Secreted Frizzled-related protein 2 as a target in antifibrotic therapeutic intervention

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Mastri M, Shah Z, Hsieh K, Wang X, Wooldridge B, Martin S, Suzuki G, Lee T. Secreted Frizzled-related protein 2 as a target in antifibrotic therapeutic intervention. Am J Physiol Cell Physiol 306: C531–C539, 2014. First published December 11, 2013; doi:10.1152/ajpcell.00238.2013.—Progressive fibrosis is a pathological hallmark of many chronic diseases responsible for organ failure. Although there is currently no therapy on the market that specifically targets fibrosis, the dynamic fibrogenic process is known to be regulated by multiple soluble mediators that may be therapeutically intervened. The failing hamster heart exhibits marked fibrosis and increased expression of secreted Frizzled-related protein 2 (sFRP2) amenable to reversal by mesenchymal stem cell (MSC) therapy. Given the previous demonstration that sFRP2-null mice subjected to myocardial infarction exhibited reduced fibrosis and improved function, we tested whether antibody-based sFRP2 blockade might counteract the fibrogenic pathway and repair cardiac injury. Cardiomyopathic hamsters were injected intraperitoneally twice a week each with 20 μg of sFRP2 antibody. Echocardiography, histology, and biochemical analyses were performed after 1 mo. sFRP2 antibody increased left ventricular ejection fraction from 40 ± 1.2 to 49 ± 6.5%, whereas saline and IgG control exhibited a further decline to 37 ± 0.9 and 31 ± 3.2%, respectively. Functional improvement is associated with a ~50% reduction in myocardial fibrosis, ~65% decrease in apoptosis, and ~75% increase in wall thickness. Consistent with attenuated fibrosis, both MSC therapy and sFRP2 antibody administration significantly increased the activity of myocardial matrix metalloproteinase-2. Gene expression analysis of the hamster heart and cultured fibroblasts identified Axin2 as a downstream target, the expression of which was activated by sFRP2 but inhibited by therapeutic intervention. sFRP2 blockade also increased myocardial levels of VEGF and hepatocyte growth factor (HGF) along with increased angiogenesis. These findings highlight the pathogenic effect of dysregulated sFRP2, which may be specifically targeted for antifibrotic therapy.

sFRP2; fibrosis; MMP; VEGF; heart failure

A common feature of most if not all chronic inflammatory diseases is excessive production and deposition of extracellular matrix (ECM) components, leading to scar formation, distorted tissue architecture, and ultimately organ failure (8, 64). Central to the fibrogenic cascade is persistent tissue injury, which activates both the innate and adaptive immune systems and perpetuates pathogenic ECM remodeling. Although there is currently no therapy on the market that specifically treats the underlying cause of fibrosis, the highly dynamic fibrogenic process is known to be regulated by multiple soluble mediators, among which transforming growth factor (TGF)-β, connective tissue growth factor (CTGF), endothelin-1, and angiotensin II have received much attention. Drug- and antibody-directed targeting of these fibrogenic mediators are currently under development as potential therapies for pathological fibrosis, which represents one of the largest groups of diseases (32, 64).

The diseased heart exhibits many pathological phenotypes, including fibrosis, which may be selectively targeted for therapeutic intervention (14). Myocardial infarction (MI) results in loss of cardiomyocytes, culminating in adverse cardiac remodeling characterized by myocyte hypertrophy and fibrosis, which in turn affect myocardial elasticity, contractile function, and flow reserve (56, 61). These architectural alterations are mediated by ECM-degrading proteases such as matrix metalloproteinases (MMPs). As ECM also serves as a reservoir of numerous growth factors and cytokines (21, 25), dysregulated turnover of myocardial ECM can further impact the survival and function of the remaining cardiomyocytes. Given that ECM expansion is a pathophysiological response of the failing heart irrespective of its etiologic origin, specific targeting of the fibrogenic signals represents a logical cardioprotective and reparative strategy. Indeed, antibody blockade, oligonucleotide interference, and small molecule inhibitors have been used to suppress the myocardial TGF-β secretome (61, 64).

A newly emerging soluble mediator implicated in myocardial fibrosis is secreted Frizzled-related protein 2 (sFRP2), which in addition to its role in embryonic development also regulates postnatal tissue function and homeostasis (9, 16, 44). In particular, Kobayashi et al. (28) found that sFRP2-null mice exhibited reduced fibrosis and significantly improved cardiac function after MI. This study demonstrated that sFRP2 may promote fibrosis through serving as an enhancer of procollagen C proteinase (PCP) activity of Tolloid (TLD)-like metalloproteinases, which convert procollagen to collagen, the major fibrillar components of the ECM (17). These findings prompted us to explore sFRP2 antagonism as an antifibrotic strategy for cardiac repair. We previously used a hamster heart failure model to investigate the mechanisms of mesenchymal stem cell (MSC)- and growth factor-mediated cardiac repair (36, 52, 69). With the use of the heart failure model, the present work shows that antibody-based sFRP2 intervention may offer a

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promising approach to heart failure therapy that specifically targets fibrosis.1

METHODS AND MATERIALS

Animals and therapeutic protocols. The TO2 strain cardiomyopathic hamsters (4-mo male) were obtained from Bio Breeders (Wertertown, MA). All procedures and protocols conformed to institutional guidelines for the care and use of animals in research and were approved by the University at Buffalo Institutional Animal Care and Use Committee. TO2 hamsters divided into three groups were injected intraperitoneally twice per week for 4 wk, each receiving 0.4 ml of normal saline, 0.4 ml of a control IgG (R&D no. AB-108-C goat IgG; 200 μg/kg) or 0.4 ml of an sFRP2 antibody (R&D no. AF1169 goat anti-mouse sFRP2; 200 μg/kg). Echocardiography was performed 1 mo after the first injection, following which animals were euthanized for tissue collection. MSC therapy for the failing hamster heart was as documented previously (36, 52, 53).

Echocardiography. Animals were anesthetized by intraperitoneal injection of xylazine (2 mg/kg) and ketamine (30 mg/kg). Multiple M-mode images were obtained from the short axis view of the left ventricle at the level of the papillary muscles with a GE Vingmed echo machine using a 10-MHz transducer. Left ventricular end-systolic dimension (LVDS), LV end-diastolic dimension (LVDD), end-systolic wall thickness (ESWT), and end-diastolic wall thickness (EDWT) were measured in an operator-blinded manner and averaged from at least two consecutive cardiac cycles. Left ventricular ejection fraction (LVEF) = (LV end-diastolic volume − LV end-systolic volume) / LV end-diastolic volume × 100. Fractional shortening (FS) = (LVDD − LVDS) / LVDD × 100. ΔWall thickness (WT) = ESWT − EDWT. %WT = ΔWT / EDWT × 100.

Cell culture. Cardiac fibroblasts were isolated from the heart of 5-mo-old Balb/c mice using our previously established protocol (30). The fibroblasts were maintained in MEM containing 10% FBS, 50 μg/ml gentamycin and 0.125 μg/ml Fungizone. Only cells receiving less than three trypsin passages were used for experiments. Cells were plated on 12-well plate at 10^3 cells per well overnight and then treated with 3 nM recombinant sFRP2 (R&D no. 1169-FR) in 5% FBS for 3 days, following which total RNA was isolated for quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis.

qRT-PCR. RNA extraction was performed using Qiagen RNA isolation kits. qRT-PCR was used to analyze gene expression as described previously (34). Bio-Rad SYBR green kit was used for PCR reactions. β2-Microglobulin was used as the reference gene. Data were analyzed by the 2ΔΔCT method. Oligonucleotides were synthesized by Midland oligo, Primer sequences were as follows: TCTCT-TGGCTCACAGGGAGGT and ATGTCAGTTGCCCACTTGTGAC, for β2-microglobulin, GTCACGTGGAGGAGATAG and GGAGATG-CGCCGACTCTC for sFRP2, GTTCAGCTCCACACACCTCA and CCATCTTCTCCGCTACTG for Axin2, TGGTTCCCTAAC-AGCTACAGT and TTTGCTTTCAGCTTCAGT for MMP-2, AACGGAATATGCAGGGTC and AAAGTCTCAGTGGTGCT- TACC for Dkk1, GTTGCAAACTCGGACACTA and CTGGTTTT- GAAACTCCCTCCTT for GATA4, and CCGGAGCTCTCTAGC CACTG and GGTGGAACCGAGAATTTTT for MeF2c.

Cloning of hamster sFRP2 cDNA and production of recombinant protein. Hamster sFRP2 cDNA was cloned from hamster heart RNA using the two primers GTGCATGTCACAGGGAGGT and ACTG- CATTGCCACGTTCGGGA. The PCR-amplified cDNA was inserted into pCR2.1-TOPO and sequenced in both orientations (GenBank Accession No. JQ911696). The sFRP2 DNA was then inserted into the NcoI and BamHI sites of the bacterial expression vector pET3d using an NcoI-tagged primer (GTGCAGTTGACAGGGAGGT- CGGGGACCCCTGCT) and a BamHI-tagged primer (CGATTGCATC-CTAGCATTGCGACTGGGA). The pET-sFRP2 construct was transferred to the BL21 strain for protein overexpression analysis. Expression of sFRP2 was induced by adding 0.5 mM isopropyl thiogalactopyranoside (IPTG) to a log phase culture for a 2 h induction. Total proteins were analyzed by 12% SDS-PAGE and Western blotting to confirm expression of sFRP2 and antibody reactivity.

Quantification of fibrosis, apoptosis, and capillary density. Quantification of capillary density is as described previously (68). OCT-embedded cryosections were fixed in acetone:ethanol mix (3:1 ratio) for 5 min. Fluorescein-labeled Griffonia Simplicifolia Lectin Isolectin B4 (GSL-B4; Vector Laboratories) diluted 1:100 was incubated with the tissue sections overnight at 4°C. Cardiomyocytes were stained with a cardiac troponin T antibody (Thermo Scientific no. MS295). Images were taken in 20 random fields at ×200 magnification. The number of capillaries was counted, and the area was quantified using ImageJ. Capillary density was normalized to total area in millimeters squared. Paraformaldehyde-fixed paraffin-embedded heart sections were processed for terminal deoxynucleotidyl transferase dUTP-mediated nick-end labeling (TUNEL) assay and Masson trichrome staining. Analysis of apoptosis was performed as documented previously (68) using the ApopTag Kit (Millipore) per manufacturer’s instructions. Apoptotic nuclei were counted and normalized to total nuclei in 20 random fields at ×200 magnification. Masson trichrome-stained sections were used for fibrosis analysis, which was performed by ImageJ-aided quantification of image pixels as described previously (53). At least 15 random fields at ×200 magnification were assessed for each slide. The ratio of fibrotic area to total tissue area was calculated as a percentage of the fibrotic areas.

ELISA analysis. Growth factors present in heart tissue extracts were measured using ELISA kits from R&D: hepatocyte growth factor (HGF) DuoSet (DY2207) and vascular endothelial growth factor (VEGF) DuoSet (DY564). Snap-frozen tissues were homogenized in an ice-cold lysis solution (normal saline supplemented with 0.1% Triton X-100 and 2 mM EDTA). Tissue homogenates were clarified, diluted to 1 mg proteins/ml, and used for ELISA and gelatin zymography.

Gelatin zymography. Heart tissue homogenates were analyzed by gelatin zymography to determine MMP activities as described previously (57). Four parts of each sample was mixed with one part of 5× sample buffer (0.25 M Tris–Cl pH 6.8, 10% SDS, 25% glycerol, and 0.25% Bromophenol blue) and fractionated by 8% SDS-PAGE gel containing 1 mg/ml porcine skin gelatin type A (Sigma). The gel was gently agitated in a wash buffer (50 mM Tris–Cl pH 8, 200 mM NaCl, 5 mM CaCl2, 5 μM ZnSO4, and 2.5% TX-100) four times, 15 min each for enzyme renaturation, following which the gel was agitated in a reaction buffer (50 mM Tris–Cl pH 8, 200 mM NaCl, 5 mM CaCl2, and 5 μM ZnSO4) at 37°C overnight. Gelatin degradation bands were revealed by staining with a 3% Coomassie Blue G250 solution prepared in 50% methanol and 10% acetic acid and quantified by densitometry.

Statistical analysis. Comparisons between two and multiple experimental groups were made with Student’s t-test and one way ANOVA, respectively. A value of P < 0.05 is considered significant. Data (n ≥ 3 in each experiment) are expressed as means ± SE.

RESULTS

Correlation between fibrosis and sFRP2 expression in the hamster heart. The δ-sarcoglycan-null TO2 strain hamster develops congestive heart failure resembling that seen in the general population of heart failure patients (13, 37, 39, 50). Functional deterioration of the TO2 hamster heart is caused by progressive loss of cardiomyocytes, leading to prominent inflammation and fibrosis at 5 mo of age (~10% fibrotic area vs. ~1% fibrotic area in the normal heart). We have been using this animal model to characterize MSc- and VEGF-mediated

1 This article is the topic of an Editorial Focus by Rennolds S Ostrom (45a).
cardiac repair (36, 52, 53, 68–70). These studies establish that myocardial fibrosis is prominently reduced by the regenerative therapies. To delineate the role of sFRP2 in heart failure and fibrosis, we cloned by RT-PCR the full-length hamster sFRP2 cDNA from the failing heart (GenBank Accession No. JQ911696). Sequence analysis reveals 99.3% (293/295) amino-acid identity between the hamster and mouse sFRP2 proteins (26). The gene sequence information allows us to design primers to quantify sFRP2 expression by qRT-PCR. Figure 1A shows that the 1-mo TO2 heart, which exhibits normal contractile function (39), displays a slight increase in sFRP2 expression compared with the age-matched F1B strain normal hamster heart. The difference in sFRP2 expression was further amplified at 5 mo (~3-fold increase compared with the F1B heart). Thus increased fibrosis and decreased function in the 5-mo TO2 heart correlates with significantly increased expression of sFRP2.

To further define the role of sFRP2 in myocardial fibrosis, we characterized sFRP2 expression in the context of MSC therapy, which attenuates myocardial fibrosis and improve function (52, 53). Since sFRP2 regulates Wnt signaling, which activates the Axin2 gene (22), we used Axin2 expression as a readout for the Wnt signaling pathway. The analysis shows that the expression of both sFRP2 and Axin2 were similarly attenuated by MSC therapy (Fig. 1C). In contrast, expression of Dkk1, a secreted inhibitor of Wnt signaling (10), was significantly upregulated by MSC therapy (Fig. 1C). These findings suggest a functional correlation between MSC therapy-induced myocardial regeneration and attenuation of Wnt signaling.

An antifibrotic therapeutic regimen based on sFRP2 blockade. We next sought to test the hypothesis that sFRP2 blockade might be beneficial for the failing heart through attenuation of myocardial fibrosis. We were interested in exploring an antibody-based sFRP2 targeting strategy analogous to that used in targeting TGF-β for antifibrotic intervention (61, 64). A goat anti-mouse sFRP2 antibody (R&D no. AF1169) was selected and tested for its ability to recognize the hamster sFRP2 protein, which was overproduced in bacteria using an IPTG-inducible pET expression vector. The Western blot shown in Fig. 2A indicates that the antibody recognized the induced hamster sFRP2 with high specificity. We then took advantage of the sFRP2/Axin2 axis to verify the sFRP2-blocking activity of the antibody. Isolated cardiac fibroblasts were treated with 3 nM recombinant sFRP2 in the presence and absence of the antibody, and expression of Axin2 was quantified by qRT-PCR. Figure 2B shows that sFRP2 significantly induced Axin2, confirming the Wnt-activating effect of sFRP2. The sFRP2-blocking activity of the antibody was revealed by the finding that sFRP2-mediated induction of Axin2 was attenuated in the presence of the antibody (Fig. 2B).

We then adopted our previous SDF1 antibody blockade protocol (69) based on administration of 200 μg antibody/kg to 4-mo-old TO2 hamsters via two intraperitoneal injections per week. Biochemical, physiological, and histological analyses were carried out after 4 wk to assess the therapeutic outcome. We found that intraperitoneal administration of sFRP2 antibody caused significant downregulation of myocardial Axin2 expression (Fig. 2C), as similarly observed following MSC therapy (Fig. 1C) and in the cultured cardiac fibroblasts (Fig. 2B). In contrast to MSC therapy, however, sFRP2 blockade did not alter the expression of endogenous sFRP2 in the heart (Fig. 2C).

Improved cardiac function by sFRP2 antibody. Echocardiography performed after 1 mo revealed that animals receiving sFRP2 antibody exhibited significantly improved cardiac function. sFRP2 antibody significantly increased left ventricular ejection fraction (LVEF) from 40 ± 1.2 to 49 ± 6.5%, whereas the saline and IgG control groups exhibited a further decline to 37 ± 0.9 and 31 ± 3.2%, respectively (Fig. 3A). Analysis of fractional shortening (FS) showed a similar functional improvement by sFRP2 antibody (from 16.5 ± 1 to 21.6 ± 2.5%) in contrast to the progressive decline in FS in the saline and IgG control groups (Fig. 3B). These functional data are similar to those obtained with MSC and VEGF therapies (36, 52, 53, 68–70).

Attenuation of myocardial fibrosis by sFRP2 antibody. Our previous regenerative studies demonstrated that cardiac functional improvement after therapy is invariably associated with attenuated fibrosis and improved angiogenesis and cardiomyogenesis (36, 53, 68). Trichrome staining was therefore used to assess the effect of sFRP2 antibody injections on myocardial fibrosis after 1 mo. Figure 4 shows that sFRP2 antibody injections significantly reduced total myocardial fibrotic areas by ~50% as similarly observed with MSC therapy (53). Thus sFRP2 blockade-mediated functional improvement is associated with normalized myocardial ECM.

Induction of myocardial MMP-2 activity by MSC and sFRP2 antibody. Reduced fibrosis after MSC therapy and sFRP2 antibody administration indicates functionally relevant activation of the ECM-degrading machinery. Since MMPs are primarily responsible for ECM degradation, we used gelatin zymography (57) to assess the activities of MMP-2 and MMP-9 in heart tissue homogenates. The enzyme assays show that MSC therapy (Fig. 5A) and sFRP2 antibody administration (Fig. 5B) increased myocardial MMP-2 activities ~3.5- and ~1.7-fold, respectively. Only weak activity of MMP-9 was detected with the homogenates, and therefore, the effect of

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**Fig. 1.** Correlation between myocardial fibrosis and expression of secreted Frizzled-related protein 2 (sFRP2). RNA was isolated from 1-mo (A) and 5-mo (B) F1B and TO2 hamster hearts. Gene expression was analyzed by quantitative (q)RT-PCR using β2-microglobulin as the reference gene (n = 4 per group). A and B: comparison of sFRP2 expression in F1B and TO2 hamster hearts. C: myocardial expression of sFRP2, Axin2, and Dkk1 in 5-mo TO2 hamster hearts after mesenchymal stem cell (MSC) therapy as demonstrated previously (52, 53). Heart tissues from saline control and MSC groups were harvested after 1 mo for qPCR analysis (n = 4/group).
therapy on MMP-9 could not be definitively ascertained (data not shown). In addition, the more robust MSC-induced MMP-2 activity was associated with increased MMP-2 gene transcription (Fig. 5C), whereas the sFRP2 blockade-induced MMP-2 activity was independent of the gene induction (Fig. 5D), indicating a major difference in the antifibrotic mechanisms of the two cardiac repair approaches.

**sFRP2 blockade promotes myocardial angiogenesis.** Since ECM can influence the bioavailability of growth factors and cytokines (21, 25), increased MMP activity caused by sFRP2 blockade may affect their function. Given that VEGF and HGF have emerged as the key therapeutic factors in regenerative repair (2, 12, 35, 52, 70), we measured by ELISA the soluble levels of VEGF and HGF in heart tissue homogenates. As similarly observed with the previous MSC therapy (52), soluble VEGF was significantly increased by sFRP2 antibody in the heart (Fig. 6A) and hamstring muscle (Fig. 6B) homogenates. HGF appeared coordinately increased in both tissue homogenates although the increase did not reach statistical significance (Fig. 6). Since VEGF and HGF possess potent and synergistic angiogenic activities (12), we further examined the effect of sFRP2 antibody administration on myocardial angiogenesis. Capillary staining as illustrated previously (68) shows that sFRP2 antibody significantly promoted myocardial capillary density by ~30% (Fig. 7), which is consistent with increased levels of angiogenic factors shown in Fig. 6.

**sFRP2 blockade promotes cardiomyogenesis and attenuates apoptosis.** Growth factors are also known for their roles in promoting myogenesis and cell survival (43, 65, 68). qPCR analysis confirms that myocardial expression of the cardiogenic transcription factors GATA4 and Mef2c were significantly increased by sFRP2 antibody administration (Fig. 8A). We further determined whether myocardial apoptosis might be affected by sFRP2 blockade. Myocardial apoptotic nuclei were quantified by TUNEL staining of tissue sections. The analysis shows that injections of sFRP2 antibody caused a ~65% decrease in myocardial apoptosis (Fig. 8B). Accordingly, sFRP2 antibody resulted in a ~75% increase in anterior wall thickness compared with the saline control as measured by echocardiography (Fig. 9). These data together reveal a pathogenic role of sFRP2 in hamster heart failure and provide evidence that perturbation of the sFRP2 axis may constitute an alternative therapeutic approach for chronic myocardial fibrosis.

**DISCUSSION**

The intriguing roles of sFRP2 in postnatal tissue regulation and disease development are beginning to unravel (4, 5, 23). The high degree of sFRP2 sequence conservation across species suggests a functionally conserved and important regulatory role. We show that increased myocardial fibrosis correlates with elevated expression of sFRP2 in the failing hamster heart, which mirrors elevated expression of sFRP3 and sFRP4 in the failing human heart (51). The significance of this observation is strengthened by the finding that MSC therapy attenuates myocardial fibrosis and expression of sFRP2. Using an antibody-based sFRP2 blockade strategy, we show here that sFRP2 interference promoted cardiac repair and function 1 mo after the antibody treatment by normalizing myocardial ECM thickness compared with the saline control as measured by echocardiography (Fig. 9). These data together reveal a pathogenic role of sFRP2 in hamster heart failure and provide evidence that perturbation of the sFRP2 axis may constitute an alternative therapeutic approach for chronic myocardial fibrosis.
remodeling through reducing fibrosis and apoptosis and promoting angiogenesis and wall thickness. These findings highlight the pathogenic effect of dysregulated sFRP2 in chronic heart failure and are consistent with the finding that sFRP2-null mice exhibit reduced fibrosis and improved function after MI (28).

In the mouse MI model, expression of sFRP2 becomes greatly elevated after the onset of the fibrotic phase (4 days after coronary artery ligation) and remains significantly elevated thereafter (28). This sustained expression pattern of sFRP2 in the infarcted mouse heart mimics that in the chronically failing hamster heart and correlates with the severity of myocardial fibrosis. The study by Kobayashi et al. (28) demonstrated that sFRP2 can serve as a direct enhancer of PCP activity of TLD-like metalloproteinases, which include bone morphogenetic protein 1 (BMP1), mTLD, mTLL1, and mTLL2 in mammals (17). In particular, two prominent activities of sFRP2 were demonstrated: one that stimulates BMP1 PCP activity by binding BMP1 through its Frizzled domain and another that enhances the interaction between BMP1 and its substrate procollagen. Since BMP1 possesses a prominent PCP activity, the sFRP2/BMP1 axis effectively promotes procollagen processing and collagen deposition in the ECM. This apparently Wnt-independent proteolytic mechanism of sFRP2 is similar to that driven by PCPE-1 in procollagen processing/maturation orchestrated by TLD-like proteinases (41).

The work presented here identified Axin2 as a downstream target of sFRP2 likely through Wnt activation. Our in vivo and in vitro studies demonstrating reduced Axin2 expression following downregulation of sFRP2 by MSC therapy and sFRP2 antagonism by antibody blockade support this conclusion. The cell culture study indicates that this sFRP2/Axin2 regulatory circuit operates in the cardiac fibroblasts as revealed by the finding that Axin2 expression was amplified in response to sFRP2. Since fibroblasts are the main source of ECM colla-

Fig. 4. sFRP2 antibody administration reduces myocardial fibrosis. Paraffin sections were prepared from TO2 ventricular tissue 1 mo after sFRP2 antibody administration and processed for digital imaging and analysis of fibrosis as detailed previously (53, 68). A: representative images of Trichrome-stained heart sections at ×50 magnification for the saline control and sFRP2 antibody groups. B: computer-assisted quantification of fibrotic areas using images at ×200 magnification. P < 0.05 vs. saline control (n = 3–4/group). C: representative images of interstitial, perivascular and pericardial fibrotic areas at ×200 magnification.
The triple-helical structure of matured collagen despite its stability is sensitive to degradation by MMPs, which are major players of ECM remodeling during chronic heart failure and following MI (55). MMP regulation involves control of MMP gene expression, proteolytic activation of pro-MMPs, and inhibition by tissue inhibitors of MMPs (TIMPs). Suppression of MMPs thus promotes ECM deposition and fibrosis. Aging-associated myocardial fibrosis, for instance, is mediated by reduced MMP expression and activity (3), and the development of liver and lung fibrosis is facilitated by reduced MMP activity (49). The fibrogenic cytokine TGF-β1 promotes fibrosis in part through inhibition and activation of MMP and TIMP expression, respectively (24, 62). Our data indicate that the increased myocardial MMP-2 activity after MSC therapy was primarily mediated by increased gene transcription. Indeed, the multiple MSC-derived cytokines are expected to exert a stimulatory effect on the expression of the MMP-2 gene (15, 46, 48). In contrast, the increased MMP-2 activity following sFRP2 blockade appears independent of the transcriptional control, which again highlights the proteolytic regulatory function of sFRP2. MMP is regulated by another ECM remodeling protein termed osteopontin (OPN), which is also elevated in hamster heart failure and MI and can suppress MMP induction during inflammation (39, 54). Mice lacking OPN exhibit reduced fibrosis and improved function after MI (54), as similarly observed in sFRP2 knockout mice. Although these findings suggest MMP activation as a potential antiblastic strategy for the failing heart, therapeutic exploration of MMP regulation may aim at up- or downregulation of MMP activity as influenced by temporal progression of the disease state. For gens, sFRP2-mediated induction of Axin2 appears to promote ECM accumulation and fibrosis through a Wnt-dependent pathway. However, studies from the group of Dzau (16) found that intracardiac injection of sFRP2 protein reduced fibrosis and improved cardiac function in a rat MI model. They also concluded that sFRP2 is the key paracrine factor released from MSCs overexpressing Akt (38). We note that a hallmark of sFRP proteins is their biphasic interactions with Wnt proteins, leading to augmentation and inhibition of Wnt signaling at low and high sFRP levels, respectively (59). Similarly, regulation of the PCP activity of BMP1 by sFRP2 exhibits a biphasic feature in that low (<1 μg/ml) and high (>6 μg/ml) levels of sFRP2 can activate and inhibit procollagen processing by BMP1, respectively (1, 16). Since we were unable to detect sFRP2 protein in the hamster heart by Western blotting, the low level of myocardial sFRP2 suggests that the protein acts to promote rather than suppress Wnt signaling in the cardiomyopathic heart. Thus, although sFRP2 has traditionally been viewed as a Wnt inhibitor, its Wnt-activating effect has been repeatedly demonstrated (31, 60). We speculate that intracardiac injection of a large quantity of exogenous sFRP2 protein as conducted by the group of Dzau (16) likely caused a favorable therapeutic outcome through direct Wnt inhibition.

Fig. 5. Induction of matrix metalloproteinases-2 (MMP-2) activity correlates with reduced myocardial fibrosis. TO2 hamster heart tissues collected from previous MSC therapy (53) were included for the enzyme assay. Ventricular tissues harvested 1 mo after MSC therapy (A) or sFRP2 antibody injections (B) were homogenized, and 40 μg proteins were loaded in each lane to analyze the activity of MMP-2 and MMP-9 by gelatin zymography. MSC- and sFRP2 antibody-treated groups were each compared with saline injection (n = 3). The 67- and 64-kDa MMP-2 bands used for quantification are illustrated. The MMP-9 bands were too faint to be reliably quantified. C and D: qPCR analysis of myocardial expression of MMP-2 gene after MSC therapy (C) and sFRP2 antibody injections (D).

Fig. 6. sFRP2 antibody administration increases levels of angiogenic factors. Heart (A) and hamstring muscle (B) tissues were homogenized 1 mo after sFRP2 antibody administration and the soluble fractions were assayed by VEGF and hepatocyte growth factor (HGF) ELISA. Growth factor concentrations are expressed as pg/mg proteins.
example, cardiac hypertrophy induced by β-adrenergic activation and cartilage degeneration was found in each one to be ameliorated by MMP inhibition (40, 45). Nonetheless, clinical trials of MMP inhibitors have so far generated disappointing results (6, 19). On the other hand, antifibrotic therapeutic intervention aiming at activation of MMP through inhibition of the TGF-β secretome (61, 64) or sFRP2 blockade as demonstrated here warrants further trials.

Tissue repair through the healing actions of trophic factors of both exogenous and endogenous sources is at the forefront of regenerative medicine (33, 52, 67). Our finding that sFRP2 blockade increases tissue levels of VEGF and HGF along with improved cardiac function is consistent with the findings that VEGF and HGF are key trophic factors mediating the therapeutic function of MSC (2, 52, 68, 70) and reveals an additional mechanism through which elevated levels of sFRP2 in the failing heart may interfere with myocardial regeneration through inhibition of angiogenesis. Along this line, sFRP4, another member of the sFRP family, has recently been found to be an angiogenic inhibitor (42). ECM components are known to interact with growth factors and cytokines, serving as a reservoir of trophic factors and influencing their degradation rate and bioactivities (18, 58). Trophic factors bound to a normalized ECM scaffold may be effectively shielded from proteolytic attack, thus promoting angiogenesis and cardiomyogenesis as demonstrated in our animal study. The inflammatory and proteolytic-rich environment of the injured and fibrotic tissues in the failing heart warrants special consideration because of the presence of diverse tissue protease activities. Tissue proteases such as elastase, cathepsin, and dipeptidylpeptidase are known to cleave and inactivate various trophic factors (7, 47, 66). VEGF is known to possess a relatively short half-life of ~3 min in the circulation (11), and the blood protease plasmin can inactivate VEGF through proteolytic cleavage (27). Given the PCP-enhancing function of sFRP2, it would be of interest to determine whether sFRP2 may also promote proteolytic degradation of VEGF. Alternatively, activation of MMPs after sFRP2 blockade or MSC therapy may release VEGF and HGF from the ECM niche as demonstrated for basic fibroblast growth factor by Whitelock et al. (63). This VEGF-antagonizing effect of sFRP2 appears in line with the emerging notion that sFRP2 may represent a novel tumor-suppressor gene, the expression of which is often silenced in cancer cells (29).

**Fig. 7.** sFRP2 antibody administration augments angiogenesis. Frozen sections were prepared from ventricular tissue 1 mo after sFRP2 antibody administration and processed for capillary staining. *A*: representative images of GSL-IB4-stained heart sections showing capillaries (green) at ×200 magnification. Myocytes were stained by a troponin T antibody (red) and total nuclei were stained by DAPI (blue). *B*: computer-assisted quantification of capillary density. *P* < 0.05 vs. saline control (*n* = 5/group).

**Fig. 8.** sFRP2 blockade promotes cardiomyogenesis and attenuates apoptosis. *A*: total RNA was isolated from TO2 hamster heart from both saline control and sFRP2 antibody injection groups. Expression of GATA4 and Mef2c was determined by qPCR (*n* = 3). *B*: quantification of myocardial apoptotic nuclei (*n* = 3/group). Terminal deoxynucleotidyl transferase dUTP-mediated nick-end labeling-stained sections were imaged at ×200 magnification (images not shown). Analysis of apoptosis was performed as documented previously (68) using the ApopTag kit.

**Fig. 9.** sFRP2 antibody administration increases myocardial wall thickening. Anterior wall thickness (Δ anterior WT) and % anterior wall thickness derived from echocardiographic data before and 1 mo after sFRP2 antibody administration are presented. **P < 0.01 vs. pretreatment; †P < 0.05 vs. saline control (*n* = 3–7/group).
While MSC therapy is currently being evaluated by multiple clinical trials, the stem cell medicine is inherently associated with several logistical hurdles such as a limited shelf-life, high cost of manufacturing, and donor-related cellular heterogeneity. Humanized monoclonal antibody has been developed and is increasingly being used for the treatments of cancer and autoimmune disease (20). Major advantages of antibody therapy include well-defined target specificity and protein stability. The current study shows that administration of sFRP2 antibody is a promising antifibrotic therapy for heart failure. The regenerative potential of this treatment may be mediated in part by therapeutically relevant trophic factors such as VEGF and HGF, which are known to contribute to the therapeutic effects of MSCs (2, 12, 35, 52, 70). Clinical utility of the sFRP2 blockade strategy will need to be validated by additional studies using different disease models.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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