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Expression Profile of WNT Molecules in Prostate Cancer and its Regulation by Aminobisphosphonates

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ABSTRACT

Skeletal metastases represent a frequent complication in patients with advanced prostate cancer (PCa) and often require bisphosphonate treatment to limit skeletal-related events. Metastasized PCa cells disturb bone remodeling. Since the WNT signaling pathway regulates bone remodeling and has been implicated in tumor progression and osteomimicry, we analyzed the WNT profile of primary PCa tissues and PCa cell lines and assessed its regulation by bisphosphonates. Prostate tissue (n = 18) was obtained from patients with benign prostate hyperplasia (BPH) and PCa patients with different disease stages. Serum samples were collected from 62 patients. Skeletal metastases were present in 17 patients of whom 6 had been treated with zoledronic acid. The WNT profile and its regulation by bisphoshonates were analyzed in tissue RNA extracts and serum samples as well as in osteotropic (PC3) and non-osteotropic (DU145, LNCaP) PCa cell lines. Several members of the WNT pathway, including *WNT5A*, *FZD5*, and *DKK1* were highly up-regulated in PCa tissue from patients with advanced PCa. Interestingly, osteotropic cells showed a distinct WNT profile compared to non-osteotropic cells. While WNT5A, FZD5, and DKK1 were highly expressed in PC3 cells, *WNT1* and *SFRP1* mRNA levels were higher in DU145 cells. Moreover, zoledronic acid down-regulated mRNA levels of *WNT5A* (−34%), *FZD5* (−60%), and *DKK1* (−46%) in PC3 cells. Interestingly, patients with skeletal metastases who received zoledronic acid had twofold higher DKK1 serum levels compared to bisphosphonate-naive patients. The WNT signaling pathway is up-regulated in advanced PCa, differentially expressed in osteotropic versus non-osteotropic cells, and is regulated by zoledronic acid. J. Cell. Biochem. 112: 1593–1600, 2011.

KEY WORDS: PROSTATE CANCER; WNT PATHWAY; DKK1; BISPHOSPHONATE

Prostate cancer (PCa) is the most common malignancy in men [Jemal et al., 2009]. It has a pronounced propensity to metastasize to bone, and 90% of patients with advanced metastatic PCa suffer from bone lesions [Bubendorf et al., 2000]. Unlike most other cancers, such as breast cancer and myeloma, which cause osteolytic bone lesions, PCa commonly forms osteoblastic lesions, which are characterized by excessive bone formation. Upon osteoclastic resorption, the highly active osteoblasts fill the sites of erosion with structurally weak sclerotic bone and although dense in radiographic appearance, these areas are at an increased risk of fracture [Clarke et al., 1993]. While the underlying mechanisms of the metastatic process and the development of osteosclerotic lesions

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are not yet fully understood, there is increasing evidence that Wingless-type (WNT) proteins play an important role in tumor progression and osteomimicry, a process in which PCa cells acquire an osteoblast-like phenotype [Hall et al., 2006]. WNT proteins either signal through "canonical" (e.g., WNT1, WNT10B) or "non-canonical" (e.g., WNT4, WNT5A) pathways and are regulated by a number of antagonist, including secreted frizzled-related proteins (sFRPs), sclerostin (SOST), and Dickkopf-1 (DKK1). Bone metabolism is strongly modulated by the WNT pathway and its antagonist DKK1, in particular, has been shown to disturb bone remodeling in rheumatoid arthritis [Diarra et al., 2007], multiple myeloma [Tian et al., 2003], breast cancer [Bu et al., 2008], and PCa [Hall et al., 2005]. Other proteins such as WNT5A have also been identified as regulators of cancer biology and may be involved in the process of osteomimicry [Kurayoshi et al., 2006]. However, the role of these WNTs may vary depending on the type of cancer and is not fully understood [McDonald and Silver, 2009].

Bisphosphonates are frequently used as potent anti-resorptive drugs to treat benign and malignant bone disease. Due to their high affinity to hydroxyapatite [Nancollas et al., 2006], plasma concentrations of bisphosphonates decline rapidly after infusion and accumulate in the skeleton, where concentrations as high as 1 mM may be reached [Sato et al., 1991]. In patients with PCa, bisphosphonates have been used to treat androgen deprivation-induced bone loss [Smith et al., 2003; Michaelson et al., 2007] and to reduce the incidence of skeletal-related events of men with castration-resistant PCa and/or subsequent bone metastases [Saad, 2002]. To date, several groups have demonstrated direct and indirect anti-tumor effects of bisphosphonates in preclinical studies including inhibition of proliferation [Verdijk et al., 2007], induction of apoptosis [Fromigue et al., 2000], inhibition of angiogenesis [Tang et al., 2010], and impaired adhesion and invasiveness [Boissier et al., 2000]. However, the effects of bisphosphonates on WNT expression have not been investigated in PCa. Here, we analyzed the expression pattern of WNT proteins in PCa and assessed its regulation by bisphosphonates in vitro and in vivo.

MATERIALS AND METHODS

PATIENTS

The studies involving human material were approved by the Institutional Review Board. Tissue and serum samples from 62 patients were examined. For patient characteristics see Table I.

CELL CULTURE

The PCa cells (PC3, DU145, LNCaP) were cultured in RPMI 1640 media (PAA) containing 10% FCS (Supreme, Lonza) and 1% streptomycin/amphotericin B (PAA). Cells were kept at 37°C in a humidified atmosphere (5% CO_2). Prior to bisphosphonate treatment, cells were starved in medium without FCS. Thereafter, cells were treated with various concentrations (10^{-7}–10^{-3} M) and durations (24–72 h) of zoledronic acid (kindly provided by Novartis) or ibandronate.

TABLE I. Characteristics of Patients

	BPH	pT2	pT3/4	Advanced
n	15	15	15	17
Age (years)	71.7 ± 1.5	65.5 ± 1.7	63.0 ± 1.6	66.6 ± 2.0
Total PSA (ng/ml)	1.9 ± 0.5*	8.3 ± 1.4*	17.9 ± 3.9*	449 ± 149

Mean ± SEM; *P*-value versus advanced by one-way ANOVA with Bonferroni's Multiple Comparison Test. BPH, benign prostatic hyperplasia; pT2, organ confined tumors; pT3/4, non-organ confined tumors; advanced, advanced disease treated by palliative transurethral resection of the prostate, including patients who received zoledronic acid.
*$P < 0.05$ vs. Advanced.

RNA ISOLATION, RT, AND REAL-TIME PCR

The tissue samples were obtained from patients with localized prostate cancer undergoing radical prostatectomy or from patients with advanced tumors who were treated by palliative transurethral resection due to urethral obstructions. Specimens were taken from macroscopically tumorous areas and immediately snap-frozen in liquid nitrogen. BPH specimens were collected and snap-frozen after removal of the prostate during a prostatic adenomectomy or radical cystectomy. Sections of the cryo-preserved tissue specimens were used for hematoxylin-eosin staining and histopathological examination as well as for RNA isolation. The content of malignant epithelial cells in the tumor specimens was at least 60%, whereas no tumor cells were present in the benign prostate tissue specimens.

RNA from the cryo-sectioned tissue samples was isolated using the Invisorb Spin Tissue RNA Mini Kit from Invitek. RNA from cell culture was isolated using the HighPure RNA extraction kit from Roche according to the manufacturer's protocol. Five-hundred nanograms RNA were reverse transcribed using Superscript II (Invitrogen) and Random hexamer primers, and subsequently used for SYBR green-based real-time PCR reactions using a standard protocol and specific primers (Supplementary Table). PCR conditions were 95°C for 10 min followed by 55 cycles with 95°C for 10 s, 56°C for 10 s and 72°C for 30 s. The results were calculated applying the ΔΔCT method and are presented in x-fold increase relative to β-actin or GAPDH expression and the internal calibrator.

WESTERN BLOT

Cells were washed with PBS and scraped in a lysis buffer (20 mM Tris/HCl pH 7.4, 1% SDS, 1:10 protease inhibitor cocktail (Sigma)). Twenty micrograms of proteins were loaded on a 10% SDS–PAGE and transferred onto a 0.2 μm nitrocellulose membrane. After blocking for with 5% non-fat dry milk in Tris-buffered saline with 1% Tween-20 (TBS-T), membranes were incubated with an anti-human WNT5A antibody (Santa Cruz), anti-human FZD5 (Abcam) or an anti-human GAPDH antibody (Cell Signaling) overnight at 4°C (1:1,000). After washing followed a 1 h incubation with HRP-conjugated anti-mouse IgG (R&D systems) or anti-rabbit IgG antibodies (Cell Signaling). Membranes were washed with TBS-T, and proteins were visualized with enhanced chemiluminescence (Pierce).

DKK1 ELISA

Conditioned medium was harvested from cultured cells before RNA and protein isolation. Samples were stored at −80°C until analysis. DKK1 protein content was determined in serum samples (1:10) and

cell culture supernatants (PC3: 1:200; DU145: 1:50) with an immunoassay from Biomedica according to the manufacturer's protocol.

STATISTICAL ANALYSIS

Data are given as means ± SEM. All experiments were repeated at least three times. Statistical analysis were performed using a one way analysis of variance (ANOVA) with Bonferroni's post hoc test, and single group comparisons using a Student's *t*-test.

RESULTS

WNT EXPRESSION PROFILE IN DIFFERENT STAGES OF HUMAN PROSTATE CANCER

The WNT profile was evaluated in tumor tissues from patients with different stages of PCa and compared with benign control tissue. The WNT ligands *WNT1* (Fig. 1A) and *WNT5A* (Fig. 1C) were stage-dependently expressed and showed approximately 100-fold higher levels in advanced PCa compared to BPH. *WNT4* was increased fourfold in advanced PCa tissue (Fig. 1B). By contrast, *WNT10B* expression was independent of the tumor stage (Fig. 1D). While the WNT receptor *ROR2* showed no difference between tumor and control tissue (Fig. 1E), *FZD5* was expressed 70-fold higher in advanced PCa (Fig. 1F). Next, we assessed the expression of specific WNT inhibitors that have been described to regulate bone mass. *DKK1* showed a 150-fold and *SOST* a 185-fold higher mRNA expression level in advanced tumor stages as compared to benign tissue (Fig. 1G,H). Finally, *SFRP1* was also stage-dependently up-regulated and was about 30-fold higher expressed in advanced PCa as compared to benign prostate control tissue and 6-fold higher compared to early stages (Fig. 1J).

WNT EXPRESSION PROFILE IN OSTEOTROPIC AND NON-OSTEOTROPIC PROSTATE CANCER CELL LINES

We next set out to determine the WNT profile in osteotropic (PC3) and non-osteotropic (DU145, LNCaP) PCa cell lines. The WNT ligands *WNT1*, *WNT4*, *WNT5A*, and *WNT10B* were differentially expressed depending on the osteotropism status. *WNT1* and *WNT4*

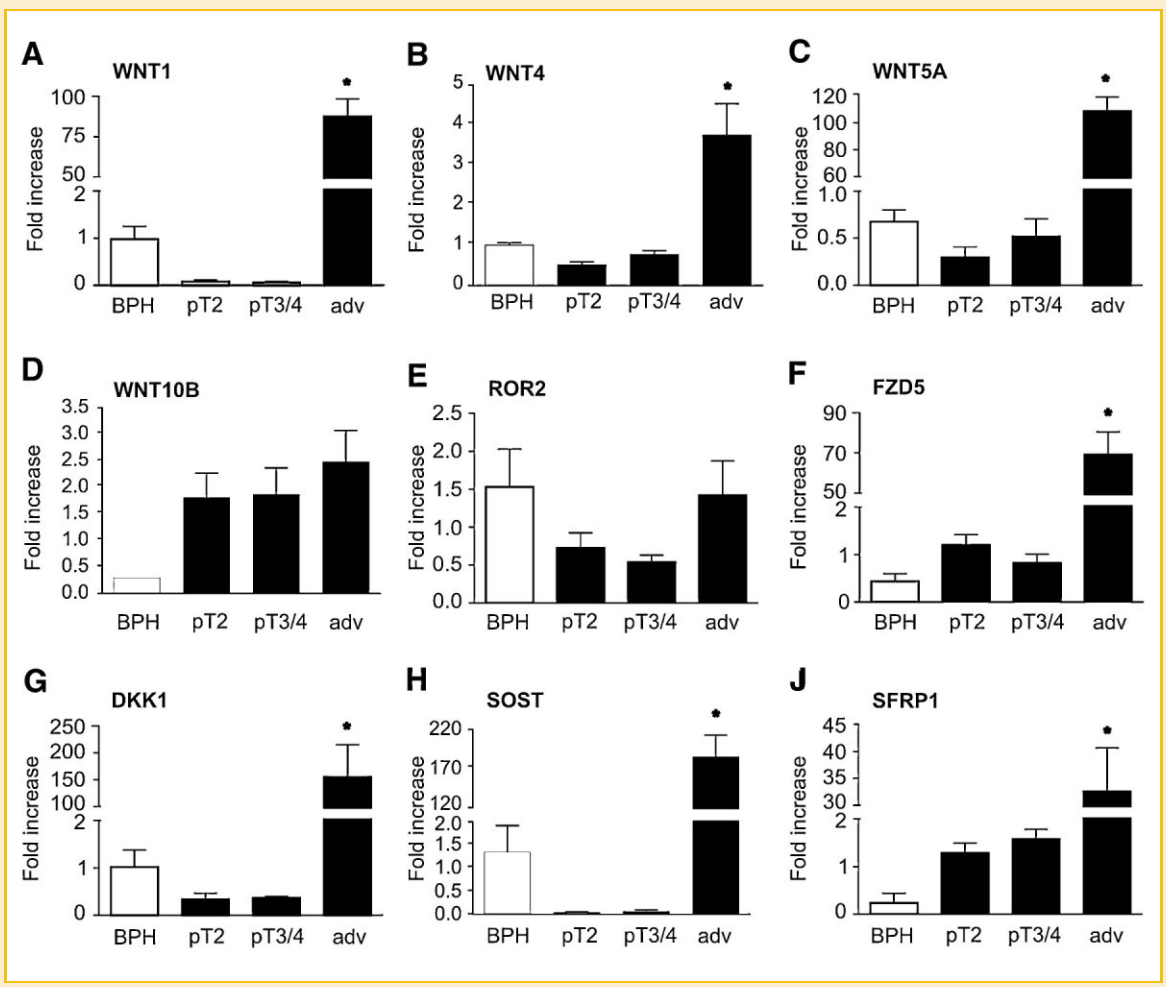

Fig. 1. WNT profile in human prostate tissue. RNA was isolated from prostate tissue of various tumor stages and subjected to quantitative gene expression analysis for (A) WNT1, (B) WNT4, (C) WNT5A, (D) WNT10B, (E) ROR2, (F) FZD5, (G) DKK1, (H) SOST, and (J) SFRP1. Gene expression levels were normalized to β-actin. Results are represented as mean ± SEM. n = 3–5. BPH, benign prostatic hyperplasia; pT2, organ confined tumors; pT3/4, non-organ confined tumors; adv, advanced disease treated by palliative prostate resection. $^{*}P < 0.05$ advanced versus other stages and BPH by ANOVA.

TABLE II. WNT mRNA Levels in Prostate Cancer Cell Lines

Gene	PC3	DU145	LNCaP
WNT1	$1.93 \times 10^{-6} \pm 3.65 \times 10^{-7}$	$6.45 \times 10^{-7} \pm 1.30 \times 10^{-7}$*	$7.83 \times 10^{-6} \pm 1.92 \times 10^{-6}$*
WNT4	$1.17 \times 10^{-5} \pm 3.65 \times 10^{-6}$	$3.26 \times 10^{-5} \pm 1.10 \times 10^{-5}$*	$5.32 \times 10^{-5} \pm 1.65 \times 10^{-5}$*
WNT10B	$1.92 \times 10^{-4} \pm 2.08 \times 10^{-5}$	$8.17 \times 10^{-6} \pm 1.75 \times 10^{-6}$*	$3.54 \times 10^{-4} \pm 7.55 \times 10^{-5}$
ROR2	$1.19 \times 10^{-4} \pm 6.04 \times 10^{-5}$	$6.00 \times 10^{-5} \pm 2.55 \times 10^{-5}$	$4.92 \times 10^{-5} \pm 2.00 \times 10^{-5}$
SFRP1	$2.94 \times 10^{-6} \pm 1.90 \times 10^{-6}$	$9.77 \times 10^{-5} \pm 1.14 \times 10^{-5}$*	$2.16 \times 10^{-6} \pm 1.07 \times 10^{-6}$
SOST	$4.70 \times 10^{-5} \pm 2.58 \times 10^{-5}$	$4.53 \times 10^{-6} \pm 2.41 \times 10^{-6}$	$1.04 \times 10^{-7} \pm 4.34 \times 10^{-8}$

Mean ± SEM; relative expression relative to β-actin.
*$P < 0.05$ PC3 versus DU145/LNCaP. n = 3.

mRNA levels were significantly higher in LNCaP and DU145 compared to PC3 cells, whereas *WNT10B* showed no differential expression (Table II). By contrast, *WNT5A* mRNA levels were 14-fold higher in the osteotropic than in the non-osteotropic cells (Fig. 2A). This expression pattern was also confirmed at the protein level (Fig. 2C). Because WNT5A expression was significantly higher in PC3 cells, two common WNT5A receptors (FZD5 and ROR2) were investigated. Whereas *ROR2* expression levels did not differ between the cell lines (Table II), the mRNA and protein expression pattern of FZD5 was similar to that of WNT5A (Fig. 2B,C), showing the highest expression in PC3 cells. Finally, the WNT inhibitors *DKK1*, *SFRP1*, and *SOST* were analyzed and found to be differentially expressed. While, *DKK1* levels were significantly higher in PC3 cells compared to DU145 cells (Fig. 2D), *SFRP1* levels were 33-fold higher in DU145 cells (Table II). In line with these data, DKK1 protein production was 55-fold higher in PC3 compared to DU145 cells (Fig. 2E), whereas sFRP1 protein levels were higher in DU145 cells (data not shown). The expression of *SOST* was low in all three cell types (Table II).

BISPHOSPHONATES REGULATE WNT EXPRESSION

As bisphosphonates are commonly used in patients with PCa, we analyzed whether the expression of WNT proteins is altered by

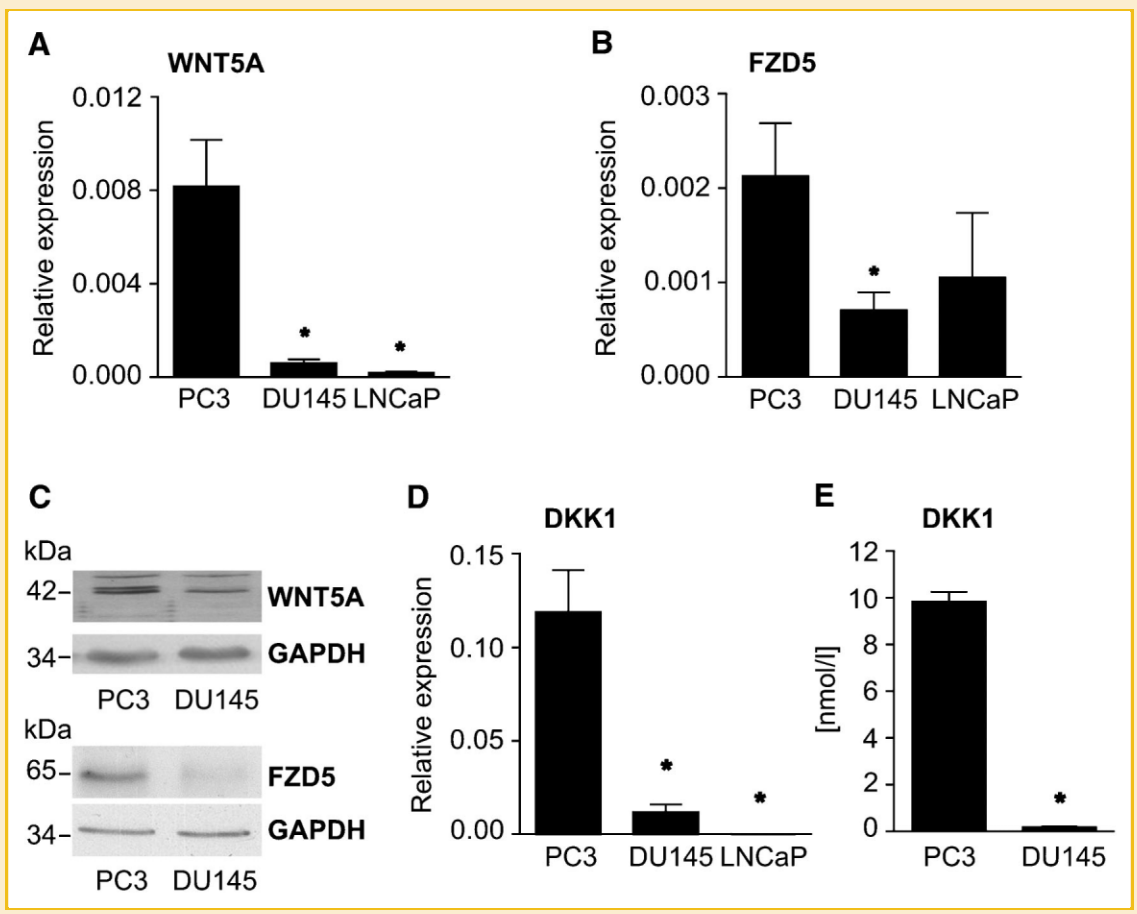

Fig. 2. WNT proteins are differentially expressed in prostate cancer cell lines. RNA from PC3, DU145, and LNCaP cells was extracted and subjected to real-time PCR analysis for (A) WNT5A and (B) FZD5. Gene expression was normalized to β-actin. C: One representative Western blot of WNT5A and FZD5 is shown. D: DKK1 mRNA levels were determined using real-time PCR and are normalized to β-actin. E: Protein levels of DKK1 were assessed using an ELISA. Results are represented as mean ± SEM. n = 3–6. *$P < 0.05$.

bisphosphonate treatment and whether this is associated with the osteotropism status of the PCa cell lines. Zoledronic acid suppressed mRNA levels of *WNT5A*, *FZD5*, and *DKK1*, which were highly expressed in the osteotropic PC3 cells, by 34%, 60%, and 46%, respectively ($P < 0.05$) (Fig. 3A–C). This effect was not observed with ibandronate. In contrast to *FZD5*, neither *WNT5A* nor *DKK1* were regulated by bisphosphonates in the non-osteotropic DU145 cells (Fig. 3A–C). The inhibition of DKK1 by zoledronic acid was also confirmed at the protein level (Fig. 3D). Of note, zoledronic acid did not regulate those WNT genes that showed a low expression in PC3 cells compared to DU145 (*WNT1* and *SFRP1*, data not shown).

To further determine the regulation of DKK1 by zoledronic acid, PC3 cells were treated with zoledronic acid at various concentrations (0.1–1,000 nM) and durations (24–72 h). DKK1 gene and protein expression levels were profoundly suppressed in PC3 cells in a dose- and time-dependent manner (Fig. 4A,B). Because zoledronic acid induces apoptosis in PCa cells and robustly suppresses DKK1 levels, we sought to determine whether DKK1 is involved in the apoptosis-inducing effects. However, the addition of recombinant DKK1 prior to the treatment with zoledronic acid did not rescue PC3 cells from apoptosis (data not shown).

SERUM LEVELS OF DKK1 ARE INCREASED IN PATIENTS RECEIVING ZOLEDRONIC ACID

Finally, we assessed whether DKK1 is also regulated by zoledronic acid in vivo. Therefore, DKK1 serum levels were determined in patients with different stages of PCa and in patients with skeletal metastases with or without zoledronic acid treatment. DKK1 levels were not altered in patients with organ-confined and non-organ-confined PCa compared to patients with BPH (Fig. 5). Strikingly, patients with advanced PCa who had received zoledronic acid displayed a twofold increase in DKK1 levels as compared to bisphosphonate-naive patients ($P < 0.05$; Fig. 5).

DISCUSSION

Prostate cancer has a strong propensity to metastasize to bone in advanced stages where it often forms osteoblastic lesions. However,

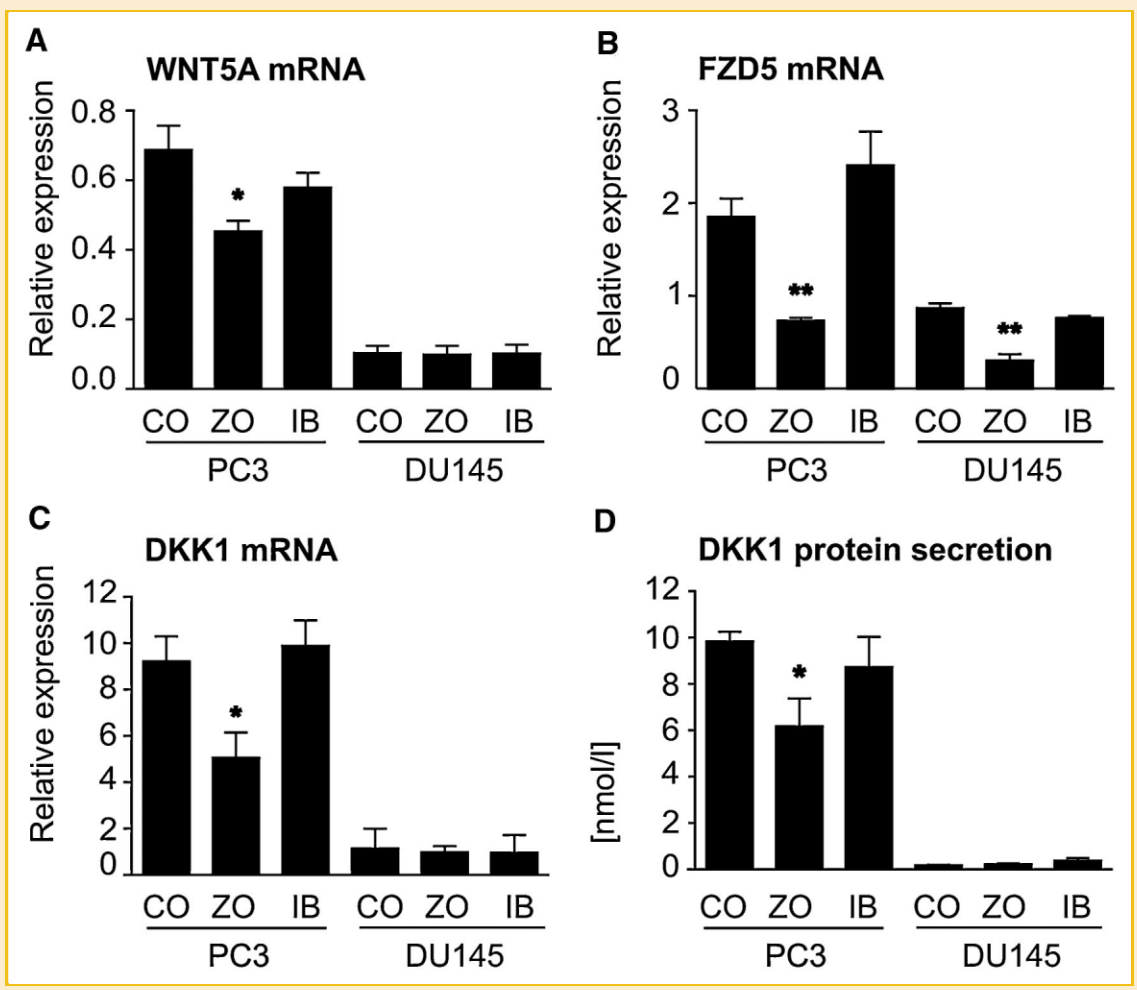

Fig. 3. Effects of bisphosphonates on WNT expression. Gene expression levels of (A) WNT5A, (B) FZD5, and (C) DKK1 were assessed in PC3 and DU145 cells that were treated with 100 μM zoledronic acid (ZO) or ibandronate (IB) for 48 h. D: DKK1 protein secretion was assessed using an ELISA. Results are represented as mean ± SEM. n = 3–5. $^{*}P < 0.05$, $^{**}P < 0.01$ versus control (CO).

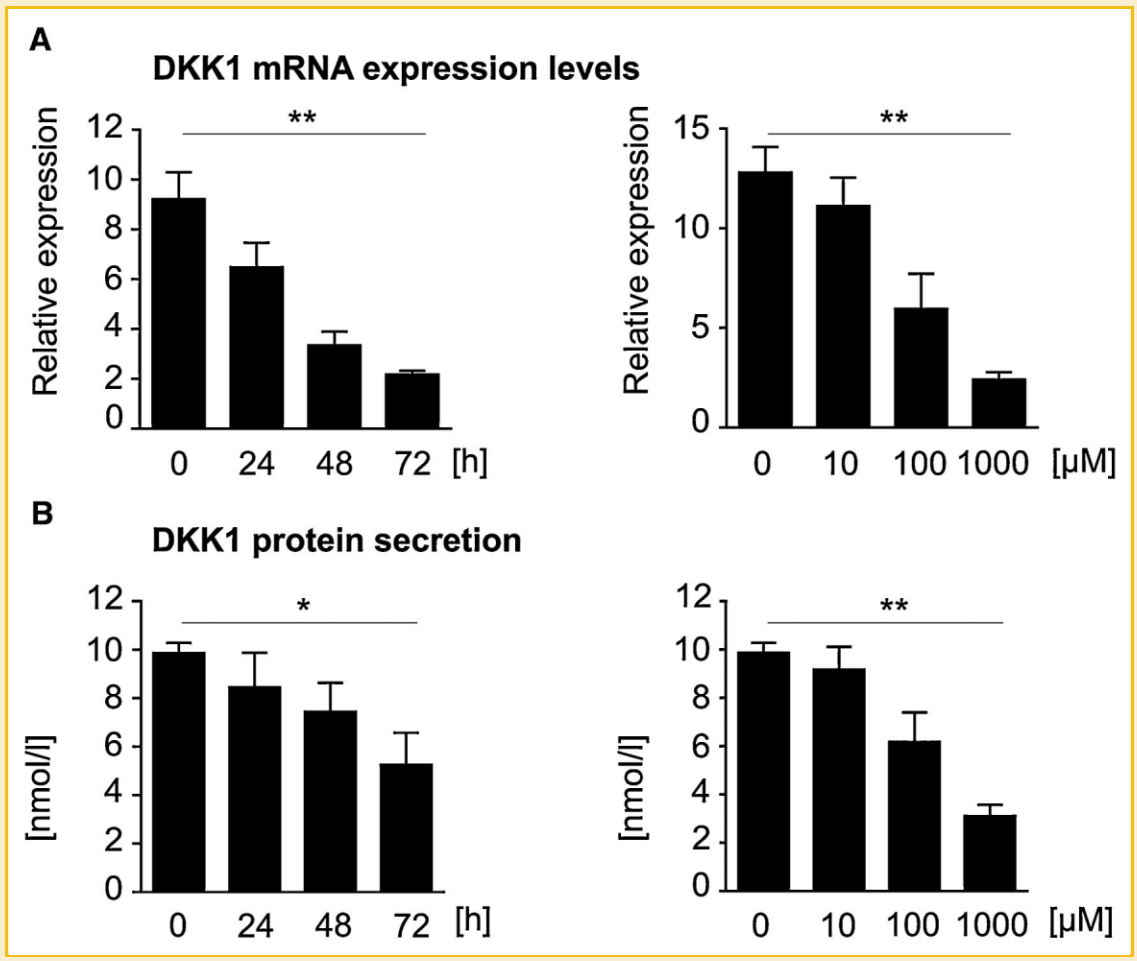

Fig. 4. Zoledronic acid decreases DKK1 levels. PC3 cells were treated with 100 μM for 24, 48, and 72 h or with 0.1–1,000 μM ZOL for 48 h. A: DKK1 mRNA levels were determined using real-time PCR and are normalized to GAPDH. B: DKK1 protein secretion was assessed using an ELISA. Results are represented as mean ± SEM. n = 4. $^{*}P < 0.05$, $^{**}P < 0.05$ by one-way ANOVA.

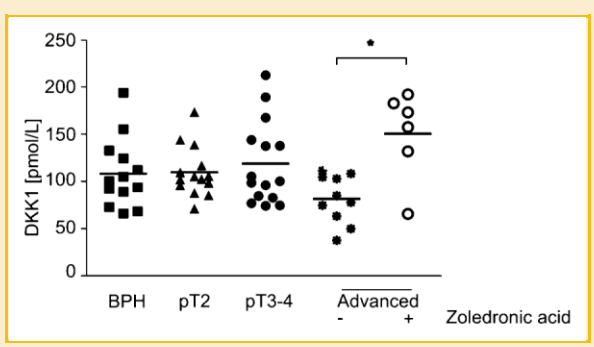

Fig. 5. DKK1 serum levels in prostate cancer and its regulation by bisphosphonates. DKK1 levels were determined in the serum of patients with benign prostatic hyperplasia (BPH) and different PCa stages without (pT2–4) or with skeletal metastasis (adv). The latter patient group underwent a transurethral resection of the prostate and in some cases were treated with zoledronic acid. Dots indicate single patients. Line displays the mean value. $^{*}P < 0.05$.

the mechanisms whereby PCa modulates the bone microenvironment and causes excessive bone formation are largely unclear. In the present study, we investigated the WNT expression profile in PCa, as this pathway is crucial for osteoblast differentiation and function and has also been implicated in the process of osteomimicry of PCa cells [Hall et al., 2005; Rentsch et al., 2009]. Moreover, a strong activation of this pathway has been observed in several tumor types, including melanoma, multiple myeloma, as well as breast and prostate cancer [Reya and Clevers, 2005]. Our results show high gene expression of WNT ligands (*WNT1*, *WNT4*, *WNT5A*) as well as receptors (*FZD5*) and inhibitors (*DKK1*, *sFRP1*, *SOST*) in terminal stages of PCa compared to BPH or earlier disease stages. In line with these findings, Chen et al. [2004] reported an increased production of WNT1 in PCa, which may contribute to the enhanced proliferation of cancer cells. While there is limited information on the regulation of WNT4 and WNT10B expression in cancer, the non-canonical ligand WNT5A is well investigated. WNT5A expression is increased in melanoma, lung, breast, and prostate cancer, in which hypomethylation and activating mutations of *WNT5A* may occur [Iozzo et al., 1995; Hall et al., 2005; Yamamoto et al., 2010]. Similar

to WNT5A, its receptor FZD5 was highly expressed in PCa, while ROR2 was not increased. Although Yamamoto et al. [2010] reported that both receptors mediate WNT5A effects in PCa cells in vitro, our data suggest that FZD5 is the main receptor for mediating WNT5A signals in primary PCa tissue. Finally, mRNA levels of three WNT signaling inhibitors that regulate bone remodeling were assessed. Of those, DKK1 was most strikingly increased in advanced PCa tissue. Similar observations were made by others who found a fivefold increase in DKK1 protein levels in primary PCa lesions as assessed by immunohistochemistry of tissue microarrays [Hall et al., 2008]. Additionally, Roato et al. [2008] reported a 50-fold increase in *DKK1* mRNA levels in PCa tissue compared to healthy control tissue. Taken together, these data suggest a general activation of the WNT pathway in primary PCa tissue. Whether the induction of WNT ligand mRNAs prevails over that of WNT inhibitors cannot be concluded from this study and will require further investigation.

Thus far, only one study has investigated serum levels of DKK1 in patients with PCa, which have found DKK1 to be increased 3.5-fold in PCa patients with skeletal metastasis [Roato et al., 2008]. In our study, patients with skeletal metastasis did not display enhanced DKK1 levels compared to BPH patients. However, we show that treatment with zoledronic acid in patients with skeletal metastasis enhanced serum levels of DKK1. Considering that most PCa patients develop osteoblastic metastasis, this may be a beneficial effect. However, as our study is limited by the small number of patients, this observation clearly needs to be verified in a larger cohort.

To determine in more detail whether certain WNTs are associated with osteotropic PCa cells, three PCa cell lines isolated from different metastatic sites were used. PC3 cells originate from a bone metastasis, whereas LNCaP are from a lymph node metastasis, and DU145 cells from a brain metastasis [Logothetis and Lin, 2005]. WNT5A, its receptor FZD5, and DKK1 were highly expressed in PC3 cells compared to DU145 and LNCaP cells, whereas *WNT1* and *SFRP1* levels were very low. The extremely high expression of DKK1 in PC3 cells, but not in non-osteotropic cells, is in line with previous studies and may account for their osteolytic rather than osteoblastic nature [Hall et al., 2005; Li et al., 2008]. With respect to WNT5A, high expression levels are often found in more aggressive cancers [Kurayoshi et al., 2006; Pukrop et al., 2006]. WNT5A is a potent inducer of matrix metalloproteases, which may contribute to tissue remodeling and support detachment of the tumor cells from the primary tissue, allowing migration to distal sites [Enomoto et al., 2009; Yamamoto et al., 2010]. Thus, the high expression of WNT5A and DKK1 in the osteotropic PC3 cells may both contribute to their aggressive metastatic and osteolytic behavior of this cell line. Whether this expression pattern applies to other highly metastatic, osteolytic cancer cells such as breast cancer cells remains to be determined.

Finally, we show that WNTs, which are preferentially expressed in osteotropic PC3 PCa cells (i.e., WNT5A, FZD5, and DKK1), are suppressed by zoledronic acid. The fact that only zoledronic acid, but not ibandronate, regulates WNT expression may depend on the potency of the bisphosphonates with zoledronic acid being the most potent one currently available. Possibly, suppressive effects on WNT signaling could also be achieved with weaker bisphosphonates at higher concentrations. Thus, further in vitro and in vivo studies are needed to clarify this discrepancy. Of note, DKK1 suppression was not involved in the apoptosis-inducing effects of zoledronic acid (data not shown). Nevertheless, these data suggest that only selective WNTs expressed in osteotropic cells are inhibited by potent bisphosphonates, which may result in beneficial effects on inhibiting invasion and the osteolytic potential of PCa cells. Although this hypothesis has to be verified in further studies using a greater variety of cancer cells, the fact that zoledronic acid regulates the WNT pathway in vitro and in vivo may also have important implications for other skeletal disorders that are treated with bisphosphonates.

In summary, we analyzed the WNT expression profile in primary PCa and PCa cell lines and assessed its regulation by bisphosphonates. Our study shows that the WNT pathway is highly upregulated in advanced PCa, differentially expressed in osteotropic versus non-osteotropic cells, and can be regulated by bisphosphonates in vitro and in vivo. These novel data encourage further studies elucidating the relevance of the WNT pathway in skeletal metastases of PCa.

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