

# p21<sup>CIP-1/WAF-1</sup> Induction Is Required to Inhibit Prostate Cancer Growth Elicited by Deficient Expression of the Wnt Inhibitor Dickkopf-1

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## Abstract

Osteoblastic bone metastases are the most common metastases produced by human prostate cancers (PCa). Deregulated activity of Wnt growth factors resulting from overexpression of the Wnt inhibitor Dickkopf-1 (DKK-1) is known to contribute to formation of the osteoblastic component of PCa skeletal bone metastases. In this study, we report that DKK-1 knockdown in osteolytic human PCa cells unexpectedly delays the development of both soft tissue and osseous lesions. PCa cells deficient in DKK-1 expression did not increase canonical Wnt signaling in target osteoblast cell lines; however, DKK-1 knockdown PCa cells exhibited increased expression of the CDK inhibitor p21<sup>CIP1/WAF1</sup> and a 32% increase in G<sub>1</sub> arrest compared with control cells. Ablating p21<sup>CIP1/WAF1</sup> in PCa cells deficient in DKK-1 was sufficient to rescue tumor growth. Collectively, our findings demonstrate that DKK-1 overexpression supports tumor growth in part by restricting expression of p21<sup>CIP1/WAF1</sup> through a mechanism independent of canonical Wnt signaling. *Cancer Res*; 70(23); 9916–26. ©2010 AACR.

## Introduction

Prostate cancer (PCa) remains a leading cause of cancer-related deaths in men within the United States (1). Men who die of PCa do so as a result of metastatic spread from the prostate to the dura, liver, lungs, and bone. Metastasis to the trabecular bone of the pelvis, femur, and vertebral bodies are the most common and occur in over 80% of all men who die of PCa (2). Growth of PCa within the bone results in primarily osteoblastic (bone forming) lesions with underlying areas of osteolysis (bone resorption; ref. 3). Numerous PCa-derived protein factors have been identified that can affect osteoblast development and differentiation *in vitro* (4), including Wnts (5), which may contribute to the formation of osteoblastic lesions *in vivo*.

Wnt proteins are a large family of soluble glycoproteins that control bone development and regulation of bone mass (6, 7). Wnts signal through 3 pathways: the canonical, the Wnt/Ca<sup>2+</sup>, and planar cell polarity pathways. In the canonical pathway, Wnts bind to a receptor complex composed of LRP 5 or 6 and

Frizzled (FZD) proteins, which leads to the stabilization of the TCF cofactor  $\beta$ -catenin. The activity of Wnt proteins is controlled by secreted antagonists including secreted FZD-related proteins (sFRP), Wnt inhibitory factor-1 (WIF-1), Cerberus, Sclerostin, and Dickkopfs (DKK; ref. 8). sFRP, WIF-1, and Cerberus act as competitive inhibitors of FZD by sequestering Wnt factors and can, therefore, block both canonical and noncanonical Wnt pathways. Sclerostin and DKK-1, 2, and 4, in contrast, bind the Wnt coreceptors LRP 5 and 6 to block canonical Wnt signaling by removing LRP from the cell surface (9).

DKK-1 was first identified in *Xenopus* for its ability to induce complete head structures (10). It is encoded by a relatively small (3 kb) gene at Chr 10q11.2 whose expression is restricted to the bone in adult mice (11). The temporal and spatial regulation of Wnt activity by DKK-1 is essential for normal bone development. In the absence of DKK-1, murine embryos display a fusion and duplication of digits whereas DKK-1 overexpression in the chick results in distal truncation of the limb bud (12, 13). The osteoblast specific expression of DKK-1 in the mouse leads to severe osteopenia underscoring the importance of Wnt signaling in bone formation (11, 14).

We previously demonstrated that blocking Wnt activity through overexpression of DKK-1 led to increased osteolytic activity and PCa tumor growth within bone *in vivo* (5). Furthermore, we demonstrated that DKK-1 expression, although strongly present in primary tumors, declines in PCa bone metastases (15). This led us to hypothesize that declining DKK-1 levels in bone metastases unmasks PCa-mediated Wnt activity that would favor development of osteoblastic lesion. To test this hypothesis, we decreased DKK-1 activity in a murine model of PCa bone metastasis.

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We found that decreased DKK-1 activity in osteolytic PC-3 PCa cells did not induce bone formation, but rather delayed the formation of osseous and subcutaneous lesions *in vivo*. Reduction of DKK-1 activity induced the expression of the cyclin-dependent kinase inhibitor p21<sup>CIP1/WAF1</sup> (CDKN1A) suggesting that DKK-1 expression in PCa cells facilitates tumor establishment by promoting cell cycle progression.

## Materials and Methods

### Cells

Human PC-3 and Du145 PCa cells were obtained from the American Type Culture Collection and were maintained as previously described (5). PC-3 luciferase cells were kindly provided by Alnawaz Rehemtulla (University of Michigan, Ann Arbor, MI). PC-3 DKK-1 shRNA 366 cells were generated from PC-3 cells as previously described (5). Additional control shRNA- and DKK-1 shRNA-transduced cells were generated by lentiviral transduction with pLKO.1 carrying a nontargeting shRNA or DKK-1 shRNA to position 796 (5'-GAAAGAAGGT-CAAGTGTGT-3'). All cells were shown to be free of *Mycoplasma* by the PlasmTest mycoplasma detection method (Invivogen).

### Generation of DKK-1/p21 double knockdown cells

pGIPZ plasmid DNA encoding a nontargeting shRNA control or p21-directed shRNAs that target p21 at positions 703 and 888 were obtained from Open Biosystems. Plasmids were packaged into virus particles according to manufacturer's instructions and used to transduce PC-3 DKK-1shRNA 796-transduced cells. Puromycin-resistant, green fluorescent protein-positive clones were then selected by limiting dilution.

### *In vivo* animal model of bone metastasis

Tumor cells ( $5 \times 10^5$  cells/50  $\mu$ L) were injected into the tibia of male nude mice at 5 to 6 weeks of age as described previously (5). Tumors were allowed to grow for 3 or 6 weeks. All animals were evaluated using Faxitron radiography (Faxitron X-ray Corp.). Radiographs were digitized and the percent osteolytic area was quantified as previously described (5). Injected tibiae and contralateral tibiae without tumors were removed, bone mineral density measured using a pDEXA Sabre scanner (Orthometrix, Inc.), and processed for histology as previously described (5).

### Subcutaneous tumor growth assay

Tumor cells ( $1 \times 10^6$  cells/100  $\mu$ L) were injected into the subcutis of male nude mice at 5 to 6 weeks of age. Tumor diameter was measured biweekly in 2 axes using a caliper and tumor volumes calculated using the formula  $(\text{min}^2 \times \text{max})/2$ . A repeated measures generalized linear model was used to test for a difference in the tumor growth rates between the 2 groups.

### Intracardiac PCa experimental metastasis model

A purified, neutralizing monoclonal antibody to DKK-1 and isotype control were provided by Eli-Lilly. Male nude mice, 5 to 6 weeks of age, received biweekly intraperitoneal injections of

5 mg/kg antibody in 0.1 mL PBS for the length of the study. One week after the start of antibody injections, DKK-1<sup>+</sup> PC-3-luc cells were injected into the left cardiac ventricle as previously described (16, 17). Six weeks post-tumor cell injection, tumor burden was measured by Bioluminescent imaging using a Xenogen IVIS imaging system (Xenogen Corporation).

### PCR analysis

The expression of p21, DKK-1, axin2, and  $\beta$ -actin was evaluated by quantitative PCR on a Roche Lightcycler 480 as previously described (18). The primers used were as follows: p21-1131F 5'-ATGAAATTCACCCCTTCC-3'; p21-1304R 5'-CCCTAGGC TGTGCTCACTTC-3'; axin2-3585F 5'-CCCAGGTTGATCCTGTGACT-3'; axin2-3823R 5'-AGGTGTGTGGAGGAAAGGTG-3'. PCR primers for DKK-1 and  $\beta$ -actin appear in (5).

### Transient transfection

DKK-1 366 siRNA (5'-GGAATAAGTACCAGACCA-3') was obtained from Thermo Scientific. Fluorescein-conjugated SignalSilence nontargeting control siRNA was used to control for transfection efficiency (Cell Signaling). On day 0,  $2.5 \times 10^5$  PCa cells per 2 mL complete medium were plated to 6-well plates and allowed to attach for, 24 hours. The following day (day 1), the cells were transfected with 125 pmole of siRNA using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. Twenty-four hours later (day 2), the media was removed and replaced with complete medium or a 72-hour conditioned medium prepared from parental PC-3 cells. On day 3, whole cell lysates or total RNA were prepared and analyzed by Western blot analysis or RT-quantitative PCR, respectively.

### Western blot for analysis

The expression of p21 and DKK-1 proteins were determined using Western blotting of total cell lysates as described previously (5). Equal amounts of protein (30  $\mu$ g per sample) were resolved using 12% SDS-PAGE. The filters were cut and separate pieces blotted with anti-p21 monoclonal antibody (BD Biosciences), anti-DKK-1 pAb (R&D Systems), anti-p27 monoclonal antibody (BD Biosciences), or anti- $\alpha$ -tubulin mAb (Sigma).

### Cell cycle analysis

A total of  $5 \times 10^5$  cells were plated to 100 mm dishes and allowed to grow for 48 hours. Following this incubation, cells were trypsinized, washed 1X in PBS and fixed for 20 minutes in 0.5 mL per tube ice-cold absolute ethanol. Fixed cells were washed then stained with propidium iodide (PI) solution (50  $\mu$ g/mL PI and 100 units RNAase A in PBS) for 20 minutes. The diploid DNA content was quantified using a Coulter FACS Scan flow cytometer (Beckman-Coulter).

### cdNA microarray analysis

The ONCOMINE database and gene microarray analysis tool, a repository for published cDNA microarray data (<http://141.214.6.50/oncomine/main/index.jsp>), was explored for mRNA expression of p21 and DKK-1 in clinical cases of prostate, breast, and ovarian cancer. Statistical analysis of differences was performed using ONCOMINE algorithms to

account for the multiple comparisons among different studies as previously described (19).

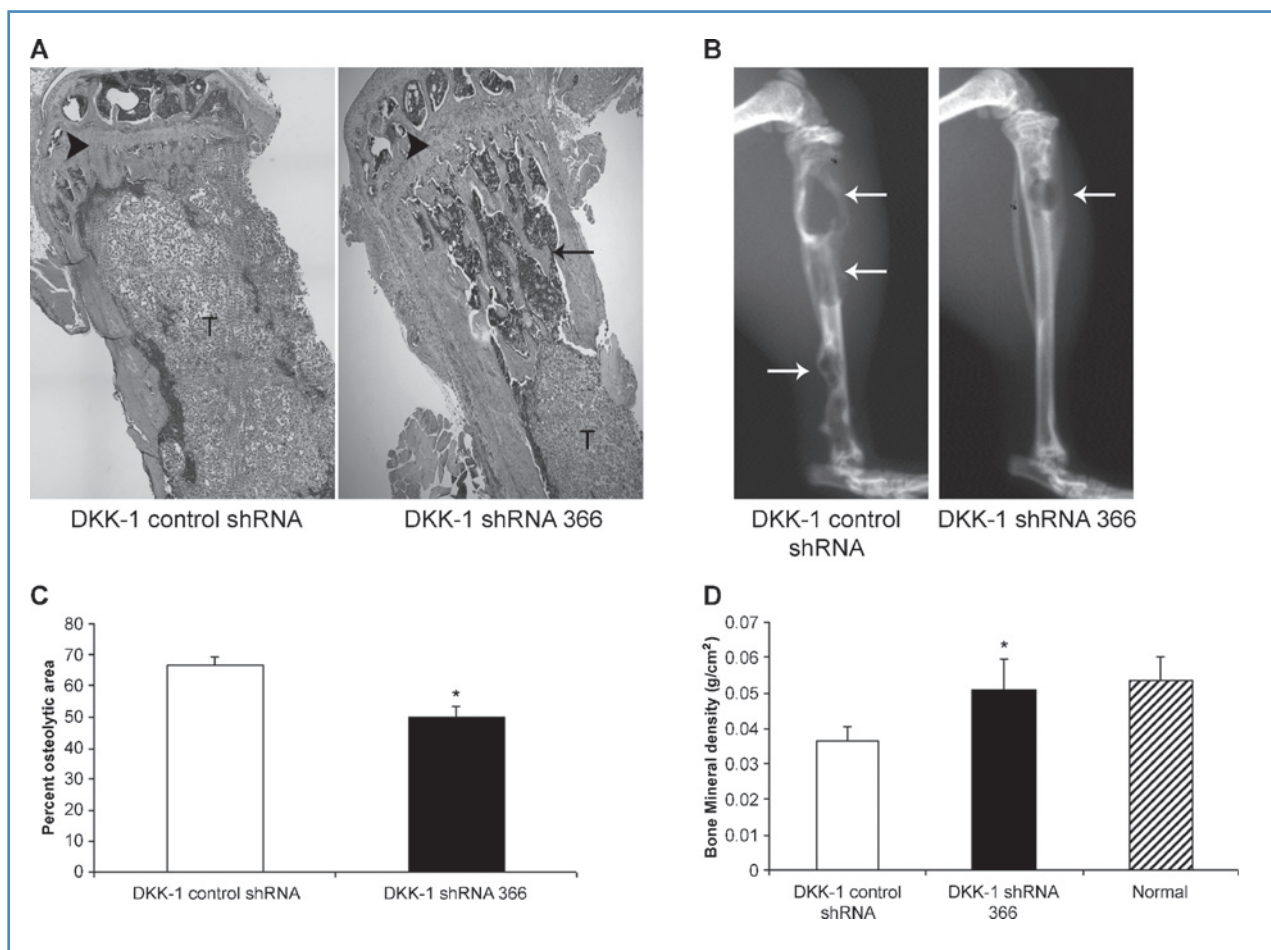
## Results

### Blocking DKK-1 delays PCa establishment within the bone

We previously demonstrated that Wnts contribute to the osteoblastic component of PCa skeletal metastases (5). This conclusion was based on 3 principal lines of evidence: (1) PCa cells express the RNA for multiple Wnts, (2) expression of the Wnt inhibitor DKK-1 in osteoblastic C4-2B PCa cells stimulated severe osteolysis *in vivo*, and (3) shRNA mediated knock-down of DKK-1 in osteolytic PC-3 PCa cells stimulated osteoblast differentiation and mineralization of cocultured

bone marrow stromal cells. PC-3 DKK-1 shRNA 366 clone 3 cells are a stable clone isolated following calcium phosphate transfection with a shRNA molecule that targets position 366 of DKK-1 (5). These cells were shown to have more than 80% downregulation of DKK-1 protein, but equal cell proliferation rates *in vitro* compared with a DKK-1 directed but noncleaving shRNA control-transfected clone (5). To characterize the effect of DKK-1 knockdown on the formation of osseous lesions, both PC-3 DKK-1 shRNA 366 and DKK-1 control shRNA-transfected cells were injected into the tibiae of male nude mice (intratibial injection).

On the basis of the data mentioned earlier, it was expected that DKK-1 shRNA 366 cells would induce bone production following intratibial injection. However, DKK-1 knockdown cells were found to grow poorly in the bone of nude mice.



**Figure 1.** Blocking DKK-1 delays PCa establishment within the bone. DKK-1-directed but noncleaving control shRNA-transfected or DKK-1 shRNA 366-transfected cells ( $5 \times 10^5$  cells per 50  $\mu$ L) were directly injected into the tibiae of anesthetized male nude mice (10 mice per group). Tumors were allowed to grow for 6 weeks at which time the mice were sacrificed and bones processed for histologic evaluation. A, representative hematoxylin and eosin histology of tibiae injected with DKK-1 control shRNA-transfected cells versus DKK-1 shRNA 366 cells. Normal trabeculae (arrow), marrow (M), tumor (T), and growth plate (arrowhead). Original magnification,  $\times 20$ . B, corresponding Faxitron X-rays of tibiae injected with DKK-1 control shRNA cells or DKK-1 shRNA 366-transfected cells. Arrows indicate osteolytic activity. C, percent osteolytic area of total tibial area. Radiographs from tumor-injected tibiae were digitized and the percent osteolytic area of the total tibial area was quantified. Data are presented as mean  $\pm$  SE within each group; \*,  $P < 0.002$  versus PC-3 DKK-1 control shRNA cells by *t* test. D, bones were subjected to DEXA to quantify bone mineral density. Normal tibiae ( $n = 5$ ), tibiae injected with PC-3 DKK-1 control shRNA cells ( $n = 8$ ), or PC-3 DKK-1 shRNA 366-transfected cells ( $n = 10$ ) were scanned. Data are presented as mean  $\pm$  SE within each group; \*,  $P < 0.005$  compared with PC-3 DKK-1 control shRNA-transfected tibiae by *t* test.

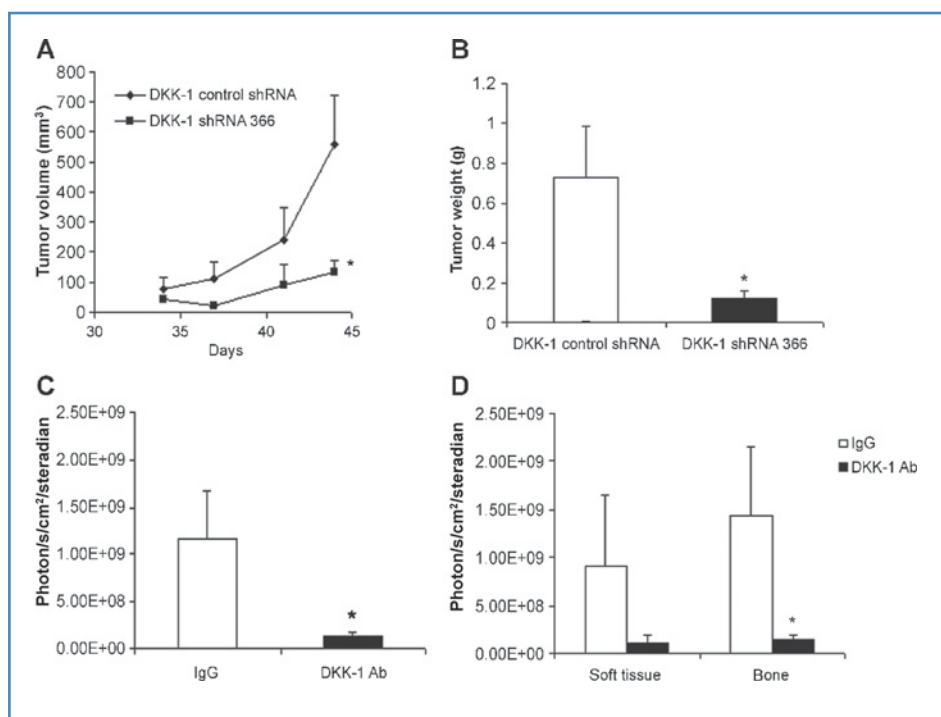
Specifically, PC-3 DKK-1 shRNA 366 cells were found to form tumors with equal incidence (incidence DKK-1 control shRNA cells, 8 of 8; incidence of DKK-1 shRNA 366 clone 3 cells, 9 of 10), but developed smaller lesions than DKK-1<sup>+</sup> control shRNA-transfected cells. Decreased tumor growth was evident by a decline in tumor volume on the basis of the histologic evaluation (Fig. 1A). Corresponding with the decreased tumor volume, there was a decline in bone remodeling as observed on radiographs (Fig. 1B), a reduction in percent osteolytic area (Fig. 1C), and an increase in bone mineral density of injected tibia (Fig. 1D). Together, these data show that blocking DKK-1 expression reduced the intraosseous growth of PC-3 PCa cells, a cell line with abundant DKK-1 expression.

### Blocking DKK-1 decreases PCa tumor burden *in vivo*

The molecular knockdown of DKK-1 using a shRNA molecule delayed PCa tumor establishment within the bone following direct injection. To determine whether the effect was specific to bone, PC-3 DKK-1 shRNA 366-transfected or DKK-1 control shRNA-transfected cells were injected into the subcutis of nude mice and the incidence and growth rate of the tumors measured. As before, the tumor incidence was equivalent in each group (4 of 4 DKK-1 control shRNA versus

10 of 10 DKK-1 shRNA 366); however, the DKK-1 shRNA-transfected cells developed tumors at a much slower rate as reflected in both a significant decreased tumor growth rate and tumor weight at the experimental endpoint (Fig. 2A and B, respectively). These data not only suggest that DKK-1 expression promotes tumor growth and establishment, but also indicate that the effect of DKK-1 knockdown was not specific to the bone.

To confirm that reduced DKK-1 expression suppresses tumor growth in soft tissue and bone, an alternative method to inhibit DKK-1 function was used. Specifically, a DKK-1 neutralizing antibody was employed to decrease systemic DKK-1 activity in an experimental metastasis model. PC-3 cells injected into the left cardiac ventricle (intracardiac injection) produce osteolytic bone lesion of the mandible, humerus, and tibia/femur as well as rare soft tissue lesions. As observed with the DKK-1 shRNA knockdown cells, DKK-1 neutralizing antibody did not alter tumor incidence compared with IgG control as the median number of lesions/mouse in each group was 3 (range 1–6). However, treatment with DKK-1 neutralizing antibody reduced the overall tumor burden 8.3-fold compared with IgG control (Fig. 2C). A similar reduction with DKK-1 antibody treatment was observed when separately



**Figure 2.** Blocking DKK-1 decreases PCa tumor burden *in vivo*. DKK-1 control shRNA-transfected or DKK-1 shRNA 366-transfected cells ( $1 \times 10^6$  cells per  $100 \mu\text{L}$ ) were directly injected into the hind flank of anesthetized male nude mice. A, tumor diameter was measured in 2 axes using a caliper twice a week and tumor volume calculated. Shown is the tumor volume over time of DKK-1 control shRNA-transfected or DKK-1 shRNA 366-transfected cells; mean  $\pm$  SE of subcutaneous tumors. \*,  $P < 0.006$  versus DKK-1 control shRNA cells by a repeat measures generalized linear model. B, subcutaneous tumor weight at experimental endpoint. After 6 weeks, the subcutaneous tumors were excised and weighed. Shown is the mean  $\pm$  SE of subcutaneous tumors; \*,  $P < 0.003$  versus DKK-1 control shRNA cells by  $t$  test. C and D, DKK-1 neutralizing antibody: beginning at day 0, male Nu/Nu mice were intraperitoneally injected with 5 mg/kg IgG isotype control or DKK-1 neutralizing antibody twice a week for 7 total weeks. At day 7, DKK-1<sup>+</sup> PC-3 luciferase cells were injected into the left cardiac ventricle ( $2 \times 10^5$  cells per 0.1 mL) to establish bone metastases. C, total tumor burden was measured using bioluminescent imaging following the injection of luciferin. The data are presented as mean number of photons/s/cm<sup>2</sup> ( $\pm$  SE), 10 mice per group. \*,  $P < 0.028$  compared with IgG control mice by  $t$  test. D, tumor burden in soft tissue or bone lesions within each treatment group; \*,  $P < 0.02$  compared with IgG control mice by  $t$  test.

comparing only bone lesions (Fig. 2D). There was also a strong trend towards decreased formation of soft tissue lesions in DKK-1 antibody-treated animals (Fig. 2D) indicating that DKK-1 activity promotes tumor establishment at any site. Taken together, the data demonstrate that knockdown of DKK-1 activity with either a shRNA or neutralizing antibody reduced PCa tumor growth *in vivo*.

### DKK-1 knockdown increases p21 expression and G<sub>1</sub> arrest

The mechanism through which DKK-1 suppression leads to a reduction in tumor growth is unknown, but may result from alterations in the rate of apoptosis and/or cell cycle. An increase in tumor cell apoptosis or a decrease in the rate of cell cycling would have a negative impact on tumor growth at any site. To investigate both of these possibilities changes in the expression of apoptosis and cell cycle genes between parental and DKK-1 shRNA 366-transfected cells were compared using PCR arrays (Table 1). The top 2 cell cycle genes upregulated on DKK-1 knockdown were the cyclin-dependent kinase inhibitors p21<sup>CIP1/WAF1</sup> and p15<sup>INK4B</sup>; however, only p21 was found to change at both the RNA and protein levels and thus was explored further. To validate the effect of DKK-1 knockout on CDKN expression independent of clonal cell selection, p21 expression was measured following the transient transfection of a DKK-1 siRNA oligonucleotide that targets position 366. Quantitative PCR analysis demonstrated that DKK-1 knockdown increased p21 RNA transcripts 2-fold in both PC-3 and Du145 DKK-1 siRNA-transfected PCa cells compared with control siRNA-transfected cells (Fig. 3A). Further Western blotting confirmed that the suppression of DKK-1 increased p21 protein levels without a corresponding increase in p27 in both cell lines suggesting that DKK-1 knockdown selectively induced p21 versus other CDKNs

(Fig. 3B). To show the specificity of DKK-1 knockdown on p21 expression, an additional stable DKK-1 shRNA cell line was generated with a shRNA that targets DKK-1 at position 796. Again, to avoid clonal selection bias, a stable viral-transduced pool of cells was used for this analysis. As in siRNA-transfected cells, transduction with the DKK-1 shRNA 796 decreased DKK-1 levels 10.8 ± 0.7-fold and increased p21 expression 5.1 ± 0.9-fold compared with control shRNA-transduced cells supporting that DKK-1 knockdown lead to increased p21 expression (Fig. 3C). To confirm that the increase in p21 message was mediated by DKK-1, the ability of DKK-1 protein to restore p21 levels was measured. The addition of PC-3 conditioned medium to add back DKK-1 protein restored p21 expression to normal levels in DKK-1 siRNA-transfected cells thus, confirming that DKK-1 directly effects p21 expression (Fig. 3D). Together, these results demonstrate that the knockdown of DKK-1 increased p21 expression in PCa cells.

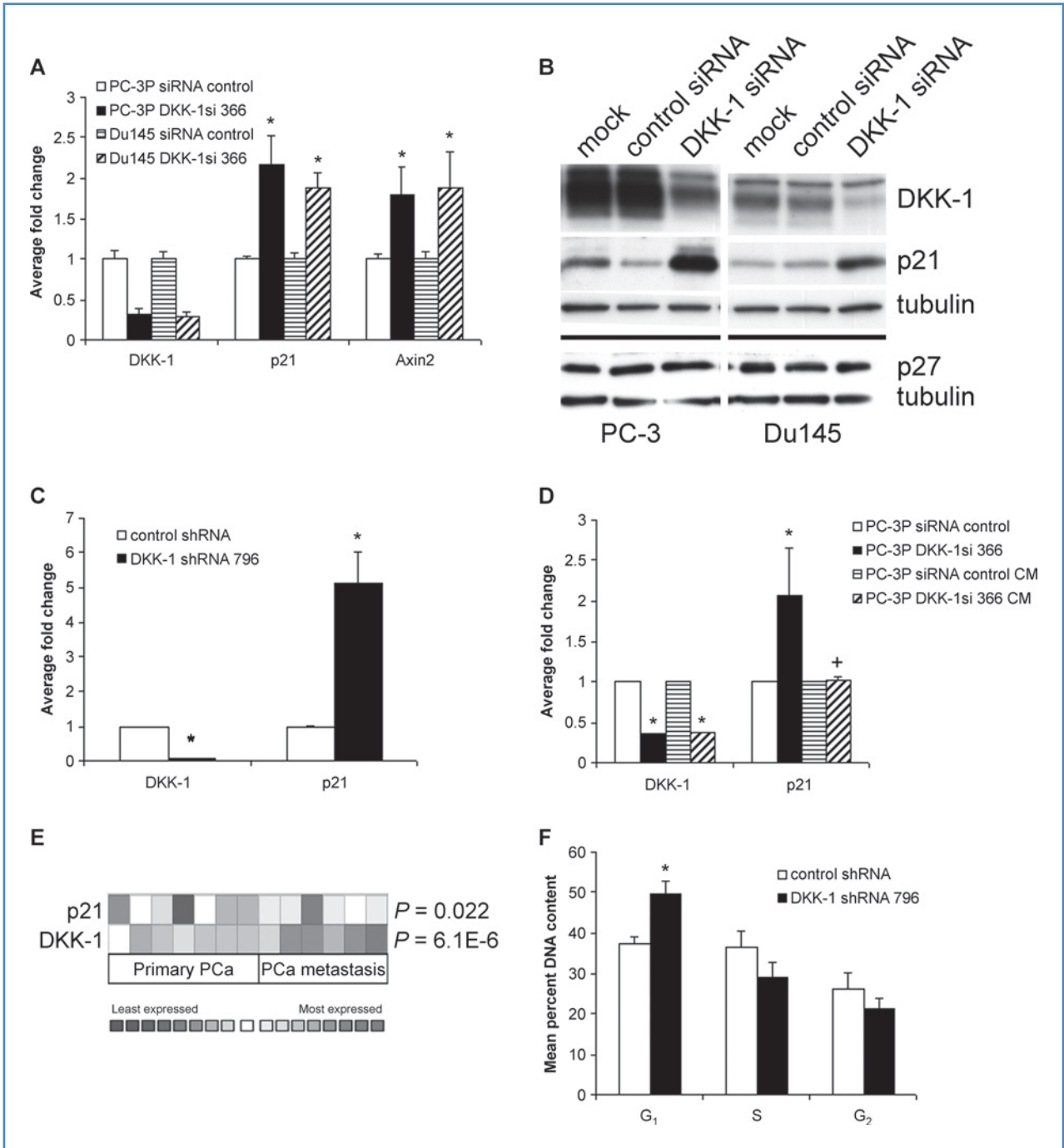
To explore whether this observation extended to clinical PCa and other cancer cell types, the Oncomine cDNA microarray database was used to evaluate p21 expression in human tumor samples where DKK-1 was found to be decreased. In a study comparing clinical cases of primary PCa versus PCa metastases, DKK-1 was dramatically decreased in PCa metastases versus primary lesions, consistent with our analysis of PCa clinical samples using tissue microarrays (ref. 15; Fig. 3E). Compared with the primary lesions, p21 was significantly increased in PCa metastases consistent with our *in vitro* data and published reports by Fizazi et al. (20). Further query of Oncomine revealed that reduced DKK-1 expression correlated with increased p21 levels in clinical cases of human breast cancer and ovarian cancer thus supporting that an inverse relationship between DKK-1 and p21 is a feature of many cancer cell types (data not shown).

To examine whether the increase in p21 contributed to suppression of cell cycle, DKK-1 shRNA 796- and control shRNA-transduced cells were compared for difference in cell cycle distribution using PI-flow cytometry. P21 is a known inhibitor of cyclin D-Cdk4/6 and cyclin E-Cdk2, which control cell cycle progression from G<sub>1</sub> to S phase. Therefore, an increase in p21 is expected to increase the number of cells in G<sub>1</sub> and decrease the number of cells in either S or G<sub>2</sub>. Consistent with this fact, compared with control shRNA-transduced cells, the diploid DNA content at G<sub>1</sub> in DKK-1 shRNA 796 knockdown cells was found to increase 32.2% ± 5.0% (Fig. 3F), indicating that increased p21 expression following DKK-1 knockdown can lead to cell cycle arrest.

To test whether DKK-1-mediated changes in cell cycle directly affect PCa cell proliferation, 2-D attachment-dependent growth rates were measured *in vitro* over 96 hours. Despite the modest yet reproducible increase in G<sub>1</sub> fraction within DKK-1 shRNA 796-transduced cells, there was no decrease in attachment-dependent growth compared with control shRNA-transduced cells (data not shown), consistent with our previous published reports (5). These data raise the possibility that p21 elevated cells are selected against during continuous culture. To remove possible selection bias, 2-D attachment-dependent growth rates were compared in PC-3

**Table 1.** Cell cycle genes elevated in DKK-1 knockdown cells

Gene name	Parental vs DKK-1 shRNA 366 (fold change)
Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	10.3
Cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)	4.2
Cyclin D2	4.1
Hectomain and RLD 5	3.1
Growth arrest and DNA-damage-inducible, alpha	2.0
Retinoblastoma-like 2 (p130)	1.9
DIRAS family, GTP-binding RAS-like 3	1.8
Tumor protein p53 (Li-Fraumeni syndrome)	1.7
Cyclin-dependent kinase inhibitor 1B (p27, Kip1)	1.6



**Figure 3.** DKK-1 knockdown increases p21 expression and G<sub>1</sub> arrest. Parental PC-3 and Du145 PCa cells were transiently transfected with a control siRNA oligo or a DKK-1 366 targeting siRNA. After 48 hours, cells were lysed for both RNA and protein isolation. A, RT-quantitative PCR analysis of transfected cells for DKK-1, p21, or axin-2, mean  $\pm$  SD of 4 experiments; \*,  $P < 0.01$  compared with control siRNA cells by *t* test. B, corresponding Western blot of transfected cells. Membranes were blotted with antisera specific to DKK-1, p21, p27, or, as a control for loading,  $\alpha$ -tubulin. C, RT-quantitative PCR analysis of DKK-1 and p21 expression in stable pools of DKK-1 shRNA 796-transduced cells. Shown is the fold change versus control shRNA-transduced cells, mean  $\pm$  SE of duplicate experiments; \*,  $P < 0.004$  versus control shRNA cells by *t* test. D, RT-quantitative PCR analysis of DKK-1 and p21 expression in PC-3 DKK-1 siRNA 366 or control siRNA-transfected cells treated with 72 hours PC-3 conditioned medium. Shown is the fold change versus control shRNA-transduced cells, mean  $\pm$  SD of duplicate experiments; \*,  $P < 0.05$  versus control siRNA cells by *t* test. E, the OncoPrint cDNA microarray database was evaluated for p21 expression in human PCa tumor samples where DKK-1 was found to be decreased. Shown is a study comparing clinical cases of primary PCa versus PCa metastases. F, cell cycle analysis. Equal number of DKK-1 shRNA 796-transduced or control shRNA-transduced cells ( $5 \times 10^5$  cells) were plated at day 0 and the percent DNA content was measured at day 2 by flow cytometry following staining with Propidium Iodide. Shown is the mean percent DNA content, mean  $\pm$  SE of 3 experiments; \*,  $P < 0.01$  versus control shRNA cells by *t* test.

and Du145 DKK-1 siRNA transiently transfected cells. Consistent with the cell cycle data, DKK-1 siRNA-transfected cells showed a modest yet reproducible decrease in attachment-dependent growth on plastic (Supplementary Fig. S1A and B). Moreover, DKK-1 knockdown did not appear to impact PCa cell survival under attachment-independent conditions (Supplementary Fig. S1C) suggesting that DKK-1 knockdown increases cell cycle arrest leading to suppression of cellular proliferation.

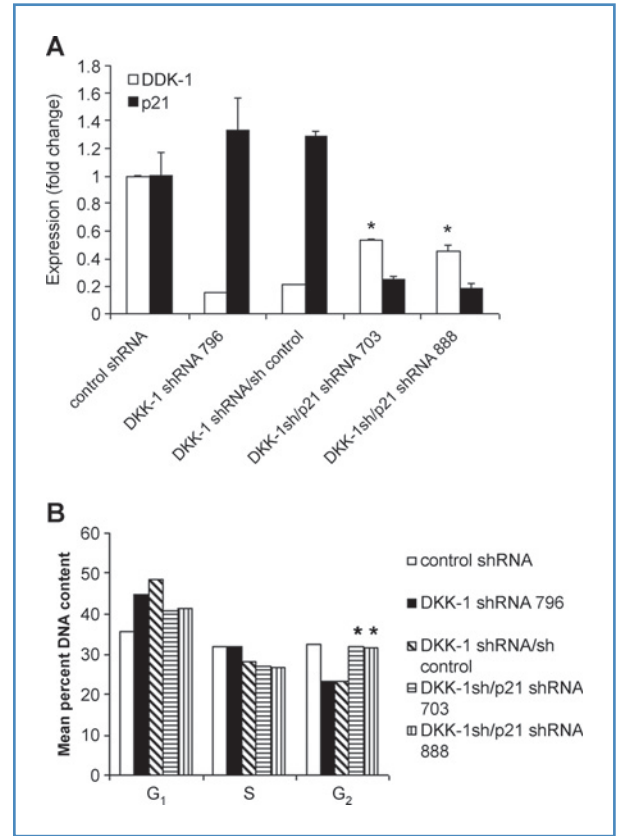
### DKK-1 knockdown increased p21 expression through a mechanism independent of canonical Wnt signaling

Given that DKK-1 is a known inhibitor of Wnt proteins that stabilize  $\beta$ -catenin, we hypothesized that  $\beta$ -catenin signaling mediates the observed increase in p21 and subsequent growth suppressive effects in PCa. Further, we previously demonstrated that PCa cells express the RNA for multiple canonical Wnts supporting that canonical Wnt signaling may be elevated in DKK-1 suppressed cells (5). Consistent with these findings, the  $\beta$ -catenin target gene axin-2 was elevated in DKK-1 knockdown cells compared with control siRNA-transfected cells (Fig. 3A). However, the conditioned media from PC-3 DKK-1shRNA 796 cells failed to increase  $\beta$ -catenin activity in cocultured ST-2 cells as measured by TOP-FLASH reporter assays; whereas, cotransfection of  $\beta$ -catenin cDNA or the addition of rWnt3a protein could activate TOP-FLASH (Supplementary Fig. S2A). PC-3 DKK-1shRNA 796 cells were further shown to have similar levels of total and phosphorylated Jun N-terminal kinase (JNK) compared with shRNA control-transduced cells suggesting that there were also no changes in noncanonical Wnt signaling within DKK-1 knockdown cells (Supplementary Fig. S2B). These findings suggest that DKK-1 knockdown increases p21 expression through a mechanism independent of canonical Wnt signaling and are consistent with those of Sato et al. (21) who showed cytotoxic effects of DKK-1 neutralizing antibody in A549 lung cancer cells without a corresponding increase in either canonical or noncanonical Wnt signaling.

Because no changes in  $\beta$ -catenin or JNK transcriptional activity were observed, we investigated whether p21 was regulated at the posttranscriptional level. Analysis of p21 RNA transcripts demonstrated an increase in the steady state levels of p21 in DKK-1 shRNA 796 cells without a corresponding increase in p21 RNA synthesis compared with control shRNA-transduced cells (Supplementary Fig. S2C). Viewed together, these data suggest that DKK-1 knockdown increased the stability of p21 RNA transcripts independent of Wnt signaling. The precise molecular mechanism through which decreased DKK-1 expression controls p21 levels via RNA stability are still under investigation. However, our data are consistent with a growth suppressive effect of DKK-1 knockdown through mRNA stabilization of p21.

### Knockdown of p21 restores *in vivo* tumorigenicity of DKK-1 knockdown cells

To directly test whether p21 participated in suppression of tumor growth following decreased DKK-1 activity, p21 expression was decreased in DKK-1 shRNA 796 cells through



**Figure 4.** Characterization of DKK-1/p21 double knockdown cells. PC-3 DKK-1 shRNA 796 cells were transfected with a control shRNA or 2 shRNAs specific to p21. A, RT-quantitative PCR analysis of transfected cells for DKK-1 or p21, mean  $\pm$  SD of a representative experiment; \*,  $P < 0.003$  versus control shRNA cells by  $t$  test. B, cell cycle analysis. Equal number of cells ( $5 \times 10^5$  cells) were plated at day 0 and the percent DNA content was measured using flow cytometry at day 2 following staining with Propidium Iodide; \*,  $P < 0.0001$  versus DKK-1 shRNA cells by  $t$  test.

transduction with 2 different p21 targeting shRNAs. Quantitative PCR confirmed the successful knockdown of both DKK-1 and p21 mRNA compared with control shRNA-transduced cells (Fig. 4A). As observed previously with DKK-1 single knockout cells, DKK-1 shRNA/control shRNA-transduced cells had a 36.3% increase in G<sub>1</sub> content compared with control shRNA cells. DKK-1 shRNA/p21 shRNA double knockout cells showed a statistically significant  $36.8 \pm 0.5\%$  increase in G<sub>2</sub> content, with modest reductions in G<sub>1</sub> and S phase content ( $11.8 \pm 0.6\%$  and  $10.1 \pm 0.5\%$ , respectively), compared with DKK-1 single knockout cells suggesting that knockdown of p21 restored cell cycle progression in cells with decreased DKK-1 expression (Fig. 4B).

On direct injection into the tibiae of male nude mice (10 mice/group), DKK-1 single knockout or DKK-1 shRNA/control shRNA-transduced cells grew poorly compared with control shRNA-transduced cells consistent with data obtained with DKK-1 shRNA 366 clones (Fig. 5A–C). As in the previous experiments, the incidence of osseous tumors was 100% in all groups. Both the DKK-1 shRNA 796-transduced and DKK-1 shRNA/control shRNA-transduced cells

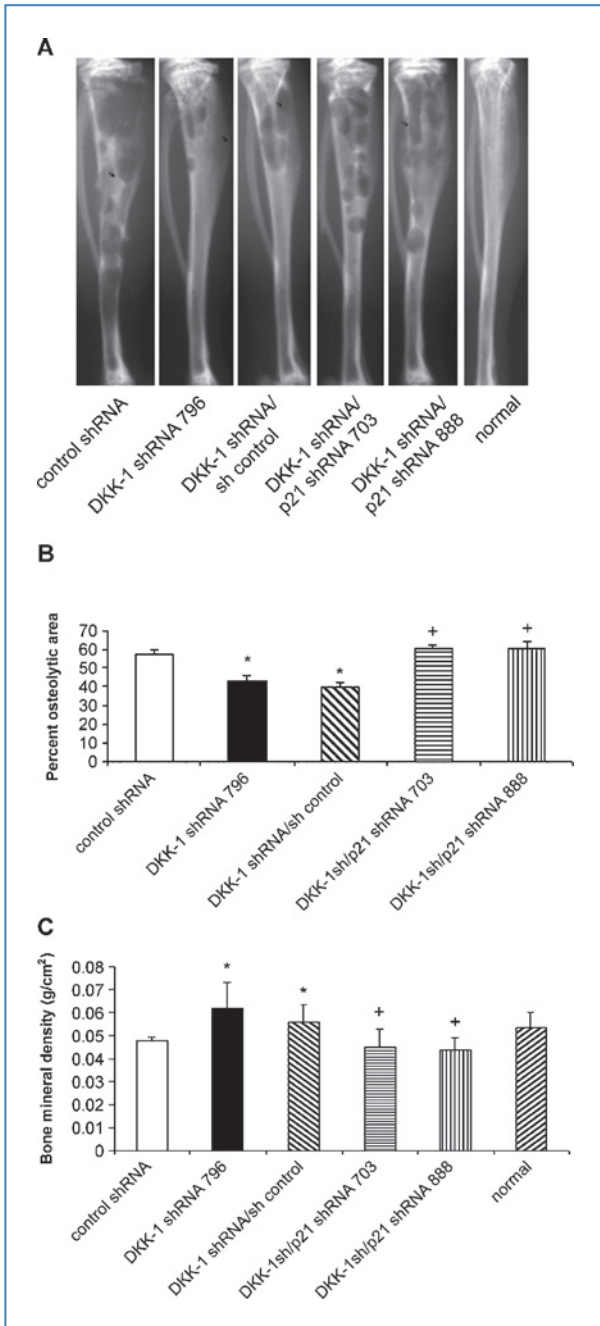
had decreased osteolysis in the bone as evident on radiographs (Fig. 5A and B) and bone mineral density (Fig. 5C). Suppression of p21 in DKK-1 knockdown cells restored the tumorigenicity of DKK-1 knockdown cells by the same measures and were indistinguishable from control shRNA-transduced cells (Fig. 5A–C). Specifically, knockdown of p21 increased osteolytic area 47.1% and decreased bone mineral density 24.4% compared with both DKK-1 shRNA 796-transduced and DKK-1 shRNA/control shRNA-transduced cells and was similar to that of control shRNA-transduced cells alone. Taken together, the data

demonstrate that targeting DKK-1 delays PCa growth *in vivo*, in part, through p21.

As reduction of p21 expression in cells with reduced DKK-1 expression restored intraosseous growth, we wanted to determine whether reduction of p21 alone could account for the restored growth in bone. Accordingly, Du145 control shRNA or p21 shRNA 888 single knockdown cells were evaluated in *in vitro* and *in vivo* growth assays. Quantitative PCR analysis confirmed that p21-directed shRNA specifically reduced p21 RNA transcript levels compared with shRNA control cells (Supplementary Fig. S3A). Despite the decrease in p21 expression, Du145 p21 shRNA 888-transduced cells did not display increased *in vitro* proliferation in cells expressing DKK-1 protein (Supplementary Fig. S3B). Consistent with this finding, p21 shRNA cells did not display increased intraosseous growth compared with control shRNA-transduced cells as indicated by no appreciable change in tumor volume, osteolytic area, or bone mineral density (Supplementary Fig. S4). Viewed together with the PC-3 cell *in vivo* data, these results suggest that knockdown of p21 alone is not sufficient to increase PCa cell growth when DKK-1 is present; however, the impact of p21 on tumor growth is realized only when DKK-1 expression is concomitantly downregulated.

### Discussion

The skeleton is the first site of reoccurrence in men who fail androgen deprivation therapy (22). Therefore, if improvements are to be realized in the treatment of men with PCa, a better understanding of the biology of bone metastases is needed. The Wnt/DKK-1 axis represents an important pathway that may contribute to tumor development within the bone and visceral sites. In this study, we demonstrate that knockdown of DKK-1 delays the growth of PCa tumors *in vivo*. We further provide evidence that DKK-1 blockade increases the expression of the cyclin-dependent kinase inhibitor p21<sup>CIP1/WAF1</sup> through a mechanism independent of canonical Wnt signaling resulting in increased cell cycle arrest. Finally, knockdown of p21 restores the tumorigenicity of DKK-1 knockdown cells supporting the hypothesis that DKK-1 expression in PCa cells facilitates tumor establishment by preventing suppression of cell cycle through induction of p21.



**Figure 5.** Knockdown of p21 restores *in vivo* tumorigenicity of DKK-1 knockdown cells. Control shRNA-transduced, DKK-1 shRNA 796-transduced, DKK-1 shRNA 796/control shRNA-transduced, or DKK-1 shRNA 796-transduced/p21shRNA-transduced cells ( $5 \times 10^5$  cells per 50  $\mu$ L) were directly injected into the tibiae of anesthetized male nude mice (10 mice per group). Tumors were allowed to grow for 3 weeks at which time the mice were sacrificed and bones radiographed and processed for histologic evaluation. A, representative Faxitron X-rays of injected tibiae. B, percent osteolytic area of total tibial area. Radiographs from tumor-injected tibiae were digitized and the percent osteolytic area of the total tibial area was quantified. Data are presented as mean  $\pm$  SE within each group; \*,  $P < 0.003$  versus PC-3 control shRNA cells; +,  $P < 0.002$  versus PC-3 DKK-1 shRNA 796 cells by *t* test. C, bones were subjected to DEXA to quantify bone mineral density. Data are presented as mean  $\pm$  SE within each group; \*,  $P < 0.01$  compared with control shRNA-transduced tibiae; +,  $P < 0.001$  versus PC-3 DKK-1 shRNA 796-transduced tibiae by *t* test.



The hypothesis that the expression of a Wnt inhibitor, DKK-1, promotes tumor growth *in vivo* is consistent with a number of pieces of clinical data. The correlation between elevated serum expression of DKK-1 in patients with osteolytic lesions of multiple myeloma (MM) is well documented (23). Subsequently, increased DKK-1 expression in patient sera or tumor tissue has been correlated with lung cancer, hepatocellular carcinoma, esophageal squamous cell carcinoma, and osteosarcoma where high DKK-1 levels were shown to be an independent predictor of diminished overall survival and/or disease-free survival within these patients (24–27). Concordantly, neutralizing antibodies to DKK-1 efficiently reduced MM-induced osteolysis and decreased MM tumor growth in bone within a rodent model of myelomatous bone disease (28–30). The mechanism for the *in vivo* inhibition of MM growth may result from inhibition of bone marrow stromal cell production of interleukin-6 or the Wnt-mediated expression of osteoprotegerin (28, 31, 32). Our data using a DKK-1 neutralizing antibody in PCa also demonstrated decreased tumor burden in bone, but are extended to soft tissue metastases suggesting a general antitumor effect that we ascribe to deregulation of cell cycle.

General antitumor activity in PCa cells following DKK-1 knockdown may seem inconsistent with the well-described pro-oncogenic effect of Wnt/ $\beta$ -catenin signaling in colon cancer; however, in the case of PCa, it appears that Wnt signaling may have an antitumor effect in transformed cells. Consistent with this possibility is that expression of the Wnt-signaling mediator,  $\beta$ -catenin, is frequently lost during PCa progression. Specifically, analysis of over 500 clinical cases in 5 separate studies found that  $\beta$ -catenin expression decreased with increasing tumor grade and was lost in advanced PCa lesions and bone metastases (33–37). Decreased  $\beta$ -catenin was a poor prognostic factor for survival in each of these studies implying that canonical Wnt signaling impedes PCa tumor progression. Consistent with these findings, activating mutations in the canonical Wnt pathway are rare in PCa occurring in only 5% to 30% of PCa primary lesions (38). In our own study,  $\beta$ -catenin was found to redistribute from the nucleus to the cytoplasm in PCa metastases, concomitant with a decrease in DKK-1 expression, further supporting  $\beta$ -catenin loss in advanced PCa (15). In this light, it is interesting to note that DKK-1 expression in the serum of patients with gastric cancer, colorectal cancer, ovarian cancer, and cervical adenocarcinoma show decreased DKK-1 levels compared with unaffected individuals (26). These data suggest that canonical Wnt signaling is more important in these cancers and provide evidence that Wnt signaling is not universally oncogenic in all lesions at all stages. Although  $\beta$ -catenin is involved in colon cancer tumorigenesis, Wnt signaling appears to be tumor suppressive in advanced PCa cells. During review of this article, Sato et al. (21) published that DKK-1 antibody reduced the *in vivo* growth of A549 lung cancer cells implanted in the subcutis. These studies suggested that DKK-1 suppression increased tumor-cell apoptosis through an undefined, Wnt-independent mechanism. Our results are consistent with these findings and suggest that stabilization of p21 is a possible mechanism for the observed antitumor effect of DKK-1 knockdown in multiple cancer types.

Our data support a model in which DKK-1 knockdown delays PCa tumor establishment through increased cell cycle arrest. Although stable DKK-1 shRNA-transduced cells maintain a modest yet reproducible increase in both p21 and G<sub>1</sub> content compared with control shRNA-transduced cells, we have been unable to demonstrate changes in attachment-dependent growth *in vitro* in DKK-1 knockdown cells. It is unclear why these cells, which show markedly reduced growth *in vivo*, would have an increase in p21 protein without a decrease in *in vitro* proliferation. One possibility is that although DKK1 increases p21, it also may impact other factors that promote cell proliferation or decrease apoptosis that results in no overall change in cell growth as measured by *in vitro* assays. The divergent results may also indicate a role of DKK-1 in the host microenvironment that suppresses tumor cell growth independent of direct effects on the tumor cell. The fact that changes in *in vitro* growth were observed in transient DKK-1 knockdown cells raises the possibility that over time we may select against p21-elevated cells in continuous culture. Which of these possibilities or combination is relevant in PCa is unclear; however, our results are consistent with the model that DKK-1 knockdown increases cell cycle arrest and suppresses tumor growth through the induction of p21.

Our finding that p21 delays tumor growth in DKK-1 knockdown PCa cells is consistent with p21's ability to induce cell cycle arrest and its ability to mediate tumor suppression induced by chemotherapeutic agents (39). Further, the overexpression of p21 in human melanoma cells suppressed (40), whereas the knockdown of p21 in human PCa cells enhanced the growth of transfected cells *in vivo* (41). Transient transfection of p21 in PCa cell line was also found to suppress *in vitro* proliferation independent of androgen sensitivity (42). However, p21 has also been shown to have antiapoptotic activity and likely plays a key role in cell survival following DNA damage by promoting the growth arrest that permits DNA repair (43). Moreover, in PCa, p21 expression in primary lesions was associated with progression to androgen independence (20) and shorter overall survival in patients treated by androgen ablation therapy (44). Expression of p21 was also found to correlate with higher Gleason score and prostate-specific antigen failure following radiotherapy (45). In our own survey of DKK-1 expression in metastatic lesions from men who died of PCa, low DKK-1 expression was associated with longer overall survival (15). Whether p21 expression is higher in DKK-1 low-expressing lesions and associated with better clinical outcome compared with DKK-1 high-expressing tumors is currently unknown. Our observation that DKK-1 knockdown led to p21 upregulation is consistent with the observation that overexpression of DKK-1 in DKK-1 transgenic mice downregulated p21 expression in hematopoietic stem cells (46). Viewed together, induction of p21 in PCa cells remains a likely mechanism for tumor suppression in DKK-1 knockdown cells.

In conclusion, we demonstrated that suppression of DKK-1 in PCa cells leads to a decrease in tumor growth through induction of p21 *in vivo*. These data suggest that DKK-1 expression in PCa is an early event necessary to support osteolysis and early tumor establishment. However, as the tumor progresses, DKK-1 expression is lost allowing Wnt

mediated bone formation. These data provide a possible mechanism for the clinical appearance of PCa bone metastases.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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