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Dickkopf 3 Inhibits Invasion and Motility of Saos-2 Osteosarcoma Cells by Modulating the Wnt-β-Catenin Pathway

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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% CO_2.

The full-length human *Dkk-3* cDNA was amplified from U2OS cells by reverse transcription-PCR using the following primer pairs: 5′-CACCATGCAGCGGCTTGGGGC-3′ (sense) and 5′-AATCTCTTCCCCTCCCAGCA-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 PcDNA3.1TOPO 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 PcDNA3.1-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.

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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 ig 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 (Oncogene Research, Boston, MA) according to the manufacturer's instructions. This assay uses terminal deoxynucleotidyl transferase and a substrate similar to deoxygenin of the original terminal deoxynucleotidyl transferase-mediated nick end labeling assay. Briefly, cells were plated in a 24-well plate at 1×10^4 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 TBS, 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-G_1 phase and other 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 (2×10^3 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^{-4} 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 CytoFluor 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 trypan blue, and viable cells were seeded in the upper chamber at 1×10^4 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% CO_2. 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:

$$\frac{\text{no. of cells invaded through a Matrigel-coated membrane}}{\text{no. of cells migrated through an uncoated (control) membrane}} \times 100$$

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 3×10^4 cells were placed 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 cells/field. All of the cell lines were assayed in triplicate for each experiment, and each experiment was repeated three times.

Immunocytochemical Analysis. Immunochemical 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. Nonspecific 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-LacZ. As shown in Fig. 1, transient transfection of *Dkk-3* (3 μg/ml) affected Wnt

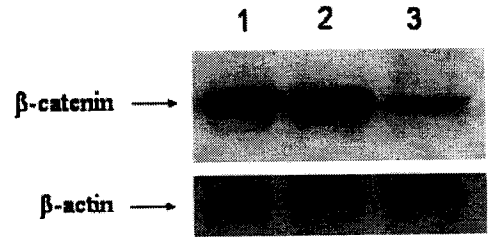

Fig. 1. Dickkopf (Dkk)-3 reduces cytoplasmic accumulation of β-catenin. After Saos-2 cells were transfected transiently with 3 μg/ml of empty control vector (EV) or *Dkk-3* expression vector, the cytoplasmic protein fractions (see "Materials and Methods") from parental (*Lane 1*), EV-transfected (*Lane 2*), and *Dkk-3*-transfected (*Lane 3*) cells were subjected to Western blot analysis using an anti-β-catenin antibody.

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. 2*A*, *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-G_1 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. 2*B*). 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-G_1 fraction of EV cells increased significantly, whereas that of *Dkk-3* transfectants remained stable (Fig. 2*B*). When exposed to doxorubicin or cisplatin, *Dkk-3*-transfected cells showed 5–25-fold higher EC_{50} than EV control cells, suggesting that *Dkk-3* reduces chemosensitivity of Saos-2 cells (Fig. 2*C*). 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. 3*A*). In contrast, *Dkk-3*-transfected cells were more compact and adherent to neighboring cells (Fig. 3*B*), 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. 3*C*, *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*-tranfected 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 proposed by Albini *et al.* (23): (*a*) adhesion to a substrate; (*b*) dissolution of the extracellular matrix; and (*c*) migration. Using this assay, we observed a significant decrease in invading capacity (expressed as percent invasion) of *Dkk-3*- and *DN-LRP5*-transfected cells. As seen in Fig. 4*A*, *Dkk*-3 and *DN-LRP5* transfectants were up to four times less invasive than EV controls ($P = 0.009$).

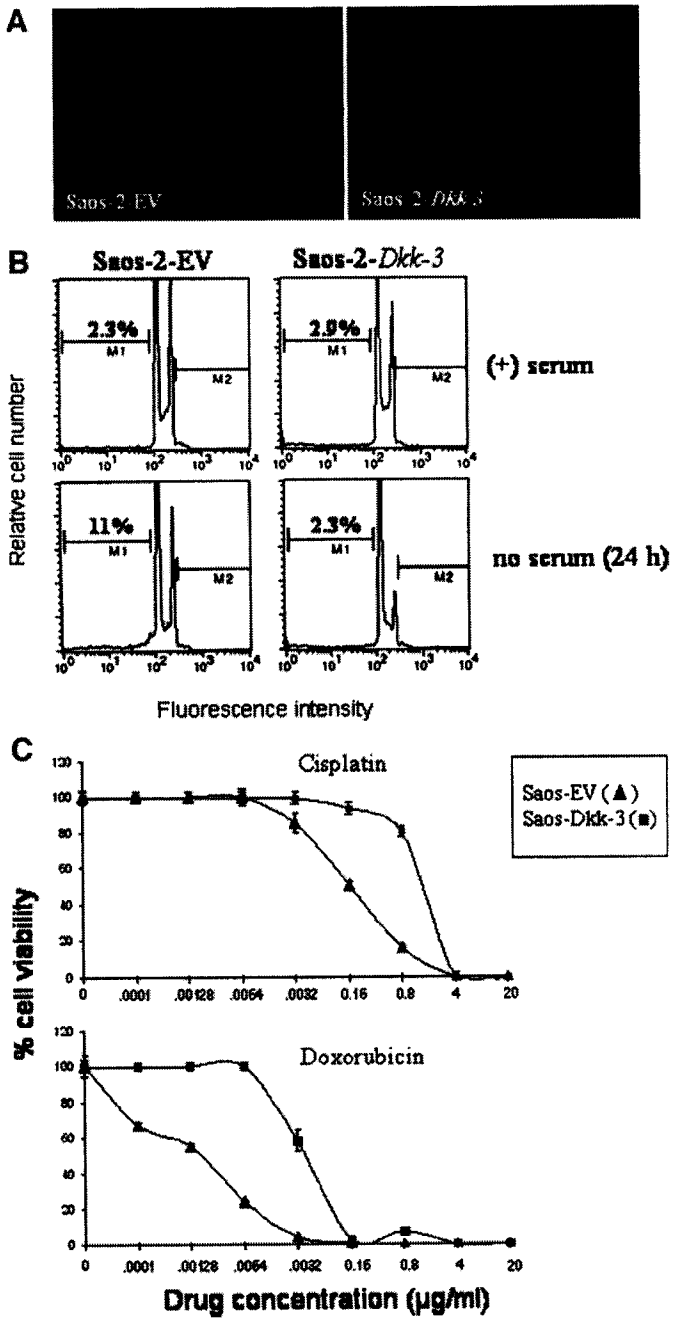

Fig. 2. Dickkopf (*Dkk*)-3 does not induce apoptosis and promote survival under nonsupportive conditions. *A*, terminal deoxynucleotidyl transferase-mediated nick end labeling staining results of empty control vector (*EV*) and *Dkk-3*-expressing Saos-2 cells. *B*, flow cytometry analysis of Saos-2 cells expressing the EV or *Dkk-3* transgene in the presence or absence of serum. Cells were cultured in serum-containing (20%) or serum-free media for 24 h and harvested for flow cytometry. Percentage of apoptotic cells with sub-G_1 DNA content (M_1 phase) was indicated in the histograms. Similar results were obtained from two independent experiments. *C*, effect of *Dkk-3* transfection on cisplatin- and doxorubicin-induced cytotoxicity in Saos-2 cells. Cell viability (after 24-h drug exposure) was determined using the alamarBlue method. Percent viability was plotted against drug concentration, and the value at each concentration represents mean ± SD of triplicate wells. Drug exposure for 72 h also showed similar results for doxorubicin and cisplatin (data not shown).

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-

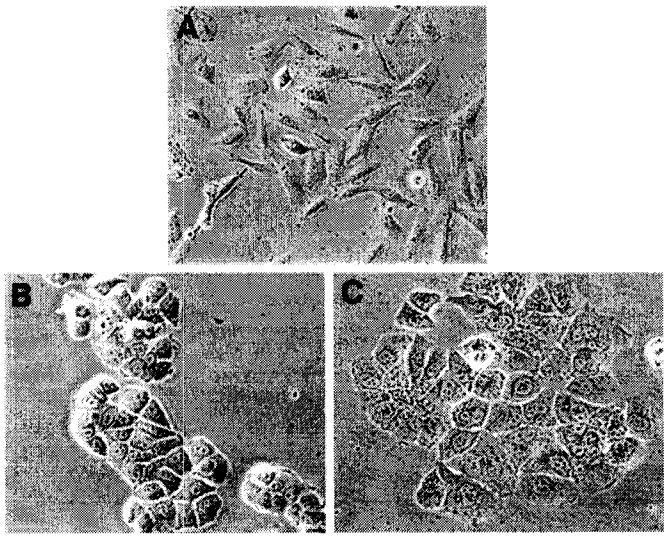

Fig. 3. Morphologic changes in osteosarcoma (OS) cells following transfection with Dickkopf 3 (*Dkk-3*) and *DN-LRP5*. Saos-2 cells were stably transfected with empty control vector (*A*), *Dkk-3* (*B*), or *DN-LRP5* (*C*) and maintained in media containing G418. Live cells were photographed using a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

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

***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. 5*A*). In contrast, β-catenin staining in *Dkk-3*-transfected cells was diffuse and less localized to the nucleus (Fig. 5*B*). 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. 5*C*). 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. 5*C*). Interestingly, transfection with the *DN-LRP5* construct also led to a similar alteration in β-catenin subcellular staining pattern (Fig. 5*D*), suggesting that Dkk-3 and DN-LRP5 might modulate cell motility and invasion via similar mechanisms.

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.

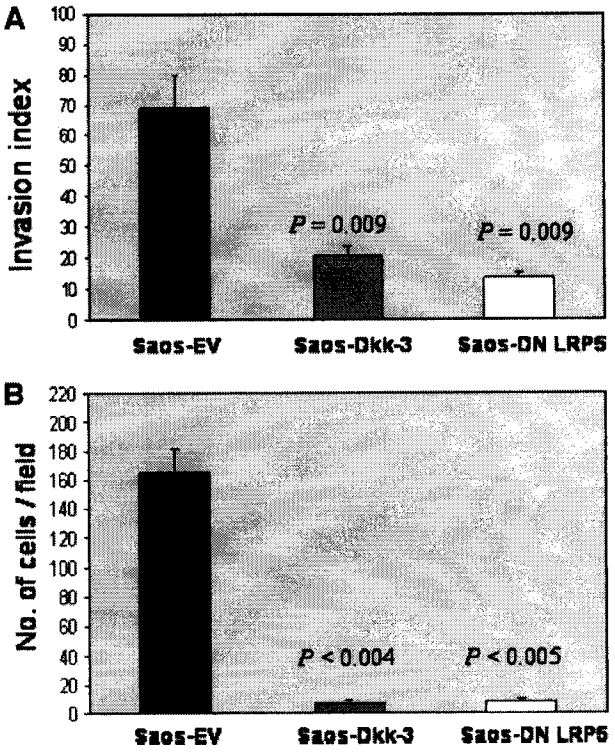

Fig. 4. *In vitro* invasive capacity and motility of Saos-2-Dickkopf (*Dkk*)-*3* and -*DN-LRP5* transfectants. *A*, *Dkk-3*- and *DN-LRP5*-transfected cells exhibited reduced invasiveness across Matrigel when compared with empty control vector (*EV*) control cells. The invasion index (see "Materials 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.

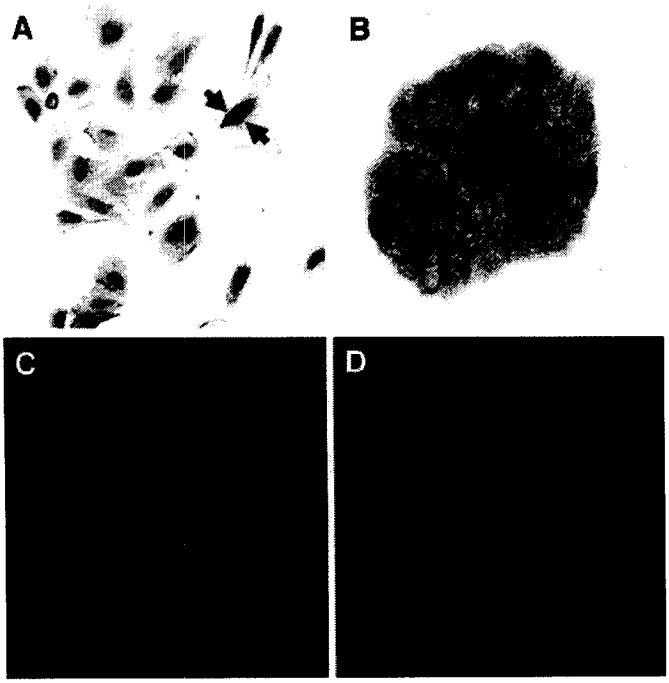

Fig. 5. Dickkopf (Dkk)-3 and dominant-negative LRP5 prevent nuclear and promote membrane localization of β-catenin. *A*, nuclear subcellular localization of β-catenin (arrows) in empty control vector (EV)-transfected Saos-2 cells (200× original magnification). *B*, *Dkk-3* transfectants exhibit diffuse, non-nuclear localization of β-catenin. *C* and *D*, immunofluorescence confocal microscopy of *Dkk-3* (*C*) and *DN-LRP5* (*D*) transfectants (63× original magnification).

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 *LRP5* construct in a cell line with low endogenous LRP5 also would be valuable to determine whether LRP5 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 LRP5 transcripts by reverse transcription-PCR (data not shown), one may not anticipate a significant effect when these cells also are transfected with an *LRP5*-expressing construct. In the future, a more comprehensive screening for OS cell lines with low-endogenous expression of LRP5 should be performed to facilitate these experiments.

In this study, we have shown that *Dkk-3* blocks nuclear accumulation of β-catenin and invasion of Saos-2 cells through Matrigel. In addition, *DN-LRP5* transfection appears to exert a similar effect on cellular invasion. These findings suggest that the Wnt pathway may play a role in regulating the invasive process in a subset of OS tumors. β-catenin, when localized to the nucleus, can activate Wnt/LEF/TCF pathway to up-regulate matrix metalloproteinase 1 (31). Metalloproteinases have long been implicated in cancer invasion and metastasis. Thus, it is conceivable that *Dkk-3* may suppress OS invasiveness by affecting the expression or activity of the metalloproteinases. Experiments are under way to assess which metalloproteinases are downstream targets of *Dkk-3* or *LRP5* in OS cells.

Although *Dkk3* expression often is reduced in tumor cell lines by promoter hypermethylation, down-regulation of expression is variable among different cell lines (32). In this study, although Saos-2 may down-regulate Dkk-3 to promote cellular invasion via the Wnt pathway, other OS cell lines may use different pathways to promote matrix invasion. For example, cathepsin B and urokinase-type plasminogen activator have been shown to promote invasive capacity of HOS cells (33, 34). Therefore, Wnt-mediated mechanisms of invasion may be relevant in only a subset of OS tumors.

In addition to reducing nuclear localization, we show that *Dkk-3* can induce a redistribution of β-catenin to the cell membrane. When localized to the membrane, β-catenin promotes cell-cell adhesion by binding to the cytoplasmic domain of E-cadherin to form the adherens junction complex (35). Binding to E-cadherin prevents β-catenin nuclear translocation and thus inhibits Wnt-mediated LEF/TCF transactivation (36). In the absence of Wnt signaling, cytoplasmic β-catenin rapidly degrades, and only β-catenin complexed with membrane cadherins is protected from degradation. Interestingly, expression of E-cadherin has been associated with decreased invasiveness of cancer cells (37). It is tempting to speculate that *Dkk-3* may transform a subset of OS cells to a less invasive phenotype by recruiting β-catenin to the cell surface to promote cell-cell adhesion.

The observation that *Dkk-3* promotes survival of Saos-2 cells during serum starvation and chemotherapy-induced DNA damage is intriguing. DNA damage has been shown to activate p53, leading to induction of p53 target genes (38). Dkk-1 has been identified recently as a direct target of p53 in response to DNA damage (39). Because Saos-2 cells have been shown to lack a functional p53 protein, ectopic expression of Dkk-3 may serve a similar function as Dkk-1 to restore the DNA damage response (40). Fan and Bertino (41) found recently that stable transfection of p53 into Saos-2 cells results in a significant decrease in sensitivity to cisplatin. In this study, a similar response to cisplatin by *Dkk-3*-transfected cells also suggests that Dkk-3 may in part restore p53-mediated damage response in p53-null OS cells. Our findings should prompt future investigations using additional p53-deficient cell lines to confirm this hypothesis.

In summary, the data suggest that modulation of the Wnt-β-catenin pathway by Dkk-3 or by a dominant-negative Wnt receptor in OS can suppress invasion and motility *in vitro*. In addition, Dkk-3 may enhance cell survival by affecting the cell cycle in the absence of p53. In OS, the consequence of down-regulating *Dkk-3* may lead to a more invasive cellular phenotype (6). In contrast, up-regulation of *Dkk-3* may confer increased resistance to DNA damage by chemotherapy.

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