Wnt Inhibitory Factor 1 Decreases Tumorigenesis and Metastasis in Osteosarcoma

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Abstract

It has been reported that the progression of osteosarcoma was closely associated with the aberrant activation of canonical Wnt signaling. Wnt inhibitory factor-1 (WIF-1) is a secreted Wnt inhibitor whose role in human osteosarcoma remains unknown. In this study, WIF-1 expression in NHOst and osteosarcoma cell lines was determined by real-time reverse transcription-PCR, methylation-specific PCR, and Western blotting analysis. In addition, tissue array from patient samples was examined for WIF-1 expression by immunohistochemistry. Compared with normal human osteoblasts, WIF-1 mRNA and protein levels were significantly downregulated in several osteosarcoma cell lines. The downregulation of WIF-1 mRNA expression is associated with its promoter hypermethylation in these tested cell lines. Importantly, WIF-1 expression was also downregulated in 76% of examined osteosarcoma cases. These results suggest that the downregulation of WIF-1 expression plays a role in osteosarcoma progression. To further study the potential tumor suppressor function of WIF-1 in osteosarcoma, we established stable 143B cell lines overexpressing WIF-1. WIF-1 overexpression significantly decreased tumor growth rate in nude mice as examined by the s.c. injection of 143B cells stably transfected with WIF-1 and vector control. WIF-1 overexpression also markedly reduced the number of lung metastasis in vivo in an orthotopic mouse model of osteosarcoma. Together, these data suggest that WIF-1 exerts potent antiosteosarcoma effect in vivo in mouse models. Therefore, the reexpression of WIF-1 in WIF-1–deficient osteosarcoma represents a potential novel treatment and preventive strategy. Mol Cancer Ther; 9(3); 731–41. ©2010 AACR.

Introduction

Osteosarcoma is the most common primary bone cancer with a propensity for local invasion and early distant metastasis. Aggressive treatment protocols including chemotherapy and wide surgical resection can achieve cure in approximately 60% to 70% of patients. Greater than 30% of patients eventually develop disease relapse, primarily in the lungs (1). The 5-year event-free survival for patients with relapse is only 20% (1). Despite intensive search for new therapies, the outcome of relapsed patients has not significantly improved during the last two decades. Currently, molecular mechanisms underlying disease progression are still lacking. Therefore, there is a great need to understand the underlying mechanisms of tumor progression to define targets for novel therapies for osteosarcoma.

Aberrant activation of Wnt signaling has been reported in a variety of bone and soft-tissue sarcomas (2–5). Haydon et al. (6) showed that osteosarcoma harbors an accumulation of β-catenin either in the cytoplasm or in the nucleus, a hallmark of Wnt signaling activation. In addition, we showed the Wnt coreceptor LRP5 as a candidate marker for disease progression in human osteosarcoma and expression of this coreceptor in osteosarcoma tissue samples correlated with metastasis and a lower rate of disease-free survival in patients (7). These findings suggest an important role for aberrant Wnt activation in sarcoma disease progression.

The Wnt signaling pathway is initiated by a combination from 19 secreted Wnt ligands, 10 Frizzled receptors, and the coreceptor lipoprotein receptor–related protein 5/6 (LRP5/6). These ligand-receptor interactions then lead to the activation of multiple intermediate Wnt effectors including β-catenin, c-Jun-NH2-kinase, and calcium-channel regulators. The accumulation of β-catenin in the cytoplasm and its translocation to the nucleus represent the hallmark of the canonical Wnt pathway activation. In the nucleus, β-catenin forms a complex with lymphocyte enhancer factor/T-cell factor family of transcription factors to activate many oncogenes, such as c-Myc, cyclin D1, metalloproteinases, c-Met, etc. We reported that osteosarcoma cell lines expressed many Wnt ligands and
receptors, whereas secreted Wnt antagonists including secreted frizzled-related protein (sFRP) and Dickkopf (Dkk) families are commonly absent in osteosarcoma cells (7). These results suggest that a complex autocrine/paracrine growth mechanism exist in osteosarcoma. Therefore, inhibition of this mechanism by the reintroduction of secreted Wnt antagonists in osteosarcoma may lead to the downregulation of Wnt signaling, which provides a novel therapeutic approach for osteosarcoma.

Secreted Wnt antagonists are divided into two classes according to their mechanisms of action. One class directly binds to Wnt ligands to cause inhibition and includes the sFRP family, Wnt inhibitory factor-1 (WIF-1), and Cerberus (8). The second class including the Dkk family exerts inhibition by endocytosis of coreceptors LRP5/6 (8). We and others recently reported that several Wnt antagonists, including Frzb/sFRP3 and Dkk-3, function as tumor suppressors (9–11). WIF-1 is a unique Wnt antagonist, structurally distinct from sFRP and Dkk families, which contains a WIF domain for Wnt binding activity and epidermal growth factor repeats (12). The WIF domain has also been found in the Ryk orphan tyrosine kinase receptor (13). WIF-1 has been implicated to play a role in normal retinal development, being highly expressed during rod photoreceptor morphogenesis and inhibiting rod production, thereby, playing a key role in homeostasis during development (14). WIF-1 silencing by hypermethylation and consequent Wnt signaling activation has been shown in numerous cancers such as nasopharyngeal cancer (3), lung cancer (15), mesothelioma (16), breast cancer (17), and gastric cancer (18). However, its role in osteosarcoma is largely unknown, but with its potential as a future target for novel therapies, it is clear that WIF-1 needs to be further explored.

Here, we show that WIF-1 is downregulated in most osteosarcoma cell lines and tumor tissues and that WIF-1 reexpression markedly reduced both tumor growth rate and lung metastasis in mouse models of osteosarcoma.

Materials and Methods

Cell Culture and Plasmid

SaOS-LM7 was a gift from Dr. Eugenie Kleinerman (M.D. Anderson Cancer Center, Houston, TX). OS160 was a gift from Dr. Richard Gorlick (Albert Einstein College of Medicine, Bronx, NY). Normal human osteoblasts (NHOst) were obtained from Cambrex Bio Science and maintained in Osteoblast Growth Media (Lonza). Human osteosarcoma cell lines 143B, 143B.89.2, SaOS-2, N-methyl-N′-nitro-N-nitrosoguanidine (MNNG)/HOS and U2OS (American Type Culture Collection), SaOS-LM7, and OS160 were maintained in MEM medium supplemented with 10% fetal bovine serum. All cells were cultured at 37°C in a humidified incubator with 5% CO2.

Stable Transfection

143B cells were plated at 1.6 × 105 per 100-mm dish. At 60% confluency, cultures were transfected with PCDNA3.1 or WIF-1 using FuGENE 6 (Roche) according to the manufacturer’s instruction. After transfection, stable clones were selected with G418 (800 μg/mL) starting at 48 h after transfection and assayed for expression of the transgene by Western blotting and real-time reverse transcription-PCR (RT-PCR). Pooled transfectants (to avoid cloning artifacts) are propagated and maintained in MEMα containing 10% fetal bovine serum and 500 μg/mL G418.

 Luciferase Reporter Assays for Wnt Inhibition

143B cells were plated in a six-well plate at a density of 1.6 × 105 per well and incubated overnight. The cells were transiently cotransfected with 2 μg of TOPFLASH luciferase reporter plasmid (Upstate Biotechnology) and 0.4 μg of β-galactosidase (Invitrogen) into WIF-1 transfectant and vector control stable cell lines, to assay T-cell factor family of transcription factor/lymphocyte enhancer factor activation. Transfection was done using the Lipofectamine Plus reagent according to the manufacturer’s protocol (Invitrogen). After 48 h, cells were harvested and luciferase and β-galactosidase activities were measured using the 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.

Quantitative Real-time RT-PCR

Total RNA was isolated using the Trizol reagent (Invitrogen) according to the manufacturer’s instructions. Real-time RT-PCR was done as previously described (9). The sequences of the primers are as below: WIF-1, forward: 5′-CACCACAAGAAAGAAGG-3′; reverse: 5′-TTGGTTACTCCTCTGATC-3′. Matrix metalloproteinase (MMP) -14, forward: 5′-ACAACCCTGTCTGAAATGGAG-3′; reverse: 5′-GTGTTCTTCCATGCAACCTT-3′. PCR condition was as follows: 45 cycles of 30 s at 95°C, 30 s at 58°C, and 60 s at 72°C. Relative fold change in mRNA expression compared with control was calculated using the comparative ΔCt method (19). Ct is the cycle number at which fluorescence intensity first exceeds the threshold level. Δ Ct = Ct (target gene) – Ct (actin). Gene-specific primer sequences are available upon request. The specificity of amplification products was verified by agarose gel electrophoresis and melting curve analysis.

Western Blotting Analysis

Twenty to 80 μg of protein lysate were separated electrophoretically on denaturing SDS-polyacrylamide gel, transferred to nitrocellulose membranes, and probed with goat polyclonal IgG antibodies against WIF-1 (N-20; Santa Cruz Biotechnology). Blots were exposed to secondary antibodies and visualized using the SuperSignal West Pico Chemiluminescent Substrate (Pierce). For loading control, the membrane used in initial Western blot.
was placed in Restore Western blotting Stripping Buffer (Thermo Scientific) for 15 min to remove the antibody (primary and secondary antibody). Then, water wash for 5 min, 5% milk block for 1 h, and probe with β-actin antibody (Santa Cruz Biotechnology) was done.

**Zymogram Assay**

To determine the proenzyme and active form of MMP-2, zymogram assay was done as previously described (20). In brief, the condition medium was collected from WIF-1–transfected 143B cells and control cells, and concentrated 20 × using centricron (Millipore). Fifteen micro-liters of concentrated condition medium was separated by electrophoresis in 0.1% gelatin-impregnated gel (Bio-Rad). After getting renatured at room temperature for 1 h in zymogram renature buffer, the gel was incubated overnight at 37°C in the zymogram development buffer (Bio-Rad). The gel was then stained with Coomassie Blue and destained according to the manufacturer’s protocols (Bio-Rad). Gelatinolytic activity was visualized as clear bands on the gel.

**Soft Agar Colony Formation Assay**

Soft agar colony formation assay was done using six-well plates. Each well contained 2 mL of 0.8% agar in complete medium as the bottom layer, 1 mL of 0.35% agar in complete medium and 6,000 cells as the feeder layer, and 1 mL complete medium as the top layer. Cultures were maintained under standard culture conditions. The number of colonies was determined with an inverted phase-contrast microscope at ×100 magnification. A group of >10 cells was counted as a colony. The data are shown as mean number of colonies ± SEM of four independent wells at 14 d after the start of cell seeding.

**Motility Assay**

Motility was assessed with a scratch assay to measure two-dimensional cellular movement. Stable WIF-1– and control-transfected cells were cultured to confluence in 24-well plates. A scratch was made on the monolayer using a sterile pipette tip. The monolayer was washed with a migration assay buffer consisting of serum-free medium plus 0.1% bovine serum albumin. At the initiation of the experiment, a digital image of the scratch wound was taken at ×10 magnification. At 12 h, the same region was imaged again. The width of the scratch wounds was measured in Photoshop 7.0 (Adobe). The relative fold change of the scratch wound width (%) at 12 h after introduction of the scratch wound compared with the control was calculated as the average of five fields (magnification, ×40).

**Methylation Studies**

Genomic DNA was obtained from multiple osteosarcoma cell lines including 143B, 143.98.2, SaOS-2, SaOS-LM7, OS160, MNNG-HOS, and U2OS as well as from NHOst cells using the Blood & cell culture DNA mini kit (Qiagen). To quantify DNA methylation, the EZ DNA Methylation-Gold kit (Zymo Research) was used. Briefly, 20 μL (2 μg) of genomic DNA were digested in C to T conversion reagent (bisulfite). The DNA was denatured and converted at 98°C for 10 min and at 64°C for 2.5 h. Bisulfite-treated DNA was then cleaned and desulfonated using the M-binding buffer, followed by wash buffer, and desulfonation buffer. The bisulfite-modified DNA was amplified by PCR using a pair of methylation-specific primers (forward: 5′-GGCCTTATTGCGGTAT-3′; reverse: 5′-AAACCAATCAACGCAGAATAC-3′) and unmethylation specific primers (forward: 5′-GGGTGGTTATTGGGTGTAT-3′; reverse: 5′-AAACCAATCAACGCAGAATAC-3′).

**Demethylation Assay by 5-Aza-2′-Deoxycytidine**

Osteosarcoma cell lines SaOS-2, SaOS-LM7, MNNG-HOS, 143B, 143.98.2, U2-OS, OS-160, and normal osteoblast were treated with demethylating agent 5-Aza-2′-deoxycytidine (5-Aza-dC; 5 μmol/L) for 2 to 5 d. Total RNA was isolated and RT-PCR and real-time PCR were done to detect the mRNA level of WIF-1 as previously described (10).

**Immunohistochemistry**

A tissue array that consisted of 50 paraffin-embedded osteosarcoma tissue specimens was procured from the Cooperative Human Tissue Network after approval was obtained from the Children’s Oncology Group and the Institutional Review Board. Tumor tissue slides were deparaffinized and dehydrated using Slide Brite (Sasco Chemical Group, Inc.). Antigen was retrieved using 0.05 mol/L Glycine-HCL buffer (pH 3.5), containing 0.01% (w/v) EDTA, at 95°C for 20 min and stained with an antibody against human WIF-1 (N-20, Santa Cruz Biotechnology). Staining was visualized with diaminobenzidine using the Cell and Tissue Staining kit (R&D Systems). The immunostaining was scored as positive or negative for WIF-1 by a pathologist experienced in immunohistochemistry of human tissue sections.

**In vivo Tumorigenesis**

Male nu/nu nude mice (Taconic), at age 4 wk on arrival, were housed in pathogen-free conditions. The animal protocol was approved by the Institutional Animal Care Utilization Committee. WIF-1–transfected and vector control-transfected 143B cells were grown to near confluence, resuspended in 0.1 mL of PBS, and injected s.c. into the flank of nude mice at ×10^6 cells/0.1 mL. Tumor size was measured every 2 d using a caliper. The tumor volume was calculated by the formula 1/6 × ab^2 (π = 3.14; a is the long axis, and b is the short axis of the tumor). Growth curves were plotted from the mean tumor volume ± SEM from 10 animals in each group. Eighteen days after injection, the animals were sacrificed and tumors were harvested, measured, weighed, and fixed in 10% formalin. Wet tumor weight of each animal was calculated as mean weight ± SD from 10 animals in each group.
Metastasis Model

Male nu/nu nude mice (Taconic), at age 4 wk on arrival, were housed in pathogen-free conditions. WIF-1– and vector control–transfected 143B stable cell lines were grown to near confluence. Cell suspension (0.03 mL; 1 × 10⁷ cells/mL PBS) was injected percutaneously into the tibia of anesthetized nude mice. Each group contains 10 mice. Three weeks later, the animals were sacrificed according to an Institutional Animal Care Utilization Committee–approved protocol. Lungs were harvested, fixed in Bouin’s solution, and the number of surface lung metastatic nodules was counted. Mean number of lung nodules was compared between WIF-1 and vector control–transfected groups. Microscopic lung metastases were visualized on 5-μm paraffin-embedded sections stained with H&E.

Statistical Analysis

Comparisons of number of colonies, fold change in levels of mRNA, tumor weight, relative luciferase activity, and the width of the wound gaps between different transfection groups were conducted using Student’s t test. For tumor growth experiments, repeated measures ANOVA was used to examine the differences in tumor volume among different time points and transfection-time interactions. Additional posttest was done to examine the differences in tumor volume between vector control and WIF-1 transfection at each time point by the conservative Bonferroni method. All statistical tests were two sided. P < 0.05 was considered statistically significant.

Results

WIF-1 Protein and mRNA Expression Is Downregulated in Osteosarcoma and Is Associated with WIF-1 Promoter Hypermethylation

Endogenous levels of WIF-1 mRNA were examined in NHOst and in 7 osteosarcoma cell lines (SaOS-2, SaOS-LM7, MNNNG-HOS, 143B, 143.98.2, U2OS, and OS160) by quantitative real-time RT-PCR. WIF-1 mRNA levels were downregulated in six of seven osteosarcoma cell lines compared with NHOst (Fig. 1A). To study the potential mechanism of WIF-1 mRNA downregulation in osteosarcoma cell lines, we examined the methylation status of the WIF-1 promoter in these cell lines. Figure 1B showed that the relative levels of WIF-1 promoter methylation in five of seven osteosarcoma cell lines were significantly higher than that in NHOst. Possible contamination was ruled out by negative “no DNA” control reaction (Supplementary Fig. S1A). In addition, the relative levels of WIF-1 promoter methylation were inversely related to their mRNA levels (Fig. 1A and B). After demethylation by 5-Aza-dC, WIF-1 mRNA levels were increased in five (Saos-2, MNNNG-HOS, 143B, 143.98.2, U2-OS) of seven osteosarcoma cell lines with WIF-1 promoter methylation. Those cell lines with more WIF-1 promoter hypermethylation (MNNNG-HOS, 143B, 143.98.2, and U2-OS) have higher WIF-1 mRNA levels than those with low WIF-1 promoter methylation levels (Saos-2, Saos-LM7, and NHOst) after 5-Aza-dC treatment. However, OS-160 with high methylation level did not respond to the same concentration and duration of 5-Aza-dC treatment (Fig. 1A).

Western blot analysis of 5-Aza-dC–treated osteosarcoma cell lines (i.e., MNNNG-HOS, 143B, 143.98.2, and U2-OS) for different time periods did not cause an increase in the expression of WIF1 protein (Supplementary Fig. S1B). These results suggest that WIF-1 downregulation in the majority of osteosarcoma cell lines is through WIF-1 promoter hypermethylation.

Consistently, WIF-1 protein levels in all osteosarcoma cell lines were also downregulated compared with that in NHOst (Fig. 1C). To further examine WIF-1 protein expression in human osteosarcoma tissue specimens, we obtained paraffin-embedded tissue microarray of 50 human osteosarcoma cases from the Cooperative Human Tissue Network (with approval from the Children’s Oncology Group). Twelve of 50 cases showed positive staining, whereas the remaining cases (76%) showed no staining. Figure 1D is a representative microphotograph of the immunostained tissue sections showing positive and negative WIF-1 staining. These results suggest that WIF-1 downregulation is a common event in human osteosarcoma.

WIF-1 Suppresses In vitro Anchorage-Independent Growth and Cellular Motility

Because 143B cells can grow tumors locally as well as form spontaneous pulmonary metastases (21), this cell line was selected for further analysis of WIF-1–mediated Wnt signaling blockade. We confirmed the protein expression of WIF-1 transgene tagged by V5 in stable 143B cell line by Western blotting analysis using an anti-V5 antibody (Invitrogen; Fig. 2A, insert). Furthermore, to examine the inhibition of canonical Wnt activity by WIF-1, Lef-1/Tcf4 transcriptional activity was assessed by the TOPFLASH luciferase reporter assay in vector- and WIF-1–transfected 143B cell lines. Adjustment of transfection efficiency was done by cotransfection of a β-galactosidase expression vector. Compared with controls, WIF-1 reduced Lef-1/Tcf4 transcriptional activity by 62.5% (Fig. 2A, Student’s t test; P < 0.05). These results indicate that WIF-1 expression inhibits canonical Wnt activity in 143B cells.

Anchorage-independent growth and the ability to resist anoikis are hallmarks of metastatic cancer cells. When anchorage-independent growth was examined in soft agar, WIF-1–transfected 143B cells formed 76% less colonies than vector control cells (P < 0.01; Fig. 2B). We next assessed the cellular motility of 143B cells stably transfected with control vector or WIF-1 using a wound-healing assay. Figures 2C and D showed that WIF-1–transfected 143B cells exhibited less migration into the wounded area when compared with control-transfected cells (P < 0.01). The knockdown of WIF-1 mRNA of >90% in 143B was confirmed by real-time PCR (Supplementary Fig. S2). We did not observe any significant
changes in cellular proliferation and colony formation after WIF-1 knockdown in 143B cells (data not shown). We are aware that endogenous WIF-1 level in 143B cells is low (Fig. 1A).

**WIF-1 Inhibits In vivo Tumor Growth and the Formation of Lung Metastasis**

To examine the *in vivo* antitumor growth effects of WIF-1 in osteosarcoma, 143B cell lines overexpressing
Figure 2. Ectopic WIF-1 expression inhibits colony formation in soft agar and cell migration of 143B cells. A, WIF-1 transfection reduced TCF4 transcriptional activity in 143B cells compared with vector control-transfected cells, measured by the TOPFLASH reporter assay and adjusted by transfection efficiency. The expression of V5-tagged WIF-1 in transfected 143B cells was confirmed by Western blotting using an anti-V5 antibody (inset). B, anchorage-independent colony formation assay showed decreased number of colonies formed by WIF-1 transfection compared with vector control transfection of 143B cells; inset, a representative photograph of soft agar colonies at 18 d after cell seeding. C, representative photomicrographs of scratch colonies at 0 and 12 h after wounds were made. D, quantitative measurement of wound gaps by Photoshop software showed a reduced cellular motility in the WIF-1–transfected 143B cell line compared with vector control cells. Columns, mean relative change (%) of wound width in WIF-1 versus vector control–transfected 143B cells; bars, SD. Experiments were replicated thrice.
WIF-1 or vector control were s.c. injected into nude mice and tumor growth was evaluated. Figure 3A showed that 143B cells expressing WIF-1 exhibited a significantly slower growth rate than that of vector control cells ($P < 0.05$). In addition, Fig. 3B showed that the average wet weight of WIF-1–expressing tumors was $\sim 70\%$ less than that of vector control tumors (Student’s $t$ test; $P = 0.001$).

To evaluate the in vivo antimetastasis effect of WIF-1, we established a clinically relevant intratibial injection model of osteosarcoma that can lead to lung metastasis formation in nude mice. Figure 4A and B showed that the WIF-1–transfected 143B cell line formed 91% fewer lung nodules than control-transfected cells (Student’s $t$ test; $P = 0.034$). In addition, the size of nodules formed by WIF-1–transfected cells was smaller by histologic examination than those from control-transfected cells (Fig. 4C). This result showed the marked antimetastasis effects of WIF-1 expression in a clinically relevant mouse model.

Because accumulated evidence suggest that Wnt signaling may play an important role in tumor metastasis by regulating the expression of MMPs in tumors (9, 20, 22, 23), we examined the effects of WIF-1 overexpression on MMP-2, MMP-9, and MMP-14. Figure 4D showed that the WIF-1–transfected 143B cell line exhibited...
reduced protein levels of MMP-9 and MMP-14. No detectable changes in MMP-2 protein levels were found between WIF-1 and vector control–transfected 143B cell lines by Western blot assay (data not shown). MMP-14 is a membrane-type MMP that functions to activate MMP-2. Therefore, MMP-2 activity was determined by a zymogram assay. Supplementary Fig. S3 A and B showed that the cleaved band (lower band, 62 kDa) of MMP-2 in the WIF-1–transfected 143B cell line was significantly reduced, compared with the control vector–transfected 143B cell line, which indicated a reduced MMP-2 activity in the WIF-1–transfected 143B cell line.

Figure 4. WIF-1 reduced lung metastasis in an orthotopic osteosarcoma mouse model and downregulated the expression of MMP-9 and 14. A, lungs from mice injected with WIF-1 and vector control–transfected 143B cells were harvested and fixed in Bouin’s solution on day 21. Arrows, surface lung metastatic nodules. B, surface lung nodules were counted under a dissecting microscope. Columns, mean number of lung surface nodules from 10 mice in each group; bars, SEM. Inset, a representative photograph of lungs from each transfection group. C, representative photographs of lung sections from WIF-1 and vector control transfection groups by H&E staining (×40 magnification). Arrows, lung metastatic nodules. D, protein expression of WIF-1, MMP-9, and MMP-14 in WIF-1, and vector control–transfected 143B cell lines was determined by Western blotting assay.
line. This result suggested that WIF downregulated MMP-14 expression, leading to the reduction of MMP-2 activity. These results further suggest the regulatory role of Wnt signaling in MMP expression and tumor metastasis.

Discussion

Aberrant Wnt signaling plays a major role in multiple cancers, including osteosarcoma (7, 24, 25). Therefore, the inhibition of Wnt effects in osteosarcoma may represent major therapeutic potentials. At present, little is known about the functional role of naturally occurring, secreted Wnt antagonists, including WIF-1 in sarcoma. In this study, we showed that the expression of WIF-1 was commonly downregulated in osteosarcoma cell lines and human tumor tissues. By reexpressing WIF-1 in osteosarcoma cell line 143B, we showed the inhibition of anchorage-independent growth and cellular motility. Furthermore, the marked inhibitory effect of WIF-1 on both tumor growth and metastasis was shown in animal models. These findings strongly suggest a tumor suppressive role for WIF-1 in human osteosarcoma.

In this study, WIF-1 promoter was found to be hypermethylated in multiple osteosarcoma cell lines, which closely related to the downregulation of WIF-1 mRNA expression in these cell lines when compared with normal osteoblasts. In addition, the immunohistochemical staining of 50 paraffin-embedded osteosarcoma patient tissue samples with a WIF-1 antibody showed reduced WIF-1 expression in the majority (76%) of the samples. These results suggest that WIF-1 may function as a common tumor suppressor in osteosarcoma. However, our study has been restricted to a small sample size of paraffin-embedded tissues obtained through the Cooperative Human Tissue Network and the Children’s Oncology Group. To determine whether WIF-1 expression has prognostic value for clinical osteosarcoma, we have obtained Institutional Review Board approval for collecting surgical specimens and clinicopathologic data to establish an osteosarcoma patient follow-up cohort. In addition, the exact mechanism by which WIF-1 is hypermethylated in osteosarcoma cell lines is not very clear at this moment. Further studies are also ongoing to examine the mechanisms of WIF-1 inactivation by promoter hypermethylation in osteosarcoma.

WIF-1 silencing due to promoter hypermethylation has also been shown in many other cancers including colorectal, prostate, bladder, melanoma, lung cancers, etc. (5, 15, 17, 26, 27). Restoring WIF-1 expression in these cancer cells to study its biological function has been done by several groups (5, 15, 17, 26, 27). The commonly described effect of WIF-1 on these cancer cells is the inhibition of cell growth (5, 15, 17, 26, 27). In this study, we showed the inhibitory effect of WIF-1 expression in osteosarcoma on cell motility, anchorage-independent growth in soft agar, and in vivo tumor growth in nude mice of 143B cells. These results are consistent with reported findings in melanoma, esophageal adenocarcinoma, and bladder cancer (28, 29). However, we are unable to show the inhibitory effect of WIF-1 on anchorage-dependent growth in cell cultures (data not shown) as reported in other cancers (28, 29). During our investigation, a publication by Kansara et al. (30) showed that recombinant WIF-1 protein can significantly decrease 143B cell proliferation at concentrations up to 2 μg/mL. We also recently reported that recombinant WIF-1 protein achieved similar anti-proliferative effect in bladder cancer cell lines (29). It is possible that high concentration of WIF-1 is needed to achieve its anti-proliferative effect or apoptosis and that gene transfection may not be as effective as a recombinant protein as a therapeutic agent. The mechanisms of cell motility and anchorage-independent growth remain unclear for the 143B cell line and likely are the result of several context-dependent processes. We are currently investigating these mechanisms through detailed microarray analysis. However, difference in cell motility did not seem to involve changes in the epithelial to mesenchymal transition, given E-cadherin level did not change significantly in 143B cells after WIF-1 transfection (data not shown).

The complexity of Wnt extracellular and membrane components that consist of at least 19 Wnts, 10 Frizzled receptors, and coreceptors LRPS and 6 suggests the existence of multiple Wnt-receptor interactions for initiation of Wnt signaling in osteosarcoma. At this moment, little is known about the exact Wnt-receptor interactions for osteosarcoma development and progression. It is reasonable to speculate that the difference between our results and others in the effect of WIF-1 anchorage-dependent growth of 143B cells may be due to the context-dependent effect of WIF-1 on Wnt activity and cell growth inhibition. Further studies are necessary to determine which Wnt-receptor interactions are predominantly affected by WIF-1 in osteosarcoma.

Patients with osteosarcoma frequently present with hematogenous metastasis to the lungs (31, 32). Despite multimodality treatment including surgery and chemotherapy, the 5-year survival of patients with relapsed osteosarcoma remains at ~20% (33–36). At present, there is no targeted antimetastatic therapy available for osteosarcoma. To our best knowledge, we are first to report that WIF-1 markedly inhibits tumor metastasis in an orthotopic animal model of osteosarcoma. This model of intratibial injection of 143B cells leading to 100% lung metastasis (21) closely recapitulates the process of osteosarcoma tumor metastasis in human. Therefore, our results suggest the potential for developing WIF-1 as a targeted antimetastatic agent for clinical use in osteosarcoma cases with aberrant Wnt signaling.

MMPs are a family of proteolytic enzymes that can degrade the extracellular matrix and facilitate cellular invasion and migration (37). High MMP-9 expression was observed in pretreatment osteosarcoma tumor samples and in most metastatic lesions, leading to the speculation that MMP-9 expression is associated with the micrometastatic behavior of osteosarcoma (38). Membrane-type
metalloproteinase (MT1-MMP), also known as MMP-14, has been shown to also play a critical role in metastasis (39). MMP-9 and 14 are transcriptional targets of Wnt signaling (23) and have been correlated with poor disease-free survival in osteosarcoma (40–43). We showed that WIF-1 overexpression reduced MMP-9 and MMP-14 protein expression in 143B cells. Taken together, these results suggest that WIF-1 exhibits its markedly antitumor metastasis effect in osteosarcoma through multiple complex mechanisms. Further studies are in progress to dissect pathways modulated by WIF-1 for metastasis in osteosarcoma.

Based on its significant antitumor and antimetastasis effects in vivo in animal models of osteosarcoma, WIF-1 represents a promising target for developing therapeutic and preventive strategies against metastatic osteosarcoma with aberrant Wnt signaling. At this point, the interaction of WIF-1 with key Wnt components at the membrane and extracellular levels needs further delineation. In addition, the study of WIF-1–regulated pathways for osteosarcoma tumor growth and metastasis may be helpful for identification of biomarkers and targets for WIF-1 deficient osteosarcoma as WIF-1 expression is commonly downregulated in this disease.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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