ABSTRACT

Osteosarcoma (OS) is a primary malignancy of bone with a tendency to metastasize early. Despite intensive chemotherapy and surgical resection, ~30% of patients still develop distant metastasis. Our previous work using clinical OS samples suggested that expression of the Wnt receptor LRP5 might be associated with tumor metastasis. In the present study, we used a Dickkopf (Dkk) family member and a dominant-negative LRP5 receptor construct to modulate Wnt signaling in OS cells. Saos-2 cells, which ectopically express Dkk-3, do not undergo apoptosis and exhibit enhanced resistance to serum starvation and chemotherapy-induced cytotoxicity. Transfection of Dkk-3 and dominant-negative LRP5 into Saos-2 cells significantly reduces invasion capacity and cell motility. This blockade is associated with changes in cell morphology consistent with a less invasive phenotype. In addition, Dkk-3 and dominant-negative LRP5 also induce changes in β-catenin localization consistent with an increase in cell-cell adhesion. Taken together, these results support a possible role for Wnt signaling in the pathobiology and progression of human OS.

INTRODUCTION

Osteosarcoma (OS) is a primary malignancy of bone with a high propensity for early micrometastasis, with the lung being the most common metastatic site. Currently, >30% of patients with localized disease eventually develop distant metastasis after intensive chemotherapy and surgical resection (1). Molecular mechanisms underlying disease progression currently are largely unknown. Despite intensive effort, the outcome of patients with OS has not improved significantly during the past decade. There is a great need to understand the underlying mechanisms of tumor progression before more targeted therapies can be realized.

Dickkopf 3 (Dkk-3), also known as Reduced Expression in Immortalized Cells (REIC), is a member of a recently identified gene family encoding secreted proteins that control cell fate during embryonic development (2, 3). Dkk-1, the prototype of this family, acts as a powerful inhibitor of the Wnt signaling pathway (4). However, Dkk-3 has not been shown to exert its action on Wnt signaling. Dkk proteins are expressed in a variety of tissues, with Dkk-3 being highly expressed in the mesenchymal condensation during skeletal formation (5). Given its involvement in normal skeletal development, we aimed to examine potential role of Dkk-3 in OS, which is a primary bone-forming malignancy. Dkk-3, also known as reduced expression in immortalized cells, has been implicated as a tumor suppressor exhibiting down-regulation in several cancer cell lines (6). However, the molecular mechanism underlying this tumor suppressor function of Dkk-3 has not been elucidated.

The Wnt pathway consists of highly conserved secreted ligands that bind cell-surface receptors called frizzled and lipoprotein receptor-related protein (LRP; Refs. 7–9). In the presence of Wnt signaling, β-catenin is accumulated in the cytosol, translocated into the nucleus, and forms a complex with lymphocyte enhancer factor (LEF)/T-cell factor (TCF) family of transcription factors to activate target genes, many of which are involved in development and oncogenesis (10–14). In contrast, Wnt inhibition leads to decreased accumulation of cytosolic and nuclear β-catenin with consequent down-regulation of Wnt-responsive genes. As such, the Wnt pathway has been implicated in the pathogenesis and progression of an increasing number of human malignancies, including melanoma, myeloma, and lung cancer (15–17).

In OS, however, involvement of the Wnt pathway in disease progression has not been clearly established. In this article, we investigate the role of Dkk-3 in the pathobiology of OS and show that overexpression of this protein can effectively reduce motility and invasion of OS cells by affecting intracellular β-catenin, a mediator of Wnt signaling. We showed previously that expression of the Wnt receptor LRP5 is associated with metastatic disease in OS (18). In this study, we found that inhibiting LRP5 by using a dominant-negative form of this receptor also inhibits tumor cell motility and invasion, suggesting that LRP5 plays an important role in promoting metastasis in OS. Taken together, these findings provide the evidence linking Wnt signaling to tumor progression in human sarcomas.

MATERIALS AND METHODS

Cell Culture and Plasmid. Saos-2 and U2OS cells were obtained from American Type Culture Collection (Manassas, VA) and maintained in MEM-α supplemented with 20% fetal bovine serum (FBS) and antibiotics. The cells were maintained at 37°C in a humidified atmosphere of 5% CO₂.

The full-length human Dkk-3 cDNA was amplified from U2OS cells by reverse transcription-PCR using the following primer pairs: 5′-CACCATG- CAGCGGCTTGGGCG-3′ (sense) and 5′-AATCTCTTCCCCCTCCAGCA-3′ (antisense; 94°C for 30 s, 55°C for 1 min, and 72°C for 1 min for 35 cycles). The amplified product was subcloned into pCedNA3.1TOPO vector (Invitrogen, Carlsbad, CA) containing a V5 tag and verified by automated DNA sequencing using standard methods. The dominant-negative LRP5-pcDNA3.1 plasmid (DN-LRP5) was a gift of Dr. Matthew Warman (Case Western Reserve University, Cleveland, OH). This plasmid, constructed by deleting the transmembrane domain and cytoplasmic tail of LRP5, encodes a secreted form of this Wnt receptor (19). It has been reported that a secreted form of LRP5 could block Wnt signaling and that an LRP5 mutant lacking the cytoplasmic tail functioned as a dominant negative (20).

Transient and Stable Transfections. For transient transfection, Saos-2 cells were plated at 1 × 10^6 cells per 100-mm dish. After 12 h, cultures were transfected with Dkk-3 or DN-LRP5 (3 μg plasmid DNA/ml of medium) using FuGene 6 (Roche, Basel, Switzerland) according to the manufacturer’s protocol. As a control, Saos-2 cells were transfected with the pCedNA3.1-LacZ empty vector only. For stable transfection, cells transfected with Dkk-3 or DN-LRP5 were selected with G418 (800 μg/ml) starting at 48 h after transfection, and all of the stable transfectants were pooled.
Detection of β-Catenin in transiently transfected Cells. Total protein lysates from transiently transfected cells were extracted for 30 min on ice with RIPA buffer (50 mM Tris, 150 mM NaCl, 1.0% NP40, 0.5% deoxycholic acid, and 0.1% SDS). Soluble (cytosolic fraction) material was recovered by ultracentrifugation at 100,000 × g as described by Shimizu et al. (21). Protein contents were determined using a protein assay kit (Bio-Rad, Hercules, CA). Thirty μg of protein lysate were separated on denaturing 10% SDS-polyacrylamide gels. Proteins were transferred from gels onto nitrocellulose filters by electroblotting and blocked overnight at 4°C in Tris-buffered saline (TBS)/0.05% Tween 20 containing 10% dry milk. Blots then were incubated with an antihuman β-catenin antibody (Upstate Biotechnology, Waltham, MA) diluted 1:1000 in TBS/0.05% Tween 20 at room temperature for 2 h. The primary antibody then was removed by washing in TBS/0.05% Tween 20 at room temperature three times for 5 min. Blots were exposed to a horseradish peroxidase-conjugated antirabbit secondary antibody and visualized using the enhanced chemiluminescence system (Amersham, Piscataway, NJ). For loading control, membranes were stripped and reprobed with β-actin.

Apoptosis Assay. Apoptosis assays were performed using the FragEL DNA fragmentation detection kit (Oncomgene Research, Boston, MA) according to the manufacturer’s instructions. This assay uses terminal deoxynucleotidyl transferase and a substrate similar to deoxyginin of the original terminal deoxynucleotidyl transferase-mediated nick end labeling assay. Briefly, cells were plated in a 24-well plate at 1 × 10^6 cells/well and allowed to adhere overnight. Cells then were fixed in 4% formaldehyde in PBS, washed in TBS, and permeabilized with proteinase K (20 μg/ml) at room temperature (RT) for 10 min. Cells were treated with a terminal deoxynucleotidyl transferase equilibration buffer and incubated at RT for 30 min. Terminal deoxynucleotidyl transferase substrate labeling reaction mix was added, and samples were incubated for 1 h at 37°C. After washing with PBS, cells were counterstained with 4′,6-diamidino-2-phenylindole, and staining was visualized with fluorescence microscopy. Saos-2 treated with doxorubicin and HL-60 cells incubated with actinomycin D to induce apoptosis were used as positive controls, and untreated HL-60 cells were used as a negative control.

Flow Cytometry for Apoptosis. Transfected Saos-2 cells were cultured for 24 h at 37°C. Adherent and floating cells were combined, washed with cold PBS, and fixed in methanol at 4°C. Cells were pelleted and treated with RNase A (200 μg/ml) and stained with propidium iodide (50 μg/ml). The proportion of cells in the sub-G1, G0/G1, and G2/M phases of the cell cycle was determined by a FACScalibur flow cytometer (Becton Dickinson, Bedford, MA) and FlowJo software (Tree Star Inc., San Carlos, CA). For serum-withdrawal experiments, 1 × 10^6 cells were seeded on day 0 in 100-mm culture dishes in MEM-α + 20% FBS and allowed to adhere overnight. On day 1, cells were washed and placed in serum-free MEM-α for 24 h. On day 2, adherent and floating cells were combined and processed for flow cytometry as described previously.

Drug Cytotoxicity Assay. On day 0, subconfluent cells were harvested by trypsinization and plated in 96-well culture plates (2 × 10^4 cells/well) in MEM-α + 20% FBS. After incubation at 37°C overnight to allow attachment, cells were treated on day 1 with cisplatin and doxorubicin at several dilutions (1 × 10^{-6} to 20 μg/ml) for 24 h. Cells then were washed and cultured in drug-free medium and allowed to grow for 72 h. On day 5, the percentage of viable cells relative to untreated controls was determined by the alamarBlue method (22). Briefly, 25 μl of alamarBlue dye (Trek Diagnostic Systems, Cleveland, OH) were added into each well, and cultures were incubated for 4 h at 37°C. The difference in absorbance between 530 and 590 nm was determined using a Cytosensor Fluor 4000 microplate reader (PerSeptive Biosystems, Foster City, CA). Each cell line was assayed in triplicate, and each experiment was repeated twice.

Matrigel Invasion Assay. Invasion assays were performed using 24-well invasion chamber system (BD Biosciences, Bedford, MA). Cells were trypsinized and counted with a hemocytometer using trypsin blue, and viable cells were seeded in the upper chamber at 1 × 10^5 cells/well in serum-free MEM-α. MEM-α supplemented with 10% FBS (used as a chemoattractant) was placed in the bottom well. Incubation was carried out for 36 h at 37°C in humidified air with 5% CO₂. Nonmigratory cells in the upper chamber then were removed with a cotton-tip applicator. Migrated cells on the lower surface were fixed with methanol and stained with hematoxylin. The number of migrating cells was determined by counting five high-powered fields (200×) on each membrane. An invasion index, corrected for cell motility, was calculated using the following formula:

\[
\text{no. of cells migrated through an uncoated (control) membrane} \\
\times 100
\]

All of the cell lines were assayed in triplicate for each experiment, and each experiment was repeated three times.

Motility Assay. This assay is a modification of the invasion assay described previously. A total of 3 × 10^5 cells were plated in the upper chamber in serum-free MEM-α. MEM-α + 10% FBS was placed in the lower chamber as a source of chemoattractant. Cells were allowed to migrate through a porous, uncoated membrane (BD Biosciences) for 12 h at 37°C. The membrane was processed as described for the invasion assay. The number of migrating cells was determined by counting five high-powered fields (200×) on each membrane and calculated as mean number of cellfields. All of the cell lines were assayed in triplicate for each experiment, and each experiment was repeated three times.

Immunocytochemical Analysis. Immunocytochemical staining of β-catenin in cultured cells was carried out using the avidin-biotin peroxidase and immunofluorescence staining methods. Cells were plated and cultured to 30–40% confluence on four-well chamber slides. Cells were fixed in ice-cold methanol for 5 min and washed in PBS. Non-specific sites were blocked with PBS-1% BSA for 1 h, followed by blocking in normal serum at RT. Cells then were incubated with a rabbit polyclonal anti-β-catenin antibody (Upstate Biotechnology) at 2 μg/ml for 1 h at RT. For the avidin-biotin method, cells were incubated with a biotinylated secondary antibody (1 μg/ml) for 1 h at RT, and staining was visualized with diaminobenzidine using the Vectastain Elite Kit (Vector Lab, Burlingame, CA) according to the manufacturer’s protocol. For the immunofluorescence staining method, cells were incubated with a secondary antibody (1 μg/ml) conjugated to Alexa-488 green fluorescence (Molecular Probes, Eugene, OR) and counterstained with 4′,6-diamidino-2-phenylindole. Membrane immunostaining was visualized by confocal microscopy at 63× magnification.

Statistical Analysis. Student’s t test was used to compare the difference between means. P < 0.05 was considered statistically significant. All of the data were analyzed using a contemporary statistical package (SPSS 10.0, Chicago, IL).

RESULTS

Transient Transfection of Dkk-3 Blocks Cytoplasmic β-Catenin Accumulation. Saos-2 cells were transfected with a Dkk-3-expressing vector or empty control vector (EV). Before transfection, three OS cell lines (U2OS, HOS, and Saos-2) were screened by reverse transcription-PCR for Dkk-3 expression. To maximize the effect of transfection, Saos-2 was selected because it showed no detectable Dkk-3 (data not shown). From preliminary experiments, we estimated the transfection efficiency at 20–25% using pcDNA3.1-LucZ. As shown in Fig. 1, transient transfection of Dkk-3 (3 μg/ml) affected Wnt

![Fig. 1. Dickkopf (Dkk)-3 reduces cytoplasmic accumulation of β-catenin. After Saos-2 cells were transfected transiently with 3 μg/ml of empty control vector (EV) or Dkk-3 expression vector, the cytoplasmic protein fractions (see “Materials and Methods”) from parental (Lane 1), EV-transfected (Lane 2), and Dkk-3-transfected (Lane 3) cells were subjected to Western blot analysis using an anti-β-catenin antibody.](image-url)
signaling by reducing the accumulation of cytosolic fraction of β-catenin. However, when whole cell lysates were examined, the levels of β-catenin were not different in Dkk-3 transfectants and control cells (data not shown). As seen in Fig. 1 (Lane 3), β-catenin often will yield doublets on Western blot analysis, perhaps as a result of being phosphorylated (most commonly tyrosine or serine phosphorylation). Dkk-3 transfection at a lower plasmid concentration (1.5 μg/ml) resulted in no significant decrease in cytoplasmic β-catenin as compared with empty vector control (data not shown).

**Dkk-3 Does Not Induce Apoptosis and Promotes Survival under Nonsupportive Conditions.** To determine whether the Dkk-3 gene stimulated apoptotic cell death, we assessed Dkk-3-transfected cells and control cells (Saos-2 transfected with EV alone) with a terminal deoxynucleotidyl transferase-mediated nick end labeling-like assay. There is no morphologic evidence of increased apoptosis in Dkk-3-transfected cells. As seen in Fig. 2A, Dkk-3 transfection did not induce more apoptosis in these cells when compared with EV controls. A positive control (i.e., Saos-2 cells treated with doxorubicin) was included to ensure the validity of the observed staining. When cell death (as reflected by the sub-G1 DNA content) was analyzed by flow cytometry, Dkk-3 transfection did not induce any significant increase in apoptosis compared with EV transfection under normal growth conditions (Fig. 2B). Similar results were obtained with DN-LRP5-transfected cells (data not shown). However, under serum deprivation, OS cells expressing Dkk-3 were much more resistant to apoptosis than EV control cells. After serum withdrawal for 24 h, the sub-G1 fraction of EV cells increased significantly, whereas that of Dkk-3 transfectants remained stable (Fig. 2B). When exposed to doxorubicin or cisplatin, Dkk-3-transfected cells showed 5–25-fold higher EC50 than EV control cells, suggesting that Dkk-3 reduces chemosensitivity of Saos-2 cells (Fig. 2C). Although our data are reproducible, the mechanisms underlying the plateau phase (doxorubicin and cisplatin concentration <0.0064 μg/ml and 0.08 μg/ml, respectively) of the cytotoxicity curves are unclear at this point.

**Dkk-3 Leads to Changes in Cellular Morphology.** Stable transfectants were selected in G418, and pooled transfectants were verified for Dkk-3 protein expression by Western blot analysis using an anti-V5 antibody (data not shown). The shape of cells expressing Dkk-3 is drastically different from that of cells transfected with EV (Fig. 3). Similar to parental Saos-2 (not shown), the EV-transfected cells were irregularly shaped and spreading with many extended processes (Fig. 3A). In contrast, Dkk-3-transfected cells were more compact and adherent to neighboring cells (Fig. 3B), suggesting a less invasive phenotype.

Given our recent data linking the expression of the Wnt receptor LRP5 to metastasis in OS, we examined the effect of transfecting a dominant-negative form of this receptor (DN-LRP5) into Saos-2 cells (18). As shown in Fig. 3C, DN-LRP5 transfection also resulted in morphologic changes reminiscent of Dkk-3 transfection, suggesting that Dkk-3 and DN-LRP5 exert their effects on OS cells by similar mechanisms.

**Dkk-3 and DN-LRP5 Lead to a Decrease in Invasion and Motility.** On the basis of the observation that Dkk-3- and DN-LRP5-transfected cells exhibit morphologic changes suggestive of a less invasive phenotype, we examined the in vitro capacity of these cells to invade through a Matrigel-coated membrane. Invasion through Matrigel has been reported to mimic the three-step hypothesis processes (Fig. 3A). In contrast, Dkk-3-transfected cells were more compact and adherent to neighboring cells (Fig. 3B), suggesting a less invasive phenotype.

Motility is a component of the invasion process of tumor cells. Because Dkk-3 and DN-LRP5 inhibited invasion, we examined whether the expression of these Wnt inhibitors also affected cell motility. Saos-2 cells were placed in the upper chamber and induced to migrate across 8-μm membrane pores in response to a chemoat-
Dkk-3 Activity in Osteosarcoma Cells

Fig. 3. Morphologic changes in osteosarcoma (OS) cells following transfection with Dickkopf 3 (Dkk-3) and DN-LRP5. Saos-2 cells were stably transfected with empty control vector (A), Dkk-3 (B), or DN-LRP5 (C) and maintained in media containing G418. Live cells were photographed using a phase-contrast inverted microscope at 200× magnification.

Fig. 4. In vitro invasive capacity and motility of Saos-2-Dickkopf (Dkk)-3 and -DN-LRP5 transfecants. A. Dkk-3 and DN-LRP5-transfected cells exhibited reduced invasiveness across Matrigel when compared with empty control vector (EV) control cells. The invasion index (see “Materials and Methods”) is expressed as mean of triplicate wells ± SD. P value was determined by comparing mean index of invasion between the EV group and Dkk-3 or DN-LRP5 group using the Student's t test. B. EV and Dkk-3- or DN-LRP5-expressing cells were evaluated for migration across a microporous membrane. Motility was expressed as the mean number of migrating cells per field ± SD. P value was determined by comparing the mean number of migrating cells per field between the EV group and Dkk-3 or DN-LRP5 group using the Student’s t test.

DISCUSSION

Although the role of Wnt signaling has been elucidated for many types of human cancer, its biological significance in sarcomas has not been examined in detail. We have shown previously that human OS cell lines express several Wnt ligand and frizzled receptor combinations, suggesting that these cells may signal through multiple Wnt-related pathways (18). The presence of the LRP5 coreceptor suggests a capacity to signal through the canonical Wnt-β-catenin pathway (24). In the present study, we provide additional evidence for Wnt involvement in OS by modulating this pathway using a Dkk family member and a dominant-negative form of LRP5.

Dkk is a family of secreted glycoproteins with powerful Wnt inhibitory activity. Recent investigations have suggested that Dkk-1 and Dkk-2 proteins exert their Wnt-blocking activity by binding to the LRP family of receptors. Although related to Dkk-1 and -2, Dkk-3 (also known as reduced expression in immortalized cells) has not been shown to affect Wnt signaling in embryonic development or in cancer (3, 25). In the present investigation, we show that the cytosolic accumulation of β-catenin is disrupted by ectopic overexpression of Dkk-3 in OS cells. In contrast to our data, Tsuji et al. (25), using a pTracer expression vector, could not demonstrate inhibition of cytosolic accumulation of β-catenin by Dkk-3. Given our findings that transfection with a lower concentration of Dkk-3 plasmid resulted in no β-catenin effect, the negative results of Tsuji et al. (25) might be because of a lower transfection efficiency, resulting in less protein expression.

Cell motility requires precise control that often is lost during tumor progression or metastasis. β-Catenin has been shown to play a dual role: formation of the adherens junction complex linking the cadherins to cytoskeletal proteins (26) and transduction of Wnt signal by nuclear translocation and interaction with LEF/TCF transcription factors (14). In this study, Dkk-3 and dominant-negative LRP5 appear to down-regulate β-catenin nuclear translocation in OS cells, suggesting that these molecules can exert Wnt-modulating activities. This apparent decrease in nuclear localization is associated with a significant reduction in cellular motility. Given that β-catenin preferentially translocates into the nucleus during cell migration, it is probable that Dkk-3 and dominant-negative LRP5 can reduce the motility of OS cells by down-regulating this nuclear translocation event.

tractant (FBS). At 12 h after seeding, Dkk-3- and DN-LRP5-transfected cells were significantly less motile than EV control cells (Fig. 4B; P < 0.005).

Dkk-3 Transfection Is Associated with Changes in β-Catenin Localization. β-Catenin, one of the main mediators of Wnt signaling, is crucial for intercellular adhesion and cellular locomotion. Given that cytosolic β-catenin was affected by Dkk-3, whereas β-catenin level from total cell lysate was unchanged, we hypothesize that Dkk-3 affects cellular motility and invasion by altering subcellular localization of β-catenin. Using immunocytochemical analysis, we examined β-catenin localization using light and confocal microscopy. Under light microscopy, EV-transfected cells exhibited localized, intense staining for β-catenin in the nucleus and only weak staining in the cytoplasm (Fig. 5A). In contrast, β-catenin staining in Dkk-3-transfected cells was diffuse and less localized to the nucleus (Fig. 5B). For more exact localization of β-catenin in Dkk-3-transfected cells, we used a laser scanning confocal microscope. Under confocal microscopy, Dkk-3 appeared to induce a marked redistribution of β-catenin to the membrane (Fig. 5C). Intense staining for β-catenin (green fluorescence) was observed along the entire cell-cell contact region among adjacent cells, whereas staining in the contact-free borders was weaker (Fig. 5C). Interestingly, transfection with the DN-LRP5 construct also led to a similar alteration in β-catenin subcellular staining pattern (Fig. 5D), suggesting that Dkk-3 and DN-LRP5 might modulate cell motility and invasion via similar mechanisms.

Dkk-3 activity in osteosarcoma cells following transfection with Dickkopf 3 (Dkk-3) and DN-LRP5. Saos-2 cells were stably transfected with empty control vector (A), Dkk-3 (B), or DN-LRP5 (C) and maintained in media containing G418. Live cells were photographed using a phase-contrast inverted microscope at 200× magnification.

To the membrane (Fig. 5). Intense staining for light microscopy, EV-transfected cells exhibited localized, intense staining in the contact-free borders, whereas staining in the contact-free borders was weaker (Fig. 5). Interestingly, transfection with the lower concentration of Dkk-3 plasmid resulted in no β-catenin effect, the negative results of Tsuji et al. (25) might be because of a lower transfection efficiency, resulting in less protein expression.

Cell motility requires precise control that often is lost during tumor progression or metastasis. β-Catenin has been shown to play a dual role: formation of the adherens junction complex linking the cadherins to cytoskeletal proteins (26) and transduction of Wnt signal by nuclear translocation and interaction with LEF/TCF transcription factors (14). In this study, Dkk-3 and dominant-negative LRP5 appear to down-regulate β-catenin nuclear translocation in OS cells, suggesting that these molecules can exert Wnt-modulating activities. This apparent decrease in nuclear localization is associated with a significant reduction in cellular motility. Given that β-catenin preferentially translocates into the nucleus during cell migration, it is probable that Dkk-3 and dominant-negative LRP5 can reduce the motility of OS cells by down-regulating this nuclear translocation event.

Although the role of Wnt signaling has been elucidated for many types of human cancer, its biological significance in sarcomas has not been examined in detail. We have shown previously that human OS cell lines express several Wnt ligand and frizzled receptor combinations, suggesting that these cells may signal through multiple Wnt-related pathways (18). The presence of the LRP5 coreceptor suggests a capacity to signal through the canonical Wnt-β-catenin pathway (24). In the present study, we provide additional evidence for Wnt involvement in OS by modulating this pathway using a Dkk family member and a dominant-negative form of LRP5.

Dkk is a family of secreted glycoproteins with powerful Wnt inhibitory activity. Recent investigations have suggested that Dkk-1 and Dkk-2 proteins exert their Wnt-blocking activity by binding to the LRP family of receptors. Although related to Dkk-1 and -2, Dkk-3 (also known as reduced expression in immortalized cells) has not been shown to affect Wnt signaling in embryonic development or in cancer (3, 25). In the present investigation, we show that the cytosolic accumulation of β-catenin is disrupted by ectopic overexpression of Dkk-3 in OS cells. In contrast to our data, Tsuji et al. (25), using a pTracer expression vector, could not demonstrate inhibition of cytosolic accumulation of β-catenin by Dkk-3. Given our findings that transfection with a lower concentration of Dkk-3 plasmid resulted in no β-catenin effect, the negative results of Tsuji et al. (25) might be because of a lower transfection efficiency, resulting in less protein expression.

Fig. 3. Morphologic changes in osteosarcoma (OS) cells following transfection with Dickkopf 3 (Dkk-3) and DN-LRP5. Saos-2 cells were stably transfected with empty control vector (A), Dkk-3 (B), or DN-LRP5 (C) and maintained in media containing G418. Live cells were photographed using a phase-contrast inverted microscope at 200× magnification.

Fig. 4. In vitro invasive capacity and motility of Saos-2-Dickkopf (Dkk)-3 and -DN-LRP5 transfecants. A. Dkk-3- and DN-LRP5-transfected cells exhibited reduced invasiveness across Matrigel when compared with empty control vector (EV) control cells. The invasion index (see “Materials and Methods”) is expressed as mean of triplicate wells ± SD. P value was determined by comparing mean index of invasion between the EV group and Dkk-3 or DN-LRP5 group using the Student’s t test. B. EV and Dkk-3- or DN-LRP5-expressing cells were evaluated for migration across a microporous membrane. Motility was expressed as the mean number of migrating cells per field ± SD. P value was determined by comparing the mean number of migrating cells per field between the EV group and Dkk-3 or DN-LRP5 group using the Student’s t test.
Although we have shown that Dkk-3 blocks cytosolic and nuclear accumulation of β-catenin, one must be cautious in identifying the Wnt pathway as the sole target for Dkk-3. There may be extensive cross-talk between multiple pathways to regulate the level of cytoplasmic β-catenin (27, 28). It remains a possibility that Dkk-3 may interact with other pathways to regulate β-catenin distribution in OS cells. However, our findings that dominant-negative LRP5 also affects β-catenin localization support a role for canonical Wnt signaling in OS cell motility. Although Dkk-1 has been shown to bind LRP5, whether secreted Dkk-3 interacts with LRP5 to influence intracellular β-catenin is unknown.

The link between morphologic changes and invasive property is not without precedent. Several authors previously have presented evidence suggesting that a change in cell shape caused by Wnt-related transfection often is associated with alterations in motility or invasive behavior (29, 30). In the current study, a direct link between morphology and invasive property cannot be made. However, when coupled with invasion and motility data, this morphologic change in Dkk-3- and DN-LRP5-transfected cells is suggestive of a less invasive phenotype. In addition to Dkk-3 and DN-LRP5, transfection experiments using an intact LRPS construct in a cell line with low endogenous LRPS also would be valuable to determine whether LRPS alone can confer an invasive capacity of OS cells. Because all of the cell lines mentioned in this study (U2OS, HOS, and Saos-2) already express abundant LRPS transcripts by reverse transcription-PCR (data not shown), one may not anticipate a significant effect when these cells also are transfected with an LRPS-expressing construct. In the future, a more comprehensive screening for OS cell lines with low-endogenous expression of LRPS should be performed to facilitate these experiments.

In this study, we have shown that Dkk-3 blocks nuclear accumula-

**REFERENCES**

1. Meyers PA, Heller G, Healey J, et al. Chemotherapy for nonmetastatic osteogenic sarcoma: the Memorial Sloan-Kettering experience. J Clin Oncol 1992;10:5–15.

2. Glinka A, Wu W, Delius H, Monaghan AP, Blumenstock C, Niehrs C. Dickkopf-1 is a member of the Dickkopf gene family. Gene 1999;238:301–13.

3. Kulp E, Sharp JD, Jiang C, et al. Functional and structural diversity of the human Dickkopf gene family. Gene 1999;238:301–13.
22. Ahmed SA, Gogal RM Jr, Walsh JE. A new rapid and simple non-radioactive assay to monitor and determine the proliferation of lymphocytes: an alternative to [3H]thymidine incorporation assay. J Immunol Methods 1994;170:211–24.

23. Albini A. Tumor and endothelial cell invasion of basement membranes. The matrigel chemoinvasion assay as a tool for dissecting molecular mechanisms. Pathol Oncol Res 1998;4:230–41.

24. Tama K, Semenov M, Kato Y, et al. LDL-receptor-related proteins in Wnt signal transduction. Nature 2000;407:530–5.

25. Tsuji T, Nozaki I, Miyazaki M, et al. Antiproliferative activity of REIC/Dkk-3 and its significant down-regulation in non-small-cell lung carcinomas. Biochem Bioph Res Commun 2001;289:257–63.

26. Ozaawa M, Baribault H, Kemler R. The cytoplasmic domain of the cell adhesion molecule Uvomorulin associates with three independent proteins structurally related in different species. EMBO J 1989;8:1711–8.

27. Matsuizawa SI, Reid JC. Siah-1, SIP, and Ebi collaborate in a novel pathway for β-catenin degradation linked to p53 responses. Mol Cell 2001;7:915–6.

28. Xiao HJ, Ghosh C, Hinchman C, et al. APC-independent regulation of β-catenin degradation via a retinoid X receptor-mediated pathway. J Biol Chem 2003;278:29954–62.

29. 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.

30. Qiang YW, Endo Y, Rubin JS, Rudikoff S. Wnt signaling in B-cell neoplasia. Oncogene 2003;22:1536–45.

31. Takahashi M, Tsunoda T, Seiki M, Nakamura Y, Furukawa Y. Identification of membrane-type matrix metalloproteinase-1 as a target of the β-catenin/Tcf4 complex in human colorectal cancers. Oncogene 2002;21:5861–7.

32. Kobayashi K, Ouchida M, Tsuji T, et al. Reduced expression of the REIC/Dkk-3 gene by promoter-hypermethylation in human tumor cells. Gene 2002;282:151–8.

33. Kariko K, Kuo A, Boyd D, Okada SS, Cines DB, Barnathan ES. Overexpression of urokinase receptor increases matrix invasion without altering cell migration in a human osteosarcoma cell line. Cancer Res 1993;53:3109–17.

34. Haeckel C, Krueger S, Roessner A. Antisense inhibition of urokinase: effect on malignancy in a human osteosarcoma cell line. Int J Cancer 1998;77:153–60.

35. Kemler R. From cadherins to catenins: cytoplasmic protein interactions and regulation of cell adhesion. Trends Genet 1993;9:317–21.

36. Orsulic S, Huber O, Aberle H, Arnold S, Kemler R. E-cadherin binding prevents β-catenin nuclear localization and β-catenin/LEF-1-mediated transactivation. J Cell Biol 1999;145:343–52.

37. Frixen UH, Behrens J, Sachs M, et al. E-cadherin-mediated cell-cell adhesion prevents invasiveness of human carcinoma cells. J Cell Biol 1991;113:173–85.

38. Levine AJ. p53, the cellular gatekeeper for growth and division. Cell 1997;88:323–31.

39. Wang J, Shou J, Chen X. Dickkopf 1, an inhibitor of the Wnt signaling pathway, is down-regulated motility and invasion of lung cancer cells through inhibition of Rac activation. J Biol Chem 2003;278:11465–70.

40. Masuda H, Miller C, Koeffler HP, Battifora H, Cline MJ. Rearrangement of the p53 gene in human osteosarcoma cell line. Cancer Res 1993;53:3109–17.

41. Frixen UH, Behrens J, Sachs M, et al. E-cadherin-mediated cell-cell adhesion prevents invasiveness of human carcinoma cells. J Cell Biol 1991;113:173–85.

42. Levine AJ. p53, the cellular gatekeeper for growth and division. Cell 1997;88:323–31.

43. Wang J, Shou J, Chen X. Dickkopf 1, an inhibitor of the Wnt signaling pathway, is down-regulated motility and invasion of lung cancer cells through inhibition of Rac activation. J Biol Chem 2003;278:11465–70.

44. Qiang YW, Endo Y, Rubin JS, Rudikoff S. Wnt signaling in B-cell neoplasia. Oncogene 2003;22:1536–45.
Dickkopf 3 Inhibits Invasion and Motility of Saos-2 Osteosarcoma Cells by Modulating the Wnt-\(\beta\)-Catenin Pathway

Bang H. Hoang, Tadahiko Kubo, John H. Healey, et al.

*Cancer Res* 2004;64:2734-2739.

**Updated version**
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/64/8/2734

**Cited articles**
This article cites 38 articles, 12 of which you can access for free at:
http://cancerres.aacrjournals.org/content/64/8/2734.full.html#ref-list-1

**Citing articles**
This article has been cited by 28 HighWire-hosted articles. Access the articles at:
/content/64/8/2734.full.html#related-urls

**E-mail alerts**
Sign up to receive free email-alerts related to this article or journal.

**Reprints and Subscriptions**
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

**Permissions**
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.