Heart failure (HF) affects >4 million people in the United States and more than 400,000 new cases are diagnosed each year, and despite recent advances in the management of HF, the incidence and prevalence continues to rise.1 HF is the most common cause of hospitalization for patients >65 years. As the number of people aged >65 years in North America is expected to double over the next 25 years, the burden of HF in the elderly will markedly increase.2 The incidence of cardiovascular disease, as well as the rate of cardiovascular morbidity and mortality, increases exponentially in the elderly, suggesting that age per se is a major risk factor for cardiovascular diseases.3

Background—The Wnt/β-catenin signaling pathway plays a central role during cardiac development and has been implicated in cardiac remodeling and aging. However, the role of Wnt modulators in this process is unknown. In this study, we examined the role of the Wnt signaling inhibitor secreted frizzled-related protein-1 (sFRP-1) in aged wild-type and sFRP-1–deficient mice.

Methods and Results—sFRP-1 gene deletion mice were grossly normal with no difference in mortality but developed abnormal cardiac structure and dysfunction with progressive age. Ventricular dilation and hypertrophy in addition to deterioration of cardiac function and massive cardiac fibrosis, all features present in dilated cardiomyopathy, were observed in the aged sFRP-1 knockout mice. Loss of sFRP-1 led to increased expression of Wnt ligands (Wnt1, 3, 7b, and 16) and Wnt target genes (Wisp1 and Lef1) in aged hearts, which correlated with increased protein levels of β-catenin. Cardiac fibroblasts lacking endogenous sFRP-1 showed increased α-smooth muscle actin expression, higher cell proliferation rates, and increased collagen production consistent with the cardiac phenotype exhibited in aged sFRP-1 knockout mice. The clinical relevance of these findings was supported by the demonstration of decreased sFRP-1 gene expression and increased Wisp-1 levels in the left ventricles of patients with ischemic dilated cardiomyopathy and dilated cardiomyopathy.

Conclusions—This study identifies a novel role of sFRP-1 in age-related cardiac deterioration and fibrosis. Further exploration of this pathway will identify downstream molecules important in these processes and also suggest the potential use of Wnt signaling agents as therapeutic targets for age-related cardiovascular disorders in humans. (Circ Heart Fail. 2015;8:362-372. DOI: 10.1161/CIRCHEARTFAILURE.114.001274.)

Key Words: cardiomyopathy, dilated heart fibrosis Wnt signaling pathway

Heart failure (HF) affects >4 million people in the United States and more than 400,000 new cases are diagnosed each year, and despite recent advances in the management of HF, the incidence and prevalence continues to rise.1 HF is the most common cause of hospitalization for patients >65 years. As the number of people aged >65 years in North America is expected to double over the next 25 years, the burden of HF in the elderly will markedly increase.2 The incidence of cardiovascular disease, as well as the rate of cardiovascular morbidity and mortality, increases exponentially in the elderly, suggesting that age per se is a major risk factor for cardiovascular diseases.3

Cardiac aging is associated with left ventricular (LV) hypertrophy and fibrosis leading to diastolic dysfunction and HF.4 The aging heart is characterized by morphological and structural changes that lead to a functional decline and diminished ability to meet increased demand. Extensive evidence, derived from both clinical and experimental studies, suggests that the aging heart undergoes significant fibrotic remodeling. Increased fibrosis is a major determinant of myocardial stiffness, which together with impaired relaxation creates the basis for the development of diastolic dysfunction.5 A common pathological feature of aged humans and rodents is dilated cardiomyopathy (DCM).6–9 DCM is characterized by impaired myocardial contractility, ventricular dilation, and replacement fibrosis that is a major determinant of myocardial stiffness,
which together with impaired relaxation create the basis for the development of diastolic dysfunction.\textsuperscript{5,10}

Wnt-proteins are a family of secreted cysteine-rich glycosylated proteins that are implicated in a variety of modeling and remodeling processes, including cell proliferation, differentiation, apoptosis, cellular polarity,\textsuperscript{11–14} and cellular senescence.\textsuperscript{15,16} There are 19 human Wnt genes, and Wnt proteins have been grouped into 2 classes: the canonical and noncanonical Wnt pathways. Canonical Wnts stabilize intracellular β-catenin, allowing β-catenin to translocate into the nucleus and influence the association of transcription factors, such as Tcf/LEF, with transcriptional corepressors to activate Wnt-dependent target genes.\textsuperscript{12,17,18} Altered Wnt signaling is implicated in various disease processes, and several studies have suggested that this pathway is involved in cardiovascular remodeling,\textsuperscript{11,14} cardiac hypertrophy,\textsuperscript{16–24} and myocardial aging,\textsuperscript{16} but evidence for a distinct role of Wnt inhibitors is less prevalent. Modulation of Wnt signaling through delivery of secreted frizzled-related proteins (sFRPs) was shown to be beneficial in improving cardiac structure and function post myocardial infarction in rodents.\textsuperscript{25,26} In addition, sFRPs were shown to be important for apoptosis-mediated cell death and vascular cell proliferation in vitro and in vivo.\textsuperscript{27} Of the 5 sFRPs, sFRP-1 is present during both cardiac development and adult life and is abundantly expressed in the mouse and human heart.\textsuperscript{18} In this, study, the role of sFRP-1 on cardiovascular structure and function was examined in the aging heart. Through the use of sFRP-1 gene deletion mice, this study identifies an important novel function of sFRP-1 in age-related cardiac remodeling.

Methods

Animal Studies

The sFRP-1 knockout mouse line was constructed by replacing 1176 bp of exon 1 with a LacZ/MC1-Neo selection cassette as described previously.\textsuperscript{28,29} Mice were analyzed over a time period of 1 year. Before euthanasia, hemodynamic and echocardiographic analysis was performed, and post-euthanasia measurements of the wet heart weight to body weight and histological analysis were performed at various time points as described. All experiments were approved by the Institute for Animal Care and Use Committee at Columbia University.

Human Heart Samples

Human heart specimens were collected during the time of LV assist device implantation and explantation at Columbia University Medical Center (New York, NY) under institutional guidelines. Nondiseased myocardium was obtained from human hearts that could not be used for cardiac transplantation. The study protocol was approved by the Institutional Review Board at Columbia University Medical Center and written and informed consent was obtained from all subjects.

Echocardiographic Analysis

Anesthetized animals were placed on a mouse bed in a shallow left lateral decubitus position. Transthoracic echocardiography was performed as previously described\textsuperscript{30} using a pediatric broad band 6 to 15 MHz linear array ultrasound transducer (Agilent Sonas 5500; Agilent Technologies, Palo Alto, CA). The ultrasound beam depth was set at 2 cm and frame rate at 150 frames/s. The 2-dimensional parasternal short-axis views were obtained at the level of the LV papillary muscles.

Hemodynamic Analysis

In vivo intraventricular hemodynamic analysis was performed as previously described\textsuperscript{30} on sFRP-1 knockout and wild-type (WT) littermate mice at 6 and 12 months. A total of 21 mice (6 months: 4 WT and 6 knockout mice; 12 months: 4 WT and 7 knockout mice) were anesthetized with 2.5% Avertin at 0.015 mL/g body weight. A midline incision in the neck exposed the trachea, and the mouse was intubated intratracheally with a 22-gauge angiocatheter (Becton Dickinson, Franklin Lakes, NJ), which was secured with 3-0 silk suture (USCC, Princeton, NJ). Mice were mechanically ventilated with a 0.5 mL ambient air tidal volume at 110 breaths/min using a small animal respirator/ventilator (Columbus Instruments, Columbus, OH). A median sternotomy was performed, and the heart was exposed. Digitized intraventricular hemodynamic measurements were obtained via a LV apical puncture with a 26-gauge fluid-filled angiocatheter (Becton Dickinson) attached to a high-fidelity pressure transducer that was connected to an 8-channel chart recorder set at 1000 Hz (MacLab 8s; ADInstruments, Mountain View, CA). The data were stored on a computer for subsequent analysis (PowerMac 5300C; Apple Computer Inc, Cupertino, CA).

Histological Analysis

Hearts were arrested in diastole with PBS/20 mmol/L KCl solution and pressure fixed at 20 mm Hg with 10% neutral buffered formalin. Paraffin-embedded tissues were sectioned (6-μm thick) and stained with hematoxylin and eosin. Masson’s trichrome staining was performed to evaluate the myocardial collagen content and distribution.

Immunohistochemistry

Sections were incubated 45 minutes at 65°C and subsequently deparaffinized in xylene. Slides were then hydrated with 100% to 80% ethanol and washed with running tap water. After antigen retrieval, the sections were incubated in 3% hydrogen peroxide, washed and blocked for 30 minutes in 2% bovine serum albumin, and then probed overnight at 4°C with sFRP-1 (1:100; Abcam) or Connexin 43 (1:100; Cell Signaling) primary antibodies. Biotinylated antirabbit secondary antibody and horseradish peroxidase streptavidin enzyme conjugate were added, and sections were incubated for 1 hour at room temperature and subsequently washed with PBS. Signal was detected by incubation with AEC substrate (Histostain SP Rabbit Primary AEC Kit; ZYMED Laboratories) and counterstained with hematoxylin and further analyzed by light microscopy. For immunohistochemistry, sections were probed overnight at 4°C with β-catenin primary antibody (1:100 dilution). Subsequently sections were incubated with fluorescent AlexaFlour antirabbit 488 secondary antibody (1:500 dilution); β-catenin and DAPI (1:1000 dilution) fluorescent signals were visualized by fluorescence microscopy.

Pathway-Focused Gene Expression Profiling Using Real-Time Polymerase Chain Reaction

Total RNA was isolated from tissue heart homogenates from sFRP-1 WT (n=3) versus knockout (n=3) mice at 1 year with QIAGEN RNeasy Mini Kit following the manufacturer’s protocols. Quantity and quality of extracted RNA were determined by Nano Drop (Fisher). cDNA was prepared using RT2 Profiler First Strand Kit (SABiosciences) according to protocol, which was then used to perform Wnt signaling mouse polymerase chain reaction (PCR) array with RT2 Profiler PCR Array system (SABiosciences) according to manufacturer’s protocol. Real-time PCR reaction was performed (Applied Biosystems) and further analyzed on the SABiosciences Web site according to the provided instructions.

mRNA Analysis by Real-Time PCR

Total RNA was isolated from tissue heart homogenates from sFRP-1 WT (n=5) versus knockout (n=5) mice at 3, 6, and 12 months with QIAGEN RNeasy Mini Kit with on-column DNase digestion following the manufacturer’s protocols. Quantity and quality of extracted RNA...
were determined using Nano Drop (Fisher), cDNA was prepared using High Capacity cDNA Reverse Transcription Kit (ABI) according to protocol, which was then used to perform real-time PCR reaction with Taqman Gene expression master (ABI). Taqman probes were used against Wnt1, Wnt3, Wnt5a, Kremen1, Wnt16, Lef1, β-catenin, and Wisp1 (all from ABI), and the results are presented as fold of regulation when compared with the housekeeping control gene β-Actin.

Western Blot Analysis
Protein samples were prepared by homogenizing whole hearts extracted from sFRP-1 knockout mice and WT littermates at 1 year. Each 50 mg of heart tissue sample was lysed in in 500 μL of protein RIPA lysis buffer (Santa Cruz Biotechnology) and centrifuged. Tissue lysates were equalized and separated by electrophoresis using a 10% polyacrylamide gel, then transferred for 1 hour to nitrocellulose membranes (Bio-Rad). Membranes were blocked 1 hour in nonfat milk (5%). After blocking, membranes were probed with primary antibodies in dilutions as follows: β-catenin, (1:1000; Cell Signaling Technology), α-smooth muscle actin (α-SMA; 1:1000; Abcam), and β-actin (1:1000; Santa Cruz Biotechnology). Primary antibody binding was detected by HRP-conjugated secondary antibodies and enhanced chemiluminescence reagents (Pierce).

Primary Cardiac Fibroblasts Isolation and Culture
Cardiac fibroblasts were isolated by enzymatic digestion using collagenase (1 mg/mL; Roche Applied Science, Indianapolis, IN) in Hank’s Balanced Salt Solution, including calcium and magnesium. Briefly, hearts were washed with PBS and dissected free of vessels and atria, washed in isolation media (Dulbecco’s modified Eagle’s medium, 20% fetal bovine serum, 10% streptomycin/penicillin). The hearts were transferred to 50 mL Falcon tubes quickly minced into small pieces. Hearts were washed with PBS and dissected free of vessels and atria, washed in isolation media (Dulbecco’s modified Eagle’s medium, 20% fetal bovine serum, 10% streptomycin/penicillin). The hearts were transferred to 50 mL Falcon tubes quickly minced into small pieces and incubated at 37°C for 25 minutes under vigorous stirring. Using a 30 mL syringe attached firmly to the 6 T75) and cultured as described above without FBS. After chunks settled, the suspension was passed through the strainer (70 μm pore size) into a 50 mL tube. The cell suspension was spun down at 400g for 8 minutes at 4°C and plated on a T75 tissue-culture flask in culture medium (DMEM/Glutamax, 10% FBS, 5% streptomycin/penicillin) and maintained in a humidified atmosphere of 5% CO₂ at 37°C. After overnight incubation, nonadherent cells were removed, and adherent cells were further cultivated.

Collagen Assay
Primary cardiac fibroblasts isolated from the hearts of 3-month-old sFRP-1 knockout and WT littermate mice were plated in equal amounts (300,000 cells per T75) and cultured as described above without FBS. Culture media was collected from sFRP-1 knockout and WT cardiac fibroblasts at passage 2. To quantify collagen production in the media of sFRP-1 knockout and WT cardiac fibroblasts, a Sircol Collagen assay was performed and used according to the manufacturer’s protocol.

Proliferation Assay
For assessment of cellular proliferation, mouse cardiac fibroblasts from passage 2 were seeded in 48-well plates. Primary cells cultured in no serum conditions were pulsed with 1.5 μCi per well [³H] thymidine (Amersham Pharmacia Biotech Ltd). The [³H] thymidine content of cell lysates was determined by scintillation counting as described previously.

Statistical Analysis
Data are expressed as means±SEM. We tested for the equal variances before all our t test and ANOVA with f test (for t test) or Levine test (for ANOVA). Two-group analysis was performed by Student t test with equal variances in all cases except in the Wnt5a comparison, where variances were unequal. In the case of unequal variance, 2-group analysis was performed by Student t test with unequal variances. For the comparison of sFRP levels in the mouse and human heart, the sample sizes were unequal and the differences between 3 groups were compared by ANOVA followed by Tukey-Kramer post-test for unequal sample size. Two-sided P values <0.05 were considered statistically significant. Statistical analysis for the Wnt-gene array was performed in Sabiosciences Web-based software automatically after input of the raw data (http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php). Western blot quantification statistics were performed with the use of Kodak software. All the graphs were generated in Graph Pad Prism 6 software.

Results
Pattern of sFRP-1 Expression in the Myocardium
sFRP-1 was abundantly present in the mouse hearts at 3 (Figure 1A), 6 and 12 months in the WT hearts as detected by immunohistochemistry. In addition, mRNA expression of sFRP-1 confirmed the abundance of sFRP-1 in the 3, 6, and 12-month-old WT hearts (Figure 1B) and exhibited the age-related increase in expression.

Loss of sFRP-1 Increases Cardiac Size
The cardiac phenotype of sFRP-1 knockout mice compared with WT littermate controls was characterized over the course

![Figure 1](http://circheartfailure.ahajournals.org/)

**Figure 1.** Expression of secreted frizzled-related protein-1 (sFRP-1) in the normal heart during aging. **A**, Immunohistochemical staining of sFRP-1 in heart sections of sFRP-1 wild-type (WT) mice aged 3 months (a and b) with IgG controls (c and d). Sections were counterstained with hematoxylin. Magnification is ×40. Scale bars represent length of 100 μm. **B**, mRNA expression of sFRP-1 in the hearts of sFRP-1 WT mice at 3, 6 (n=3), and 12 months (n=4) analyzed by real-time polymerase chain reaction with β-actin used as a housekeeping gene control. Data are presented as fold of regulation and statistically significant differences are denoted by *P<0.05.
of 1 year (Figure 2). The sFRP-1 KO mice appeared normal at birth and at 3 months. Although there was no significant difference in survival at 1 year, sFRP-1 knockout mice exhibited a marked increase in heart size (Figure 2A) when compared with WT mice. At 6 months, sFRP-1 knockout mice developed cardiac hypertrophy when compared with WT littermates as shown by an increased heart weight/body weight ratio, which further progressed at 1 year (Figure 2B; n=5).

**Loss of sFRP-1 Leads to Cardiac Remodeling With Impaired Cardiac Function**

Although little or no changes were detected in heart function at 3 and 6 months, at 1-year time point sFRP-1 knockout mice developed significant changes in cardiac structure and function. Histological analysis using hematoxylin and eosin staining of the sFRP-1 knockout hearts revealed mild hypertrophy and ventricular dilation compared with WT hearts (Figure 3A) at 1 year. Echocardiographic analysis of 1-year-old littermates confirmed that the sFRP-1 knockout mice developed LV chamber dilation compared with WT mice (LV end-diastolic dimension Figure 3B) with an increase in LV posterior wall thickness (PW; Figure 3C) above the dilatation seen already at 6 months (Figure 3B). The increase in LV wall thickness was sustained at 12 months (Figure 3C). A significant decrease in fractional shortening (FS; Figure 3D) represents a decrease in the LV contractility in the sFRP-1 knockout mice at 1 year. Furthermore, hemodynamic analysis of sFRP-1 knockout hearts at 1 year revealed significant worsening of LV systolic functional parameters (Figure 4A and 4B), such as LV systolic pressure (Figure 4A) and LV dP/dT max (Figure 4B). In addition, the diastolic functional parameters (Figure 4C and 4D) of sFRP-1 knockout mice were decreased, such as LV end diastolic pressure (Figure 4C) and dP/dT peak negative (dP/dT min; Figure 4D). In addition, supporting the deterioration of cardiac function in aged sFRP-1 knockout mice, we observed age-dependent downregulation of connexin 43 (Cx43; Figure 4E). Gap junction channels composed of Cx43 are essential for normal heart formation and function, and this age-dependent decrease in Cx43 in the sFRP-1 KO heart is in line with the cardiac deterioration observed in the aged sFRP-1 knockout mice. The heart rates for the animals during hemodynamic and echocardiographic analysis were not...
Loss of sFRP-1 Leads to Abnormal Wnt/β-Catenin Signaling in the Aging Heart

To determine whether the canonical Wnt signaling pathway mediated through β-catenin was disrupted in sFRP-1 knockout mice, mRNA abundance of Wnt ligands was examined in aged sFRP-1 knockout hearts (Figure 5). Interestingly, the main ligands responsible for inducing the canonical Wnt signaling pathway and targets of sFRP-1 for inhibition are significantly induced, such as Wnt1, Wnt3, and Wnt7b (Figure 5A–5C). In addition, we observed suppression of Kremen1 (Wnt signaling antagonist; Figure 5D) and Wnt5a (noncanonical Wnt ligand; Figure 5E). Interestingly Wnt16, the aging and senescence marker, was upregulated in aged sFRP-1 knockout hearts (Figure 5F).

Next, the expression level of β-catenin in sFRP-1 knockout hearts at 6 and 12 months (Figure 6A and 6B) was determined. As expected, a significant increase in the protein level of β-catenin in total heart homogenates of sFRP-1 knockout hearts at 6 months (Figure 6A) was sustained until 12 months (Figure 6B) when compared with the hearts of their WT littermates. To further localize β-catenin protein, an immunohistofluorescent study was performed. We observed a uniform increase of β-catenin throughout the myocardium with increased localization to the cell membrane in the myocardium of the sFRP-1 knockout hearts at 6 months, as well as at 12 months (Figure 6C) when compared with their WT littermates, consistent with Western blot analysis.

We did not detect significant changes in mRNA expression of β-catenin at 12 months (Figure 6D) but did demonstrate the upregulation of Lef1 in the sFRP-1 knockout hearts at 12 months (Figure 6E), which is a prominent target of Wnt/β-catenin signaling pathway, all together suggesting that the canonical Wnt signaling pathway is operative in aged sFRP-1 knockout hearts at 12 months. Additionally, the accumulation of β-catenin in the cell membrane of intercalated disks of the myocardium of the sFRP-1 knockout hearts at 1 year is likely to contribute to the increase in wall stiffness in the sFRP-1 knockout hearts.

Loss of sFRP-1 Leads to Increased Cardiac Fibrosis in Aged Mice

Trichrome staining was performed on the histological sections of the hearts at 6 and 12 months time point. A slight increase in development of fibrosis was observed in the sFRP-1 knockout mice at 6 months (Figure 7A); however, by 12 months, a massive increase in interstitial and perivascular fibrosis was noted in the sFRP-1 knockout hearts compared with hearts from WT littermates (Figure 7A). These results together with
the above-mentioned hemodynamic observations suggest that sFRP-1 plays an important role in maintaining normal cardiovascular structure and function during aging, and that the loss of sFRP-1 leads to the development of fibrotic cardiac remodeling with deterioration of cardiac function in the aged mice.

The increase in fibrosis development in aged sFRP-1 knockout hearts coincides with the enrichment of the sFRP-1 knockout heart with canonical Wnt ligands, such as Wnt1. The main profibrotic Wnt1 target gene, Wnt1-inducible signaling protein 1 (Wisp1), which has previously been shown to be involved in fibrosis development in the lung and heart is age-dependently upregulated in the sFRP-1 knockout hearts at 6 and 12 months when compared with their WT littermates (Figure 7B). In addition to increased fibrosis in the hearts of sFRP-1 knockout mice accompanied by significant upregulation of Wisp1, the treatment of cardiac fibroblast for 24 hours with Wisp1 was able to induce collagen 1 and 3 expression in the primary cardiac fibroblast isolated from WT mice (Figure 7C). We hypothesized that the loss of sFRP-1 in the heart regulated collagen production, as well as the activation of cardiac fibroblasts. To test this hypothesis, primary murine cardiac fibroblasts were isolated from sFRP-1 knockout and WT mice, and collagen production and proliferation capacity were measured. As expected, sFRP-1 was completely absent in the sFRP-1 knockout cardiac fibroblasts (Figure 7D). Cardiac fibroblasts isolated from sFRP-1 knockout hearts displayed a significant increase in collagen production (Figure 7E) and proliferation capacity (Figure 7F). Most interestingly, a significant increase in protein expression of α-SMA was observed (Figure 7G and 7H) in cardiac fibroblasts isolated from sFRP-1 knockout hearts when compared with WT cardiac fibroblasts.

**Loss of sFRP-1 and Increased Wisp1 in Human Cardiomyopathy**

To evaluate the relevance of the proposed hypothesis in human diseased hearts, gene expression analysis was performed on LVs from healthy (n=8), DCM (n=8), and ischemic dilated cardiomyopathy (n=7) patients. Interestingly, we observed that sFRP-1 gene expression was significantly suppressed in LVs of cardiomyopathic patients, suggesting that the loss of sFRP-1 correlates with cardiomyopathic changes also in the human hearts (Figure 8A). In addition, induction of Wisp1 as shown in the myocardium of sFRP-1–deficient mice at 12 months was also found in human cardiomyopathic hearts (Figure 8B), suggesting that Wnt signaling is operative in human cardiac failure suggesting that suppression of sFRP-1 might be crucial player in this process.

**Discussion**

This study identifies a novel role of sFRP-1, an endogenous inhibitor of the Wnt signaling pathway in an animal model of cardiac failure and human cardiomyopathies demonstrating that patients with this disease exhibit decreased levels of sFRP in their heart. Additionally, the experiments directly demonstrate that loss of sFRP-1 results in cardiac dilatation, myocardial fibrosis with deterioration of cardiac function, and increased LV end-diastolic pressure characteristic of...
DCM. These structural changes are accompanied by an age-dependent increase in the expression of the canonical Wnt ligands, β-catenin protein levels, and mRNA levels of Wnt target genes, such as Lef1, age-related Wnt16, and profibrotic Wisp1. In addition, the loss of endogenous sFRP-1 in cardiac fibroblasts led to increased α-SMA expression and collagen production when compared with WT cardiac fibroblasts. Taken together, these data suggest that the loss of sFRP-1 and the subsequent increase in canonical Wnt signaling leads to an age-dependent impairment of cardiac function and progressive cardiac fibrosis.

Global overexpression of sFRP-1 post myocardial infarction improves cardiac function and reduces infarct size and rupture. However, studies using a cardiac conditional transgenic mouse expressing sFRP-1 specifically in cardiomyocytes reported a larger infarct size and diminished cardiac function in transgenic mice compared with WT cardiac fibroblasts. Taken together, these data suggest that the loss of sFRP-1 and the subsequent increase in canonical Wnt signaling leads to an age-dependent impairment of cardiac function and progressive cardiac fibrosis.

Figure 6. Age-related increase in canonical Wnt signaling in secreted frizzled-related protein-1 (sFRP) knockout (KO) hearts. A and B, Protein expression of β-catenin analyzed by Western blotting in control sFRP-1 WT hearts vs sFRP-1 KO hearts at 6 months (A) and 12 months (B), β-Actin was used as a housekeeping loading control. C, Representative immunofluorescent staining for β-catenin merged with DAPI in the myocardium of sFRP-1 KO compared with WT hearts at 6 and 12 months. Magnification is ×20. Scale bars represent length of 100 μm. D and E, mRNA analysis of β-catenin and Lef1 in sFRP-1 KO vs WT hearts at 6 and 12 months of age (n=5). β-actin was used as a housekeeping control, data are presented as fold of regulation. Significant differences were denoted by *P<0.05 vs control littermate hearts. All values were expressed as means±SEM.

sFRP-1 led first to mild cardiac hypertrophy and subsequently to cardiac fibrosis and deterioration of heart function as the mice aged. The disruption of cardiac function was associated with a decrease in Cx43, which is a functional gap junction channel protein that positively correlates with normal cardiac function. A downregulation of cardiac Cx43 has been identified during normal cardiac development, cardiac aging, and in several cardiac disorders in mice and humans, such as DCM or ischemic cardiomyopathy. The fact that Cx43 is downregulated in the hearts of aged sFRP-1 knockout mice resembles human DCM.

The modulation of the Wnt/β-catenin signaling pathway is known to play a role in cardiac development and remodeling. The function of the downstream target of canonical Wnt signaling, β-catenin, depends on its subcellular localization. Membrane-bound β-catenin maintains tissue architecture and cell polarity at adherens junctions by linking the cadherin
In the presence of canonical Wnt signaling ligands, β-catenin is not targeted for proteosomal degradation and accumulates within the cell. Cytoplasmic β-catenin translocates into the nucleus and forms a complex with transcription factors of the Tcf/Lef family and regulates expression of specific canonical Wnt signaling target genes, such as Lef1, axin2, Wisp1, and also amplifies the Wnt signal by upregulating canonical Wnt ligands, such as Wnt1. Both positive and negative Wnt signaling is observed during cardiac remodeling of the adult heart because of stress or injury; Wnt ligands, as well as sFRPs inhibiting Wnts, are expressed. Both β-catenin loss and gain of function mutations have been characterized, demonstrating that inhibition of canonical Wnt signaling by β-catenin loss attenuates LV remodeling and improves ventricular function. Conversely, increased mortality, severely impaired fractional shortening, and a phenotype of DCM were observed in β-catenin gain of function studies. We demonstrate that indeed the phenotype observed in the sFRP-1 knockout mice was accompanied by an increase in canonical Wnt ligand expression, such as Wnt1, 3, and 7b, known to promote β-catenin–dependent transcriptional activity that was in line with age-related cardiac function impairment. In addition, a decrease in the noncanonical Wnt5a and

Figure 7. Development of cardiac fibrosis in myocardium of aged secreted frizzled-related protein-1 (sFRP-1) knockout (KO) hearts. A, Representative pictures for Masson staining in the heart myocardium of sFRP-1 KO at 6 months or 12 months old mice vs wild-type (WT) littermates. Magnification is ×40 and ×20 (inset). Scale bars length is 100 μm. B, Wisp1 mRNA expression by Taqman real-time polymerase chain reaction in 6 and 12 months old sFRP-1 KO hearts in vs WT hearts (n=5). C, Expression of collagens (1 and 3) in primary cardiac fibroblasts, isolated from sFRP-1 WT hearts (n=3). D, mRNA expression of sFRP-1 in cardiac fibroblast isolated from sFRP-1 WT and KO mice (n=3). E, Protein concentration of collagen in the media of sFRP-1 WT and KO fibroblasts measured by Sircol assay (n=5). F, Proliferation capacity of cardiac fibroblasts isolated from sFRP KO mice vs WT mice measured by [3H]-Thymidine uptake (n=6). Values are expressed in counts per minute (cpm). α-Smooth muscle actin (α-SMA) protein level in primary cardiac fibroblast isolated from sFRP-1 WT and KO mice analyzed by Western blot (G) and subsequently quantified by densitometry (H). β-actin was used as a housekeeping control, data are presented as fold of regulation. Significant differences were denoted by **P<0.01 or ***P<0.001 vs control littermate hearts. All values were expressed as means±SEM.
The upregulation of canonical Wnt ligands correlates with the age-dependent increase in total β-catenin protein levels in heart homogenates of the sFRP-1 knockout mice. Most importantly Lef1, a transcriptional target of the Wnt/β-catenin pathway, was upregulated, suggesting that the canonical Wnt pathway was operational in the aged hearts of the sFRP-1 knockout mice. Although β-catenin accumulation in the cell membrane of cardiomyopathic hearts correlates with increased myocardial wall stiffness and LV end-diastolic pressure, it is likely to be playing an important role in the maintenance of the cardiac structure and function as seen in aged sFRP-1 knockout mice.

Progressive cardiac fibrosis as we saw in the sFRP-1 knockout mice is a hallmark of aging and associated with increased deposition of matrix proteins in the myocardium. Age-dependent accumulation of collagen in the heart leads to a progressive increase in ventricular stiffness and impaired diastolic function seen in DCM. The Wnt signaling pathway was previously connected with fibrosis during cardiac remodeling, but its potential role in age-related cardiac fibrosis was previously unknown. Interestingly, RT-PCR–based analysis performed on heart homogenates of sFRP-1 knockout mice revealed upregulation of several Wnt target genes crucial for fibrosis, as represented by an increase in α-SMA and collagen production. In addition, the fact that we observed downregulation of sFRP-1 and upregulation of Wisp1 in human DCM and ischemic cardiomyopathy suggests that the above-described activation of Wnt signaling by the loss of sFRP-1 might be a crucial factor for the development or acceleration of human cardiac disorders, such as DCM or ischemic cardiomyopathy.

This study suggests that the induction of the Wnt pathway is a potentially crucial pathway in the development of cardiomyopathy during aging with sFRP-1 being a critical factor in maintaining normal cardiovascular structure and function during this process. The cardiac phenotype represented in the mice lacking sFRP-1 provides us with novel insight into age-related cardiac function maintenance because still little is known of the molecular mechanisms prevent fibrosis and cardiac dysfunction in response to cumulative hemodynamic stress related to aging. Additional studies elucidating the role of sFRP-1 in the heart and its regulation of the Wnt signaling pathway could help to understand the molecular mechanisms of cardiomyopathy and may support the potential clinical applications of new strategies to protect the heart during aging and disease.

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Disclosures

None.

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**CLINICAL PERSPECTIVE**

This study identifies a novel role for secreted frizzled-related protein-1 (sFRP-1) in age-related cardiac deterioration and fibrosis. Through the studies described in this article, we have gained significant insight into the role of Wnt signaling in cardiac function and its role in the development of dilated cardiomyopathy in mice. In this study, we demonstrated that loss of sFRP-1 lead to an increase in cardiac fibrosis and dilated cardiomyopathy like phenotype by an age-dependent upregulation of Wnt/β-catenin signaling pathway. In addition, loss of sFRP-1 in cardiac fibroblasts leads to higher susceptibility of these cells for activation as represented by increased α-smooth muscle actin protein levels, collagen production, and proliferation. In addition, we demonstrated that in sFRP-1 and main Wnt target gene, Wisp-1 are dysregulated in human dilated and ischemic cardiomyopathy suggesting that loss of sFRP-1 might lead to development of human cardiomyopathies. Thus, this work provides important new insights into the poorly understood role of Wnt signaling in cardiac fibrosis and loss of cardiac function in the cardiomyopathic heart.
Loss of Secreted Frizzled-Related Protein-1 Leads to Deterioration of Cardiac Function in Mice and Plays a Role in Human Cardiomyopathy

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