

Blockade of Wnt Signaling Inhibits Angiogenesis and Tumor Growth in Hepatocellular Carcinoma

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Abstract

Aberrant activation of Wnt signaling plays an important role in hepatocarcinogenesis. In addition to direct effects on tumor cells, Wnt signaling might be involved in the organization of tumor microenvironment. In this study, we have explored whether Wnt signaling blockade by exogenous expression of Wnt antagonists could inhibit tumor angiogenesis and control tumor growth. Human Wnt inhibitory factor 1 (WIF1) and secreted frizzled-related protein 1 (sFRP1) were each fused with Fc fragment of human IgG1 to construct fusion proteins WIF1-Fc and sFRP1-Fc. The recombinant adenoviral vectors carrying WIF1-Fc and sFRP1-Fc driven by cytomegalovirus promoter were constructed. Ad-WIF1-Fc or Ad-sFRP1-Fc induced the expression and correct conformation of WIF1-Fc and sFRP1-Fc fusion proteins. These molecules caused down-regulation of *E2F1*, *cyclin D1*, and *c-myc* and promoted cell apoptosis in hepatocellular carcinoma cells. Treatment of established hepatocellular carcinoma tumors with Ad-WIF1-Fc and/or Ad-sFRP1-Fc resulted in significant inhibition of tumor growth and prolonged animal survival. The antineoplastic effect was associated with increased apoptosis of tumor cells, reduced microvessel density, and decreased expression of vascular endothelial growth factor and stromal cell-derived factor-1. Tube formation and migration of human microvascular endothelial cells and mouse endothelial progenitor cells (EPC) were significantly inhibited by both WIF1-Fc and sFRP1-Fc. In addition, these molecules blocked EPC differentiation and caused EPC apoptosis. Our data indicate that Wnt antagonists WIF1-Fc and sFRP1-Fc inhibit Wnt signaling and exert potent antitumor activity by increasing the apoptosis rate in tumor cells and by impairing tumor vascularization. [Cancer Res 2009;69(17):6951–9]

Introduction

The Wnt/Frizzled (Fzd) signaling cascade is important for cell fate determination during embryonic liver development as well as for maintaining tissue homeostasis in the adult (1, 2). Hepatocellular carcinoma (HCC) is a tumor with frequent aberrant activation of Wnt signaling (3, 4). Nuclear and/or cellular β -catenin accumulation has been observed in most of HCC tumors. However,

genetic mutations of *β -catenin*, *APC*, or *Axin* genes are found only in some of these cases (5, 6). Accumulating evidence suggest that regulatory mechanisms other than mutations involving β -catenin or proteins in its destruction complex are important in HCC (7, 8). Functional loss of Wnt antagonists [secreted Fzd-related protein (sFRP), Wnt inhibitory factor 1 (WIF1), and Dickkopf] can contribute to activation of the Wnt pathway (9). Down-regulation by promoter hypermethylation of the Wnt antagonist sFRP has been identified in human HCC and in a *c-myc/E2F1* transgenic mouse model of HCC (10–12). Down-regulation of other Wnt antagonists WIF1 and Dickkopf has been found in other types of cancers (13, 14). Thus, through various mechanisms, Wnt signaling pathway is implicated at various stages of hepatocarcinogenesis, making this pathway an attractive therapeutic target.

Angiogenesis is a key issue for the maintenance of tumor growth, particularly for HCC, a neoplasia characterized by marked hypervascularization (15–17). Tumor angiogenesis can occur locally by proliferation and migration of endothelial cells from surrounding existing vessels or systemically by mobilization of bone marrow-derived endothelial progenitor cells (EPC), which enter the peripheral blood circulation, migrate to sites of angiogenesis, and incorporate into growing vessels (18–20). Functional studies suggest that Wnt signaling is required for angiogenesis (21, 22). Mice with targeted disruptions of Wnt/Fzd genes, such as *Wnt2*, *Wnt4*, *Wnt7b*, *Fzd4*, and *Fzd5*, displayed abnormalities in the development of vasculature. Mutations in the *Fzd4* gene are linked to familial exudative vitreoretinopathy, a hereditary ocular disorder characterized by a failure of peripheral retinal vascularization (21, 22). Numerous Wnt signaling components, including *Wnt*, *Fzd*, *sFRP*, and *Dickkopf*, are expressed by human umbilical vein and microvascular endothelial cells (23, 24). The stabilization of cytosolic β -catenin and activation of a T-cell factor (TCF)-luciferase promoter were observed in cultured endothelial cells and endogenous TCF activity was inhibited by transfection with a secreted inhibitor of canonical Wnt signaling (23–26). It has been shown recently that Wnt2 is an autocrine growth and differentiation factor specific for hepatic sinusoidal endothelial cells (27). Although these data point to a critical role for Wnt/Fzd signaling in embryonic vascular development and in the function of endothelial cells, it is unclear whether this pathway is involved in tumor angiogenesis.

In this study, we have explored if the blockade of Wnt signaling by exogenous expression of Wnt antagonists *WIF1* and *sFRP1* could inhibit tumor angiogenesis and induce antitumor activity in HCC.

Materials and Methods

Cell lines and antibodies. Human cell lines 293 (embryonic kidney containing the E1A region of Ad5), HepG2, PLC/PRF/5, Huh7, Hep3B (human HCC cell lines), and BJ (human normal fibroblast) were obtained from the American Type Culture Collection. Cell lines 293, PLC/PRF/5,

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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Huh7, Hep3B, and BJ were cultured in DMEM (Invitrogen/Life Technologies) supplemented with 10% heat-inactivated fetal bovine serum, 2 mmol/L glutamine, 100 units/mL penicillin, and 100 mg/mL streptomycin. HepG2 cells were grown in RPMI 1640 (Invitrogen/Life Technologies) supplemented with 10% fetal bovine serum, 2 mmol/L glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin. Primary culture of human microvascular endothelial cells (HMVEC) were obtained from Cascade Biologics and cultured in medium 131 supplemented with Microvascular Growth Supplement (Cascade Biologics). HMVEC were used between the fourth and the seventh passages.

The primary antibodies used were goat anti-WIF1 (AF134), goat anti-sFRP1 (AF1384), and goat anti-β-catenin (AF1329; R&D Systems); mouse anti-caspase-3 (BD Biosciences); and polyclonal anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Biogenesis).

Animals. BALB/c nude mice and C57BL/6 mice, 4 to 6 weeks old, were purchased from Harlan. Animals were kept under standard pathogen-free conditions and received care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" by the National Academy of Sciences. The experiments were done in accordance with the local animal commission.

Adenoviral vector construction. Human *WIF1*, *sFRP1*, and *IgG-Fc* cDNA were cloned into pBluescript by *SpeI/BamHI* to form pBS/WIF1-Fc and pBS/sFRP1-Fc. The WIF1-Fc and sFRP1-Fc were inserted into the pTrack-CMV by *NotI/XhoI* to make pTrack-CMV-WIF1-Fc and pTrack-CMV-sFRP1-Fc. The latter plasmids were linearized by *PmeI* and cotransformed into BJ5183 cells by electroporation with pAdEasy-1. The resultant recombinant plasmids were transfected into 293 cells with Lipofectamine 2000 (Invitrogen) to generate adenoviral vectors. Vectors were propagated in 293 cells and purified by centrifugation on cesium chloride by standard procedures. Titers of adenoviruses (IU) were determined by immunoassay with anti-hexon antibody to the 293 cells.

Differentiation of EPC from mononuclear cells of bone marrow. Mononuclear cells from bone marrow of C57BL/6 mice were cultured on fibronectin-coated dishes in EMB2 medium supplemented with 5% fetal bovine serum, 10 ng/mL mouse epidermal growth factor (R&D Systems), 1 ng/mL basic fibroblast growth factor (R&D Systems), 10 ng/mL rat vascular endothelial growth factor (VEGF; R&D Systems), 10 ng/mL mouse insulin-like growth factor-I (R&D Systems), and 1 µg/mL hydrocortisone and antibiotics (Clonetics) for 7 days. At this time point, EPC were used for tube formation, migration, and cell survival assays.

TCF activity assay. Tumor cells were seeded in 24-well plates and transfected by Lipofectamine LTX (Invitrogen) with TOP flash and FOP flash plasmids. A *Renilla* luciferase reporter plasmid (Promega) was mixed as an internal control. Luciferase activity was measured using a Dual-Luciferase kit (Promega).

Analysis of gene expression by real-time PCR. Total RNA was purified from cells using a Trizol reagent (Life Technologies). First-strand cDNA was synthesized using 2 µg RNA and Moloney murine leukemia virus reverse transcriptase (Promega). For determination of the levels of the WNT/β-catenin targeted genes, primers for PCR of *E2F1*, *cyclin D1*, *c-myc*, *Axin2*, and *GAPDH* were designed by TaqMan program as follows: *E2F1*, atgtttctgtgcctgag and atctgtggtgaggatgagg; *cyclin D1*, gtgctgcaagt-gaaacc and atccaggtggcagcatct; *c-myc*, taccctctcaacgacagcag and tcttgacattctctcgggtg; *Axin2*, agccaagcgatctcaaaaagg and ggtaggcattttctccatcac; and *GAPDH*, ccaaggtcatcatgacaac and tgtcataccaggaaatgagc. A SYBR Green PCR Master Mix (Applied Biosystems) was employed, and GAPDH was coamplified as an endogenous control to standardize the amount of the sample RNA added to the reaction. The PCR conditions were as follows: 95°C for 5 min to activate the hot-start DNA polymerase followed by 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s.

Treatment of established HCC tumor. S.c. HCC tumors were established by inoculation of 5×10^6 Huh7 or Hep3B cells into the right flank of male BALB/c nude mice. When the tumors reached 60 to 150 mm³, mice were treated by intratumor injection of adenoviral vectors at 1×10^9 IU or saline as control. Tumor size was measured in two perpendicular diameters by using a caliper every 3 days and tumor volume (*V*) was calculated using the formula: $V \text{ (mm}^3\text{)} = \text{length} \times \text{width}^2/2$.

Orthotopic liver HCC tumors were established by injection of 2×10^6 Huh7 cells into the left liver lobe of BALB/c nude mice during laparotomy with anesthesia of ketamine-xylazine. A single tumor nodule (~3 mm in diameter) was observed in liver 7 days after inoculation of tumor cells. Mice were randomized into five groups receiving Ad-sFRP1-Fc or/and Ad-WIF1-Fc (1×10^9 IU/animal), Ad-GFP, or saline as controls by i.v. injection. Survival was checked daily and animals were killed if moribund. Median survival analysis was done by using the Kaplan-Meier survival analysis, which was done by using GraphPad Prism software (GraphPad Software).

Terminal deoxynucleotide transferase-mediated dUTP nick end labeling and immunohistochemistry. To detect apoptotic rate in tumor specimens, *In Situ* Cell Apoptosis Detection Kit (Chemicon) was used according to the manufacturer's protocol. Immunohistochemistry for detection of vessel density was carried out in tumor samples using anti-CD31 antibody as described before (28).

Tube formation assay. A 48-well plate was coated with 150 µL Matrigel (Becton Dickinson). Then, 10,000 HMVEC or 100,000 EPC were dispensed into each well and incubated with different concentration of fusion proteins in medium 131 containing 10% low-serum growth supplement for 4 to 6 h. Each well was photographed and tube formations were quantified by counting the number of connecting branches between two discrete endothelial cells.

Migration assay. Inserts (8 µm pores; Costar) for 24-well culture plates were coated with 100 µg/mL rat tail collagen type I (Becton Dickinson). HMVEC and EPC were resuspended in medium 131 containing 1% bovine serum albumin (Sigma) and seeded into the upper chamber. The lower chamber was filled with medium 131 containing 1% bovine serum albumin and human VEGF₁₆₅ (Peprotech) at a final concentration of 5 ng/mL. The chambers were incubated for 4 to 6 h at 37°C to allow cells to migrate through the collagen-coated membranes. Five to eight representative fields in each well were counted under the microscope to determine the number of migrated cells.

Statistical analysis. Data were presented as the mean ± SE or SD (as appropriate) of three independent experiments. When indicated, the statistical differences were calculated by using a nonparametric test (unpaired *t* test, two-tailed) for unpaired samples. Differences between groups were compared by the ANOVA test.

Results

Inhibition of tumor cell proliferation by WIF1-Fc and sFRP1-Fc *in vitro*. We constructed recombinant adenoviral vectors carrying fusion proteins comprising human WIF1 or sFRP1 coupled via a 5-amino acid flexible linker (Gly-Ser-Gly-Gly-Ser) to the NH₂ termini of the Fc domains of human IgG1 (Supplementary Fig. S1A). The encoded recombinant fusion proteins were purified from conditioned medium of vector-infected A549 cells by Hi-trap protein A column. WIF1-Fc and sFRP1-Fc were present in a monomer and dimer conformation in SDS-PAGE under reduced and nonreduced conditions, respectively (Supplementary Fig. S1B). The expression of these proteins was confirmed by Western blotting using specific antibodies against WIF1-Fc and sFRP1-Fc (Supplementary Fig. S1C).

We first evaluated whether Ad-WIF-Fc and Ad-sFRP1-Fc had a direct inhibitory effect on human HCC cells. Figure 1A shows significant inhibition of cell survival in a time-dependent manner in Huh7, PLC/PRF/5, and HepG2 cells treated with different adenovirus compared with cells treated with Ad-GFP. However, this inhibitory effect was more pronounced in those cells treated with a combination of Ad-WIF1-Fc and Ad-sFRP1-Fc. To exclude whether the inhibitory effect was due to different sensitivity of tumor cell to adenovirus infection, we measured WIF1-Fc and sFRP1-Fc secretion in supernatants from vector-infected cells. As shown in Supplementary Fig. S2, all tumor and normal cells expressed

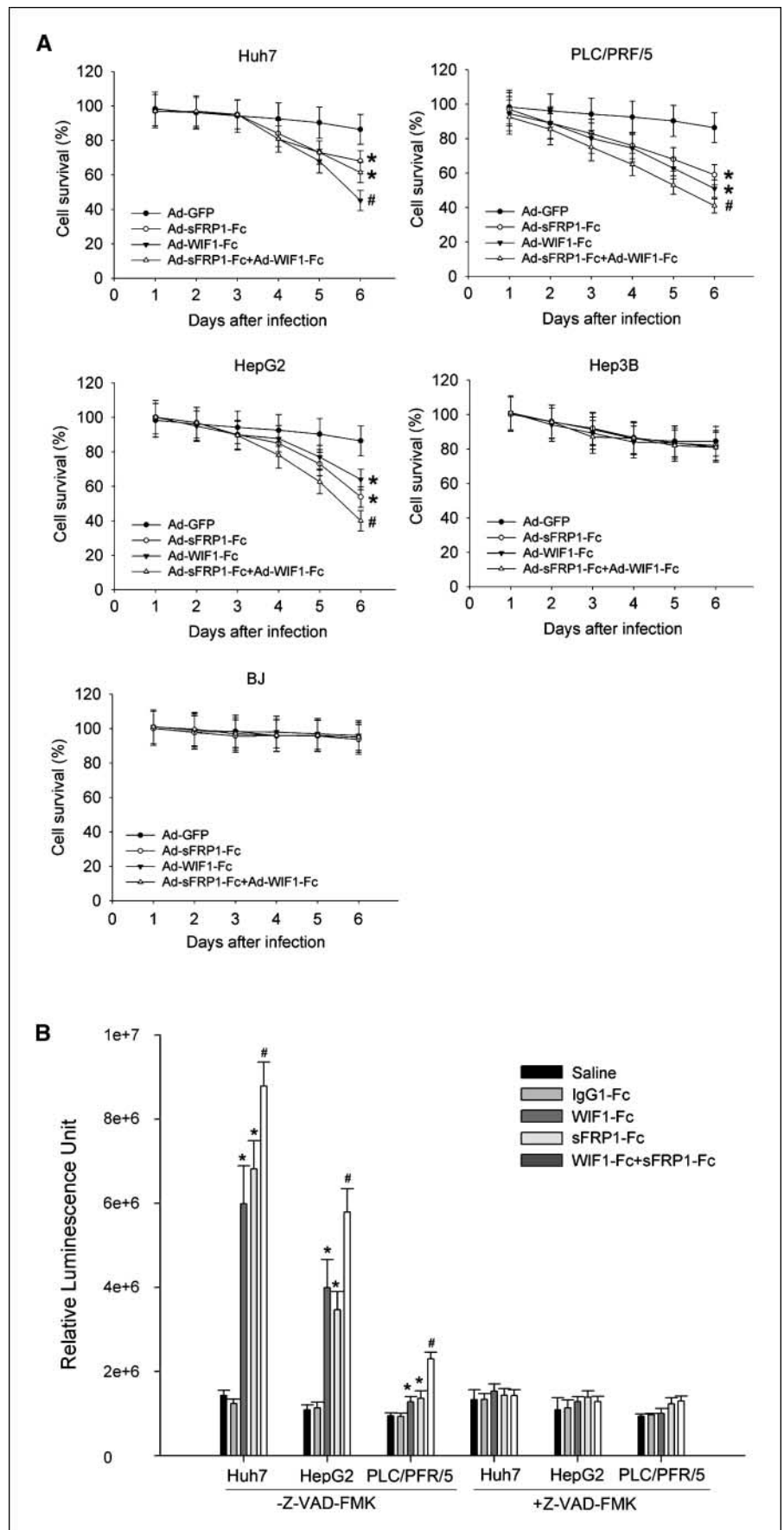


Figure 1. WIF1-Fc and sFRP1-Fc inhibit cell growth in part by inducing apoptosis. *A*, inhibition of HCC cell growth by adenoviral vectors expressing WIF1-Fc and sFRP1-Fc. Tumor and normal cells were infected with different adenoviral vectors at a multiplicity of infection of 5. Cell proliferation was measured by MTT assay at different time points postinfection. *B*, induction of apoptosis by purified fusion proteins. Huh7, HepG2, and PLC/PRF/5 cells were cultured with recombinant fusion proteins at 1 μ g/mL in the absence or presence of caspase inhibitor Z-VAD-FMK (20 μ mol/L). Activation of caspase-3/7 was measured after 24 h of incubation. Mean \pm SD of three independent experiments. *, $P < 0.01$, compared with saline or IgG1-Fc control groups; #, $P < 0.05$, compared with WIF1-Fc or sFRP1-Fc.

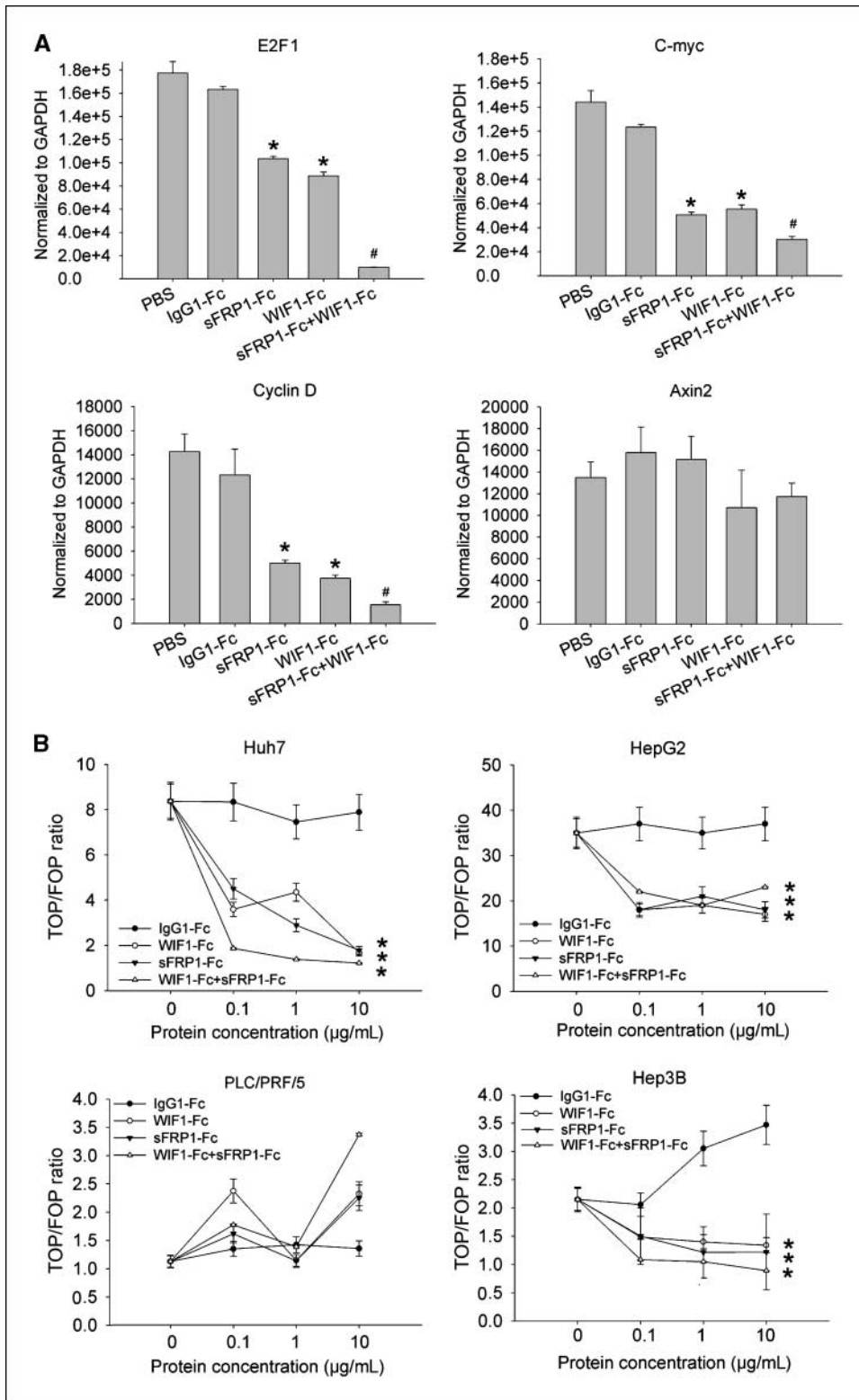


Figure 2. Inhibition of Wnt signaling by WIF1-Fc and sFRP1-Fc. **A**, inhibition of Wnt targeting genes expression. Huh7 cells were cultured with recombinant fusion proteins at 1 µg/mL and expression of E2F1, cyclin D1, c-myc, and Axin2 was evaluated by real-time PCR. Results were normalized to GAPDH. **B**, inhibition of TCF activity by recombinant fusion proteins. Tumor cells were cotransfected with TOP flash and FOP flash reporter plasmids and followed by incubation in medium containing different concentrations of recombinant fusion proteins. Mean ± SD of three independent experiments. *, $P < 0.01$, compared with PBS or IgG1-Fc control groups; #, $P < 0.05$, compared with WIF1-Fc or sFRP1-Fc.

comparable level of WIF1-Fc and sFRP1-Fc. Similar inhibitory effect on cell proliferation was also observed when tumor cells were cultured with purified fusion protein WIF1-Fc or sFRP1-Fc in a dose-dependent manner (Supplementary Fig. S3). To determine whether increased cell death is one of the mechanisms responsible for the inhibitory effects of fusion proteins, caspase activation was

evaluated in Huh7, PLC/PRF/5, and HepG2 cells treated with WIF1-Fc and sFRP1-Fc. As shown in Fig. 1B, both WIF1-Fc and sFRP1-Fc fusion proteins induced significant caspase activation in tumor cells. A more dramatic effect was observed when both proteins were used. Caspase activation was completely inhibited when the general caspase inhibitor Z-VAD-FMK was added.

Effect of WIF1-Fc and sFRP1-Fc on the expression of Wnt target genes and on TCF activity. To investigate if our fusion proteins were able to block Wnt signaling pathway, we cultured Huh7 cells in the presence of WIF1-Fc and/or sFRP1-Fc and examined the expression of Wnt target genes by real-time PCR. We found that *E2F1*, *cyclin D1*, and *c-myc* were down-regulated significantly in tumor cells treated with fusion proteins (Fig. 2A). We also used TOP flash and FOP flash reporters to further assess the effect of the fusion proteins on TCF activity, the hallmark of canonical Wnt signaling activity. We found that both Huh7 and HepG2 cells exhibited high TCF activity that was efficiently abrogated by WIF1-Fc and sFRP1-Fc fusion proteins. However, PLC/PRF/5 had a low TCF activity and neither WIF1-Fc nor sFRP1-Fc had any significant effect in these cells. Hep3B had also a relatively low TCF activity and the effects of Wnt blockers was moderate (Fig. 2B).

Suppression of tumor growth in animal models with s.c. and orthotopic HCC tumors. To explore the therapeutic effect of fusion proteins, we established s.c. and orthotopic HCC tumors in nude mice. In the s.c. models based on implantation of Huh7,

tumors were treated by intratumoral injection of Ad-WIF1-Fc and/or Ad-sFRP1-Fc. As shown in Fig. 3A, animals receiving saline or control vector Ad-GFP had progressive tumor growth. Treatment with Ad-WIF1-Fc or Ad-sFRP1-Fc led to significant inhibition of tumor growth. The effect was more profound when combination therapy with Ad-WIF1-Fc plus Ad-sFRP1-Fc was used. Similar antitumor effect was also observed in the s.c. models based on implantation of Hep3B cells that were not responsive to Ad-WIF1-Fc and/or Ad-sFRP1-Fc *in vitro* (Fig. 3B). In orthotopic HCC tumor model, the treatment was given by i.v. administration. As shown in Fig. 3C, treatment with Ad-sFRP1-Fc, Ad-WIF1-Fc, or both could significantly increase survival of tumor-bearing animals compared with animals receiving saline or control vector. The median survival was 51 and 55 days for the saline and Ad-GFP groups and 60.5, 60, and 63 days for mice treated with Ad-WIF1-Fc, Ad-sFRP1-Fc, or both (Fig. 3C).

To determine the duration of transgene expression, serum levels of WIF1-Fc and sFRP1-Fc were determined by ELISA after i.v. administration of Ad-WIF1-Fc and Ad-sFRP1-Fc in normal BALB/c nude mice. As shown in Supplementary Fig. S4A, WIF1-Fc and

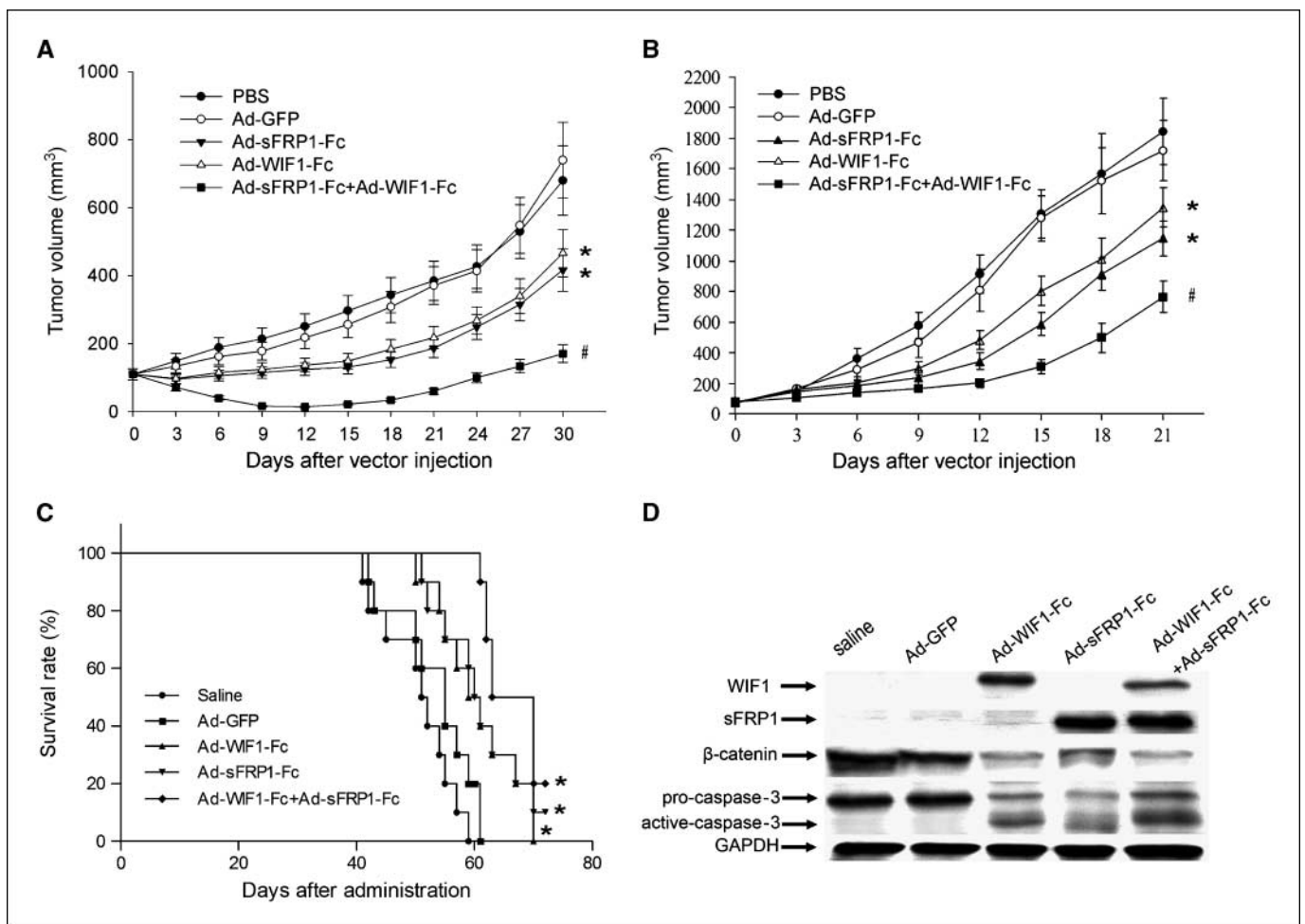


Figure 3. Antitumor activity in animal models and transgene expression *in vivo*. A and B, s.c. models were established on BALB/c nude mice by implantation of Huh7 (A) and Hep3B (B) tumor cells. The tumors were treated by intratumoral injection of adenoviral vectors. Size of tumors was measured and presented as mean \pm SE ($n = 12$ mice per group in the Huh7 tumor model and $n = 8$ mice per group in the Hep3B tumor model). C, orthotopic liver tumor model was established by intrahepatic implantation of Huh7 tumor cells. The animals were treated by i.v. administration of adenoviral vectors. Survival of animals was monitored daily. The survival rate is presented ($n = 10$ mice per group). D, intratumoral expression of WIF1-Fc and sFRP1-Fc. Huh7 s.c. tumors were treated with adenoviral vectors and tumor tissue was collected at day 7 after therapy. Tumor tissues were homogenized and lysates were collected after centrifugation. Same amount of protein was analyzed by Western blotting to detect transgene expression, β -catenin, and active caspase-3.

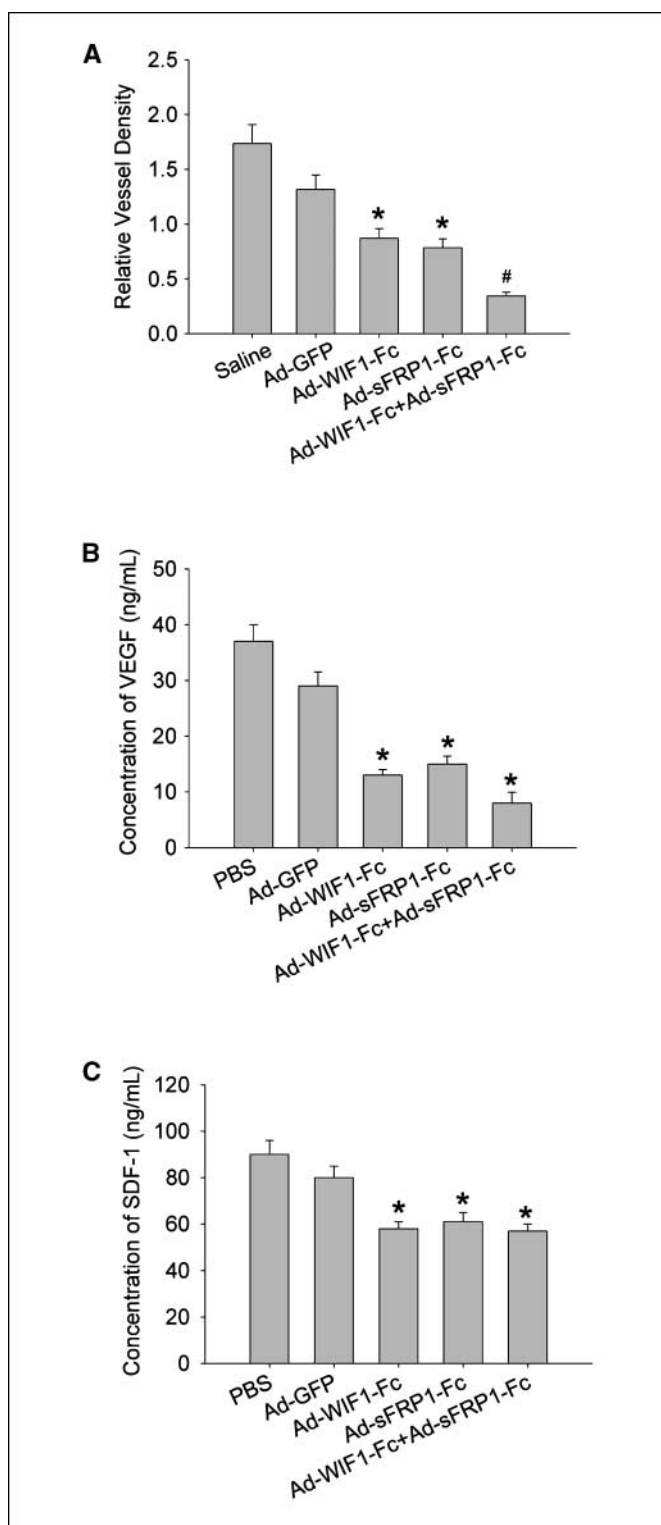


Figure 4. Reduction of microvessel density and determination of VEGF and SDF-1 levels in tumors after therapy. Huh7 s.c. tumors were treated with adenoviral vectors and tumor tissues were collected at day 7 after therapy. A, tumor tissue sections were analyzed by immunohistochemistry using anti-CD31 antibody for detection of microvessel density. Quantitative analysis of microvessel density was made by counting the positive stained cells in 10 high-power fields (HPF). Magnification, $\times 400$. Mean \pm SD ($n = 5$ per group). *, $P < 0.01$, compared with PBS and Ad-GFP-treated group; #, $P < 0.01$, compared with Ad-WIF1-Fc and Ad-sFRP1-Fc group. Tumor lysates were obtained for determination of VEGF (B) and SDF-1 (C) by ELISA. Mean \pm SE ($n = 5$ per group). *, $P < 0.01$, compared with control PBS and Ad-GFP.

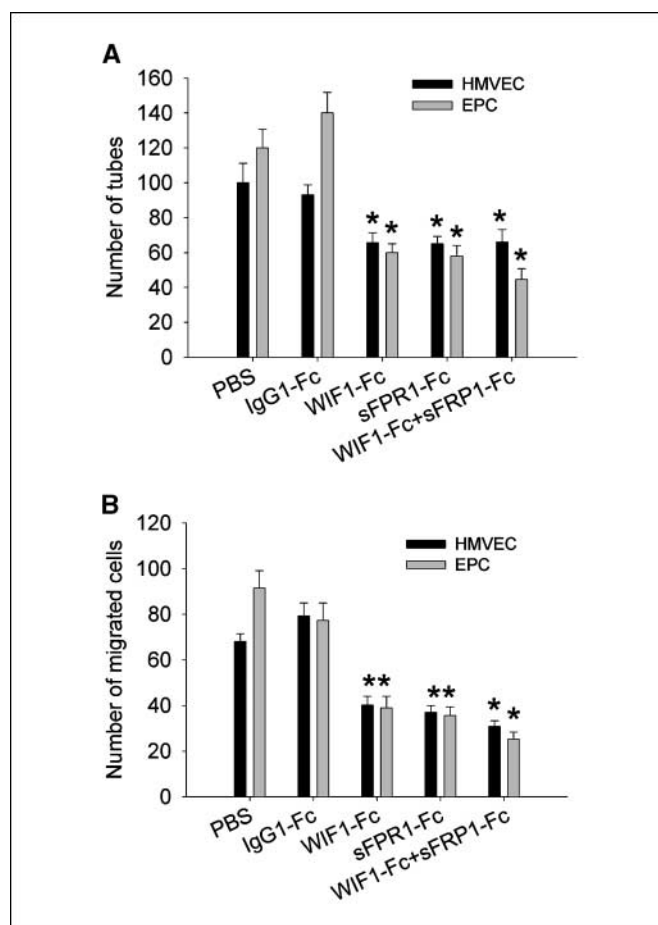


Figure 5. Inhibition of tube formation and migration of HMVEC and EPC by recombinant fusion proteins. A, HMVEC and EPC were cultured on Matrigel-coated dishes in the presence of $10 \mu\text{g/mL}$ recombinant fusion proteins or IgG-Fc control. Tube formations were quantified. B, HMVEC and EPC were preincubated in the top chamber in the presence of $10 \mu\text{g/mL}$ recombinant fusion proteins for 30 min, and cell migration was stimulated by addition of VEGF (final concentration of 5 ng/mL) to the bottom chamber. Migrated cells were counted after 4 h of incubation. Mean \pm SD of four independent experiments. *, $P < 0.01$, compared with control PBS and IgG-Fc.

sFRP1-Fc could be detected in serum at 24 h, reached peak values at 4 to 5 days, and became undetectable at week 3 after vector administration.

In animals that received the vectors encoding Wnt inhibitors, there was no change in serum alanine transaminase or aspartate transaminase level for up to 30 days after treatment (data not shown). In addition, no changes in body weight or gross appearance or behavior were observed in the treated animals, indicating absence of significant systemic toxicity.

Induction of tumor cell apoptosis, reduced microvessel density, and diminished angiogenic factors in tumors treated with Wnt antagonists. To investigate the mechanisms of antitumor activity induced by Wnt blocking molecules, we sampled tumor tissue from animals with Huh7 s.c. tumors after therapy. Terminal deoxynucleotide transferase-mediated dUTP nick end labeling assay showed significantly higher levels of apoptotic cell death in tumors from animals treated with Ad-WIF1-Fc, Ad-sFRP1-Fc, or both than those in controls (Supplementary Fig. S5). WIF1-Fc and/or sFRP1-Fc expression was confirmed by Western blotting in tumor specimens (Fig. 3D) or by ELISA in serum (Supplementary Fig. S4B)

from animals receiving either AdWIF1-Fc, Ad-sFRP1-Fc, or both. Decreased β -catenin level and increased activation of caspase-3 were observed in tumors treated with Ad-WIF1-Fc, Ad-sFRP1-Fc, or both (Fig. 3D).

To explore whether tumor angiogenesis was inhibited by Wnt blocking molecules, we performed immunostaining with anti-CD31 in tumor tissue and microvessel density was measured. Quantitative analysis showed 41%, 45%, and 78% reduction in intratumor microvessel density in mice that received Ad-WIF1-Fc, Ad-sFRP1-Fc, and combination therapy, respectively, compared with control animals given Ad-GFP (Fig. 4A). Furthermore, we measured the levels of angiogenic factors in tumor extracts after therapy. We found that VEGF and stromal cell-derived factor-1 (SDF-1) levels from animals given Ad-WIF1-Fc, Ad-sFRP1-Fc, or combination therapy were significantly lower than those from animals received saline or Ad-GFP (Fig. 4B and C).

Inhibition of angiogenesis by fusion proteins. To determine whether fusion proteins have a direct effect on the function of endothelial cells and EPC that are responsible for tumor angiogenesis, we analyzed tube formation and cell migration in the presence of fusion proteins. Our data showed that WIF1-Fc, sFRP1-Fc, or the combination of both significantly inhibited tube formation by HMVEC and EPC compared with IgG1-Fc (Fig. 5A). Similarly, a significant inhibition of cell migration of HMVEC and EPC was observed in the presence of WIF1-Fc, sFRP1-Fc, or both (Fig. 5B). These data indicated an inhibitory role of WIF1-Fc and sFRP1-Fc on the function of both endothelial cells and EPC.

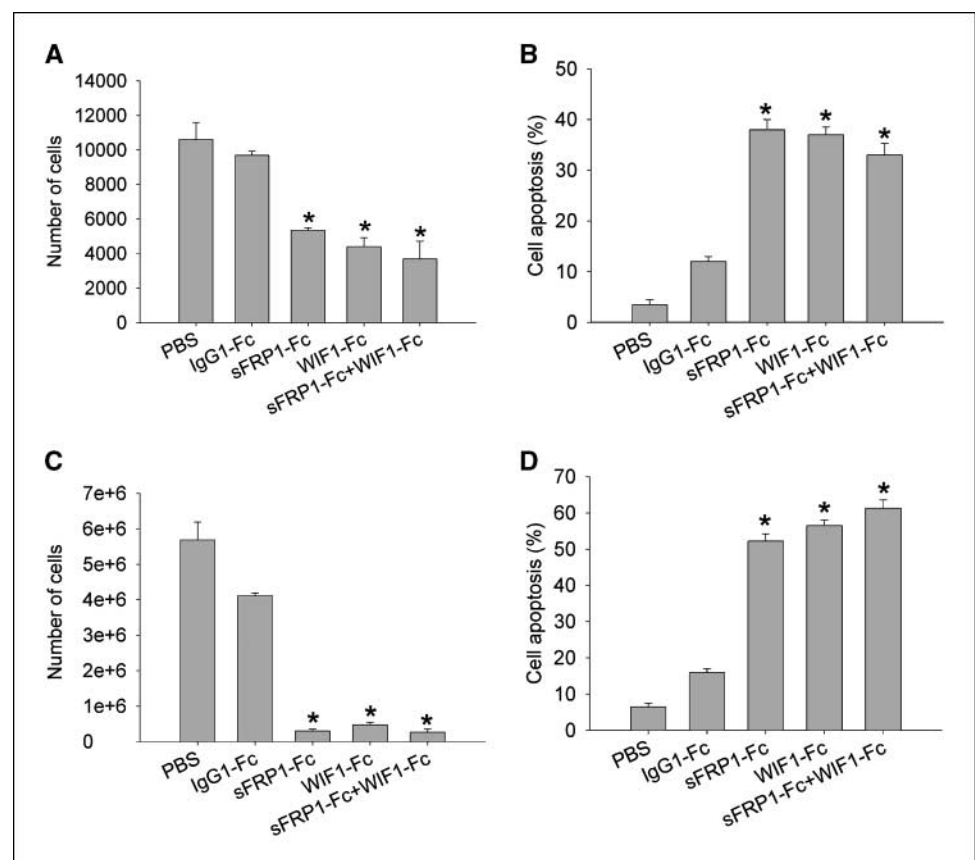
Induction of apoptosis in EPC and inhibition of differentiation of EPC from bone marrow-derived mononuclear cells. To further characterize the effect of Wnt antagonists on EPC

function, we investigated cell viability on differentiated EPC in the presence of WIF1-Fc and sFRP1-Fc for 2 days. As shown in Fig. 6A, incubation of EPC with WIF1-Fc, sFRP1-Fc, or both reduced cell number. Cytometry analysis with anti-Annexin V indicated that there were significant more apoptotic EPC when they were cultured in the presence of WIF1-Fc, sFRP1-Fc, or both (Fig. 6B). We also investigated the effect of Wnt antagonists on the process of EPC differentiation from bone marrow-derived mononuclear cells. When WIF1-Fc, sFRP1-Fc, or the combination was added to the EPC differentiation medium, we observed a significant reduction in EPC numbers and a significant increase in cells undergoing apoptosis (Fig. 6C and D). These findings indicate that WIF1-Fc and sFRP1-Fc not only affect viability of differentiated EPC but also block EPC differentiation from bone marrow-derived mononuclear cells.

Discussion

Data presented here show that high levels of WIF1-Fc and sFRP1-Fc are expressed and secreted from HCC and normal cell lines after infection with adenoviral vectors encoding WIF1-Fc and sFRP1-Fc. Infection with Ad-WIF1-Fc or Ad-sFRP1-Fc inhibited cell growth in Huh7, PLC/PRF/5, and HepG2 cells in a dose-related manner. Combination of Ad-WIF1-Fc and Ad-sFRP1-Fc had additive effect on the inhibition of tumor cell growth. These vectors, however, displayed little antitumor activity on Hep3B cells. The reduction of tumor cell growth provoked by Ad-WIF1-Fc or Ad-sFRP1-Fc in the sensitive HCC cell lines was seemingly due to the effect of secreted Wnt antagonists because similar antitumor effect was observed using purified recombinant WIF1-Fc or sFRP1-Fc proteins. The exposure to these molecules resulted in reduced β -catenin activity

Figure 6. Induction of apoptosis of EPC and differentiation of EPC from bone marrow-derived mononuclear cells by recombinant fusion proteins. EPC was cultured in the presence of 10 μ g/mL recombinant fusion proteins for 48 h. The number of cells was determined (A). Apoptosis was assayed by staining cells with anti-Annexin V (B). Mononuclear cells from bone marrow were cultured on fibronectin-coated dishes in complete culture medium in the presence of 10 μ g/mL recombinant fusion proteins or IgG-Fc as control. After 7 d of differentiation, adherent EPC were collected for cell counting (C) and apoptosis was analyzed by staining with anti-Annexin V (D). Mean \pm SD of three independent experiments. *, $P < 0.01$, compared with control PBS and IgG-Fc.



as reflected in the TOP flash reporter assay and in a significant decrease in the expression of Wnt target genes such as *E2F1*, *c-myc*, and *cyclin D1*. Down-regulation of these cell cycle-related genes appears to be a critical event explaining the observed inhibition of tumor cell growth. However, there is not a simple correlation between the inhibition of β -catenin activity and the regulation of cell survival by the Wnt antagonist fusion proteins. Because both PLC/PRF/5 and Hep3B have a relatively low TCF activity, canonical pathway of Wnt signaling may not be important for regulation of cell survival for these cells. Interference with the noncanonical pathway or with other signal transduction pathways, such as Hedgehog (29), may be involved in growth inhibition by Wnt blockade.

To explore the therapeutic potential of the Wnt antagonists employed in this study, we have used s.c. and orthotopic HCC models to evaluate the antitumor efficacy of Ad-WIF1-Fc and/or Ad-sFRP1-Fc. In the treated tumors, we could confirm the expression of WIF1-Fc and/or sFRP1-Fc together with a dramatic reduction in β -catenin levels. In s.c. tumors, we observed a significant inhibition of tumor growth with Ad-WIF1-Fc and Ad-sFRP1-Fc and an enhancement of the antitumor effect in the animals that received both vectors. The reasons for additive effect are unclear. It may be presumed that WIF1 and sFRP1 may bind different Wnt proteins or different sites of Wnt proteins or that cells infected with the two adenoviruses could produce a heterodimer formed by WIF1-Fc and sFRP1-Fc, which can block Wnt signaling more efficiently. In the orthotopic HCC tumor model, treatment with Ad-WIF1-Fc and Ad-sFRP1-Fc resulted in prolonged animal survival, indicating that both WIF1-Fc and sFRP1-Fc can be used as therapeutic agents for liver cancer.

The inhibition of tumor growth induced by blocking Wnt signaling was associated with enhanced caspase-3 activity and a marked increase in the apoptotic rate in tumors, indicating the critical role of Wnt signaling in the maintenance of tumor growth. However, the transient nature of the antitumor effect is related to the short duration of transgene expression when using first-generation adenoviruses. These vectors have provided the proof-of-concept for the therapeutic benefit of Wnt antagonists in HCC, but long-term expression vectors should be employed to halt HCC progression for longer periods. A previous study showed that i.v. administration of Ad-Dkk1 resulted in disruption of small intestine and colonic tissue architecture (30). Although we did not observe apparent deleterious effect in our study, extensive toxicology studies should be undertaken before considering systemic administration of Wnt antagonists in cancer patients. In addition, if gene therapy is contemplated to express Wnt blockers in livers with HCC, it would be adequate to use tissue-specific promoters and inducible expression systems to ensure fine control of transgene expression.

A key observation in our study is the dramatic reduction of microvessel density observed in tumor sections from animals treated with Ad-WIF1-Fc and/or Ad-sFRP1-Fc. This finding was accompanied by a significant reduction in the levels of VEGF and SDF-1 in those tumors. These data are in keeping with previous

reports showing that inhibition of β -catenin activity reduced VEGF expression in colon cancer cells (31, 32). The marked effect of Wnt antagonists on tumor vasculature observed in this study explains the fact that although Hep3B cells did not respond to Ad-WIF1-Fc and/or Ad-sFRP1-Fc *in vitro*, a significant antitumor activity was observed in *in vivo* tumor models based on Hep3B cells. These findings indicate that the inhibitory effect on tumor neovascularization exerted by WIF1-Fc and sFRP1-Fc might constitute a relevant mechanism underlying the antitumor activity of these molecules.

Previous reports have shown that endothelial cells express various Wnt signaling components and that activation of Wnt1 signaling induces endothelial cell proliferation and formation of capillary-like networks (23–26). In our study, we have found that both WIF1-Fc and sFRP1-Fc inhibited tube formation and migration of HMVEC and mouse EPC. The most significant finding was that both WIF1-Fc and sFRP1-Fc caused apoptosis of EPC and inhibition of differentiation of EPC from bone marrow mononuclear cells. The CXCR4 receptor signaling profoundly modulates the angiogenic activity and homing capacity of EPC (33). The chemokine SDF-1 and its unique receptor CXCR4 play a critical role in postnatal vasculogenesis by increasing EPC number and formation of vascular structures from bone marrow progenitor cells (34). Our data point to the concept that Wnt antagonists impair EPC differentiation and expansion possibly, at least in part, by inhibiting SDF-1 production.

It has been shown that tumor neovascularization may arise from proliferation and migration of endothelial cells from surrounding existing vessels (angiogenesis) but also from incorporation of mobilized bone marrow-derived EPC (vasculogenesis; refs. 15, 18, 20). Our data indicate that both WIF1-Fc and sFRP1-Fc have direct effect on these two cell types, which are important in the formation of tumor neovessels. In other words, blockade of Wnt signaling by WIF1-Fc and sFRP1-Fc may impair both tumor angiogenesis and tumor vasculogenesis.

In conclusion, Wnt antagonists WIF1-Fc and sFRP1-Fc inhibit Wnt signaling and exert antitumor activity by inducing apoptosis of tumor cells and inhibition of tumor neovascularization. Wnt antagonists, as those used in the present study, are promising molecules for the treatment of HCC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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