Functional analysis of NKX3.1 in LNCaP prostate cancer cells by RNA interference

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Abstract. The function of the androgen-regulated homeobox protein NKX3.1 in prostate cancer is controversial. NKX3.1 is necessary for correct prostate development and undergoes frequent allelic loss in prostate cancer. However, no mutations occur in the coding region and some particularly aggressive cancers over-express the protein. Nevertheless NKX3.1 is often referred to as candidate tumor suppressor gene. Recent findings suggest a function in protection against oxidative damage involved in prostate carcinogenesis. Thus NKX3.1 may act differently at various stages of prostate cancer. Unlike a classical tumor suppressor NKX3.1 is up-regulated by androgens and down-regulated by phytoestrogens. In this study we performed RNAi based functional analysis by knocking down NKX3.1 expression in LNCaP prostate cancer cells and analyzing the impact of NKX3.1 on gene expression and cell proliferation. Knock-down of NKX3.1 evoked a massive down-regulation of NKX3.1 expression, followed by reduction in mRNA expression of the androdrogen receptor (AR) and the insulin-like growth factor receptor (IGF-1R). Western blot analysis showed strong decreases of NKX3.1, AR, and IGF-1R protein expression. Concomitantly, cell proliferation decreased and expression of prostate-specific antigen (PSA) mRNA and its secretion were diminished, whereas expression of IGF binding protein 3 (IGFBP-3) and MMP tissue inhibitor 3 (TIMP-3) was up-regulated. In tumor cells not deprived of NKX3.1 expression this gene still has a function which might differ from its role in prostate development and carcinogenesis. NKX3.1 knock-down altered the expression of genes highly relevant in prostate cancer cell proliferation and apoptosis. In LNCaP NKX3.1 most probably plays the role of an androgen-regulated transcription factor whose down-regulation is paralleled by anti-proliferative and pro-apoptotic effects. Since NKX3.1 can regulate AR expression it may become a target for interference in hormone refractory prostate carcinoma.

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Introduction

In Western societies prostate cancer is the most common malignancy in men. There is a striking 10-fold gradient in the incidence of clinically significant prostate cancer between Western industrialized and East Asian countries, which can not be explained by genetic factors, but is most probably due to Western diet (1). Prostate cancer is characterized by a long latency period of the disease. This time affords opportunities for intervention with therapies that are designed to delay disease initiation or progression (2). For prostate cancer, no consistent cancer pathway is known as for other malignancies such as colorectal cancer. Instead, in the recent past a multitude of genes aberrantly expressed in prostate cancer have been discovered.

One of the genes controversially discussed concerning its role in the initiation of prostate cancer is NKX3.1. This homeobox protein is believed to represent a tumor suppressor. NKX3.1 was primarily described in mice, in which it is expressed only in prostatic lobes and the bulbourethral gland (3). The first isolation of this gene in humans was achieved by He and his colleagues in 1997 (4). In humans expression of NKX3.1 was primarily detected in the testis, in rare mucous glands of the lung and ureteral epithelial cells, but the strongest expression was found in prostate epithelia (5). There is considerable agreement on NKX3.1 being partly responsible for the development and differentiation of prostate tissue, since it is expressed in the developing mouse rostral urogenital sinus as early as 15.5 days post-coitum (6). Accordingly, mice deficient in NKX3.1 show defects in prostate epithelial differentiation (3). NKX3.1 maps to

chromosome 8p21, a region that undergoes loss of heterozygosity (LOH) in 50-85% of prostate cancers (7,8). Due to this frequent allelic loss it is widely believed that NKX3.1 functions as a tumor suppressor. However, there are controversial findings about correlation of loss of this gene and tumor progression. Mogal *et al* even provided the first functional evidence in support of the stochastic, dosage-sensitive gene regulation model (on NKX3.1) of haploinsufficient tumor suppression in prostate cancer (9). In a recent study Bethel *et al* report a reduced expression of NKX3.1 in focal prostatic atrophy and intraepithelial neoplasia which is, however, not related to 8p allelic loss. The authors conclude that non-genetic mechanisms reduce NKX3.1 protein levels in prostate carcinogenesis (10). Asatiani *et al* observed less intense immunohistochemical staining for NKX3.1 in carcinoma compared to normal prostatic epithelium and reduced expression correlated with the loss of one NKX3.1 copy, CpG hypermethylation near the putative promoter region, and Gleason grade (11). Notably, complete loss of expression, as expected for a canonical tumor suppressor was rare. Likewise, Bowen *et al* found that expression of NKX3.1 protein decreased with disease severity, indicating that loss of expression correlates with tumor progression (5). In contrast, arguments for an elevated expression of NKX3.1 in a more aggressive phenotype of prostate cancer were presented by Xu *et al* (12). They found a rather indifferent distribution of NKX3.1 m-RNA expression between normal and tumor tissue of the prostate but NKX3.1 over-expression was detected in a higher percentage of non-organ-confined tumors than in organ-confined cases. Korkmaz *et al* even reported a lack of significant changes in NKX3.1 mRNA levels during prostate cancer development. They also pointed out that NKX3.1 may not be associated with the allelic loss of chromosomal band 8p21 (13). This corroborates the statement by Voeller *et al* who did not find mutations in the coding region of NKX3.1, which might inactivate its presumed tumor suppressor function (14). Lack of mutations in the coding sequence further emphasizes the notion that NKX3.1 may function in a different fashion as a classical tumor suppressor gene. Ouyang *et al* found loss-of-function of NKX3.1 in mutant mice to lead to deregulated expression of several antioxidant and pro-oxidant enzymes. According to their findings one of the principal roles of NKX3.1 in prostate cancer suppression is to maintain the integrity of the prostatic epithelium by regulating the expression of genes that provide protection against oxidative damage (15).

Stimulation of hormone sensitive LNCaP prostate cancer cells with steroids raised further doubts concerning the supposed role of NKX3.1 being a tumor suppressor. Treatment of LNCaP cultures with synthetic androgens R1881 or mibolerone resulted in a dose-dependent increase of NKX3.1 mRNA (4,12,16). In contrast, quercetin, a flavonoid and phytoestrogen with anti-cancer properties including inhibition of expression and function of the androgen receptor (AR) evoked down-regulation of NKX3.1 expression (17). Thus, NKX3.1 expression was up-regulated by androgens and was down-regulated by phytoestrogens. Taken together there are arguments in favor of a tumor suppressor function for NKX3.1 but also much evidence against this conception. Doubtless, NKX3.1 is expressed differently in various stages of prostate cancer with a tendency to over-expression in non-organ-confined tumors. Furthermore, NKX3.1 expression is positively regulated by androgens and down-regulated by phytoestrogens which otherwise have chemopreventive properties. Therefore, NKX3.1 may have different functions in various states of prostate cancer.

In this study we report that at different stages of prostate cancer development the isoflavone tectorigenin causes a down-regulation of NKX3.1 expression in LNCaP prostate cancer cells similar to quercetin (17). In previous studies we found down-regulated expression of AR, insulin-like growth factor receptor (IGF-1R) and prostate-specific antigen (PSA) and up-regulated expression of tissue inhibitor of matrix metalloproteinases-3 (TIMP-3) in prostate cancer cells after stimulation with tectorigenin (18). These results raised the following questions: i) How could the down-regulation of a putative tumor suppressor be reconciled with the otherwise beneficial tectorigenin induced rectification of aberrant gene expression in prostate cancer, namely down-regulation of pro-proliferative AR, IGF-1R and up-regulation of pro-apoptotic and anti-invasive TIMP-3? ii) Where in the sequence of events caused by tectorigenin treatment occurs the down-regulation of NKX3.1 expression and is it causatively involved in down-stream alteration of gene expression? Therefore, we performed functional analysis by siRNA-mediated specific knock-down of NKX3.1 expression to compare its effects to that of the phytoestrogen tectorigenin. For these experiments we used AR-expressing LNCaP prostate cancer cells. This cell line originates from a lymph node metastasis and represents an advanced state of prostate cancer with androgen-responsive NKX3.1 expression (4,12). Our study revealed that the knock-down of NKX3.1 expression evokes anti-proliferative effects in LNCaP cells, concomitantly with altered expression of genes relevant for proliferation and apoptosis. Therefore, in tumor cells not deprived of NKX3.1 due to 8p21 deletion, this gene may have functions different from those of a tumor suppressor.

Materials and methods

Cell culture

Transfection. LNCaP cells were grown at 37˚C in a humidified incubator at 5% CO_2. The day before transfection cells were seeded in 6-well plates at a density of $5x10^5$ per well using RPMI-1640 medium (PAN-Systems GmbH, Nuremberg, Germany) containing 10% fetal calf serum (PAA, Coelbe, Germany), 1% L-glutamine, 2% amino acid solution and 1% penicillin-streptomycin.

Transfection of LNCaP cells was performed using Oligofectamine™ reagent (Invitrogen, Karlsruhe, Germany) and Opti-MEM® (Invitrogen-Gibco, Karlsruhe, Germany) following the supplier's instructions. We used siRNA against NKX3.1 (Stealth™ siRNA duplex oligoribonucleotides, Invitrogen, Paisley, UK) with the following sequences: 5'-GGAGACUUGGAGAAGCACUCCUCUU-3' and 5'-AAG AGGAGUGCUUCUCCAAGUCUCC-3'. In control transfections we used siRNA against luciferase gene (Eurogentec, Seraing, Belgium). For both siNKX3.1 and siLuc we used concentrations of 20 μM. Forty-eight hours after transfection RPMI medium, containing the transfection reagents, was

removed and cells were washed with 800 µl RPMI medium. Another 1.5 ml of RPMI medium was added and cells were returned to the incubator for an additional 17 h. After a total of 61 h incubation cells were harvested.

Stimulation of LNCaP cells. LNCaP cells were stimulated with dihydrotestosterone (DHT, Sigma, Taufkirchen, Germany). The androgen was dissolved in ethanol and used at concentrations of 10 nM. Control stimulations were performed with solvent ethanol alone. Substrates were added at the time of cell plating and removed 24 h later.

The phytochemical tectorigenin (Girindus, Bensberg Germany) dissolved in DMSO was used at a concentration of 100 µM. For controls we used DMSO which was adjusted to 0.1% in all experiments. Time of incubation was 48 h.

Cell viability test. Transfection was carried out plating cells at a density of $5x10^3$ cells per well in a 96-well plate following the supplier's instructions. Viability of LNCaP cells after siRNA mediated down-regulation of NKX3.1 was quantified using the AlamarBlue assay (Serotec GmbH, Düsseldorf, Germany). Control assays were performed using parental LNCaP cells and transfected cells with siRNA against luciferase. After 59 h, 10 µl of AlamarBlue substrate per well were added and cells were incubated for an additional two hours. Finally samples were analyzed in a spectrophotometer.

RNA extraction and real-time RT-PCR analysis. Total cellular RNA from pelleted LNCaP cells was extracted with the RNeasy Mini Kit (Qiagen, Hilden, Germany). The Agilent Bioanalyzer 2100 with an RNA 6000 Nano LabChip-Kit was used to assess RNA integrity and quantity (Agilent Technologies, Waldbronn, Germany). Reverse transcription of 500 ng total cellular RNA was performed by the Omniscript RT Kit (Qiagen, Hilden, Germany). mRNA expression of NKX3.1, ARP, AR, PSA, IGF-1R, IGFBP-3 and TIMP-3 was quantitated by real-time RT-PCR (iCycler, Bio-Rad, Munich, Germany) using an QuantiTect™ SYBR® Green RT-PCR Kit (Eurogentec, Seraing, Belgium). Fluorescence signals were monitored on the iCycler and terminated when all reactions reached an amplification plateau, while template-free controls remained at basal levels. The iCycler real time detection software was used to analyze the data (Bio-Rad). In all experiments ARP served as an internal control, as housekeeping gene. Primers were designed using the primer3 on-line primer design program (www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). These primers were evaluated by the Operon oligo tool kit (http://www.operon.com). Secondary DNA structure during PCR was ruled out using the Mfold web server program for nucleic acid folding and hybridization prediction (http://www.bioinfo.rpi.edu/applications/mfold/old/dna/). Primers for AR, PSA, IGF-1R, TIMP-3 and IGFBP-3 were used as described in previous experiments. Further primers used for quantitative RT-PCR were: ARP forward primer, 5'-CGA CCT GGA AGT CCA ACT AC-3'; ARP reverse primer, 5'-ATC TGC TGC ATC TGC TTG-3'. NKX3.1 forward primer, 5'-CCG AGA CGC TGG CAG AGA CC-3'; NKX3.1 reverse primer: 5'-GCT TAG GGG TTT GGG GAA G-3'. AR forward primer, 5'-AGG AAC TCG ATC CTA TCA TTG C-3'; AR reverse primer: 5'-CTG CCA TCA TTT CCG GAA-3'.

Western blot analysis and PSA secretion. Protein expression was assessed by Western blot analysis using 3 µg/ml mouse monoclonal antibodies, such as anti-NKX3.1 (Zymed Laboratories, USA), anti-AR (Neomarkers, Westinghouse, USA), anti-IGF-1R and anti-α-tubulin (Sigma-Aldrich, Deisenhofen, Germany). After 61 h of incubation parental and transfected LNCaP cells were homogenized with lysis buffer containing 150 mM NaCl, 1 mM EDTA, 50 mM Tris-HCl, pH 7.4, 1% NP-40, 0,25% sodium deoxycholate, 1 mM PMSF, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin A, 1 mM Na_3VO_4, 1 mM NaF. After centrifugation cell lysates were boiled and denaturated in sample buffer containing SDS and dithiothreitol (DTT, Invitrogen). NuPage 4-12% Bis-Tris precast gel and MES buffer (Invitrogen) were used to perform electrophoresis. After electrotransfer the PVDF membrane (GE Healthcare, Munich, Germany) was hybridized with the above mentioned antibodies. For visualization we used horseradish peroxidase-coupled secondary antibodies (Dianova, Hamburg, Germany) and the ECL Plus kit (GE Healthcare).

For measuring PSA secretion RPMI medium containing transfection reagents was collected after a total of 44-h incubation. PSA secretion from LNCaP cells was measured with a PSA ELISA Kit (R&D Systems, USA).

Statistical analysis. For statistical analysis experiments were repeated three times. Means and standard deviation were calculated. The statistical significance of differences was determined by using unpaired t-test with $p<0.05$ considered as statistically significant.

Results

Induction and suppression of NKX3.1 mRNA expression in LNCaP cells. Stimulation of LNCaP cells with DHT for 24 h evoked an up to 1.8-fold increase of NKX3.1 mRNA expression compared to cells stimulated with ethanol (Fig. 1A). However, stimulation with the phytoestrogen tectorigenin caused a down-regulation of NKX3.1 mRNA to 19% (Fig. 1B).

mRNA and protein expression after knock-down of NKX3.1. Treatment with siRNA against NKX3.1 evoked a marked knock-down of mRNA expression to 5% (Fig. 2A), whereas the housekeeping gene ARP remained unaffected (Fig. 3A). To demonstrate a consecutive down-regulation of NKX3.1 at the protein level, Western blot analyses were performed. Using anti-NKX3.1 and anti-α-tubulin mouse monoclonal antibodies we found a down-regulation of NKX3.1-protein, whereas the housekeeping protein α-tubulin remained at a similar expression level (Figs. 2B and 3B).

Concomitantly with NKX3.1 knock-down, expression of the androgen receptor decreased to 34% (Fig. 4A). Western blot analysis also confirmed reduced expression of AR at the protein level (Fig. 4B).

In addition mRNA expression of IGF-1R was diminished to 18% (Fig. 5A). Western blot analyses also revealed a slightly diminished expression of IGF-1R protein (Fig. 5B).

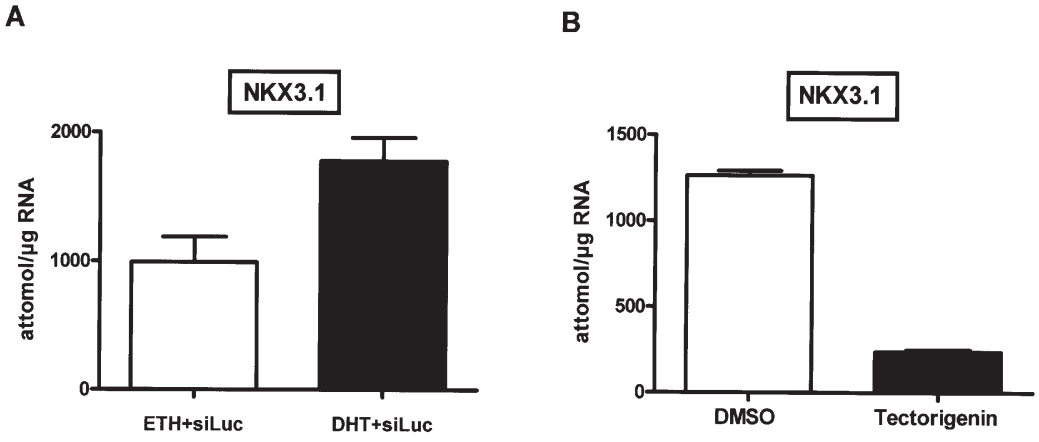

Figure 1. A, NKX3.1 mRNA expression after stimulating LNCaP cells with dihydrotestosterone (DHT), compared to control stimulation performed with ethanol. mRNA expression is shown as means of three independent experiments with error bars for standard deviation. B, NKX3.1 mRNA expression after stimulating LNCaP cells with tectorigenin, compared to control stimulation performed with DMSO. mRNA expression is shown as means of three independent experiments with error bars for standard deviation.

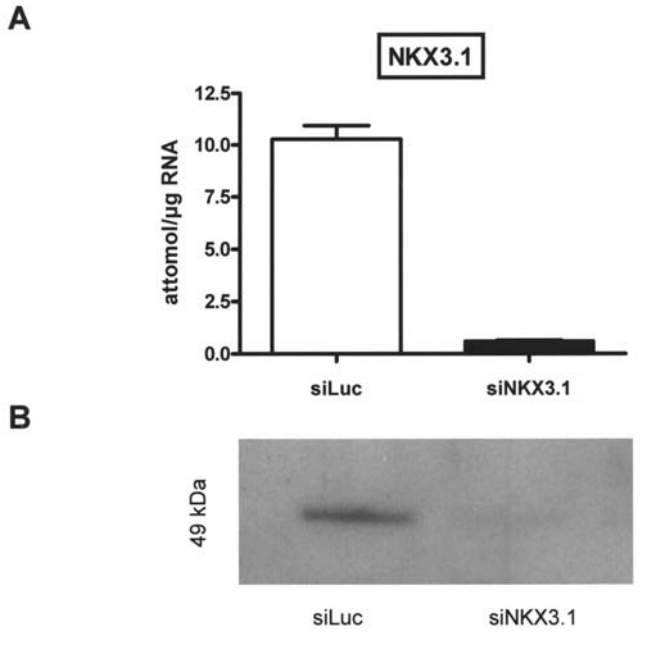

Figure 2. A, Suppression of NKX3.1 mRNA in LNCaP cells 61 h after transfection with siNKX3.1 compared to control transfections with Luc siRNA. mRNA expression is shown as means of three independent experiments with error bars for standard deviation. B, Western blot analysis of NKX3.1 protein expression after siRNA mediated knock-down of NKX3.1 compared to control transfections.

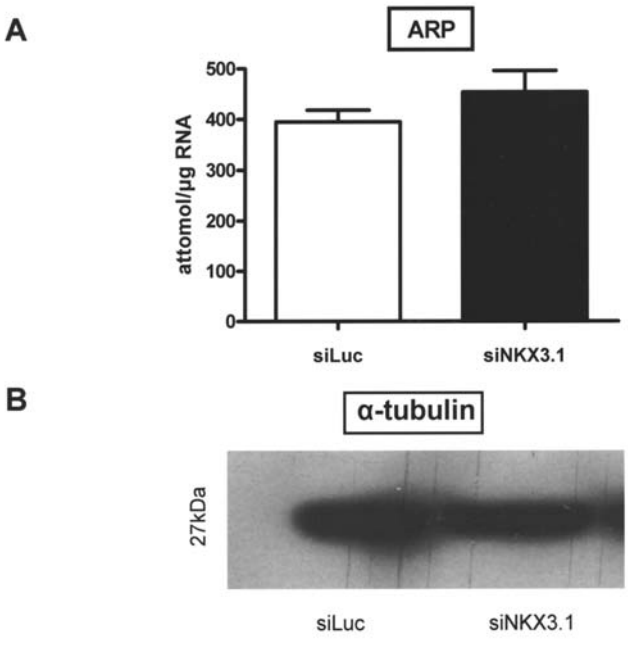

Figure 3. A, mRNA expression of housekeeping gene ARP after transfection of LNCaP cells with siNKX3.1. mRNA expression is shown as means of three independent experiments with error bars for standard deviation. B, Western blot analysis of housekeeping gene α-tubulin after transfecting cells with siNKX3.1.

Furthermore, mRNA expression of the tumor marker PSA was markedly reduced to 10% (Fig. 6A). Forty-four hours after incubation of LNCaP cells with transfection reagents, RPMI medium was collected and PSA secretion measured. The quantity of PSA secretion was diminished to 44% compared to control transfections (Fig. 6B).

We also analyzed mRNA expression of IGF binding protein 3 (IGFBP-3) which was up-regulated 4-fold following treatment with NKX3.1 siRNA (Fig. 7). Similarly, expression of the tissue inhibitor of matrix metalloproteinases and pro-apoptotic factor TIMP-3 increased 11-fold (Fig. 8).

NKX3.1 knock-down evokes a decrease in LNCaP cell viability. Cell viability test was carried out after a total of 61 h of incubation. Control assays were performed using parental LNCaP cells as well as cells that had undergone transfection with siRNA against luciferase gene.

Following NKX3.1 knock-down cell proliferation decreased slightly, albeit significantly to 93% compared to cells trans-

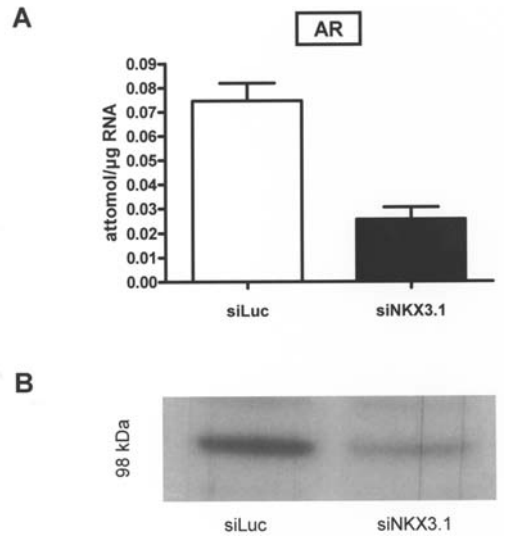

Figure 4. A, mRNA expression of androgen receptor after siRNA mediated knock-down of NKX3.1. mRNA expression is shown as means of three independent experiments with error bars for standard deviation. B, Down-regulation of androgen receptor protein after transfection with siNKX3.1 in Western blot analysis.

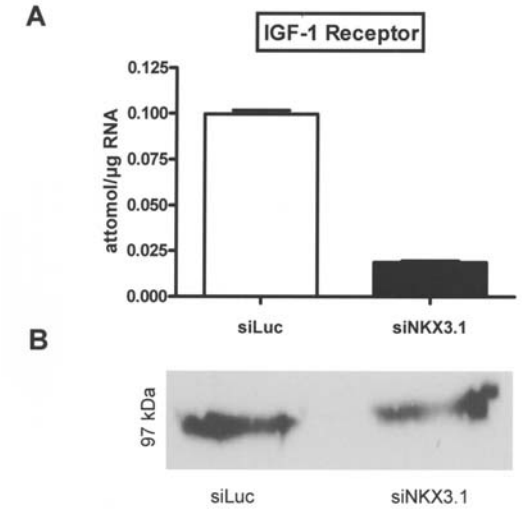

Figure 5. A, mRNA expression of IGF-1R after siRNA mediated knock-down of NKX3.1. mRNA expression is shown as means of three independent experiments with error bars for standard deviation. B, Down-regulation of IGF-1R protein after transfection with siNKX3.1 in Western blot analysis.

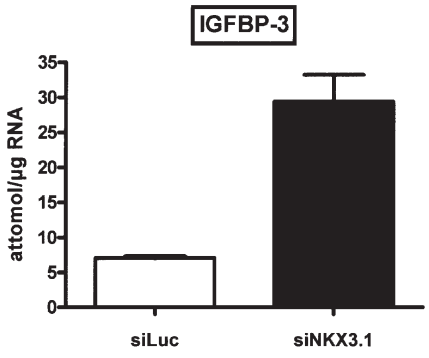

Figure 7. Up-regulation of IGFBP-3 upon knock-down of NKX3.1. RT-PCR analyses demonstrate mRNA expression of IGFBP-3 as means of three independent experiments with error bars for standard deviation.

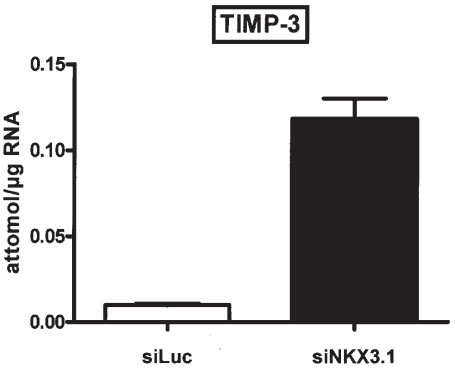

Figure 8. Up-regulation of TIMP-3 upon knock-down of NKX3.1. RT-PCR analyses demonstrate mRNA expression of TIMP-3 as means of three independent experiments with error bars for standard deviation.

fected with siLuc (Fig. 9). There was no significant difference in cell growth between parental LNCaP cells and cells transfected with siLuc, indicating no damage of cells by siRNA transfections *per se*.

Discussion

The role of the androgen-regulated transcription factor NKX3.1 in prostate cancer is controversially discussed. It is widely

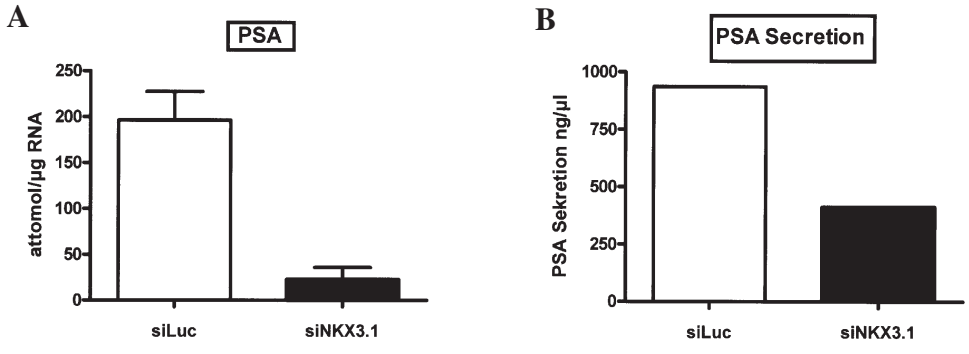

Figure 6. A, mRNA expression of PSA after knock-down of NKX3.1 compared to control cells transfected with siLuc. mRNA expression is shown as means of three independent experiments with error bars for standard deviation. B, PSA secretion from LNCaP cells after knock-down of NKX3.1 compared to secretion from cells treated with siLuc. PSA secretion was measured as described in Materials and methods.

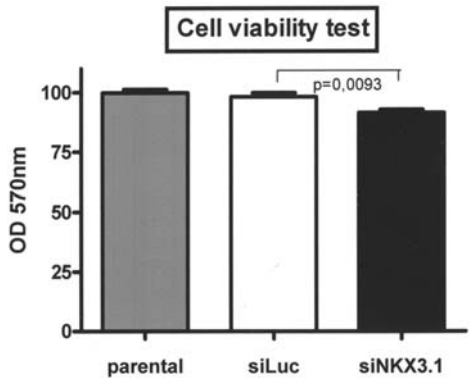

Figure 9. Decrease of cell viability to 93% after NKX3.1 knock-down compared to siLuc transfected cells and parental cells. Cell viability assay was carried out after 44-h exposure with transfection reagents. Control assays were performed using untransfected parental LNCaP cells and cells transfected with siLuc (p=0.0093).

believed that NKX3.1 functions as a tumor suppressor, its loss being involved in initiation and progression of prostate cancer. However, there is considerable evidence inconsistent with an archetypical tumor suppressor role for NKX3.1 in prostate cancer, such as the lack of somatic mutations and reports of increased expression in advanced stage cancers (14). Thus, the pathogenic role of NKX3.1 might differ with various stages of prostate carcinoma.

In this study we showed that expression of NKX3.1 is up-regulated after stimulating hormone sensitive LNCaP cells with DHT, and therefore coincides with growth stimulating events in these cells. These results confirm earlier studies reporting a dose-dependent increase of NKX3.1 mRNA levels after addition of the synthetic androgens R1881 or mibolerone (4,12,16). In contrast, quercetin (17) and, as reported here, tectorigenin, two phytoestrogens with chemopreventive properties evoked a dramatic down-regulation of NKX3.1 expression. In addition, our previous studies showed that tectorigenin treatments of LNCaP cells influenced AR-, IGF-1R-, and PSA-, and TIMP-3-expression (18). To elucidate the function of NKX3.1 in cells such as LNCaP, which have not lost this gene due to deletion and actually express NKX3.1 protein, we performed functional analysis by siRNA mediated down-regulation and investigated the impact of the NKX3.1 knock-down on the expression of the genes regulated by tectorigenin.

We showed that NKX3.1 knock-down caused a diminished expression of AR at mRNA and protein level. The androgen receptor is a ligand-activated transcription factor of the nuclear receptor superfamily (19), which is expressed in the majority of prostate cancers, regardless of clinical stage or hormone status (20). Androgen ablation is part of many prostate cancer treatments, however it is rarely curative. Diverse mechanisms appear to be responsible for the development of hormone-refractory disease: e.g. several peptide growth factors and cytokines, which can activate the AR synergistically (21-23). Another mechanism is amplification of the AR gene, which occurs in ≥30% of prostate carcinomas growing under androgen depletion leading to increased sensibility towards minimal levels of androgens and other signals activating the receptor (24). The androgen receptor is the crucial factor in the process of prostate cancer cells becoming refractory to antiandrogenic therapy, indicating that androgens are still required for growth in hormone-refractory tumors (25). Therefore, elimination of excess AR or interference with AR-activation might be efficacious in fighting hormone refractory prostate cancer (26). Interestingly, in prostate cancers represented by LNCaP cells, the loss rather than the gain of the putative tumor suppressor NKX3.1 evoked a beneficial effect.

Concomitantly with NKX3.1 suppression the expression of PSA mRNA and secretion of this tumor marker were strongly diminished. In androgen depletion therapy this would be a sign for the demise of tumor cells. There are diverse explanations for the mechanisms of regulation of PSA. Oettgen *et al* demonstrated that the prostate-derived Ets factor (PDEF) is capable of synergizing with AR to activate transcription of the PSA promoter, presumably by physically interacting with the DNA binding domain of AR (27). More recently NKX3.1 was found to interact with PDEF and suppress the ability of PDEF to transactivate the PSA promoter. Furthermore, NKX3.1 was suggested to be a modulator of AR function (28). NKX3.1 may compete with AR for binding to PDEF, or it may participate in formation of a complex that includes PDEF and AR, where it could exert its transcriptional repressor function (28). We showed that NKX3.1 knock-down lead to a decline of PSA and reduced carcinoma cell proliferation.

In addition, after NKX3.1 knock-down the expression of genes involved in proliferation and apoptosis in prostate cancer was altered significantly. As a result of NKX3.1 knock-down we found a diminished expression of IGF-1R. In the majority of prostate cancers this receptor is up-regulated and up-regulation persists in metastatic disease (29). Suppression of IGF-1R is associated with inhibition of tumor cell growth and inhibition of invasion *in vivo* (30,31). Down-regulation of AR inhibits expression of the IGF-1R, which in turn causes an up-regulation of IGFBP-3 (18,31). IGFBP-3 is the major regulatory binding protein for IGFs (32). Elevated levels of plasma IGF-1 and reduced levels of IGFBP-3 are associated with an increased risk of prostate cancer and IGFBP-3 is a negative regulator of cell proliferation and an inducer of apoptosis in prostate cancer cells (33-36). Furthermore, Nickerson *et al* described that a combination of increased IGF-1 and IGF-1R and decreased IGFBP-3 in LNCaP cells leads to androgen independence *in vivo* (37). With NKX3.1 knock-down we found a decrease of IGF-1R and an increase of IGFBP-3 expression, indicating anti-proliferative and pro-apoptotic effects on prostate cancer cells.

NKX3.1 knock-down was also followed by a significant up-regulation of TIMP-3 expression. In order to become invasive, tumor cells need to overcome extracellular matrix barriers for which they use matrix metalloproteinases (MMPs). Accordingly prostate cancer progression is accompanied by an imbalanced MMP to TIMP ratio. We found a loss of TIMP-3 expression in prostate cancer - mainly in central tumor and capsule invasive areas as compared to tumor free tissue samples generated from radical prostatectomies by laser microdissection (18). Besides its MMP-inhibiting capacity, TIMP-3 also functions as a pro-apoptotic factor (38,39). This indicates that up-regulation of this gene, which can be elicited by NKX3.1 knock-down, is associated with inhibition of

invasive growth of prostate cancer cells and pro-apoptotic effects.

The changes of expression after NKX3.1 knock-down were accompanied by a decreased proliferation of LNCaP cells. Even after a very short time (44 h) of NKX3.1 knock-down proliferation of LNCaP was significantly inhibited compared with cells transfected with siLuc. Further experiments with a longer time of incubation are warranted to verify and potentially enlarge the differences of proliferation in these transfected cells. When LNCaP cells were treated with the isoflavon tectorigenin which also caused a down-regulation of NKX3.1 a more excessive impact on cell proliferation was revealed (18).

Likewise after down-regulating the AR Eder *et al* found growth inhibition of a prostate carcinoma cell line (40). We reported similar effects when expression of IGF-1R was inhibited by antisense RNA and found suppression of cell proliferation and reduction of cellular invasive capacity in PC-3 cells (31). Since NKX3.1 leads to down-regulation of AR and IGFR-1, the diminished growth of LNCaP cells after NKX3.1 knock-down is supported by the aforementioned findings.

Phytoestrogens, e.g. genistein, quercetin and tectorigenin, have been shown to negatively influence development and progression of prostate cancer (17,18,41). Stimulation of LNCaP cells with tectorigenin or quercetin caused reduced AR expression and PSA secretion (17,18). Upon treatments of LNCaP cells with genistein, Takahashi *et al* demonstrated an inhibition of androgen-inducible genes and induction of androgen-suppressed genes, suggesting an overall inhibitory effect of genistein on AR-mediated events (41). However they did not find reduced levels of AR mRNA. With increasing concentrations of tectorigenin we observed a significant decrease of cell proliferation concomitant with altered expression of genes involved in tumor growth, apoptosis, survival, and invasion (18). Interestingly NKX3.1 knock-down has similar effects on LNCaP cells as phytoestrogens such as quercetin and tectorigenin, suggesting a crucial role of NKX3.1 down-regulation in the effects of phytoestrogen treatments of LNCaP cells.

In conclusion, we found siRNA mediated down-regulation of NKX3.1 in LNCaP cells to beneficially alter the expression of genes highly relevant in tumor cell proliferation and apoptosis. Most likely NKX3.1 is involved in down-stream alteration of gene expression mentioned above, still further experiments are warranted to analyse the exact process. Our data suggest that in androgen-sensitive LNCaP prostate cancer cells, which retain NKX3.1 expression, this gene still has a function which might differ from its role in prostate development and carcinogenesis. This assumption does not contradict the statement of Ouyang *et al* who found that one of the principal roles of NKX3.1 is to maintain the integrity of the prostatic epithelium by regulating the expression of genes that provide protection against oxidative damage (15). In LNCaP cells NKX3.1 most probably plays the role of an androgen-regulated transcription factor and its down-regulation elicits anti-proliferative and pro-apoptotic effects. Since NKX3.1 can regulate AR and IGF-1R expression it may become a target for tumor interference in hormone-refractory prostate carcinomas expressing this gene.

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