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Frzb, a Secreted Wnt Antagonist, Decreases Growth and Invasiveness of Fibrosarcoma Cells Associated with Inhibition of Met Signaling

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Abstract

Soft tissue sarcomas (STS) have a strong propensity for aggressive growth and metastasis. We showed that the secreted Wnt antagonist Frzb exhibited potent antitumor activity against prostate cancer, an epithelial type of malignancy. In this study, we further showed the antitumor efficacy of Frzb in STS, a mesenchymal group of cancer. Frzb transfection of HT1080 (fibrosarcoma) and SW872 (liposarcoma) cell lines and their conditioned media resulted in a significant reduction in cellular invasion, motility, and colony formation in soft agar compared with vector control-transfected cells. In a xenograft mouse model, Frzb dramatically suppressed tumor growth of HT1080 cells in nude mice. In a tail-vein injection metastatic model, Frzb-transfected HT1080 cells formed fewer and smaller lung nodules than vector control cells. In addition, we identified new mechanisms for Frzb antitumor activities. Frzb reduced c-Met expression and inhibited Met-mediated signaling, associated with up-regulation of epithelial markers (i.e., keratins 8 and 18) and down-regulation of mesenchymal markers (i.e., vimentin, N-cadherin, fibronectin, Slug, and Twist). Similar to Frzb, silencing of c-Met by short hairpin RNA or using a dominant-negative LRP5 receptor also suppressed Met signaling, leading to reduced cellular motility, invasion, and *in vivo* tumor growth. Given recent studies indicating an important role of c-Met in sarcoma development and progression, our data showed that Frzb expression was significantly inversely correlated with Met expression in both STS cell lines and tissues. These results suggested the usefulness of Frzb in modulating Met signaling as a new treatment strategy for STS.

Introduction

Soft tissue sarcomas (STS) are malignant tumors of mesenchymal origin, among which liposarcoma, fibrosarcoma, and leiomyosarcoma are the most common histologic subtypes in adults (1). The mortality rates of STS approach 50% (1). Despite advances in cancer therapy, the mainstay of treatment for STS is still surgical resection. Complete response to conventional chemotherapy is rare, and the prognosis for patients with unresectable or metastatic disease

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remains dismal. Radiation therapy improves local control for high-grade tumors. However, local control does not always translate into better overall survival as systemic disease continues to be a major prognosticator (1). Currently, there is no convincing evidence that conventional chemotherapy significantly alters the natural history of STS. Therefore, more effective strategies are needed to improve systemic and local control.

In the canonical Wnt pathway, Wnt ligands bind to Frizzled receptors (Fz) and coreceptors low-density lipoprotein receptor-related proteins (LRP5/LRP6), leading to activation of disheveled (Dsh), inactivation of glycogen synthase kinase 3 α (GSK3 α), and disaggregation of adenomatous polyposis coli (APC), Axin, and GSK3 α . As a consequence, degradation of β -catenin is blocked, leading to cytoplasmic accumulation and nuclear translocation of this protein. Inside the nucleus, β -catenin binds to lymphoid enhancer factor (LEF)/T-cell factor (TCF), inducing the transcription of Wnt target genes (2).

Wnt signaling has been implicated in several human tumors, among which are skin, connective tissue, colon, gastric, lung, breast, and prostate (3–9). In addition, recent studies highlighted the potential role for Wnt signaling in STS. Sporadic mutations of Wnt components, such as β -catenin and APC, have been reported in STS (10,11). Recently, Wnt5A and Fz10 have been shown to be highly expressed in synovial sarcomas (12). Wnt-1 blockade by a monoclonal antibody or small interfering RNA (siRNA) can induce cell death in rhabdomyosarcoma (13). These findings strongly suggest that Wnt activation at the membrane level plays an important role in STS pathobiology.

Frzb (also known as sFRP3) belongs to the secreted Fz-related protein family, whose members share a characteristic cysteine-rich domain (CRD) with Fz receptors (14). Frzb blocks receptor signaling by binding to extracellular Wnt ligands and thus preventing ligand-receptor interaction (15,16). The Gly324 variant of Frzb with attenuated ability to antagonize Wnt has been associated with an increased risk of colon cancer (17). Recently, Mandal et al. (18) reported that Frzb expression was down-regulated in osteosarcoma tissue and cell lines. Together, these findings strongly suggest a tumor suppressor role for Frzb. To examine the role of Frzb in STS, we established stable fibrosarcoma and liposarcoma cell lines expressing this protein. Blocking Wnt signaling by Frzb led to a marked reduction in cell motility, invasiveness, and tumorigenesis. These effects were associated with decreased expression and activity of c-Met and matrix metalloproteinase-2 (MMP-2). In liposarcoma cell line SW872, decreased invasiveness was also associated with increased expression of epithelial markers (i.e., keratins 8 and 18) and decreased expression of mesenchymal markers (i.e., vimentin, N-cadherin, fibronectin, Slug, and Twist). Our results implicated an important tumor suppressive function for Frzb in sarcomas.

Materials and Methods

Cell culture and plasmid

Normal human dermal fibroblasts (NHDF) and skeletal muscle stromal cells (SMSC) were obtained from the Clonetics collection (Cambrex Corp.) and maintained in the following Cambrex media: FGM-2 medium (NHDF cells) and ScGM medium (SMSC cells). HT1080 cell line (fibrosarcoma), SW872 cell line (liposarcoma), and SK-LMS-1 cell line (leiomyosarcoma) were obtained from American Type Culture Collection. SYO-1 cell line (synovial sarcoma) was a gift from Marc Ladanyi (Memorial Sloan-Kettering Cancer Center). HT1080 cells were maintained in DMEM with 10% fetal bovine serum (FBS; Invitrogen). SK-LMS-1 and SYO-1 cells were maintained in MEMa with 10% FBS. SW872 cells were grown in Liebovitz's L-15 medium with 10% FBS. All cell lines were maintained at 37°C in humidified atmosphere of 5% CO₂, except for SW872 cells, which was cultured without CO₂. PcDNA3.1 expression vector was obtained from Invitrogen. The PcDNA3.1 vector

expressing bovine Frzb was described previously (3). The dominant-negative *LRP5* plasmid (*DNLRP5*, a generous gift from Dr. Matthew Warman, Children's Hospital Boston) encodes a secreted form of LRP5 (Δ TM; ref. 19). The TCF4 luciferase reporter (*Super TOPFLASH* and *Super FOPFLASH*) and full-length β -catenin plasmids were from Dr. Marian Waterman (University of California). β -Galactosidase plasmid was obtained from Invitrogen.

Stable transfection

HT1080 cells and SW872 cells were plated at 2×10^5 per well in six-well plates. After 24 h, 60% confluent cultures were transfected with *Frzb* or *DNLRP5* construct using Fugene 6 (Roche) as reported (20). As controls, HT1080 and SW872 cells were also transfected with PcDNA3.1. Transfected cells were then selected with G418 (800 μ g/mL) starting at 24 h after transfection, and all stable transfectants were pooled to avoid cloning artifacts.

RNA interference

The SureSilencing Met short hairpin RNA (shRNA) plasmids were obtained from SuperArray Bioscience Corporation. Each vector contains a U1 promoter and neomycin resistance gene for stable selection. A human c-Met shRNA (5'-gcgaagtcctcttaacatctaCTTCCTGTCA-tagatgtaagaggacttcgc-3') was used to achieve stable knockdown. A scrambled artificial sequence (5'-ggaatctcattcgatgcatacCTTCCTGTCAgtatgcacgaatgagattcc-3'), which does not match any human or mouse gene, was used as a negative control. All constructs were transfected using Lipofectamine 2000 (Invitrogen) and selected with G418. The pooled stable transfectants were examined.

Quantitative real-time reverse transcription-PCR

Total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Real-time reverse transcription-PCR (RT-PCR) was performed as previously described (3). PCR condition was as follows: 95°C for 5 min, 40 cycles of 30 s at 95°C, 30 s at 68°C, 60 s at 72°C. Relative fold change compared with control was calculated using the comparative C_t method (21). C_t is the cycle number at which fluorescence intensity first exceeds the threshold level. ΔC_t is C_t (target gene) – C_t (actin). Gene specific primer sequences are available upon request. Specificity of amplification products were verified by agarose gel electrophoresis and melting curve analysis.

Western blot analysis

Twenty to 80 μ g of protein lysate were separated electrophoretically on denaturing SDS-polyacrylamide gel, transferred to nitrocellulose membranes, and probed with antibodies against E-cadherin and N-cadherin (BD Bioscience); vimentin, keratin 8, keratin 18, and fibronectin (Lab Vision); AKT, phosphorylated AKT, mitogen-activated protein kinase (MAPK), phosphorylated MAPK, phosphorylated Met, and Frzb (Cell Signaling, Danvers, MA); and Slug, Twist, Met, and β -actin (Santa Cruz Biotechnology). Blots were exposed to secondary antibodies and visualized using the SuperSignal West Pico Chemiluminescent Substrate (Pierce). For loading control, membranes were stripped and reprobed with β -actin.

Immunofluorescence

Transfected cells were grown overnight in Lab-Tek II Chamber Slides (Nalge Nunc International) and fixed in 4% paraformaldehyde solution for 20 min and methanol for 2 min. After blocking with PBS containing 3% bovine serum albumin for 1 h, cells were incubated with primary antibodies (dilution, 1:50 to 1:100) for 1 h. After washing in PBS, cells were incubated for 45 min with an Alexa-488-conjugated secondary antibody (Molecular Probes) and mounted in Vectashield. Immunostaining was analyzed with a Nikon Eclipse TE2000-S fluorescent microscope (magnification, 200 $\times$) using the 488-excitation wavelength.

Gelatin zymography

Conditioned medium was collected by culturing cells in serum-free medium at 90% confluence for 48 h. The medium was concentrated 20× using Centricon filters (Millipore). Samples were applied to non-denaturing 10% polyacrylamide gels containing 1 mg/mL gelatin. After electrophoresis, gels were washed with 2.5% Triton X-100, incubated overnight at 37°C in zymography buffer, and stained with Coomassie brilliant blue (3). Gelatinolytic activity was visualized as clear areas of lysis in the gel.

Luciferase and β -galactosidase assays

STS cells stably expressing PcDNA3.1, Frzb, or DNLRP5 were grown in six-well plates and transiently cotransfected with 1 μ g Super TOPFLASH or Super FOPFLASH and 0.1 μ g cytomegalovirus (CMV)- β -galactosidase plasmids (Invitrogen) using FuGENE 6. After 24 h, cells were harvested, and the luciferase and β -galactosidase activities were measured using Bright-Glo luciferase assay system and β -galactosidase enzyme assay system (Promega). The relative luciferase unit for each transfection was adjusted by β -galactosidase activity in the same sample.

Motility and Matrigel invasion assays

These assays were performed as previously described (20). For motility, cells were allowed to migrate through uncoated filters for 8 to 12 h at 37°C. The number of migrating cells was determined by counting 10 fields (100×) on each membrane and calculated as mean number of cells per field. For invasion, incubation was carried out for 16 h (HT1080 cells) and 28 h (SW872 cells) at 37°C in humidified air with 5% CO₂. The number of invading cells was determined by counting 10 fields at $\times 100$ magnification. All cell lines were assayed in triplicate for each experiment, and the mean $\pm$ SE was determined.

Transient transfection

For Met rescue experiments, a cDNA clone containing a full-length human Met ORF was purchased from Invitrogen (Ultimate ORF Clone, Invitrogen) and subcloned into a PcDNA40 destination vector (Invitrogen). The resulting Met expression construct was transiently cotransfected into Frzb-transfected HT1080 and SW872 cells using Lipofectamine 2000 (Invitrogen). PcDNA40 empty vector was cotransfected into PcDNA-transfected cells as a control. At 24 h after transfection, cells were harvested and used for motility and Matrigel invasion assays.

To examine β -catenin-mediated effects, HT1080 and SW872 cells stably transfected with Met shRNA or control shRNA were transiently cotransfected with β -catenin expression construct (a gift from Dr. Marian Waterman, University of California-Irvine) using Lipofectamine 2000 (Invitrogen). PcDNA empty vector was cotransfected as a control. At 24 h after transfection, cells were harvested and used for motility and Matrigel invasion assays.

Colony formation assay

This assay was done in six-well plates with each well containing 2 mL of 0.8% agar in complete medium as the bottom layer, 1 mL of 0.38% agar in complete medium with 4,000 cells as the feeder layer, and 1 mL of complete medium as the top layer. The number of colonies was determined using an inverted microscope at $\times 100$ magnification. A group of >10 cells was considered a colony. The data represent means $\pm$ SE of four independent wells at 18 d after seeding.

***In vivo* tumor growth and metastasis assays**

NCR-nu/nu (nude) mice were obtained from Taconic. Stably transfected HT1080 cells ($1 \times 10^6/200 \mu\text{L}$ PBS) were injected s.c. into the right flank. Tumor size was measured every 3 d with a caliper. The tumor volume was calculated by the formula $1/6\pi ab^2$ ($\pi = 3.14$, a = long axis, and b = short axis of the tumor). Growth curves were plotted from the mean tumor volume $\pm$ SE from 10 animals in each group.

For lung metastasis, stably transfected HT1080 cells ($1 \times 10^6/200 \mu\text{L}$ PBS) were injected i.v. via the tail vein. Animals were sacrificed on day 40 after injection. Lungs were harvested and fixed in Bouin's solution. Surface lung nodules were counted under a dissecting microscope. Microscopic lung metastases were visualized on 5- μm paraffin-embedded sections. All animal studies were approved by the Institutional Animal Care and Use Committee at the University of California-Irvine.

Immunohistochemistry

Flank tumors and lung tissues were fixed in formalin and sectioned. Antigen retrieval was done using 10 mmol/L sodium citrate (pH 6.0) at 95°C for 15 min. Slides were incubated with an anti-Flag M2 antibody (Stratagene) at 1:50 dilution for 12 h at room temperature. Slides were then incubated with a biotinylated secondary antibody. Staining was performed using a Cell and Tissue Staining kit (R&D Systems) according to the manufacturer's instructions. Slides were counter-stained with hematoxylin and photographed using a light microscope. Negative control samples were exposed to a secondary antibody with a similar IgG isotype (Cell Signaling) to the primary antibody.

Paired human STS tissue array slides were purchased from Cybrdi, each containing paraffin-embedded STS samples that were pathologically confirmed: leiomyosarcoma ($n = 11$), fibrosarcoma ($n = 7$), liposarcoma ($n = 13$), and rhabdomyosarcoma ($n = 1$). No paired normal tissues were available for these samples. The histologic diagnoses were confirmed by a musculoskeletal pathologist, and immunohistochemical staining of tissue array slides were done using human Met (C-28, 1:250 dilution) or Frzb (C-20, 1:100 dilution) primary antibody (Santa Cruz Biotechnology) and biotinylated secondary antibodies. Stained sections were scored by two independent investigators (one of whom is a musculoskeletal pathologist) and classified as 0 (negative), 1+ (low), 2+ (moderate), or 3+ (high) according to the staining intensity. Xenograft tumor tissues were stained with IgG and Frzb or Met antibodies for negative and positive controls, respectively.

Statistical analysis

Comparisons of number of invading cells, agar colonies, levels of mRNA expression, levels of protein expression, and luciferase activity between different transfection groups were done using Student's t test. To correlate Met and Frzb protein expression on Western blots, Pearson's correlation coefficient was calculated from densitometry measurements of protein bands. For tumor growth, repeated measures ANOVA were used to examine the differences in tumor size among different time points. Additional posttest was performed to examine the difference in tumor size between vector control and other transfection at each time point by using conservative Bonferroni method. All statistical tests were two-sided. $P < 0.05$ was considered statistically significant. Because tissue immunostaining intensity is an ordinal categorical variable, Spearman's rank-order correlation coefficients were calculated and the test of the correlation coefficient equals 0 was performed using Student's t test.

Results

Frzb expression is down-regulated and inversely related to Met expression in STS cell lines and tissues, and Frzb transfection decreases anchorage-independent growth of HT1080 cells associated with Wnt inhibition

Endogenous levels of Frzb mRNA were examined in two normal human primary cell lines (NHDF and SMSC) and four STS cell lines (fibrosarcoma, HT1080; liposarcoma, SW872; leiomyosarcoma, SK-LMS-1; synovial sarcoma, SYO-1) by quantitative real-time RT-PCR. Frzb mRNA levels in all STS cell lines were down-regulated when compared with NHDF control (Fig. 1A, *left*; $P < 0.04$). In contrast, mRNA levels of LRP5 (a coreceptor required for canonical Wnt signaling) were significantly up-regulated in HT1080, SW872, and SYO-1 cell lines compared with NHDF control (Fig. 1A, *right*; $P < 0.01$). Due to low endogenous levels of Frzb and high LRP5 expression, HT1080 and SW872 cell lines were selected for further analysis of Wnt signaling blockade by secreted Wnt antagonists Frzb and DNLRP5. By Western blotting, we found a very significant inverse relationship between Frzb and Met protein expression in three STS (HT1080, SW872, Sk-LMS-1) and two normal control cell lines (NHDF, SMSC; Fig. 1B, *top* and *bottom*). Correlation of densitometric measurement of Frzb and Met protein bands (Pearson correlation coefficient, -0.68 ; $P < 0.01$) suggested that Frzb may negatively influence Met expression at least in a subset of STS cells. In addition, immunohistochemical analysis of tissue arrays (Fig. 1C; Supplementary Table S1) comprising of leiomyosarcoma, liposarcoma, fibrosarcoma, and rhabdomyosarcoma ($n = 32$) revealed a significant inverse correlation between Frzb and Met levels of expression (Spearman rank order correlation coefficient, -0.47 ; Student's t test, $P < 0.05$).

By Western blot analysis, we confirmed expression of Frzb and DNLRP5 fusion proteins using anti-FLAG and anti-Myc antibodies, respectively (Fig. 1D). To evaluate the levels of LEF/TCF transcriptional activity, HT1080 and SW872 cells expressing PCDNA3.1, Frzb, or DNLRP5 were transiently cotransfected with reporter plasmids containing either wild-type (Super TOPFLASH) or mutant (Super FOPFLASH) consensus TCF/LEF binding elements and CMV-galactosidase plasmids. Compared with controls, Frzb reduced LEF/TCF transcriptional activity by 68% (HT1080) and 73% (SW872; Student's t test; $P < 0.05$; Fig. 1D). Similarly, DNLRP5 decreased transcriptional activity in HT1080 and SW872 cells by 83% and 82% of controls, respectively (Student's t test; $P < 0.05$; Fig. 1D).

In soft agar, Frzb-transfected HT1080 cells formed 83.1% less colonies than vector control cells (Student's t test; $P = 0.01$; Supplementary Fig. S1), whereas DNLRP5 transfection abrogated colony formation (Supplementary Fig. S1). These results suggest that Frzb and DNLRP5 can reduce anchorage-independent tumor growth, parallel with their ability to inhibit canonical Wnt signaling (Fig. 1D). We were unable to study anchorage-independent growth of SW872 cell line given this line did not form any colony in soft agar.

Frzb inhibits *in vivo* tumor growth of fibrosarcoma

We next performed xenograft experiments to determine the ability of secreted Wnt antagonists Frzb and DNLRP5 in inhibition of *in vivo* tumor growth in nude mice. Figure 2A shows that tumor volume in Frzb-transfected HT1080 cell line is significantly lower than that of vector control cell line (Student's t test, $P = 0.03$; Fig. 2A). In addition, tumors from Frzb-transfected cell line did not enlarge 15 days after mice bearing tumors from vector-transfected cells were sacrificed (data not shown). Immunohistochemical analysis using an anti-FLAG antibody confirmed Frzb-transfected tumors expressed the FLAG-tagged Frzb protein compared with vector control-transfected tumors (Fig. 2B). Consistent with its Wnt inhibitory activity (Fig. 1D), DNLRP5 transfection resulted in a complete suppression of tumor growth in nude mice (Fig. 2C). No tissue is available in the DNLRP5 transfection group for immunohistochemical

analysis. Parental and control-transfected SW872 cells did not show *in vivo* tumorigenic or metastatic potential in nude mice (data not shown). Therefore, we cannot measure reduced tumorigenicity or metastatic potential by Frzb using this particular cell line.

Frzb suppresses *in vitro* motility and invasiveness

To examine whether Wnt antagonists Frzb and DNLRP5 also affect the invasiveness of STS cell lines, we have performed transwell motility and Matrigel invasion assays. Figure 3A showed that at 8 hours (HT1080) and 12 hours (SW872) after seeding, Frzb-transfected and DNLRP5-transfected cell lines exhibited significantly lower motility compared with vector control-transfected cells ($P = 0.03$, Student's *t* test). The *in vitro* Matrigel transwell assay showed that Frzb decreased the number of invading cells by 80% (HT1080) and 60% (SW872) compared with vector control cells (Student's *t* test, $P < 0.01$; Fig. 3A). Similarly, DNLRP5 reduced invasive capacity of HT1080 and SW872 cells by 48.5% and 45.5%, respectively (Student's *t* test, $P < 0.03$; Fig. 3A). To examine the physiologic effect of Frzb protein, we collected conditioned media from stable SW872/Frzb and SW872/PcDNA cell lines and treated HT1080 and SW872 parental cells. Figure 3 showed that Frzb conditioned media (50% final concentration) decreased the Matrigel invasive capacity of HT1080 and SW872 cells by 57% and 61%, respectively, compared with control conditioned media (Student's *t* test, $P < 0.01$).

Frzb modulates Met signaling, expression of epithelial-to-mesenchymal transition-related markers, and MMP-2 activity in STS cells

Based on the information that Wnt signaling affects multiple target genes (2,3), we predicted that Frzb may affect several pathways responsible for tumor invasion, metastasis, and growth. First, given that c-Met, a potential Wnt target gene, plays an important role in sarcoma progression (22,23), we examined whether Frzb modulates Met signaling in HT1080 and SW872 cell lines. Frzb and DNLRP5 transfection decreased protein expression and phosphorylation of c-Met (Fig. 4A). Consistently, Frzb and DNLRP5 also inhibited Met-mediated downstream events AKT and MAPK phosphorylation in both cell lines (Fig. 4A). Frzb conditioned media (50% final concentration) decreased Met protein expression in both HT1080 and SW872 cell lines compared with conditioned media from PcDNA control culture (Supplementary Fig. S2A). Met downstream event AKT phosphorylation was decreased in SW872 cell line. In HT1080 cells, however, phosphorylated AKT band was not detected in either control or Frzb-treated cells (Supplementary Fig. S2A), perhaps partially due to a low concentration of HGF in the mixture containing growth medium and serum-free conditioned media, low autocrine HGF secretion, and no constitutive Met receptor activation in HT1080 cell line.

To examine whether Wnt stimulation can positively modulate Met signaling, we treated parental SW872 cells with 50% conditioned media from Wnt3a secreting L cells. Both Met protein expression and downstream event phosphorylated AKT were up-regulated in response to exogenous Wnt3a (Supplementary Fig. S2B). To examine the effects of other Wnt antagonist, such as Dkk-1 on Met signaling, we transiently transfected SW872 cells with a human Dkk-1 expression construct. Our preliminary results showed that Dkk-1 overexpression decreased Met protein expression and phosphorylation similar to the effects of Frzb.⁴

Second, the epithelial-to-mesenchymal transition (EMT) has been shown to be important for cancer progression and metastasis. We have shown that Frzb caused a reversal of the EMT in prostate cancer PC-3 cells (3). Therefore, we examined whether Frzb can modulate EMT-related markers associated with tumor invasion in STS cells. Figure 4B showed that Frzb-transfected SW872 cells exhibited up-regulation of epithelial markers keratin 8 and keratin 18

⁴Unpublished data.

by Western analysis and E-cadherin by real-time RT-PCR. We were unable to detect E-cadherin protein using Western blot or immunofluorescence, perhaps due to the low endogenous protein level of E-cadherin in SW872 cells. Conversely, mesenchymal markers (i.e., N-cadherin, vimentin, and fibronectin) were markedly down-regulated in Frzb-transfected SW872 cells (Fig. 4B). Transcription factors Slug and Twist, both known to repress E-cadherin and promote a mesenchymal phenotype (24), were also down-regulated (Fig. 4B). Immunofluorescent staining showed down-regulation of keratin 18 and up-regulated vimentin (Fig. 4B) without noticeable changes in cellular morphology. We were unable to detect any changes in EMT-related markers in Frzb-transfected HT1080 cell line (data not shown). Our data further support the role of Frzb in modulating EMT-related events, depending on specific cellular context.

Third, several MMPs are additional Wnt target genes that play an important role in promoting sarcoma invasion (25–28). Thus, we also examined the effects of Frzb and DNLRP5 on MMPs expression. Frzb reduced expression of pro-MMP-2 protein in both HT1080 and SW872 cell lines (Fig. 4C), whereas expression of MMP-9 and MMP-14 were not affected (data not shown). Figure 4E showed that Frzb consistently reduced MMP-2 enzyme activity by zymography. DNLRP5 transfection showed similar effects on pro-MMP-2 expression and enzymatic activity (Fig. 4C). These results suggest that down-regulation of MMP-2 is also one of the mechanisms by which secreted Wnt antagonists suppress the invasiveness of STS.

Taken together, our data suggested that inhibition of Wnt signaling by secreted antagonists Frzb and DNLRP5 affected multiple Wnt-related pathways in STS cell lines, leading to reduced cellular invasiveness.

Inhibition of c-Met by RNA interference reduces *in vitro* invasion and *in vivo* tumor growth

We next examined whether c-Met expression is at least in part required for *in vitro* and *in vivo* tumor growth of STS cells. Figure 5A showed that c-Met protein expression in HT1080 and SW872 cell lines was efficiently inhibited up to 90% using a predesigned plasmid-based shRNA compared with a control shRNA construct encoding a scrambled artificial sequence. Down-regulation of c-Met protein by shRNA led to a marked reduction in cellular motility and invasion compared with control shRNA-transfected cells (Fig. 5B; $P < 0.01$). The control shRNA-transfected HT1080 and SW872 cell lines did not show any difference in c-Met expression and cellular invasion with their parental cell (data not shown). Consistently, c-Met silencing by shRNA led to inhibition of its downstream events, AKT and MAPK phosphorylation (Fig. 5A). Met shRNA also caused induction of epithelial markers (keratin 8/keratin 18) and down-regulation of mesenchymal markers (N-cadherin and vimentin) in SW872 cell line (Supplementary Fig. S3), suggesting that c-Met act downstream of Frzb to partially modulate the EMT.

To determine the relative contribution of c-Met to Wnt-mediated invasive activity of STS cells, we transiently cotransfected a β -catenin expression construct into Met shRNA-transfected HT1080 and SW872 cells. In control-transfected HT1080 cells, β -catenin increased cellular invasiveness by 63%, whereas in HT1080/Met-ShRNA cells, β -catenin only increased invasiveness by 6% (Student's *t* test, $P < 0.05$; Supplementary Fig. S4A). Similarly, β -catenin increased invasiveness by 221% in control SW872 cells, whereas in SW872/Met-ShRNA cells, β -catenin only increased invasiveness by 71% (Student's *t* test, $P < 0.05$; Supplementary Fig. S4A). In contrast, β -catenin-mediated cell motility was less dependent on Met expression. β -catenin transfection increased motility by 62% and 66% in control and Met ShRNA-transfected SW872 cells, respectively (Student's *t* test, $P = 0.77$; Supplementary Fig. S4B). Similarly, β -catenin increased motility by 21% in control and 37% in Met shRNA-transfected HT0180 cells (Student's *t* test, $P = 0.23$; Supplementary Fig. S4B). Together, these data suggested that Met expression was at least partially required for Wnt-mediated cellular invasiveness.

To further examine whether the effect of Met siRNA is independent of the Wnt pathway, overexpression of a full-length Met construct was used to rescue the phenotype found in Frzb and DNLRP5-transfected cells. In Frzb-transfected HT1080 cells, Met overexpression significantly increased motility and invasiveness compared with control-transfected cells (Student's *t* test, $P < 0.05$; Fig. 5C). Similarly, overexpression of Met significantly increased motility and invasiveness of DNLRP5-transfected HT1080 cells compared with controls (Student's *t* test, $P < 0.01$; Fig. 5C). In SW872 cells, overexpression of Met either restored (Student's *t* test, $P < 0.9$; Fig. 5C) or significantly increased (Student's *t* test, $P < 0.05$; Fig. 5C) motility and invasiveness of Frzb-transfected or DNLRP5-transfected cells. These results indicated that Met can overcome the repression induced by Wnt antagonists, therefore suggesting that Met acts as a downstream target for Wnt signaling in STS.

In addition, we showed that HT1080 cell line with stable Met suppression by shRNA did not form overt tumors in nude mice (Fig. 5D), whereas HT1080 cell line stably transfected with a control shRNA grew as rapidly as the parental cell line (data not shown). Together, these results suggest that inhibition of Met signaling by Frzb and DNLRP5 may at least in part be responsible for the antiinvasion and antitumor growth activities of these two antagonists.

Role of Frzb in the formation of lung metastasis *in vivo* and tissue immunostaining

Given that Frzb decreased STS cell motility and invasion *in vitro*, we examined whether Frzb can affect metastasis formation *in vivo*. For these experiments, HT1080 cells were used due to their propensity to form lung metastasis in nude mice by tail vein injection. Figure 6A showed that Frzb-transfected cell line formed 67.3% fewer lung nodules than control-transfected cells (Student's *t* test, $P < 0.01$). In addition, nodules formed by Frzb-transfected cells seem smaller by histologic examination than nodules from control-transfected cells (Fig. 6B). Consistent with its Wnt inhibitory activity (Fig. 1C), DNLRP5 transfection abrogated the formation of metastatic foci in tail-vein injected animals (data not shown). These results suggested that secreted Wnt antagonists negatively regulated the formation of lung metastasis by reducing the ability of STS cells to colonize lung tissue.

Discussion

Frzb/sFRP3, originally isolated as a chondrogenic factor from articular cartilage, is a potent antagonist of the Wnt signaling pathway (14,29). We previously showed that Frzb had a dramatic tumor suppressive activity *in vivo* on prostate cancer cell line PC-3, a cell type of epithelial origin (3). In this study, we further showed that Frzb markedly suppressed tumorigenicity of HT1080 cell line, which is of mesenchymal origin. These results suggest the generality of Frzb tumor suppressive function in cancer.

In addition to *in vivo* data, our *in vitro* studies showed that Frzb reduced cell motility and invasion associated with inhibition of Met signaling, up-regulation of epithelial markers (i.e., keratins 8 and 18), down-regulation of mesenchymal markers (i.e., vimentin, N-cadherin, fibronectin, Slug, and Twist), and reduced expression and activity of MMP-2. Given c-Met was reported as a Wnt target gene (6) and silencing of c-Met by shRNA achieved similar *in vitro* and *in vivo* effects as Frzb (Fig. 5), our data suggest that Met may serve as a downstream event of Frzb-induced effects in STS cell lines. Recent reports indicate that c-Met expression is regulated by Wnt signaling, at least in certain cellular context (6). Consistent with these reports, Frzb and DNLRP5 transfection led to down-regulation of c-Met, with a concomitant decrease in invasiveness of STS cell lines. ShRNA-mediated silencing confirmed that c-Met was partially responsible for the invasive activity and a reversal of EMT-related events (i.e., up-regulation of keratin 8/keratin 18 and down-regulation of fibronectin, N-cadherin, and Twist) in SW872 cell line. It is intriguing that although Wnt/TCF can induce c-Met expression,

LEF/TCF binding sites in the Met promoter have not yet been identified (6). Further studies are necessary to elucidate detailed mechanisms of c-Met regulation by Wnt signaling.

Frequent dysregulation of Met expression has been reported in mesenchymal tumors (22), and overexpression of c-Met can transform normal osteoblasts into osteosarcoma cells (23). Met signaling can be activated in tumor cells through several mechanisms. Overexpression of c-Met can lead to spontaneous dimerization of this receptor and subsequent activation, even in the absence of ligand (30). Another mechanism of Met activation is by ligand-dependent autocrine or paracrine stimulation, as seen in many types of mesenchymal tumors (31). For instance, osteosarcoma and rhabdomyosarcoma cells often coexpress c-Met and HGF, forming an autocrine circuit for constitutive Met signaling (22,32,33). This overactivity of Met signaling then promotes tumor growth and invasion (34). Conversely, down-regulation of c-Met by shRNA has been shown to inhibit proliferation, invasion, and anchorage-independent growth of rhabdomyosarcoma (35). Here, we showed that suppression of c-Met by shRNA in HT1080 cell line resulted in a complete inhibition of tumor growth in nude mice. In addition, c-Met knockdown also reduced motility and invasion of HT1080 and SW872 cell lines (Fig. 5B). Together, these results suggest that c-Met is a critical target for treatment of mesenchymal tumors. In addition, our study indicates that secreted Wnt antagonists Frzb and DNLRP5 can serve as a means to down-regulate c-Met and its mediated signaling in STS tumors.

The mechanism of Wnt activation have not been ascertained in the majority of sarcomas. Haydon et al. (36) have shown that cytoplasmic and nuclear accumulation of β -catenin occurred in osteosarcoma without any activating mutation. Sievers et al. (37) reported that APC gene promoter was hypermethylated without mutations in myxoid liposarcoma. Protocols are in progress in our laboratory to obtain clinical specimens to determine whether down-regulation of secreted Wnt antagonists or APC by hypermethylation or deletion are responsible for enhanced Wnt activity in STSs.

Oncogenic EMT is a well known phenomenon for invasive cancers, characterized by a conversion of epithelial cells into highly motile mesenchymal cells (38). On a molecular level, EMT is defined by loss of cell-cell adhesion, loss of epithelial differentiation, and induction of mesenchymal markers. Here, we showed that liposarcoma cells underwent a partial reversal of the EMT after Frzb transfection, shown by induction of epithelial markers (keratin 8/keratin 18) and down-regulation of mesenchymal markers (vimentin, N-cadherin, and fibronectin) without significant change in E-cadherin protein expression and EMT-related cell morphology. Lack of E-cadherin changes may be due to complex posttranscriptional and/or translational regulation in this cell line (39). This partial EMT reversal after Frzb transfection is accompanied by reduced motility and invasive capacity of liposarcoma cells. These data confirmed our recent reports on EMT reversal in prostate cancer PC-3 and osteosarcoma Saos-2 cell lines by Frzb and DNLRP5 (3,40). Slug and Twist, major transcriptional factors regulating the EMT process, have been shown to function as Wnt target genes (41,42). Thus, our results strongly suggest that Slug and Twist are the transcriptional factors responsible for Frzb-induced EMT reversal.

In addition to c-Met, several studies have indicated that MMP-2 plays an important role in tumor growth and invasiveness (43,44). The suppression of cellular invasion *in vitro* and lung metastasis formation *in vivo* by secreted Wnt antagonists Frzb and DNLRP5 is associated with significant down-regulation of pro-MMP-2 and its enzyme activity (Fig. 4C). A recent investigation by Wu et al. (25) suggests that MMP-2 is also a direct transcriptional target of Wnt. Therefore, secreted antagonists, such as Frzb or DNLRP5, may inhibit MMP-2 through direct modulation of LEF/TCF transcriptional activity in STS cells.

In conclusion, secreted Wnt antagonists Frzb and DNLRP5 inhibited Wnt signaling, leading to down-regulation of several potential Wnt target genes (e.g., c-Met, Slug, Twist, MMP-2) in

STS cell lines. Given the involvement of Wnt target genes in sarcoma progression (11,32,45) and the pronounced inhibitory effects of Frzb and DNLRP5 on tumorigenesis, invasion, and EMT reported in this study, secreted Wnt antagonists deserve further investigation as a new strategy for treatment of human sarcoma. Importantly, a secreted Wnt antagonist consisting of the CRD of Fz8 fused with human IgG has recently showed potent antitumor efficacy in animal models of teratocarcinoma (46). These results strongly suggest the feasibility of developing Frzb fusion protein as a new therapeutic agent. Further studies in our laboratories are in progress to establish the stability, bioavailability, and antitumor efficacy of Frzb fusion protein in animal models.

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Figure 1.

Frzb expression is down-regulated in STS cell lines, and Frzb transfection decreases anchorage-independent growth of HT1080 cells associated with Wnt inhibition. **A**, mRNA levels of Frzb and LRP5 in STS (HT1080, SW872, SK-LMS-1, SYO-1) and normal (NHDF, SMSC) cell lines were determined by real-time RT-PCR. Experiments were performed thrice. **B**, expression of Frzb and Met proteins were detected by Western blotting with anti-Frzb and Met antibodies. **C**, representative photographs of immunohistochemical detection of Frzb and Met expression in human sarcoma tissues showing negative (0), 1+, 2+, and 3+ staining intensity; magnification, 200×. Xenograft tumor tissues were used as negative and positive controls. **D**, expression of Frzb and DNLRP5 proteins were detected by Western blotting with

anti-Flag (Frzb) and anti-Myc (DNLRP5) antibodies (*insert*). For TCF/LEF transcriptional activity, stable transfectants (HT1080/PcDNA, HT1080/Frzb, HT1080/DNLRP5, SW872/PcDNA, SW872/Frzb, and SW872/DNLRP5) were cotransfected with *Super TOPFLASH* and *Super FOPFLASH* luciferase reporters with β -galactosidase plasmid as an internal control for transfection efficiency. The relative TCF/LEF transcriptional activity in Frzb and DNLRP5 transfectants was compared with vector control transfectants after normalizing transfection efficiency by the activity of β -galactosidase. *Columns*, means of relative luciferase activity (*Luc*/ β -gal) from three independent experiments; *bars*, SE.

Figure 2.

Frzb and DNLRP5 inhibit *in vivo* tumor growth. 1×10^6 HT1080 cell lines were inoculated s.c. into the right flank of NCR nu-nu (nude) mice. **A**, tumor growth curve. *Points*, mean tumor volume (each group contains 10 mice); *bars*, SE. A representative photograph of tumors harvested on day 15 after inoculation with Frzb or vector control-transfected HT1080 cells (*insert*). **B**, immunohistochemical staining of FLAG-tagged Frzb protein expression with anti-FLAG antibody in tumor tissue sections. Control immunostaining was performed with IgG isotype alone. **C**, tumor growth curve. *Points*, mean tumor volume (each group contains 10 mice); *bars*, SE. A representative photograph of tumors harvested on day 25 after inoculation with DNLRP5 or vector control-transfected HT1080 cells (*insert*).

Figure 3.

Frzb and DNLRP5 decrease motility and invasiveness of STS cells. Invasive capacity and motility were determined by transwell invasion assays with and without Matrigel. *A*, porous membranes without Matrigel were used to assess motility. HT1080 and SW872 cells were allowed to migrate for 8 and 12 h, respectively. Matrigel-coated membranes were used to assess invasion activity. HT1080 and SW872 cells were allowed to invade through Matrigel for 16 and 24 h, respectively. *B*, Matrigel invasion assays were performed with control and Frzb conditioned media. Nonmigrating cells in the upper chamber were removed with cotton swabs. Cells on the lower surface of the membrane were fixed in methanol and stained with hematoxylin. Motility and invasion activity was determined by counting 10 fields at 100×

magnification on each membrane and calculated as mean number of cells per field. *Columns*, mean number of migrating or invading cells per field; *bars*, SE.

Figure 4.

Frzb modulates Met signaling, expression of EMT-related markers, and MMP-2 activity in STS cells. **A**, expression of Met, phosphorylated Met at Tyr¹²³⁴ and Tyr¹²³⁵, phosphorylated AKT, total AKT, phosphorylated MAPK, total MAPK in Frzb-transfected and DNLRP5-transfected H1080 and SW872 cells were determined by Western blotting. **B**, expression of EMT-related markers in SW872 cell lines. Expression of E-cadherin was determined by real-time RT-PCR, as described in Materials and Methods. *Columns*, mean from three independent experiments; *bars*, SE. Expression of other epithelial markers (keratin 8/keratin 18) and mesenchymal markers (N-cadherin, fibronectin, vimentin), as well as transcriptional factors (Twist, Slug), was determined by Western blotting. Immunofluorescent microscopy of keratin

18 and vimentin staining in transfected SW872 cells; magnification, 400×. C, expression of pro-MMP-2 protein in stable transfectants was determined by Western blotting. Gelatinolytic activity of MMP-2 was evaluated by zymographic analysis.

Figure 5.

Inhibition of c-Met by shRNA reduces *in vitro* invasion and *in vivo* tumor growth of STS. *A*, the efficiency of c-Met inhibition by shRNA was evaluated by Western blot analysis of c-Met protein expression and its downstream events, AKT and MAPK activation. *B*, invasive capacity and motility of cells transfected with Met siRNA were determined by transwell invasion assays with and without Matrigel, as described in Fig. 3. *C*, motility and invasion assays were performed after Met overexpression in Frzb-transfected and DNLRP5-transfected cells. Motility and invasion activity was determined by counting 10 fields at 100× magnification on each membrane and calculated as mean number of cells per field. *Columns*, mean number of migrating or invading cells per field; *bars*, SE. *D*, tumor growth curve. *Points*, mean tumor

volume (each group contains 10 mice); *bars*, SE. A representative photograph of tumors harvested on day 12 after inoculation with HT1080/Met shRNA cells and control shRNA cells (*insert*).

Figure 6.

Frzb reduces the formation of lung metastasis *in vivo* and Met and Frzb expression in sarcoma tissues. Frzb or PcDNA stably transfected HT1080 cell lines were injected into tail vein of NCR nu-nu (nude) mice. *A*, lungs from mice injected with Frzb and PcDNA stable transfectants were harvested and fixed in Bouin's solution on day 28. Surface lung nodules were counted under a dissecting microscope. *Columns*, mean number of lung surface nodules from 10 mice in each group; *bars*, SE. A representative photograph of lungs from each transfection group (*insert*). *B*, representative photographs of lung sections from Frzb and PcDNA-transfected groups by H&E staining; magnification, 40 $\times$.