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Wnt Inhibitory Factor 1 (WIF-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 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 normal human osteoblast and osteosarcoma cell lines was determined by real-time RT-PCR, methylation-specific PCR (MSP), and Western blotting analysis. In addition, tissue array from patient samples was examined for WIF-1 expression by immunohistochemistry. Compared to normal human osteoblasts, WIF-1 mRNA and protein levels were significantly down-regulated 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 subcutaneous 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 anti-osteosarcoma effect *in vivo* in mouse models. Therefore, re-expression of WIF-1 in WIF-1 deficient osteosarcoma represents a potential novel treatment and preventive strategy.

Keywords

osteosarcoma; Wnt; WIF-1; tumor growth; metastasis

Introduction

Osteosarcoma (OS) 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 60–70% of patients. Greater than 30% of patients eventually develop disease relapse, primarily in the lungs (1). The five-

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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 in order to define targets for novel therapies for OS.

Aberrant activation of Wnt signaling has been reported in a variety of bone and soft-tissue sarcomas (2–5). Haydon et al. (6) demonstrated that OS harbors an accumulation of beta-catenin either in the cytoplasm or in the nucleus, a hallmark of Wnt signaling activation. In addition, we demonstrated the Wnt co-receptor LRP5 as a candidate marker for disease progression in human OS and expression of this co-receptor in OS 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 co-receptor Lipoprotein Receptor-Related Protein 5/6 (LRP5/6). These ligand-receptor interactions then lead to activation of multiple intermediate Wnt effectors including beta-catenin, JNK, and calcium-channel regulators. The accumulation of beta-catenin in the cytoplasm and its translocation to the nucleus represent the hallmark of the canonical Wnt pathway activation. In the nucleus, beta-catenin forms a complex with lymphocyte enhancer factor/T cell factor family of transcription factors (LEF/TCF) to activate many oncogenes, such as c-Myc, cyclin D1, metalloproteinases, c-Met, etc. We reported that OS 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 OS cells (7). These results suggest that a complex autocrine/paracrine growth mechanism exist in OS. Therefore, inhibition of this mechanism by re-introduction of secreted Wnt antagonists in OS may lead to downregulation of Wnt signaling which provides a novel therapeutic approach for OS.

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 co-receptors 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 (EGF) 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 demonstrated in numerous cancers such as nasopharyngeal cancer (3), lung cancer (15), mesothelioma (16), breast cancer (17), and gastric cancer (18). However, its role in OS 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 down-regulated in a majority of OS cell lines and tumor tissues and that WIF-1 re-expression markedly reduced both tumor growth rate and lung metastasis in mouse models of OS.

Materials and Methods

Cell culture and plasmid

SaOS-LM7 was a gift from Dr. Eugenie Kleinerman (MD Anderson Cancer Center, Houston, TX). OS160 was a gift from Dr. Richard Gorlick (Albert Einstein College of Medicine, Bronx, NY). Normal Human Osteoblasts (NHOb) were obtained from Cambrex Bio Science and maintained in Osteoblast Growth Media (Lonza). Human OS cell lines 143B, 143.89.2, SaOS-2, MNNG/HOS and U2OS (American Type Culture Collection), SaOS-LM7, and OS160 were maintained in MEM α medium supplemented with 10% fetal bovine serum (FBS). All cells were cultured at 37°C in a humidified incubator with 5% CO₂. *PCDNA3.1* Directional TOPO Expression vector was obtained from Invitrogen. An Ultimate ORF WIF-1 clone (ID: IOH11153) was obtained from Invitrogen and subcloned into the *PCDNA3.1* TOPO vector.

Stable transfection

143B cells were plated at 1.6×10^5 per 100-mm dish. At 60% confluency, cultures were transfected with *PCDNA3.1* or *WIF-1* using FuGENE6 (Roche) according to 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 RT-PCR. Pooled transfectants (to avoid cloning artifacts) are propagated and maintained in MEM α containing 10% FBS and 500 µg/mL G418.

Luciferase Reporter Assays for Wnt inhibition

143B cells were plated in a 6-well plate at a density of 1.6×10^5 per well and incubated overnight. The cells were transiently co-transfected 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 TCF/LEF activation. Transfection was performed 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 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 TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Real-time RT-PCR was performed as previously described (9). The sequences of the primers are as below: WIF-1, forward: 5'-CACTGCCTACGAGAGGAAGG-3'; reverse: 5'-TTGGGGTACTCGCTATCCAC-3'. MMP-14, forward: 5'-ACAACCCTGTGCGAAATGGAG-3'; reverse: 5'-GTGTCTTCCATGCCAACCTT-3'. PCR condition was as follows: 45 cycles of 30 seconds at 95°C, 30 seconds at 58°C, 60 seconds at 72°C. Relative fold change in mRNA expression compared to control was calculated using the comparative C_t method (19). 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 blotting analysis

Twenty to 80 µg of protein lysate was 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 hour, and probe with β -actin antibody (Santa Cruz Biotechnology)

Zymogram assay

To determine proenzyme and active form of MMP-2, zymogram assay was performed as previously described (21). In brief, the condition medium was collected from WIF-1 transfected 143B cells and control cells, and concentrated 20 $\times$ using centricon (Millipore). 15 μ L concentrated condition medium was separated by electrophoresis in 0.1% gelatin-impregnated gel (Bio-Rad). After re-natured at room temperature for one hour in zymogram re-nature buffer, the gel was incubated overnight at 37°C in zymogram development buffer (Bio-Rad). 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 performed 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 6000 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 $\times 100$ magnification. A group of >10 cells was counted as a colony. The data are shown as mean number of colonies $\pm$ SE of four independent wells at 14 days 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 migration assay buffer (MAB) consisting of serum-free medium plus 0.1% BSA. At the initiation of the experiment, a digital image of the scratch wound was taken at 10X 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 hour after introduction of the scratch wound compared to the control was calculated as the average of 5 fields (40X magnification).

Methylation studies

Genomic DNA was obtained from multiple OS cell lines including 143B, 143.98.2, SaOS-2, SaOS-LM7, OS160, MNNG-HOS, and U2OS as well as from NHOst cells using Blood & cell culture DNA mini kit (Qiagen). In order to quantify DNA methylation, the EZ DNA Methylation-Gold Kit (Zymo Research) was used. Briefly, 20 μ L (2 μ g) of genomic DNA was diluted in C to T conversion reagent (bisulfite). The DNA was denatured and converted at 98°C for 10 minutes and 64°C for 2.5 hours. Bisulfite-treated DNA was then cleaned and desulphonated using the M-binding buffer, followed by wash buffer, and desulphonation buffer. The bisulfite modified DNA was amplified by PCR using a pair of methylation specific primers (MSP, forward: 5'-GGGCGTTTTATTGGGCGTAT-3'; reverse: 5'-AAACCAACAATCAACGAAC-3') and un-methylation specific primers (UMSP, forward: 5'-GGGTGTTTTATTGGGTGTAT-3'; reverse: 5'-AAACCAACAATCAACAAAAC-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 μ mol/L) for 2–5 days.

Total RNA was isolated and reverse transcription and real time PCR were performed to detect the mRNA level of WIF-1 as described previously (10).

Immunohistochemistry

A tissue array consisted of 50 paraffin-embedded OS tissue specimens was procured from the Cooperative Human Tissue Network after approval was obtained from the Children's Oncology Group (COG) and the Institutional Review Board. Tumor tissue slides were de-paraffinized and dehydrated using Slide Brite (Sasco Chemical Group, Inc.). Antigen was retrieved using 0.05M 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 diaminobenzadine 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), 4-week old on arrival, were housed in pathogen-free conditions. The animal protocol was approved by the Institutional Animal Care Utilization Committee (IACUC). WIF-1 transfected and vector control transfected 143B cells were grown to near confluence, resuspended in 0.1 ml of PBS and injected subcutaneously into the flank of nude mice at 1×10^6 cells/0.1 ml. Tumor size was measured every 2 days using 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. 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 $\pm$ SD from 10 animals in each group.

Metastasis model

Male nu/nu nude mice (Taconic), 4-week old on arrival, were housed in pathogen-free conditions. WIF-1 and vector control transfected 143B stable cell lines were grown to near confluence. 0.03 ml of cell suspension (1×10^7 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 IACUC-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- μ m 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 post-test 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 down-regulated in OS and associated with WIF-1 promoter hypermethylation

Endogenous levels of WIF-1 mRNA were examined in normal human osteoblasts (NHOS) and in 7 OS cell lines (SaOS-2, SaOS-LM7, MNNG-HOS, 143B, 143.98.2, U2OS, OS160) by quantitative real-time RT-PCR. WIF-1 mRNA levels were down-regulated in 6 of 7 OS cell lines as compared to NHOS (Fig. 1A). To study the potential mechanism of WIF-1 mRNA downregulation in OS cell lines, we examined methylation status of the WIF-1 promoter in these cell lines. Figure 1B showed that the relative levels of WIF-1 promoter methylation in 5 of 7 OS cell lines were significantly higher than that in NHOS. 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-2'-deoxycytidine, WIF-1 mRNA levels were increased in 5 (Saos-2, MNNG-HOS, 143B, 143.98.2, U2-OS) out of 7 osteosarcoma cell lines with WIF-1 promoter methylation. Those cell lines with more WIF-1 promoter hypermethylation (MNNG-HOS, 143B, 143.98.2, U2-OS) have higher WIF-1 mRNA levels than those with low WIF-1 promoter methylation levels (Saos-2, Saos-LM7, NHOS) 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 OS cell lines (i.e. MNNG-HOS, 143B, 143.98.2, U2-OS) for different time periods did not cause an increase in the expression of WIF1 protein (supplementary Fig. S1B). These results suggest WIF-1 downregulation in the majority of OS cell lines is through WIF-1 promoter hypermethylation.

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

WIF-1 suppresses *in vitro* anchorage-independent growth and cellular motility

Since 143B cells can grow tumors locally as well as form spontaneous pulmonary metastases (20), 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 TOPFLASH luciferase reporter assay in vector and WIF-1 transfected 143B cell lines. Adjustment of transfection efficiency was performed by co-transfection of a β -galactosidase expression vector. Compared to controls, WIF-1 reduced LEF-1/TCF4 transcriptional activity by 62.5% (Fig. 2A, Student's *t* test; $P < 0.05$). These results indicate 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 (Fig. 2B, $P < 0.01$). We next assessed 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 knock-down of WIF-1 mRNA of more than 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 knock-down 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* anti-tumor growth effects of WIF-1 in OS, 143B cell lines overexpressing WIF-1 or vector control were subcutaneously 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, Figure 3B showed that the average wet weight of WIF-1 expressing tumors was about 70% less than that of vector control tumors (Student's *t* test; $P = 0.001$).

To evaluate the *in vivo* anti-metastasis effect of WIF-1, we established a clinically relevant intra-tibial injection model of OS that can lead to lung metastasis formation in nude mice. Figure 4A, 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 histological examination than those from control transfected cells (Fig. 4C). This result demonstrated the marked anti-metastasis effects of WIF-1 expression in a clinically relevant mouse model.

Since accumulated evidence suggest that Wnt signaling may play an important role in tumor metastasis by regulating the expression of MMPs in tumors (9,21–23), we examined the effects of WIF-1 overexpression on MMP-2, 9, and 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, 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 to the control vector transfected 143B cell line, which indicated a reduced MMP-2 activity in the WIF-1 transfected 143B cell line. This result suggested that WIF down-regulated MMP-14 expression, leading to 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 OS (7,24,25). Therefore, inhibition of Wnt effects in OS 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 down-regulated in OS cell lines and human tumor tissues. By re-expressing WIF-1 in OS cell line 143B, we demonstrated inhibition of anchorage independent growth and cellular motility. Furthermore, the marked inhibitory effect of WIF-1 on both tumor growth and metastasis was demonstrated in animal models. These findings strongly suggest a tumor suppressive role for WIF-1 in human OS.

In this study, WIF-1 promoter was found to be hypermethylated in multiple OS cell lines, which closely related to the down-regulation of WIF-1 mRNA expression in these cell lines when compared to normal osteoblasts. In addition, immunohistochemical staining of 50 paraffin-embedded OS patient tissue samples with a WIF-1 antibody showed reduced WIF-1 expression in the majority (76%) of the samples. These results suggest WIF-1 may function as a common tumor suppressor in OS. 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 OS, we have obtained IRB approval for collecting surgical specimens and clinicopathologic data to establish an OS patient follow-up cohort. In addition, the exact mechanism by which WIF-1 is hypermethylated in OS 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 OS.

WIF-1 silencing due to promoter hypermethylation has also been shown in many other cancers including colorectal, prostate, bladder, melanoma, lung cancers, etc. (26–30). Restoring WIF-1 expression in these cancer cells to study its biological function has been done by several groups (26–30). The commonly described effect of WIF-1 on these cancer cells is the inhibition of cell growth (26–30). In this study, we showed the inhibitory effect of WIF-1 expression in OS 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 (31–33). 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 (31–33). During our investigation, a publication by Kansara et al. (34) 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 (33). 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 as effective as recombinant protein as a therapeutic agent. The mechanisms of cell motility and anchorage independence remain unclear for 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 appear to involve changes in the epithelial to mesenchymal transition (EMT), 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 which consist of at least 19 Wnts, 10 Frizzled receptors, and co-receptors LRP5 and 6 suggest the existence of multiple Wnt-receptor interactions for initiation of Wnt signaling in OS. At this moment, little is known about the exact Wnt-receptor interactions for OS 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 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 OS.

Patients with OS frequently present with hematogenous metastasis to the lungs (35,36). Despite multi-modality treatment including surgery and chemotherapy, five-year survival of patients with relapsed OS remains approximately 20% (37–40). At present, there is no targeted anti-metastatic therapy available for OS. To our best knowledge, we are first to report that WIF-1 markedly inhibits tumor metastasis in an orthotopic animal model of OS. This model of intra-tibial injection of 143B cells leading to 100% lung metastasis (20) closely recapitulates the process of OS tumor metastasis in human. Therefore, our results suggest the potential for developing WIF-1 as a targeted anti-metastatic agent for clinical use in OS cases with aberrant Wnt signaling.

Matrix metalloproteinases (MMPs) are a family of proteolytic enzymes that can degrade the ECM and facilitate cellular invasion and migration (41). High MMP-9 expression was observed in pre-treatment OS tumor samples and in a majority of metastatic lesions, leading to speculation that MMP-9 expression is associated with the micrometastatic behavior of OS

(42). Membrane-type metalloproteinase (MT1-MMP), also known as MMP-14, has been shown to also play a critical role in metastasis (43). MMP-9 and 14 are transcriptional targets of Wnt signaling (23) and have been correlated with poor disease-free survival in OS (44–47). We demonstrated 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 anti-tumor metastasis effect in OS through multiple complex mechanisms. Further studies are in progress to dissect pathways modulated by WIF-1 for metastasis in OS.

Based on its significant anti-tumor and anti-metastasis effects *in vivo* in animal models of OS, WIF-1 represents a promising target for developing therapeutic and preventive strategies against metastatic OS 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, study of WIF-1 regulated pathways for OS tumor growth and metastasis may be helpful for identification of biomarkers and targets for WIF-1 deficient OS as WIF-1 expression is commonly down-regulated in this disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations list

OS	osteosarcoma
WIF-1	Wnt Inhibitory Factor-1
MMP	metalloproteinase
ECM	extracellular matrix
NHOS	Normal Human Osteoblast

References

1. Link M, Goorin A, Miser A, et al. The effect of adjuvant chemotherapy on relapse free survival in patients with osteosarcoma of the extremity. *N Engl J Med* 1986;314:1600–6. [PubMed: 3520317]
2. Barker N, Clevers H. Mining the Wnt pathway for cancer therapeutics. *Nat Rev Drug Discov* 2006;5:997–1014. [PubMed: 17139285]
3. Lin YC, You L, Xu Z, et al. Wnt signaling activation and WIF-1 silencing in nasopharyngeal cancer cell lines. *Biochem Biophys Res Commun* 2006;341:635–40. [PubMed: 16427602]
4. Weeraratna AT, Jiang Y, Hostetter G, et al. Wnt5a signaling directly affects cell motility and invasion of metastatic melanoma. *Cancer Cell* 2002;1:279–88. [PubMed: 12086864]
5. Wissmann C, Wild PJ, Kaiser S, et al. WIF-1, a component of the Wnt pathway, is down-regulated in prostate, breast, lung, and bladder cancer. *J Pathol* 2003;201:204–12. [PubMed: 14517837]
6. Haydon RC, Deyrup A, Ishikawa A, et al. Cytoplasmic and/or nuclear accumulation of the beta-catenin protein is a frequent event in human osteosarcoma. *Int J Cancer* 2002;102:338–42. [PubMed: 12402302]

7. Hoang BH, Kubo T, Healey JH, et al. Expression of LDL receptor-related protein 5 (LRP5) as a novel marker for disease progression in high-grade osteosarcoma. *Int J Cancer* 2004;109:106–11. [PubMed: 14735475]
8. Kawano Y, Kypka R. Secreted antagonists of the Wnt signalling pathway. *J Cell Sci* 2003;116:2627–34. [PubMed: 12775774]
9. Zi X, Guo Y, Simoneau AR, et al. Expression of Frzb/secreted Frizzled-related protein 3, a secreted Wnt antagonist, in human androgen-independent prostate cancer PC-3 cells suppresses tumor growth and cellular invasiveness. *Cancer Res* 2005;65:9762–70. [PubMed: 16266997]
10. Guo Y, Xie J, Rubin E, et al. Frzb, a secreted Wnt antagonist, decreases growth and invasiveness of fibrosarcoma cells associated with inhibition of Met signaling. *Cancer Res* 2008;68:3350–60. [PubMed: 18451162]
11. Abarzua F, Sakaguchi M, Takaishi M, et al. Adenovirus-mediated overexpression of REIC/Dkk-3 selectively induces apoptosis in human prostate cancer cells through activation of c-Jun-NH2-kinase. *Cancer Res* 2005;65:9617–22. [PubMed: 16266978]
12. Hsieh JC, Kodjabachian L, Rebbert ML, et al. A new secreted protein that binds to Wnt proteins and inhibits their activities. *Nature* 1999;398:431–6. [PubMed: 10201374]
13. Lu W, Yamamoto V, Ortega B, Baltimore D. Mammalian Ryk is a Wnt coreceptor required for stimulation of neurite outgrowth. *Cell* 2004;119:97–108. [PubMed: 15454084]
14. Hunter DD, Zhang M, Ferguson JW, Koch M, Brunken WJ. The extracellular matrix component WIF-1 is expressed during, and can modulate, retinal development. *Mol Cell Neurosci* 2004;27:477–88. [PubMed: 15555925]
15. Mazieres J, He B, You L, et al. Wnt inhibitory factor-1 is silenced by promoter hypermethylation in human lung cancer. *Cancer Res* 2004;64:4717–20. [PubMed: 15256437]
16. Batra S, Shi Y, Kuchenbecker KM, et al. Wnt inhibitory factor-1, a Wnt antagonist, is silenced by promoter hypermethylation in malignant pleural mesothelioma. *Biochem Biophys Res Commun* 2006;342:1228–32. [PubMed: 16516163]
17. Ai L, Tao Q, Zhong S, et al. Inactivation of Wnt inhibitory factor-1 (WIF-1) expression by epigenetic silencing is a common event in breast cancer. *Carcinogenesis* 2006;27:1341–8. [PubMed: 16501252]
18. Yamashita S, Tsujino Y, Moriguchi K, Tatematsu M, Ushijima T. Chemical genomic screening for methylation-silenced genes in gastric cancer cell lines using 5-aza-2'-deoxycytidine treatment and oligonucleotide microarray. *Cancer Sci* 2006;97:64–71. [PubMed: 16367923]
19. Tsai CN, Tsai CL, Tse KP, Chang HY, Chang YS. The Epstein-Barr virus oncogene product, latent membrane protein 1, induces the downregulation of E-cadherin gene expression via activation of DNA methyltransferases. *Proc Natl Acad Sci USA* 2002;99:10084–9. [PubMed: 12110730]
20. Luu HH, Kang Q, Park JK, et al. An orthotopic model of human osteosarcoma growth and spontaneous pulmonary metastasis. *Clin Exp Metastasis* 2005;22:319–29. [PubMed: 16170668]
21. Guo Y, Zi X, Koontz Z, et al. Blocking Wnt/LRP5 signaling by a soluble receptor modulates the epithelial to mesenchymal transition and suppresses met and metalloproteinases in osteosarcoma SaOS-2 cells. *J Orthop Res* 2007;25:964–71. [PubMed: 17318900]
22. Marchenko GN, Marchenko ND, Leng J, Strongin AY. Promoter characterization of the novel human matrix metalloproteinase-26 gene: regulation by the T-cell factor-4 implies specific expression of the gene in cancer cells of epithelial origin. *Biochem J* 2002;363:253–62. [PubMed: 11931652]
23. Wu B, Crampton SP, Hughes CC. Wnt signaling induces matrix metalloproteinase expression and regulates T cell transmigration. *Immunity* 2007;26:227–39. [PubMed: 17306568]
24. Polakis P. Wnt signaling and cancer. *Genes Dev* 2000;14:1837–51. [PubMed: 10921899]
25. Hoang BH, Kubo T, Healey JH, et al. Dickkopf 3 inhibits invasion and motility of SaOS-2 osteosarcoma cells by modulating the Wnt-beta-catenin pathway. *Cancer Res* 2004;64:2734–9. [PubMed: 15087387]
26. Taniguchi H, Yamamoto H, Hirata T, et al. Frequent epigenetic inactivation of Wnt inhibitory factor-1 in human gastrointestinal cancers. *Oncogene* 2005;24:7946–52. [PubMed: 16007117]
27. Mazieres J, He B, You L, et al. Wnt inhibitory factor-1 is silenced by promoter hypermethylation in human lung cancer. *Cancer Res* 2004;64:4717–20. [PubMed: 15256437]
28. Lin YC, You L, Xu Z, et al. Wnt inhibitory factor-1 gene transfer inhibits melanoma cell growth. *Hum Gene Ther* 2007;18:379–86. [PubMed: 17472570]

29. Ai L, Tao Q, Zhong S, et al. Inactivation of Wnt inhibitory factor-1 (WIF-1) expression by epigenetic silencing is a common event in breast cancer. *Carcinogenesis* 2006;27:1341–8. [PubMed: 16501252]
30. Wissmann C, Wild PJ, Kaiser S, et al. WIF-1, a component of the Wnt pathway, is down-regulated in prostate, breast, lung, and bladder cancer. *J Pathol* 2003;201:204–12. [PubMed: 14517837]
31. Lin YC, You L, Xu Z, et al. Wnt inhibitory factor-1 gene transfer inhibits melanoma cell growth. *Hum Gene Ther* 2007;18:379–86. [PubMed: 17472570]
32. Clement G, Guilleret I, He B, et al. Epigenetic alteration of the Wnt inhibitory factor-1 promoter occurs early in the carcinogenesis of Barrett's esophagus. *Cancer Sci* 2008;99:46–53. [PubMed: 18005197]
33. Tang Y, Simoneau AR, Liao WX, et al. WIF-1, a Wnt pathway inhibitor, regulates SKP2 and c-myc expression leading to G1 arrest and growth inhibition of human invasive urinary bladder cancer cells. *Mol Cancer Ther* 2009;8:458–68. [PubMed: 19174556]
34. Kansara M, Tsang M, Kodjabachian L, et al. Wnt inhibitory factor 1 is epigenetically silenced in human osteosarcoma, and targeted disruption accelerates osteosarcomagenesis in mice. *J Clin Invest* 2009;119:837–51. [PubMed: 19307728]
35. Bacci G, Avella M, Picci P, Briccoli A, Dallari D, Campanacci M. Metastatic patterns in osteosarcoma. *Tumori* 1988;74:421–7. Review. [PubMed: 3055577]
36. Ferrari S, Briccoli A, Mercuri M, et al. Post relapse survival in osteosarcoma of the extremities: prognostic factors for long-term survival. *J Clin Oncol* 2003;21:710–715. [PubMed: 12586810]
37. Mialou V, Philip T, Kalifa C, et al. Metastatic osteosarcoma at diagnosis: prognostic factors and long-term outcome--the French pediatric experience. *Cancer* 2005;104:1100–9. [PubMed: 16015627]
38. Harris MB, Gieser P, Goorin AM, et al. Treatment of metastatic osteosarcoma at diagnosis: a Pediatric Oncology Group Study. *J Clin Oncol* 1998;16:3641–3648. [PubMed: 9817286]
39. Meyers PA, Heller G, Healey JH, et al. Osteogenic sarcoma with clinically detectable metastasis at initial presentation. *J Clin Oncol* 1993;11:449–453. [PubMed: 8445419]
40. Marina NM, Pratt CB, Rao BN, Shema SJ, Meyer WH. Improved prognosis of children with osteosarcoma metastatic to the lung(s) at the time of diagnosis. *Cancer* 1992;70:2722–27. [PubMed: 1423203]
41. Nelson AR, Fingleton B, Rothenberg ML, Matrisian LM. Matrix metalloproteinases: biologic activity and clinical implications. *J Clin Oncol* 2000;18:1135–49. [PubMed: 10694567]
42. Himelstein BP, Asada N, Carlton MR, Collins MH. Matrix metalloproteinase-9 (MMP-9) expression in childhood osseous osteosarcoma. *Med Pediatr Oncol* 1998;31:471–4. [PubMed: 9835898]
43. Itoh Y. MT1-MMP: a key regulator of cell migration in tissue. *IUBMB Life* 2006;58:589–96. [PubMed: 17050376]
44. Foukas AF, Deshmukh NS, Grimer RJ, Mangham DC, Mangos EG, Taylor S. Stage-IIB osteosarcomas around the knee: A study of MMP-9 in surviving tumour cells. *J Bone Joint Surg Br* 2002;84:706–11. [PubMed: 12188489]
45. Kido A, Tsutsumi M, Iki K, et al. Overexpression of matrix metalloproteinase (MMP)-9 correlates with metastatic potency of spontaneous and 4-hydroxyaminoquinoline 1-oxide (4-HAQO)-induced transplantable osteosarcomas in rats. *Cancer Lett* 1999;137:209–16. [PubMed: 10374843]
46. Heikkila P, Teronen O, Hirn MY, et al. Inhibition of matrix metalloproteinase-14 in osteosarcoma cells by clodronate. *J Surg Res* 2003;111:45–52. [PubMed: 12842447]
47. Uchibori M, Nishida Y, Nagasaka T, Yamada Y, Nakanishi K, Ishiguro N. Increased expression of membrane-type matrix metalloproteinase-1 is correlated with poor prognosis in patients with osteosarcoma. *Int J Oncol* 2006;28:33–42. [PubMed: 16327977]

Figure 1.

WIF-1 expression is down-regulated in OS cell lines, which is associated with promoter methylation. **A**, mRNA levels of WIF-1 in 7 OS cell lines (SaOS-2, SaOS-LM7, MNNG-HOS, 143B, 143.98.2, U2-OS, OS-160) and normal human osteoblasts (NHOst) were determined by real-time RT-PCR. After all these osteosarcoma cell lines and normal osteoblast were treated with 5-Aza-2'-deoxycytidine (5-Aza-dC; 5 μ mol/L) for 72 h, real time RT-PCR were performed to detect the WIF-1 mRNA level. Experiments were replicated three times. **B**, WIF-1 promoter hypermethylation is evident in 5 of 7 OS cell lines. Methylation status of WIF-1 promoter was determined by disulfite treatment of cell lines followed by methylation-specific PCR. **C**, Expression of WIF-1 proteins were detected by Western blotting with an anti-

hWIF-1 antibody. **D**, Representative photograph (200X magnification) of negative versus positive immunohistochemical staining of human OS tissue microarray using an anti-hWIF-1 antibody.

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 to vector control transfected cells, measured by 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 (insert). **B**, Anchorage-independent colony formation assay showed decreased number of colonies formed by WIF-1 transfection compared to vector control transfection of 143B cells; A representative photograph of soft agar colonies at 18 days after cell seeding (insert). **C**, Representative photomicrographs of scratch wounds at 0 hours and 12 hours 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 to vector control cells. Columns represent mean relative change (%) of wound width in WIF-1 vs vector control transfected 143B cells. Bars, standard deviation (SD). Experiments were replicated three times.

Figure 3.

WIF-1 inhibits *in vivo* tumor growth in nude mice. 1×10^6 143B cells per mice were inoculated subcutaneously into the left flank of NCR nu-nu (nude) mice. **A**, Tumor growth curve. Points, mean tumor volume (each group contains 10 mice); Bars, standard error (SE). **B**, Tumor wet weight. Tumors were harvested at 18 days after inoculation. Columns, mean wet tumor weight from 10 mice in each group; Bars, SE. A representative photograph of harvested tumors is shown (insert)

Figure 4.

WIF-1 reduces lung metastasis in an orthotopic OS mouse model and down-regulated 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. Surface lung metastatic nodules were pointed out by arrows. **B**, 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 is shown (insert). **C**, representative photographs of lung sections from WIF-1 and vector control transfection groups by H&E staining ($\times 40$ magnification). Lung metastatic nodules were

pointed out by arrows. **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.