanswered, which specific Wnt pathway controlled myofibroblast formation. Inhibition of myofibroblast differentiation by blocking Wnt/β-catenin axis with JW55, FH-535, or ICG-001 clearly pointed to the critical role of the canonical Wnt pathway in this process. In contrast to tankyrase inhibitor JW55, which inhibits canonical Wnt signalling by stimulating β-catenin degradation, FH-535 and ICG-001 specifically inhibit TCF/

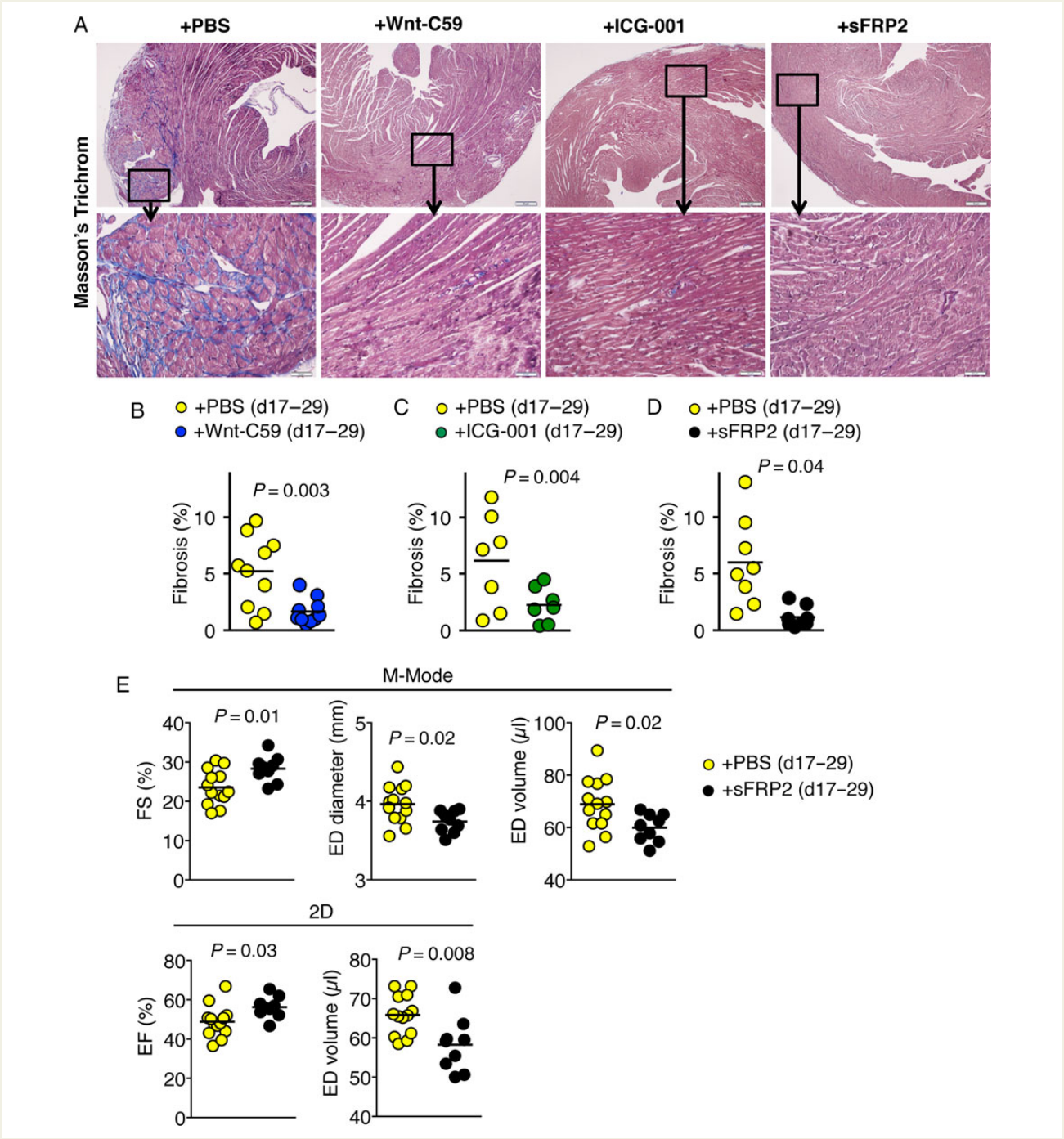

Figure 8 Wnt pathway promotes the development of post-inflammatory fibrosis and ventricular dysfunction in EAM. BALB/c mice were immunized at Days 0 and 7 with αMyHC/CFA and treated as indicated between Days 17–29. Bar = 50 μm. (*A*) Representative Masson's trichrome staining of left ventricle at Day 40 of EAM. (*B–D*) The graphs show quantification of fibrotic areas. (*E*) Echocardiographic parameters in αMyHC/CFA-immunized mice at Day 60 treated with secreted Frizzled-related protein 2 or PBS at Days 17–29. Percentage of fractional shortening and percentage of ejection fraction represent measurements for cardiac systolic function. End diastolic diameter and end diastolic volume show preserved left ventricular dimension after secreted Frizzled-related protein 2 treatment. *P*-values were computed using the unpaired two-tailed Student's *t*-test; line shows mean value.

β-catenin-mediated transcription. Our data suggest that expression of myofibroblast-specific genes is under control of the canonical Wnt pathway. In a mouse model of pulmonary fibrosis, ICG-001 was also reported to control transcription of myofibroblast-specific genes and to revert fibrosis,[21] whereas FH-535 suppressed transformation of retinal epithelial cells into myofibroblasts.[29]

In our study, we investigated TGF-β-induced Wnt secretion in resident and inflammatory precursor cells, which represent Wnt target cells in cardiac fibrosis. Given the fact that TGF-β-dependent myofibroblast differentiation is mediated by secreted Wnts, autocrine and paracrine signalling seem to play a key role in cardiac fibrosis *in vivo*. All these data suggest that cardiac fibrosis depends on bioavailability of extracellular Wnts, which potentially can be produced by most of cell types in the inflamed heart.

Taken together, TAK1-dependent Wnt protein secretion represents a novel downstream key mechanism of TGF-β-mediated cardiac fibrosis and remodelling in human and mouse myocarditis (Supplementary material online, *Figure S8*). Our data also show that inhibition of Wnt signalling improved cardiac function and thus indicate that pharmacological targeting of extracellular Wnts or the canonical Wnt pathway might represent a promising therapeutic option. Such anti-fibrotic therapy in inflammatory cardiomyopathy patients potentially could prevent disease progression. Therefore, inhibition of their *de novo* formation could eventually lead to resolution of the established fibrotic tissue and consequently to improvement of heart function in some patients. Furthermore, targeting of Wnts or Wnt pathway might represent an attractive alternative for anti-TGF-β treatment by offering a broader range of available pharmacological compounds with well-defined function. Future studies are, however, needed to address their specific anti-fibrotic activity and potential side effects.

Supplementary material

Supplementary material is available at *European Heart Journal* online.

Authors' contributions

P.B., B.M.-E., T.V., E.O., M.S., and G.K. performed statistical analysis. P.B., T.F.L., U.E., and G.K. handled funding and supervision. P.B., B.M.-E., T.V., E.O., M.S., S.B., and G.K. acquired the data. P.B., B.M.-E., T.V., E.O., M.S., U.E., and G.K. conceived and designed the research. P.B., B.M.-E., T.V., E.O., M.S., S.B., K.G., K.B., T.F.L., O.D., U.E., and G.K. drafted the manuscript. P.B., B.M.-E., T.V., E.O., M.S., S.B., K.G., K.B., T.F.L., O.D., U.E., and G.K. made critical revision of the manuscript for key intellectual content.

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Conflict of interest: disclosure statement of O.D. is available in the Supplementary material online.

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