Differential effects of *Nucleostemin* suppression on cell cycle arrest and apoptosis in the bladder cancer cell lines 5637 and SW1710

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Abstract

Objectives: The *Nucleostemin* (NS) gene encodes a nucleolar protein enriched in adult and embryonic stem cells. NS is thought to regulate cancer cell proliferation, but the mechanisms involved are poorly understood. In this study, we have investigated the role of NS in bladder cancer.

Materials and methods: Expression of NS was determined by quantitative reverse transcription–polymerase chain reaction in bladder carcinoma cell lines and in normal uro-epithelial cell cultures. We used an RNAi strategy to investigate the function of NS in two selected carcinoma cell lines.

Results: High NS expression was found in most bladder carcinoma cell lines and normal uro-epithelial cells. Knockdown of NS expression induced a severe decline in cell proliferation in 5637 and SW1710 cell lines, both with mutant p53. Apoptosis was more strongly enhanced in 5637 cells lacking *RB1* than in SW1710 cells lacking p16^{INK4A}. Moreover, NS-siRNA-treated 5637 cells accumulated mainly in G₂/M, whereas SW1710 cells arrested in G₀/G₁.

Conclusion: Our data indicate that NS expression is necessary for cell proliferation and evasion of apoptosis in bladder cancer cells, independent of its effect on p53. Also, we speculate that the precise effect of NS on cell cycle regulation may relate to functional status of RB1 and CDKN2A/p16^{INK4A}.

Introduction

Nucleostemin (NS) is a newly characterized nucleolar protein, primarily located in the nucleoli of embryonic and adult stem cells, but not in differentiated cells of most adult tissues (1–5). Expression of NS is abruptly down-regulated during differentiation prior to terminal stages (1,6,7). However, observations of NS expression in adult renal tissue (8) or its re-induction in the myocardium following cardiomyopathic injury (7,9) call for re-evaluation of NS expression and function in normal and pathological conditions.

Several knockdown experiments suggest that NS functions to maintain the proliferative capacity of stem and cancer cells. It is generally agreed that suppression of NS protein by siRNA blocks cell cycle progression and inhibits cell proliferation both in cultured stem cells and cancer cell lines (1,4,10–12). However, the precise effects of this vary between different cell lines. For example, knockdown of NS in PC3, a human prostate cancer cell line, results in increased numbers of apoptotic cells (13), whereas apoptosis was not enhanced in NS-mutant mouse embryos (14). Likewise, in most cell lines, NS knockdown causes G₀/G₁ arrest, whereas in some others, G₂/M arrest was observed (4,11–13,15). The reasons for these differences are not yet understood.

Co-immunoprecipitation and pull-down experiments have suggested that NS interacts with p53 (1). However, whether NS controls cell proliferation only in a p53-dependent manner remains unclear. It seems plausible at present that there are p53-dependent and p53-independent mechanisms by which NS might influence cell proliferation (4,11). NS may be important for proliferation of cancer cells, since its over-expression has been reported in a number of human cancer cell lines and in several malignant tissues (1,8,16,17). However, if NS functions solely through p53, it should not be essential in p53-mutant cancer cells.

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In this study, we examined expression of NS in a series of bladder carcinoma cell lines. A considerable fraction of invasive bladder cancers (18,19) and most bladder cancer cell lines (20) have mutant p53. In addition, all bladder cancer cell lines harbour defects in key regulators of the cell cycle, either loss of p16^{INK4A}, or the more severe loss of RB1 (20,21). We used an RNAi strategy to investigate the function of NS in two bladder cancer cell lines with different genetic alterations; the 5637 line lacks RB1, whereas SW1710 lacks CDKN2A/p16^{INK4A}. Both cell lines have mis-sense mutations inactivating p53.

Materials and Methods

Cell lines and cell culture

All bladder carcinoma cell lines were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ), Braunschweig, Germany, and were cultured in Dulbecco's modified Eagle's medium or RPMI-1640 (Gibco Life Technologies, Karlsruhe, Germany), supplemented with 10% foetal calf serum and 100 mg/ml penicillin/streptomycin, as described elsewhere (22). Normal uro-epithelial cells were prepared from ureters of nephrectomy patients by a standard method (23) with minor modifications (22). Normal cells were routinely maintained in keratinocyte serum-free medium (KSFM, Gibco Life Technologies), supplemented with 50 mg/ml bovine pituitary extract (BPE), 5 ng/ml epidermal growth factor (EGF) and 30 ng/ml cholera toxin. After the first passage, at which non-epithelial cells were removed, they were used for further experiments.

RNA interference

Double-stranded, short (21-mer) interfering RNA (siRNA) corresponding to NS mRNA and an irrelevant siRNA were designed with the following sense and anti-sense sequences as described previously (4), and were purchased from MWG (Ebersberg, Germany):

NS:

Sense: GAACUAAAACAGCAGCAGdTdT

Anti-sense: UCUGCUGCUGUUUUAGUUCdTdT

Irrelevant:

Sense: CUGAUGCAGGUAAUCGCGUdTdT

Anti-sense: ACGCGAUUACCUGCAUCAGdTdT

Cells were harvested with 0.25% trypsin, 1 mM EDTA in phosphate-buffered saline (PBS) without Ca²⁺ and Mg²⁺, and were plated in six-well plates at 20 × 10⁴ cells per well. Next day, when cultures were 30–50% confluent, siRNAs were transfected into cells using LipofectamineTM RNAiMAX Transfection Reagent (Invitrogen, Carlsbad, CA, USA). In brief, 5 µl siRNA (20 µM solution) was

mixed with 6 µl RNAiMAX reagent in 489 µl OptiMem (Invitrogen) for 20 min; the mixture was then added to cells at final volume of 2.5 ml. For some assays, cells were transfected again after 3 days and cultured for 2 further days before harvesting. Furthermore, to rule out possible false results due to toxicity or off-target effects of siRNAs as such, a blank control (without siRNA treatment) was run in parallel in all assays.

RNA extraction

Total RNA was isolated using Qiazol reagent and purified using RNeasy columns (both from Qiagen, Hilden, Germany). cDNA synthesis was performed using SuperScript^{II} reverse transcriptase (Promega, Mannheim, Germany) with oligo-dT primers, as described elsewhere (24).

Reverse transcription–polymerase chain reaction (RT-PCR)

Real-time RT-PCR assays were performed using the LightCycler II apparatus (Roche, Mannheim, Germany). Real-time RT-PCR for *Nucleostemin* and *TBP* mRNAs was performed using specific Quantitect primer assays (Qiagen) using the QuantiTect SYBR Green PCR Kit (Qiagen). Real-time RT-PCR for *GAPDH* and *BTG2* mRNAs was performed using specific primers (*GAPDH*, Forward: TCCCATCACCATCTTCCA and Reverse: CATCACGCCACAGTTTCC, *BTG2*, Forward: GAAGGGAA CCGACATGCTC and Reverse: CCAGTGGTGTGTTG-TAGTGCTC). LightCycler-FastStart DNA Master PLUS SYBR Green I kit (Roche) was used for measurement of *GAPDH* mRNA and the QuantiTect SYBR Green PCR Kit (Qiagen) was used for the other three genes. PCR for the genes included an initial denaturation step at 95 °C for 15 min (10 min for *GAPDH*), followed by 40 amplification cycles consisting of denaturation at 94 °C for 15 s (95 °C for *GAPDH*), 55 °C for 20 s (59 °C for 10 s for *GAPDH*), and extension at 72 °C for 20 s (10 s for *GAPDH*). All measurements were performed in at least duplicates, until there was less than 10% difference. Relative gene expression was calculated using the standard curve method. During initial pilot experiments, we also ran agarose gels from the finished PCRs that proved the products had the correct expected sizes.

Viability assay

Cell viability was determined using Cell Titer-Glo Luminescent Cell Viability Assay (Promega), performed in accordance with the manufacturer's instructions. This is a homogeneous assay for determining number of viable, metabolically active cells in a culture, based on quanti-

fication of ATP level. The assay involves adding a reagent that in a single step generates a luminescent signal proportional to amount of ATP present in cells and is linear across many orders of magnitude. Results were based on four separate experiments and quantity of viable cells is reflected by number of relative luminescence units.

Cell cycle analysis

For fluorescence-activated cell sorting analysis, cells were harvested by trypsinization 48 h after the second transfection, washed in PBS, then stained with 50 µg/ml propidium iodide solution containing 0.1% Triton X-100 and sodium citrate, as described elsewhere (25), and were analyzed for cell cycle distribution using FACS Calibur instrumentation (Becton Dickinson, Heidelberg, Germany). Cell cycle profiles were analysed using WinMDI version 2.8 software.

Analysis of apoptosis

Apoptosis was quantified by measuring caspase-3 and caspase-7 activities using Caspase-Glo 3/7 reagent (Promega). Briefly, after adding Caspase-Glo 3/7 reagent to cell suspension aliquots in quadruplicate, and incubating them at room temperature for 1 h, luminescence of each sample was measured using a plate reader luminometer (Victor2, PerkinElmer, Waltham, MA, USA). Following subtraction of blank, values were normalized to the cell numbers.

Western blotting

Cells were lysed 48 h after the second siRNA transfection in RIPA buffer containing 1% Triton X-100, 0.5%

sodium deoxycholate, 0.1% sodium dodecyl sulphate and complete protease inhibitor cocktail (Sigma, Munich, Germany). Lysates containing equivalent of 30 µg protein per lane were electrophoresed on 12% sodium dodecyl sulphate–polyacrylamide gel, and were blotted on Immobilon-P membranes (Millipore, Hamburg, Germany). Blots were blocked for 1 h with 5% milk powder in Phosphate buffer saline containing 0.2% Tween 20 (PBST). Samples were then probed with polyclonal antibodies against NS (1 : 500; Santa Cruz Biotechnology, Santa Cruz, CA, USA), α -tubulin (1 : 10000; Sigma) and subsequently, with horseradish peroxidase-conjugated anti-goat (1 : 5000; Jackson ImmunoResearch, Hamburg, Germany) or anti-mouse (1 : 5000; Santa Cruz Biotechnology) antibodies. Immunoreactivity was visualized using a chemiluminescence kit (Amersham Biosciences, Freiburg, Germany).

Statistical analyses

All experiments were replicated at least in triplicate, and statistical significance was measured using Student's *t*-test. *P*-values of <0.05 were considered statistically significant.

Results

NS expression in bladder carcinoma cell lines and normal uro-epithelial cells

We compared relative expression of NS in several established bladder carcinoma cell lines and several independent primary cultures of normal uro-epithelial cells (Fig. 1). Interestingly, we observed high NS expression in most examined normal uro-epithelial cells. Ten out

Figure 1. Expression of Nucleostemin (NS) in bladder cancer cell lines and normal urothelial cells. Histograms comparing relative gene expression of NS to GAPDH as determined by quantitative real-time RT-PCR in various bladder cancer cell lines (black columns) and several independent cultures of normal uro-epithelial cells (NUEC, white columns). Cancer cell lines with reported wild-type p53 are indicated by an asterisk. Values shown represent mean $\pm$ standard deviation.

of 18 carcinoma cell lines displayed comparable or higher *NS* relative expression than the mean $\pm$ standard deviation of normal cells. Nevertheless, *NS* expression was very low in some other lines, especially T24, RT4, EJ and TSU-PR1. For knockdown experiments, we initially selected the 5637 cell line, with mutant *TP53* and *RB1* but wild-type *CDKN2A*, with high *NS* expression. Subsequently, we used another cell line, SW1710, with mutant *TP53* and homozygous deletion of *CDKN2A* but wild-type *RB1*, with nearly the same level of *NS* expression.

Specific down-regulation of *NS* expression after RNAi treatment

Application of NS-siRNA resulted in a dramatic reduction in *NS* mRNA expression in both 5637 ($P = 0.003$) and SW1710 ($P = 0.03$) cell lines compared to cells treated with irrelevant (IR)-siRNA (Fig. 2a,b). Western blotting performed on cell lysates of IR-siRNA- or NS-siRNA-treated cells demonstrated that NS protein was likewise down-regulated in both cell lines following siRNA treatment (Fig. 2c,d).

Cell viability reduction after *NS* suppression

The effect of *NS* knockdown on cell proliferation was measured using cell Titer-Glo luminescent cell viability assay kit, which determines number of viable cells based on overall amounts of ATP present in the culture, determined by luciferase luminescence. There was a dramatic reduction of luminescence (82.5%) in NS-siRNA-treated 5637 cells compared to the cells treated with IR-siRNA (Fig. 3a, $P = 0.02$). A likewise significant but weaker decrease (40.6%) was observed on *NS* down-regulation in SW1710 cells (Fig. 3b, $P = 0.04$).

Detection of apoptotic cell death in 5637 and SW1710 cell lines treated with NS-siRNA

To compare levels of apoptotic cell death after NS-siRNA treatment, relative activities of caspase-3 and caspase-7 were measured using a luminescence-based assay. NS-siRNA-treated 5637 cells showed 6.43-fold elevation in caspase-3 and caspase-7 activities compared to cells treated with IR-siRNA (Fig. 4a, $P = 0.007$). Assessment of relative activities of caspase-3 and caspase-7 in NS-siRNA-treated SW1710 cells showed a still significant, albeit weaker elevation of apoptosis (Fig. 4b, $P = 0.01$).

Differential cell cycle redistribution of 5637 and SW1710 cell lines following *NS* suppression

Cell cycle alterations in 5637 and SW1710 cells treated with NS- and IR-siRNAs were investigated by flow cytometry. Figure 5a shows that NS-siRNA treatment of 5637 cells resulted in an increase in percentage of cells in G₂/M phase ($41.8 \pm 0.01\%$ versus $35.2 \pm 0.2\%$ in IR-siRNA-treated cells; $P = 0.011$). Moreover, the fraction of cells appearing in 'sub-G₁', a characteristic feature of apoptotic cells, increased from $17.9 \pm 2.5\%$ in IR-treated cells to $33.6 \pm 1.6\%$ (Fig. 5a, upper panel, $P = 0.025$).

Accordingly, as evident in Fig. 5b, NS-siRNA treatment in SW1710 cells caused significant increase in percentage of sub-G₁ cells compared to IR-treated cells ($10.4 \pm 0.7\%$ versus $4.6 \pm 0.5\%$; $P = 0.014$). In contrast to 5637 cells, no increased G₂/M fraction was seen, but fraction of G₀/G₁ cells was strongly and significantly elevated ($84.2 \pm 0.5\%$ versus $45.2 \pm 0.5\%$; $P = 0.011$).

In addition, we measured relative mRNA levels of *BTG2*, as an anti-proliferative and a cell cycle regulatory

Figure 2. Downregulation of Nucleostemin (NS) expression by siRNA in bladder cancer cell lines. Knocking down expression of *NS* by means of siRNA caused efficient down-regulation of *NS* mRNA in 5637 (a) and SW1710 (b) cell lines as determined by quantitative RT-PCR, and of protein levels (c and d, respectively) as determined by Western blotting, after 72 h. In (a) and (b), relative expression of *NS* in IR-siRNA-treated controls was adjusted to 100. α -tubulin was used as loading control for Western blotting in (c) and (d).

Figure 3. Effect of Nucleostemin (NS) knockdown on cell viability in bladder cancer cell lines. Suppression of NS caused significant reduction in number of viable 5637 (a) and SW1710 (b) cells as assessed by luminescence-based ATP measurements, represented as relative luminescence units (RLU). Values shown are mean $\pm$ standard deviation.

gene. NS-siRNA-treated 5637 cells showed 1.5-fold elevation in *BTG2* mRNA expression level compared to cells treated with IR-siRNA (Fig. 6a, $P = 0.047$). Relative mRNA levels of *BTG2* increased more strongly, 6.8-fold in NS-siRNA- versus IR-siRNA-treated SW1710 cells (Fig. 6b, $P = 0.032$).

Discussion

Nucleostemin (NS) has been recently cloned and characterized as a novel p53-binding protein that is highly expressed in stem/progenitor cells and some cancer cell lines (1). Recently, its expression has also been observed in normal differentiated renal tissues (8) and differentiated heart cells following pathological stresses (7). According to original reports, NS has a functional role in proliferation of both stem and cancer cells and it may exert a novel control mechanism of cell cycle progression, especially in late S and G₂ phases (1). NS expression has been further reported in several human cancer cell lines and tissues (8,16,17). However, its exact mechanism of action and whether it has a similar or different role in normal stem cells as opposed to malignant cells are not well understood.

Figure 4. Effect of Nucleostemin (NS) suppression on caspase activity. Elevation of caspase-3/caspase-7 relative activities in response to NS knockdown in 5637 (a) and SW1710 (b) cells was determined using Caspase-Glo 3/7 kit, as a measurement of the apoptotic level. Values were normalized to viable cell numbers as measured in Fig. 3 and represented as mean $\pm$ standard deviation.

Using quantitative RT-PCR, we compared relative expression of *NS* in several bladder carcinoma cell lines and independent primary cultures of normal uro-epithelial cells. While overexpression of *NS* was found in most urothelial carcinoma cell lines, there was only low expression of *NS* in several others, suggesting that involvement of *NS* differs even between tumours of the same type, such as invasive bladder cancers. Expression of *NS* is particularly low in all derivatives of the T24 line, including EJ and TSU-PR1 cells, which have non-sense mutations in *TP53* (20,26,27), and in the RT4 cell line, which is one of the few cell lines that has been derived from a superficial bladder tumour and retains wild-type *TP53* (28).

Interestingly, we also observed high relative expression of *NS* in most examined normal uro-epithelial cells. This finding may be explained as follows: cultured normal uro-epithelial cells proliferate spontaneously and mimic rapid and dramatic regeneration of urothelium during wound healing, growing at similar rates as bladder cancer cell lines (22,23). However, they do not differentiate spontaneously and do not express markers of fully

Figure 5. Effect of Nucleostemin (NS) knockdown on cell cycle distribution in bladder cancer cell lines. Cell cycle re-distribution of 5637 (a) and SW1710 (b) cell lines after NS-siRNA treatment. Upper panels show sub-G₁ population of cells as depicted by the FL3 filter of FACS instrumentation and lower panels show distribution and percentage of cells in the G₀/G₁, S and G₂/M phases.

differentiated urothelial cells, such as uroplakins. Therefore, NS may function to maintain the proliferative capacity of these precursor cells.

We next investigated whether NS plays a functional role in proliferation of bladder carcinoma cell lines; we chose 5637 and SW1710 cell lines with similarly high expression of NS. Like almost all cell lines from invasive bladder cancers, they harbour mutant p53, but differ in the genetic defect, leading to disturbed cell cycle regulation (that is, RB1 and p16^{INK4A} loss, respectively). Successful suppression of NS in both lines, as confirmed by real-time RT-PCR and Western blotting, lead to significant decrease in number of viable cells, indicative of an important regulatory role for NS in maintaining proliferation of these

Figure 6. Relative mRNA expression changes of *BTG2* in bladder cancer cell lines in response to Nucleostemin (NS) knockdown. Knocking down expression of NS by means of siRNA caused up-regulation of *BTG2* mRNA in 5637 (a) and more strongly in SW1710 (b) cells as determined by relative quantitative real-time RT-PCR. Values shown represent mean ± standard deviation.

cells. Decline in population of proliferating cells after NS suppression is in accordance with previous reports (1,4,10–12).

Both cell lines showed evidence for cell cycle arrest. Interestingly, while NS-siRNA-treated 5637 cells showed primarily accumulation of cells in G₂/M, SW1710 cells accumulated mostly in G₀/G₁. While most reports have shown G₁ arrest after NS depletion in normal stem and malignant cell lines (4,11–13), Zhu *et al.* (15) reported an increase in sub-G₁ and G₂/M fractions of NS^{+/-} cultured mouse embryonic fibroblasts from passage 3 to 5. Furthermore, Tsai and McKay (1) reported that over-expression of GTP-deleted mutants of NS blocked cell cycle progression in late S phase, which lead to increased level of apoptosis in U2OS cell line, with wild-type p53. They concluded that NS may function through known tumor suppressor genes in late S and G₂ phases. Our study suggests a differential mechanism of action for NS in our two cell lines, which may not only depend on p53, but also on status of RB1. However, further detailed studies need to be performed to unveil the molecular mechanisms responsible for influences of NS on RB signalling pathway and/or *vice versa*.

Increased percentage of sub-G₁ cells after NS-siRNA treatment in both cell lines indicates a potential role for NS in regulating apoptosis. This conclusion was further

supported by increased activities of caspase-3 and caspase-7 in NS-suppressed cells. Elevated apoptotic level following NS suppression was also observed in some previous reports (13,15), but not in others. For instance, Beekman *et al.* (14) reported that decreased cell numbers of NS^{+/-} E3.5–4.5 mouse embryos were not associated with increased level of apoptotic cells. This discrepancy could be due to possible intrinsic differences, such as presence of intact or else mutant p53.

Additionally, we measured relative expression of *BTG2* (*TIS21*) after treating the cells with NS-siRNA. *BTG2* is recognized as an antiproliferative gene that regulates cell cycle transitions between the G₁/S and G₂/M phases in malignant cells lacking active pRB1 and/or p53 (29,30). In normal cells with wild-type p53 and pRB1, *BTG2* inhibits expression of cyclin D1, resulting in arrest of cells at the G₁/S transition in pRB- and p53-dependent manner. Moreover, *BTG2* inhibits degradation of cyclin A and cyclin B1 in G₂/M, and directly binds to cdc2. In p53 null tumour cells, this function results in failure of mitotic exit and increased tumour cell death (20,31). NS suppression in both cell lines led to a significant increase in *BTG2* expression, which was consistent with the increased fraction of cells in sub-G₁, G₂/M and G₀/G₁ phases in NS-siRNA-treated 5637 and SW1710 cells.

It was at first proposed that proliferation-promoting role of NS is only due to its property of binding and inactivating p53 (1). Like most bladder carcinoma cell lines, 5637 and SW1710 cell lines have mutant p53 (20,32). Therefore, promotion of proliferation by NS and its anti-apoptotic function cannot be merely due to its p53 binding activity. Accordingly, down-regulation of NS decreased the level of proliferation of p53 mutant HeLa and PC3 cells and increased percentages of apoptotic cells in PC3 cell line (10,13). Therefore, our observations lend further support to the notion that NS exerts its role *via* mediators additional to p53. Intriguingly, a recently published paper also revealed that p53 knockout did not rescue the lethality of NS knockout in mice (14). Based on previous studies and the current investigation, it seems plausible that there are p53-dependent and p53-independent mechanisms of actions for NS to regulate cell proliferation and apoptosis.

To further clarify the dependency of NS on p53, RB1 or p16, it would be helpful to investigate bladder cancer cell lines with mutations in each individual gene at a time. However, in the case of bladder malignant cell lines, this strategy is not straightforwardly feasible, because disturbances of both the p53 and RB1 regulatory networks are fundamental in this cancer type (19,33). The only commonly available bladder cancer cell lines with wild-type p53, RT4 and 253J, have very low expression of NS; however, they harbour deletions in *CDKN2A*, which compromise p53 activation through

p14^{ARF}. In the other bladder cancer cell lines, re-expression of wild-type p53 induces rapid apoptosis (34). Identification of NS expression in normal uro-epithelial cells at a level comparable to many urothelial carcinoma cells suggests that NS over-expression and knock-down approaches in these cells may eventually lead to insights into NS function in normal cells.

In conclusion, our data show that NS, in a p53-independent manner, differentially regulates proliferation and apoptosis in two human bladder cancer cell lines (5637 and SW1710). Moreover, the precise effect of NS on controlling cell cycle distribution seems to be dependent on functional status of *RB1* and *CDKN2A* genes in cancer cells. These insights lay the ground for identification of the p53-independent mechanism of NS in regulation of cell proliferation and apoptosis.

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