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Shi-Qi Ji (jishiqi09@163.com)
Lin Yao (yaolin01@163.com)
Xiao-Yu Zhang (zhang_xiaoyu08@163.com)
Xue-Song Li (li_xuesong01@163.com)
Li-Qun Zhou (zhoulqmail@china.com)

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Shi-Qi Ji,^{Aff1 Aff2 Aff3}

Email: jishiqi09@163.com

Lin Yao,^{Aff1 Aff2 Aff3}

Email: yaolin01@163.com

Xiao-Yu Zhang,^{Aff1 Aff2 Aff3}

Email: zhang_xiaoyu08@163.com

Xue-Song Li,^{Aff1 Aff2 Aff3}

Email: li_xuesong01@163.com

Li-Qun Zhou,^{Aff1 Aff2 Aff3 Aff4}

Corresponding Affiliation: Aff4

Email: zhoulqmail@china.com

Aff1 Department of Urology, Peking University First Hospital, Beijing 100034, China

Aff2 The Institute of Urology, Peking University, Beijing 100034, China

Aff3 National Urological Cancer Center, Beijing 100034, China

Aff4 Department of Urology, First Hospital of Peking University, Peking University, No. 8, Xishiku Street, West District, Beijing 100034, China

Abstract

Background

The nucleosome binding protein 1 (HMGN5/NSBP1) is a member of the HMGN protein family and is highly expressed in several kinds of cancer. Nevertheless, the role of NSBP1 in clear cell renal cell carcinoma (ccRCC) remains unclear. This study aimed to confirm the oncogenic role of NSBP1 in ccRCC using in vitro and in vivo models and explore the mechanism by which NSBP1 contributes to ccRCC tumorigenesis.

Methods

NSBP1 expression was detected in renal tissues from 152 ccRCC patients by immunohistochemistry, and examined in ccRCC cell lines by RT-PCR and Western blot analysis. ccRCC cells were transfected by NSBP1 RNAi and cell viability, apoptosis and invasion were detected by cell vitality test, flow cytometry and transwell assay in vitro.

Xenograft in nude mice was also employed to examine the tumorigenesis of ccRCC cells depleted of NSBP1.

Results

Immunohistostaining showed strong immunoreactivity of NSBP1 in all ccRCC tissues and NSBP1 expression level was associated with tumor grade ($p = 0.04$). NSBP1 expression at mRNA and protein levels was high in ccRCC cell lines. Knockdown of NSBP1 induced cell cycle arrest and apoptosis, and inhibited invasion in 786-O cells. Western blot analysis demonstrated increased expression of Bax and decreased expression of Bcl-2, CyclinB1, VEGF, VEGFR-2, MMP-2, MMP-9, c-fos and c-jun in 786-O cells depleted of NSBP1. In vivo study further showed that knockdown of NSBP1 affected the tumorigenesis of ccRCC cells in nude mice.

Conclusions

NSBP1 plays oncogenic role in ccRCCs by promoting cell proliferation and invasion, and could be exploited as a target for ccRCC treatment.

Keywords

Clear cell renal cell carcinoma, NSBP1, Apoptosis, Cell cycle, MMPs

Introduction

Renal carcinoma is the 13th most common cancer worldwide, with clear cell and clear cell renal cell carcinoma (ccRCC) accounting for most of the renal cell carcinoma (RCC) [1]. Radical nephrectomy is effective to cure early and local ccRCCs, but advanced or metastatic ccRCCs barely respond to chemotherapy or radiotherapy and have poor prognosis. Therefore, it is important to better understand the pathogenesis of aggressive RCC in order to develop effective strategies for the prevention and treatment of RCC.

NSBP1 is a new member of the high mobility group N (HMGN) protein family that modulates the structure and function of chromatin and plays an important role in transcription, histone modifications, DNA replication and DNA repair in living cells[2]. Early study showed that nucleosome binding protein 1 (HMGN5/NSBP1) was abundantly expressed in prostate cancer [3]. In addition, NSBP1 expression was upregulated in squamous cell carcinoma, metastatic MDA-MB-435HM breast cancer cell line and adenocarcinoma, suggesting that NSBP1 may promote tumorigenesis [4-7].

Our previous studies showed that downregulation of NSBP1 expression caused G2 cell cycle arrest, decreased proliferation rate and increased apoptosis rate in prostate cancer cells in vitro [8,9]. Nevertheless, the role of NSBP1 in ccRCC development remains unknown.

Tumor invasion and metastasis are complicated processes, among which proteolytic degradation of extracellular matrix (ECM) and angiogenesis (VEGF) are essential steps. ECM degradation can be promoted by the imbalance between proteolytic proteases and their

inhibitors. Extensive studies have shown that matrix metalloproteinases (MMPs) play crucial role in the degradation of ECM to promote tumor invasion and metastasis [10,11].

Therefore, in this study we investigated the role of NSBP1 in ccRCC. First we detected NSBP1 expression in clinical ccRCC tissues and ccRCC cell lines. Then we examined the effects of lentivirus mediated NSBP1 knockdown on the growth and invasion of ccRCC 786-O cells and xenograft tumor growth in nude mice. The results showed that NSBP1 expression was upregulated in ccRCC tissues and ccRCC cell lines, and NSBP1 knockdown could induce apoptosis and inhibit the proliferation and invasion of ccRCC cells, and further decrease ccRCC tumor growth in nude mice.

Methods

Clinical samples

A total of 152 patients (aged 52 to 90 years old, median age of 64 years) who underwent surgery from January 2008 to January 2011 in Peking University First Hospital were enrolled in the present study. All patients were of Chinese origin. Paraffin wax-embedded blocks of tumor tissues from each patient were assembled from the archival collections at the Department of Pathology. Survival data of all patients were collected. Among these patients, 20 patients were randomly selected and paired cancer and adjacent tissues were collected from them for Western blot analysis of NSBP1 expression. All adjacent tissues were confirmed to be normal by experienced pathologists. The protocols for the present study were approved by the Ethics Committee of Peking University First Hospital.

Cell culture

The ccRCC cell lines Caki-2, A498, 786-O and the normal renal tubular epithelial line HK-2 were purchased from American Type Culture Collection (ATCC, Manassas, VA). HK-2 cells were cultured in K-SFM medium (Gibco™ Life Technologies, Grand Island, NY), and other cells were cultured in RPMI-1640 (HyClone, Logan, UT) medium supplemented with 10% Gibco™ FBS (Life Technologies, Grand Island, NY). All cells were cultured at 37°C in a standard humidified incubator containing 5% CO₂ and 95% O₂.

Lentivirus RNAi construct and transfection

The siRNA targeting the human NSBP1 (NM_030763) transcript was designed using the software developed by Ambion (Foster, CA, USA) with the following sequence: PscSI616 CACAGCCTTTCTTTAGCATTTC AAGAGAATGCTAAAGAAAGG-CTGTG/CACAGCCTTTCTTTAGCATTCTCTTGAAATGCTAAAGA-AAGGCTGTG. NSBP1 siRNA or control scramble siRNA was cloned into vector. 786-O cells were seeded onto 6-well plates and grown to 60% confluence on the day of transfection. 4 h before transfection, cells were placed in serum-free media. Cells were transfected with 100 nM siRNA vector diluted in RPMI-1640 according to the manufacturer's protocol. Successful knockdown of NSBP1 was analyzed by Western blot analysis and real-time PCR.

Immunohistochemistry

Paraffin-embedded tissues were cut into 4 μm -thick consecutive sections and were then dewaxed in xylene and rehydrated in graded ethanol solutions. Antigen retrieval was performed following the standard procedure. Sections were cooled and immersed in a 0.3% hydrogen peroxide solution for 15 min to block endogenous peroxidase activity, and then rinsed in PBS for 5 min. Non-specific labeling was blocked by incubation with 5% bovine serum albumin at room temperature for 30 min. Sections were then incubated with primary rabbit anti-human antibody against NSBP1 (diluted in 1:100, Abcam, ab56031, Cambridge, MA) at 4°C overnight, rinsed with PBST, incubated with horseradish peroxidase-conjugated Santa Cruz™ goat anti-rabbit IgG secondary antibody (Santa Cruz, CA), developed by peroxidase-conjugated streptavidin and DAB, and counterstained by hematoxylin. All slides were examined independently by two pathologists, who were not informed about patients' clinical data. Specimens were then grouped according to stage (T1–T4) and specific staining intensity. The staining intensity was scored as “–” for negative, “+” for moderate, and “++” for strong staining.

Quantitative real-time PCR assay

Total RNA was extracted from the cells using Trizol (Invitrogen) according to the manufacturer's protocol. First-strand cDNA was generated using 2 μg total RNA via MMLV-reverse transcriptase using High Capacity RNA-to-cDNA kit (Promega) with random primers. A final reaction of 20 μl was used to determine the mRNA level by real-time PCR using an ABI Prism 7300 (Applied Biosystems, Foster City, CA, USA). The specific primers were as follows: NSBP1, 5'-TCGGCTTTTTTCTGCTGACTAA-3'(forward) and 5'-CTCTTTGGCTCCTGCCTCAT-3'(reverse); Actin, 5'-GTGGACATCCGCAAAGAC3'(forward) and 5'-ATCAACGCAATGTGGGAAA-3'(reverse). Thermal cycling was initiated with a denaturation step for 5 min at 94°C followed by 36 cycles done in three steps: 30 s at 94°C, 30 s at 58°C and 1 min at 72°C.

Cell proliferation assay

Cell proliferation was assessed using the CellTiter 96 Aqueous assay kit (Promega, Madison, WI). After transfection, the cells (10,000/well) were seeded in 96-well plates and incubated at 37°C, and cell proliferation was assessed after 96 h based on the absorbance measured at 570 nm using a multiwell spectrophotometer.

Flow cytometry

Apoptosis was evaluated by Annexin V-PE/7-AAD staining followed by flow cytometry analysis. After cells were plated in 6-well plates at a density of 1×10^5 /well and cultured at 37°C in 5% CO₂ incubator for three days, they were transfected with NSBP1 siRNA or scramble siRNA vector, the cells were gently trypsinized and washed with ice-cold PBS after 72 h. At least 20,000 cells were resuspended in 500 μL 1 \times binding buffer, stained with 5 μL 7-AAD (25 $\mu\text{g mL}^{-1}$) and 1 μL Annexin V-PE and immediately analyzed with a FACScalibur flow cytometer (Becton Dickinson, Erembodegem, Belgium).

Western blot analysis

inhibitors. Protein samples (40 µg) were separated in 10% SDS–polyacrylamide gels and transferred to PVDF membranes. The membranes were blocked with nonfat milk in TBST, and probed with primary antibodies CyclinB1 (CST-4138), CyclinD1 (CST-2978), Proliferating Cell Nuclear Antigen (PCNA, CST-2586), Bax (CST-2772), Bcl-2 (CST-2876), VEGF (CST-2445), VEGFR-2 (CST-2472), MMP-2 (CST-4022), MMP-9 (CST-3852) (CST indicated Cell Signaling Technology, Beverly, MA, USA). c-fos (santa cruz-52), c-jun (santa cruz-1694), GAPDH (santa cruz-137179), or β-Actin (santa cruz-81178), and secondary antibodies goat anti-mouse IgG (santa cruz), goat anti-rabbit IgG (santa cruz) (santa cruz indicated Santa Cruz Biotech, Santa Cruz, CA, USA). Immunoreactivity signals were developed using ECL kit (GE Healthcare Bioscience, Piscataway, NJ, USA). protease and phosphatase with Whole-cell extracts were prepared in RIPA buffer

Cell invasion assay

Cell invasion assay was performed with 24-well Transwell insert (pore size 8 µm, Corning, NY). After transfection, 786-O cells were starved in serum free medium overnight, and $3-5 \times 10^4$ cells were resuspended in 200 µl serum-free medium and placed in the upper chambers with 8 µm filter pores in triplicate. The membrane undersurface was coated with 30 µl ECM gel from Engelbreth-Holm-Swarm mouse sarcoma (BD Biosciences, Bedford, MA, USA) mixed with RPMI-1640 serum free medium in 1:5 dilution for 30 min at 37°. The lower chamber was filled with 500 µl 10% FBS as the chemoattractant and incubated for 48 h. At the end of the experiments, the cells on the upper surface of the membrane were removed by cotton buds, and the cells on the lower surface were fixed in 4% paraformaldehyde and stained with 0.1% crystal violet. Five visual fields were chosen randomly for each insert and photographed under a light microscope at 200× magnification. The cells were counted and the data were summarized by means ± standard deviation and presented by a percentage of controls.

Gelatin zymography assay

After transfection, the cells were cultured in serum free medium for 24 h. Then the medium was collected by centrifugation at 4,000 rpm for 15 min at 4°C, and subjected to zymographic SDS-PAGE containing 0.1% gelatin (w/v). The gels were washed and incubated in incubation buffer for 48 h, then stained with Coomassie Brilliant Blue and destained. The zones of gelatinolytic activity were shown by negative staining.

Tumourigenesis assay in nude mice

Female BALB/c^{nu/nu} mice (4–6 weeks old, weighed 25–30 g) were maintained in a germ-free environment in the animal facility. NSBP1 knockdown or control 786-O cells were cultured in 100-mm dishes and trypsinized. The cells (10^6 in 100 µl medium) were infused subcutaneously in the armpit area. Tumor diameter was measured every 5 days, and tumor volume was calculated by length × width² × 0.5. Mice were sacrificed after 1.5 months.

Statistical analysis

Values were represented as mean \pm SD for at least triplicate determination, and analyzed using Fisher's exact test and Kruskal-Wallis test. All statistical analyses were performed using SPSS 13.0 and $P < 0.05$ was considered as statistically significant.

Results

NSBP1 expression is high in ccRCC tissues

We examined NSBP1 expression in ccRCC tissue by immunohistochemistry. As shown in Figure 1A, NSBP1 staining was weak in the normal renal tissues but strong in ccRCC tissues. Western blot analysis of 20 paired adjacent normal renal tissues and ccRCC tissues confirmed the high expression of NSBP1 in ccRCC tissues ($p = 0.006$) (Figure 1B). Most importantly, we found that NSBP1 staining intensity was correlated with the clinical and pathologic characteristics of ccRCC (Table 1). NSBP1 expression was positively correlated with the tumor grade and pathologic stage.

Figure 1 NSBP1 expression is high in ccRCC tissues and cells. (A), Representative immunohistochemistry staining of NSBP1 in control renal tissue and ccRCC tissue. NSBP1 immunoreactivity in brown was predominantly localized in the nucleus. Original magnification: X10 (a, c), X40 (b, d). (B) Ratio between protein expression levels of NSBP1 and β -Actin in pairs of ccRCC and normal tissue from 20 patients was calculated based on Western blot analysis. (C), Western blots demonstrating the expression of NSBP1 in different ccRCC cells. Actin served as loading control. (D), Real-time PCR assay showing the relative NSBP1 mRNA level in different ccRCC cells. * $p < 0.05$, ** $p < 0.01$, versus HK-2 cells

Table 1 Correlation of NSBP1 expression with clinical and pathological characteristics of renal carcinoma

Characteristics	Cases	NSBP1 immunoreactivity						P
		-		+		++		
		Cases (%)	(%)	Cases (%)	(%)	Cases (%)	(%)	
Gender								0.653
Male	129	18	11.8	33	21.7	78	51.3	
Female	23	3	2.0	8	5.3	12	7.9	
Age (years)	60.4 \pm 8.9	59.8 \pm 9.7		60.2 \pm 9.8		61.3 \pm 11		
Grade								0.040*
1	0	0		0		0		
2	63	14	9.2	16	10.5	33	21.7	
3	89	7	4.6	25	16.5	57	37.5	
Pathologic stage								0.002**
T1	18	10	6.6	5	3.3	3	2.0	
T2	35	6	3.9	12	7.9	17	11.1	
T3	47	3	2.0	15	9.9	29	19.1	
T4	52	2	1.3	9	5.9	41	27.0	

Correlation significant at the 0.05 level (one-tailed)

* $P < 0.05$; ** $P < 0.01$

NSBP1 expression is high in ccRCC cells

We examined NSBP1 expression in ccRCC cell lines and the normal renal tubular epithelial line cells by quantitative real-time RT-PCR and Western blot. NSBP1 protein level was higher in ccRCC cell lines than normal renal tubular epithelial line cells (Figure 1C). Similarly, NSBP1 mRNA level was increased in ccRCC cell lines compared to normal renal tubular epithelial line cells (Figure 1D).

NSBP1 knockdown decreases the proliferation of ccRCC cells

To investigate the role of NSBP1 in the proliferation of ccRCC cells, we employed the loss of function approach. 786-O cells were transfected with NSBP1 siRNA or scramble siRNA as control and cell proliferation was evaluated by MTT assay. The results showed that NSBP1 knockdown significantly reduced proliferation of ccRCC cells over the 72 h period (Figure 2A). Lentivirus short hairpin constructs against NSBP1 (PscSI616) was efficient and specific in the knockdown of NSBP1 in 786-O cells and the inhibitory efficiency at protein level was $74.8 \pm 2.1\%$ based on Western blot analysis (Figure 2C).

Figure 2 NSBP1 knockdown decreases the proliferation of ccRCC cells. (A), MTT assay showing that NSBP1 knockdown significantly reduced the proliferation of ccRCC cells over the 96 h period. (B), Annexin V-PE/7-AAD staining in FCM assays showing the ratio of apoptosis in different 786-O cells. (Data were presented as mean \pm SEM, n = 3). (C), Representative blots demonstrating the reduced protein level of NSBP1 in NSBP1 siRNA transfected 786-O cells compared to scramble siRNA transfected cells. In addition, Bax protein level was increased and CyclinB1 and Bcl-2 levels were decreased in NSBP1 siRNA transfected 786-O cells compared to scramble siRNA transfected cells

The apoptosis of ccRCC cells was examined by FCM after the cells were transfected with NSBP1 siRNA or scramble siRNA as control. The apoptotic ratio was increased in NSBP1 knockdown 786-O cells compared to control (Figure 2B). To confirm that NSBP1 knockdown could inhibit proliferation and induce apoptosis in ccRCC cells, we examined the expression of apoptosis and cell cycle related proteins and found that Bax protein level was significantly increased while CyclinB1 and Bcl-2 protein levels were decreased in NSBP1 knockdown cells compared with control (Figure 2C). These data provide evidence that NSBP1 modulates cell cycle and antagonizes apoptosis to promote the oncogenic potential of ccRCC cells.

NSBP1 knockdown inhibits the invasion of ccRCC cells

Next we assessed the role of NSBP1 in cell invasion, an important aspect of ccRCC metastasis. By transwell assay we found that NSBP1 knockdown cells showed few number of invading cells compared to control group which expressed high level of NSBP1 (Figure 3A). The number of cells crossing the matrigel was 62.3 ± 3.1 in NSBP1 siRNA group versus 110.7 ± 3.1 in scramble siRNA control group ($P < 0.05$). Moreover, gelatin zymography assay demonstrated that NSBP1 knockdown efficiently decreased MMP-2 and MMP-9 enzymatic activity, especially MMP-9 enzymatic activity (Figure 3B). To address whether decreased MMP-9 and MMP-2 activity is due to the downregulation of their expression after NSBP1 knockdown, we examined the expression of MMP-9, MMP-2 and their upstream transcription factors c-fos and c-jun. Western blot analysis demonstrated that NSBP1 knockdown downregulated the expression of VEGF, VEGFR-2, MMP-2, MMP-9, c-fos and

c-jun (Figure 3C). Taken together, these data suggest that NSBP1 upregulates the expression of MMP-2 and MMP-9 via c-fos and c-jun. The increased MMPs activity and angiogenesis then contributes to the migration and invasion of ccRCC cells.

Figure 3 NSBP1 knockdown inhibits the invasion of ccRCC cells. (A), Representative photos showing the invasion of ccRCC cells into the lower chamber of transwell. $\times 200$. (B), Gelatin zymography assay showing that MMP-9 and MMP-2 activities were decreased in NSBP1 knockdown 786-O cells. Data shown were mean \pm SEM from three independent experiments. (C), Western blot analysis showing that the expression of VEGF, VEGFR-2, MMP-2, MMP-9, c-fos and c-jun were significantly decreased in NSBP1 knockdown 786-O cells. Data shown were mean \pm SEM from three independent experiments. * $p < 0.05$, ** $p < 0.01$, versus the scramble siRNA transfected control group

NSBP1 knockdown inhibits ccRCC growth in xenograft nude mice

To further investigate the role of NSBP1 in ccRCC *in vivo*, we established xenograft ccRCC by subcutaneous injection of 1×10^6 NSBP1 knockdown 786-O cells or the corresponding scramble siRNA transfected control cells into the flanks of BALB/c nude mice ($n = 10$). After 6 weeks, we observed that the volume of tumor derived from NSBP1 knockdown cells was significantly smaller than that derived from control cells (Figure 4). These data demonstrate that NSBP1 knockdown inhibits the tumorigenicity of ccRCC cells *in vivo*.

Figure 4 NSBP1 knockdown inhibits the tumorigenicity of ccRCC cells *in vivo*. (A), A representative nude mice showing the different morphology of the tumors derived from NSBP1 siRNA transfected 786-O cells (left side) and scramble siRNA transfected control cells (right side). (B), the growth curve of the tumors ($n = 10$)

Discussion

NSBP1 was identified as a new member of the HMGN protein family in 2001 [12,13]. As a nuclear protein, NSBP1 modulates the structure and function of chromatin and plays an important role in transcription, DNA replication and repair [14-16]. Interestingly, recent studies demonstrated that NSBP1 expression was abnormally high in a variety of solid tumors, indicating the oncogenic role of NSBP1 [4-7],

In this study, we found that NSBP1 expression was significantly higher in ccRCC tissues and cell lines than normal renal tissue and cell lines. These data suggest that NSBP1 overexpression is correlated with the progression of ccRCC.

To elucidate the role of NSBP1 in the tumorigenesis of ccRCC, we employed loss of function approach via the knockdown of endogenous NSBP1 expression in ccRCC cells. Notably, we found that NSBP1 knockdown inhibited the proliferation rate of ccRCC cells through MTT assay. Furthermore, our experiments showed that knockdown of NSBP1 led to increased Bax expression and decreased CyclinB1, Bcl-2 expression. These results suggest that downregulation of NSBP1 expression causes G2 cell cycle arrest, decreases the proliferation rate and increases apoptosis rate in ccRCC cells *in vitro* [17-20].

Metastasis is an important aspect of ccRCC. To characterize the role of NSBP1 in ccRCC metastasis, we employed *in vitro* invasion assay and found that NSBP1 knockdown led to

decreased invasion of ccRCC cells. Tumor invasion and metastasis are crucially dependent on MMPs and VEGF [10,11,20]. MMP-2 and MMP-9 play important roles in the degradation of the ECM, including type IV collagen, and their activity and expression are correlated with metastatic abilities and prognosis of cancer[21,22]. Our results showed that silencing of NSBP1 in 786-O cells decreased MMP-2 and MMP-9 activity based on zymography assay. In addition, we found that MMP-2 and MMP-9 expression as well as the expression of transcription factors c-fos and c-jun were decreased in NSBP1 knockdown cells. These data suggest that NSBP1 modulates the expression of MMPs and VEGF/VEGFR-2 thus influencing the invasion behavior of ccRCC cells.

Finally, to demonstrate that NSBP1 contributes to ccRCC development in vivo, we employed xenograft nude mice model and found that NSBP1 knockdown suppressed tumor growth of ccRCC cells, indicating that NSBP1 promotes the tumorigenicity of ccRCC cells in vivo.

In summary, here we present both in vitro and in vivo evidence that NSBP1 promotes ccRCC cells growth and invasion. NSBP1 plays important role in the regulation of apoptosis and invasion of ccRCC cells by regulating the expression of Bcl-2, Bax, CyclinB1 VEGF/VEGFR-2 and MMPs. Based on these findings, intervention with NSBP1 expression may provide a therapeutic approach in ccRCC development and metastasis.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

SQJ supervised research project, participated in the data collection, drafted the manuscript. LY participated in the data collection, supervised ICH. XYZ participated in the data collection. XSL carried out the operation. LQZ carried out the operation, acted as corresponding author and did the revisions. All authors read and approved the final manuscript.

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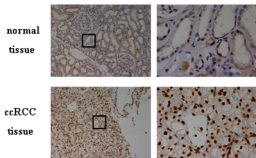
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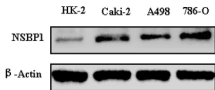
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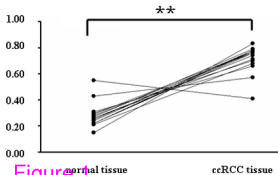
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B.



D.

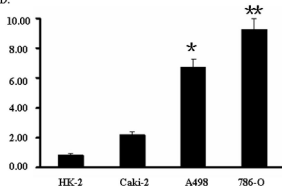


Figure 1

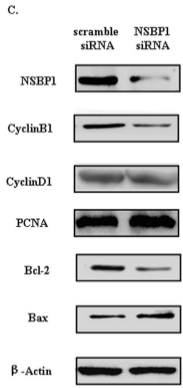
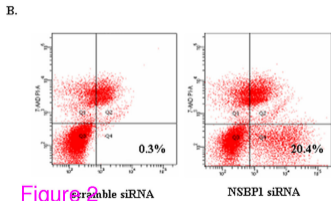
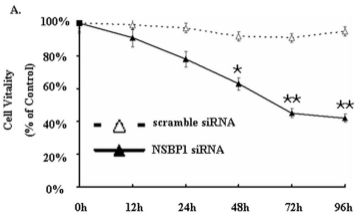
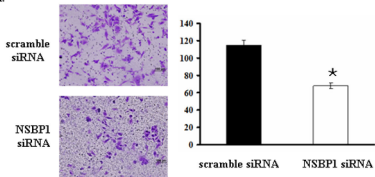
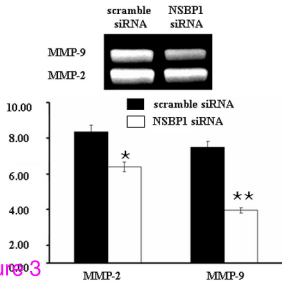


Figure 2

A.



B.



C.

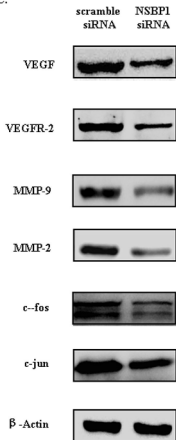
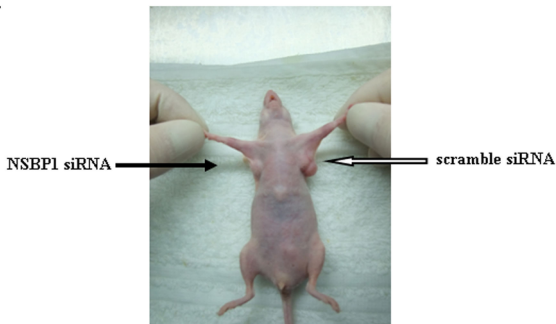


Figure 3

A.



B.

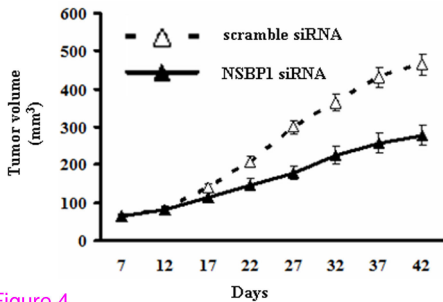


Figure 4