

Original Article

Tumor suppressor gene *NGX6* induces changes in protein expression profiles in colon cancer HT-29 cells

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Nasopharyngeal carcinoma-associated gene 6 (*NGX6*; syn. transmembrane protein 8B, *TMEM8B*) is a recently identified tumor suppressor gene. The underlying mechanisms by which the gene inhibits tumor development are not completely known. To further understand the function of the gene's protein product *NGX6*, in the present study, we employed two-dimensional difference gel electrophoresis to analyze the protein expression profiles of colon cancer HT-29 cells stably transfected with the gene *NGX6*. The differentially expressed proteins were selected and identified by matrix-assisted laser desorption/ionization coupled with time-of-flight tandem mass spectrometry. The results showed that 12 proteins were down-regulated and 4 were up-regulated in *NGX6*-transfected HT-29 cells, compared with vector-transfected HT-29 cells. The MS results were verified by western blot. Bioinformatic analysis showed that these proteins are involved in cell proliferation, metastasis, apoptosis, cytoskeletal structure, metabolism, and signal transduction, suggesting that *NGX6* may inhibit colon cancer through the regulation of these biological processes.

Keywords *NGX6*; colon cancer; differential in-gel electrophoresis; mass spectrometry

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Introduction

Colon cancer is a common malignancy whose incidence and mortality continue to rise every year. The initiation and development of colon cancer involve both the activation of oncogenes and the inactivation of tumor suppressor genes. Nasopharyngeal carcinoma-associated gene 6 (*NGX6*) is a putative tumor suppressor gene that encodes an eponymous 338-amino acid transmembrane protein

containing two trans-membrane structures, an epidermal growth factor (EGF)-like domain and a tyrosine kinase phosphorylation site [1]. Previous studies have shown that *NGX6* is down-regulated in colon cancer tissues. Particularly, the expression of *NGX6* is significantly reduced or absent in colon cancer with metastasis, and the rate of *NGX6* down-regulation is as much as 93.7% [2]. To characterize the tumor suppressor function of *NGX6* in colon cancer, in a previous study, we constructed a colon cell line that over-expresses *NGX6* and found that the protein inhibited the proliferation and invasion of human colorectal cancer cells *in vitro* and *in vivo* [3]. A cDNA array analysis showed that *NGX6* negatively regulates Wnt/beta-catenin- and epidermal growth factor receptor (EGFR)-mediated mitogen-activated protein kinase (MAPK) signaling [3,4].

To provide new clues regarding the mechanisms of *NGX6* function at the protein level, in the present study, we employed difference gel electrophoresis (DIGE) and matrix-assisted laser desorption/ionization coupled with time-of-flight tandem mass spectrometry (MALDI-TOF-MS/MS) to analyze the protein expression profiles of colon cancer HT-29 cells stably transfected with *NGX6*. The differentially expressed proteins were selected and identified and their biological functions were analyzed to elucidate the nature of *NGX6*'s function in tumor suppression. The proteomic study we report here will help identify new targets for the diagnosis, therapy, and prognosis of colon cancer.

Materials and Methods

Materials

The HT-29 cell line was provided by the Cancer Research Institute of Xiangya Medical College. The stable cell lines pcDNA3.1(+)/HT-29 and pcDNA3.1(+)/*NGX6*/HT-29 were established in our lab as described previously [4].

Dulbecco's modified Eagle's medium (DMEM) was purchased from Hyclone (Waltham, USA). The 2D clean-up protein purification, 2D Quant kits, the CyDye™ DIGE fluorescence labeling dye kit, solid phase pH (pH 3–10) ladder dry strip (24 cm), IPG buffer solution (pH 3–10), and strip cover fluid were purchased from GE Healthcare (Pittsburgh, USA). Coomassie Brilliant Blue G-250, acrylamide, *N,N'*-methylene diacrylamide, sodium dodecyl sulfate (SDS), tris(hydroxymethyl)aminomethane (Tris), ammonium persulfate, tetramethylethylenediamine, dithiothreitol (DTT), and iodoacetamide (IAA) were from Amresco (Solon, USA). Trypsin (TPCK modification, Klenow Sequencing), α -cyano-4-hydroxycinnamic acid, dimethylformamide, and trifluoroacetic acid were obtained from Sigma (St Louis, USA).

Sample preparation

Cells were cultured in 75 cm² flasks in DMEM supplemented with 10% fetal bovine serum in a 5% CO₂ atmosphere at 37°C. Three flasks for each cell line were collected and the cells were washed three times with phosphate buffered saline (PBS; 20 mM K-phosphate buffer, pH 7.2, containing 150 mM NaCl), then washed twice with distilled water and twice with tris-sugar for 3 min each. The cells in each flask were extracted with 100 μ l DIGE lysis buffer [7 M urea, 2 M thiourea, 4% 3-(3-cholamidopropyl)]. After lysis for 40 min on ice, the suspension was collected and transferred to a pre-chilled 2-ml microcentrifuge (Eppendorf) tube, and centrifuged for 30 min at 14,000 *g* at 4°C. The supernatant was transferred to a new microcentrifuge tube. The concentration of protein was determined with 2D Quant kit.

Sample labeling

Proteins were labeled with fluorescence dyes Cy2, Cy3, and Cy5 using the DIGE™ kit following the manufacturer's protocol. Fifty microgram protein samples prepared from pcDNA3.1(+)/HT-29 and pcDNA3.1(+)/NGX6/HT-29 cells were labeled with 400 pmole Cy3 dye and 400 pmole Cy5 dye, respectively. At the same time, a mixture of 25 μ g protein from pcDNA3.1(+)/HT-29 cells and 25 μ g protein from pcDNA3.1(+)/NGX6/HT-29 cells was labeled with 400 pmole Cy2 dye as an interior label. The experiments were repeated four times for cross-marked protein samples. Each gel contained the pooled standard and two other samples. The two samples were analyzed in replicates labeled with Cy3 and Cy5 by running four gels. After labeling, the protein samples were placed on ice and incubated for 10 min after the addition of 1 μ l of lysine (10 mM).

Two-dimensional difference gel electrophoresis

Fifty microgram protein samples from pcDNA3.1(+)/HT-29, pcDNA3.1(+)/NGX6/HT-29, and the internal

standard were labeled with the CyDye DIGE Fluor minimal cyanine dyes (GE Healthcare) Cy3, Cy5, and Cy2, respectively, following the manufacturer's protocol. Fifty-microgram aliquots from each of the labeling mixtures were subjected to first-dimension isoelectric focusing. The strips were equilibrated with gentle shaking in two steps of 15 min each, first in equilibration buffer I (1% DTT, 50 mM Tris-HCl, pH 6.8, 6 M urea, 30% glycerol, 2% SDS-bromophenol blue) and then in equilibration buffer II (identical to buffer I except that DTT was replaced with 2.5% IAA). Afterward, the IPG strips were electrophoresed on 12.5% acrylamide gels in the second dimension, with the Amresco-electrophoresis parameter set at 3 W/gel for 30 min and then 16 W/gel. The second-dimension electrophoresis was run for ~6 h until the bromophenol blue reached the gel bottom (gel size is 24 cm). The gels were scanned with a Typhoon 9400 fluorescence scanner (GE Healthcare). DeCyder 2D differential analysis software was used to match and select the differentially expressed proteins (fold change >1.8).

Preparative gel electrophoresis for protein identification

Preparative gel electrophoresis was run according to the procedures described above except that the loading materials were 1 mg protein. After electrophoresis, the gel was fixed in 40% methanol, 10% acetic acid, and 50% H₂O for 30 min and washed with distilled water four times for 15 min each. Then the gel was stained with Colloidal Coomassie Brilliant Blue G (0.12% G250, 20% methanol, 10% phosphoric acid, and 10% ammonium sulfate) overnight. The gel was washed with distilled water for 15 min one time, 30 min twice, and 1 h twice until the background of the gel was clear.

Protein identification by MALDI-TOF-MS/MS

Proteins spots were cut from the gels and destained in 50% acetonitrile and 25 mM ammonium bicarbonate for 30 min. After acetonitrile dehydration and freezing, the spots were incubated with 10 μ l of reducing agent (25 mM NH₄HCO₃, 10 mM DTT) for 1.5 h at 57°C, then incubated with an alkylating agent (25 mM NH₄HCO₃, 55 mM IAA) for 30 min at room temperature. The spots were kept in 0.2 M NH₄HCO₃ for 20 min and then lyophilized. Each spot was digested overnight in 12.5 ng/ml trypsin in 0.1 M NH₄HCO₃. The digested protein was separated and identified by using a MALDI-TOF-TOF MS (UltraFlex I; Bruker Daltonics, Munich, Germany). NCB Inr-human database searches, through Mascot, using combined peptide mass finger (PMF) and MS/MS datasets were performed via Bio Tools 2.2 software (Bruker) with mass tolerance in PMF of 0.3 Da, MS/MS tolerance of 1.0 Da, one

missing cleavage site and cysteines modified by carbamidomethylation. The protein identification was considered to be confident when the protein score of the hit exceeded the threshold significance score of 58 ($P < 0.05$). The first 'hit' protein was selected.

Western blot analysis

Fifty micrograms of total protein were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. The membranes were blocked with 5% (w/v) skimmed milk powder for 1 h at room temperature, then incubated with heat shock protein 90 kDa (HSP90) and guanine nucleotide-binding protein subunit β -2 (GNB2), fatty acid binding protein (FABP) and Annexin II (ANXA2) antibodies (1:1000; Abcam, Cambridge, UK) at 4°C overnight. The membranes were washed by Tris-buffered saline with Tween20 (10 mM Tris-HCl, pH7.5, 150 mM NaCl, 0.1% Tween20) three times for 15 min each. The membranes were then incubated with secondary antibody for 1 h at room temperature. The membrane was developed using an enhanced chemiluminescence kit (Pierce, Rockford, USA) and exposed to X-ray film.

Statistical analysis

SPSS 11.5 software (SPSS Inc., Chicago, USA) was used for Student's *t*-test analysis. All data were shown as mean \pm standard deviation. $P < 0.05$ was considered statistically significant.

Results

Protein expression profiles in pcDNA3.1(+)/NGX6/HT-29 and pcDNA3.1(+)/HT-29 cells

We performed 2D-DIGE analysis to examine the alteration in protein levels between pcDNA3.1(+)/NGX6/HT-29 and pcDNA3.1(+)/HT-29 cells. After 2D fluorescent gel images were obtained via a Typhoon scanner, protein spots were detected, quantified, matched, and analyzed with DeCyder software. The use of the internal standard effectively eliminated gel-to-gel variation, allowing the detection of small differences in protein levels. We compared the differentially expressed proteins in each group, and the results of the matched spots from the different gels were analyzed using Student's *t*-test. We found 16 protein spots differentially expressed (i.e. fold change > 1.8) between pcDNA3.1(+)/NGX6/HT-29 and pcDNA3.1(+)/HT-29 cells (Fig. 1 and Table 1) established the 3D simulation of these differentially expressed proteins (Fig. 2). Close-up images of partial differential expression protein spots were shown in Fig. 3.

Protein identification by MALDI-TOF-TOF MS

The differentially expressed proteins were selected for automated spot picking and 16 of them were identified with

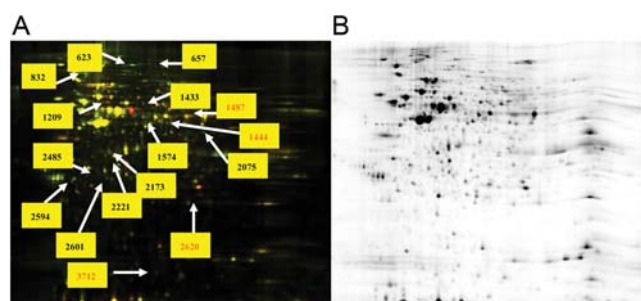


Figure 1 2D-DIGE gel images of proteins from pcDNA3.1(+)/NGX6/HT-29 and pcDNA3.1(+)/HT-29 cells (A) Overlapping image of three different emission filters. (B) 2D map of pcDNA3.1(+)/HT-29 and pcDNA3.1(+)/NGX6/HT-29 labeled with saturation dyes. The numbers present the differentially expressed protein detected and their Master spot No. The red numbers represent up-regulated proteins, and the white numbers represent down-regulated proteins.

confidence by MALDI-TOF-TOF MS (Fig. 4). According to DIGE analysis, compared with the levels in pcDNA3.1(+)/HT-29 cells, four proteins in the pcDNA3.1(+)/NGX6/HT-29 cells were up-regulated, 12 proteins were down-regulated. By bioinformatics analysis, these 16 identified proteins' functions include: metabolic enzymes, proteins related to signal transduction, proteins related to cellular proliferation, metastasis and apoptosis, and cytoskeletal protein (Table 1).

Validation of MS results by western blot analysis

Among the proteins listed in Table 1, HSP90, ANXA2, GNB2, and FABP5 are implicated in carcinogenesis. HSP90 is a responsive protein usually induced by stimuli such as infection, and is involved in the development of cancer cell resistance to chemotherapy and radiotherapy [5]. ANXA2 takes part in a variety of biological processes including migration and invasion, signal transduction, cell proliferation, and apoptosis of colorectal cancer [6]. GNB2 is closely related to tumor metastasis and invasion [7]. FABP5 is involved in the proliferation, metastasis, and apoptosis of various cancer cells [8–10]. To validate the results of MALDI-TOF-MS/MS, the expression of these four proteins was detected by western blot in pcDNA3.1(+)/HT-29 and pcDNA3.1(+)/NGX6/HT-29 cells. The results showed that the expression of HSP90, GNB2, and ANXA2 was down-regulated and the expression of FABP5 was up-regulated in pcDNA3.1(+)/NGX6/HT-29 cells compared with those in pcDNA3.1(+)/HT-29 cells (Fig. 5), which was consistent with the results of MALDI-TOF-MS/MS.

Discussion

Tumorigenesis of colon cancer is a complicated process that involves a wide array of molecules. Previous studies have shown that oncogenes such as K-ras and c-myc, and

Table 1 The identified proteins with differential expression between NGX6/HT-29 and HT-29 cells

Spot	Accession*	Protein	Function	Fold change
623	gi 119591368	NCL, isoform CRA_c	Apoptosis, Pre-rRNA	− 1.96
657	gi 306891	HSP90	Metastasis, apoptosis	− 1.85
832	gi 20149594	HSP90B	Metastasis, apoptosis	− 3.25
1209	gi 109096484	TUBA, ubiquitous isoform 10	Cytoskeleton, apoptosis	− 1.87
1433	gi 48145673	hnRPH1	Metabolism, signal transduction	− 1.97
1484	gi 2183299	ALDH1	Metabolism	+2.25
1487	gi 66361514	G6PD	Metabolism	+3.04
1574	gi 48375173	MAP6 isoform 1	Cytoskeleton	− 1.89
2075	gi 18645167	ANXA2	Cell proliferation, apoptosis	− 1.81
2173	gi 91992949	HCRTR1	Metabolism	− 2.26
2221	gi 13937391	GNB2	Signal transduction	− 2.15
2485	gi 39654881	CLIC1, Chain A	Unknown	− 2.05
2594	gi 83754467	YWHAZ	Signal transduction	− 2.07
2601	gi 29726390	Human phosphoserine phosphatase	Signal transduction	− 2.29
2620	gi 4505753	PGAM1 (brain)	Intermediate metabolism	+1.96
3712	gi 4557581	FABP5	Apoptosis, cell proliferation, metastasis	+1.94

*Accession number refers to the NCB Inr Database.

HSP90B, HSP90-beta; '−' indicates down-regulated; '+' indicates up-regulated.

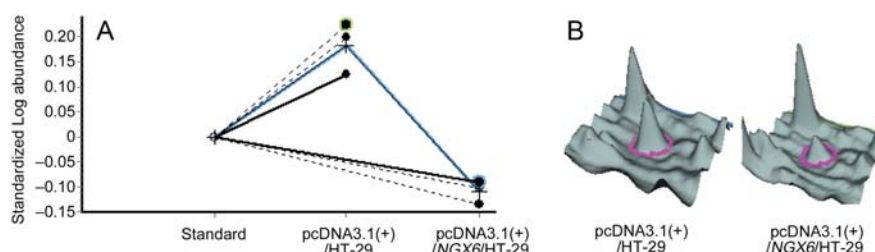


Figure 2 Relative protein abundance and three-dimension graphics of spot 1433 (A) Linear graphics of the spot 1433. (B) Three-dimensional graphics of the spot 1433. The peak marked by the red circle indicated that spot 1433 was down-regulated in pcDNA3.1(+)/NGX6/HT-29 cells compared with the control pcDNA3.1(+)/HT-29 cells.

tumor suppressor genes such as APC, DCC, MCC, p53, and nm 23 are implicated in colon cancer. Recently, *NGX6* was identified as a colon cancer metastasis suppressor gene. However, the mechanisms underlying *NGX6*-mediated tumor suppression are not completely understood. In the current study, we employed a proteomic approach to investigate alterations at the protein level associated with overexpression of *NGX6* in pcDNA3.1(+)/HT-29 colon cancer cells. These results may provide some novel insights into the molecular mechanisms of the tumor suppressor function of *NGX6*.

According to DIGE analysis, 4 up-regulated and 12 down-regulated proteins were identified in the pcDNA3.1(+)/*NGX6*/HT-29 cells when compared with pcDNA3.1(+)/HT-29 cells. These differentially expressed proteins were classified into four groups based on their biological functions: (1) metastasis and apoptosis-related proteins: nucleolin (NCL), HSP90, ANXA2, and FABP5; (2) signal

transduction proteins: GNB2, chloride intracellular channel 1 (CLIC1) Chain A, 14-3-3 protein zeta/delta (YWHAZ), human phosphoserine phosphatase, and heterogeneous nuclear ribonucleoprotein H1 (hnRPH1); (3) cytoskeletal proteins: α -tubulin (TUBA) and microtubule-associated protein 6 (MAP6) isoform 1; and (4) cell metabolism-related enzymes: phosphoglycerate mutase (PGAM1), aldehyde dehydrogenase-1 (ALDH1), glucose-6-phosphate dehydrogenase (G6PD), and hypocretin (orexin) receptor 1 (HCRTR1).

HSP90 is a stress-induced protein that is often up-regulated in cancer [5]. HSP90 indirectly affects the c-Jun N-terminal kinase (JNK) MAPK pathway by maintaining the activation of Raf-1 to regulate the proliferation, differentiation and survival of cancer cells [11–13]. In addition, HSP90 activates P13K/AKT and the target transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (nuclear factor kappa B, NF- κ B) in cancer

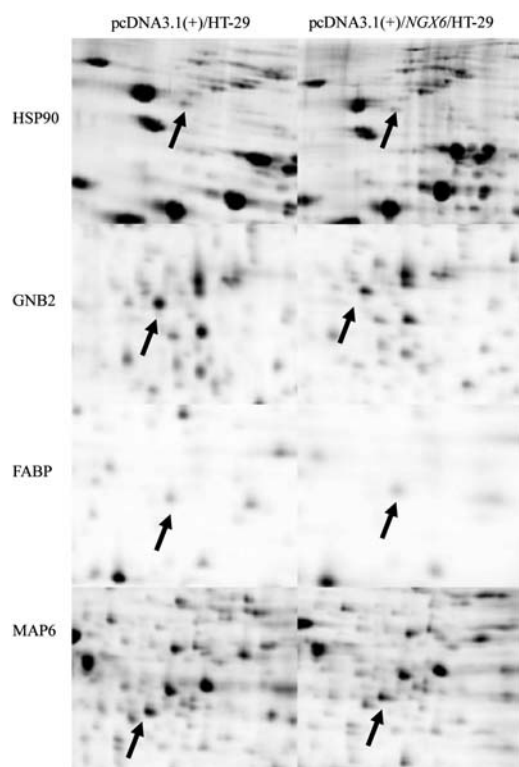


Figure 3 Close-up images of partial differentially expressed protein spots. Arrows indicate spots of interest. HSP90, GNB2, and MAP6 were down-regulated in pcDNA3.1(+)/NGX6/HT-29 compared with the control pcDNA3.1(+)/HT-29 cells; FABP was up-regulated in pcDNA3.1(+)/NGX6/HT-29 cells.

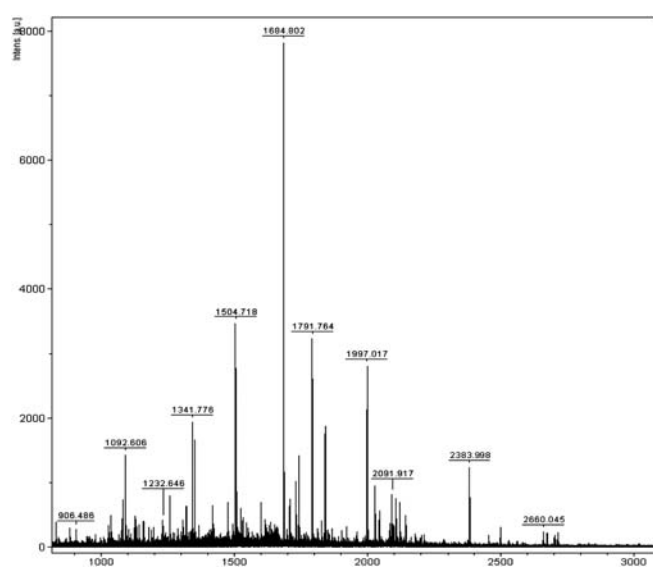


Figure 4 A representative peptide mass fingerprint profile of spot 1433 (hnRPH1). NCBI database was searched according to Mascot scores, the number of matching fragments and coverage. The protein was identified as hnRPH1.

cells [14–16]. Our present results showed that HSP90 protein was down-regulated in NGX6-overexpressing pcDNA3.1(+)/HT-29 cells, which suggests that perhaps

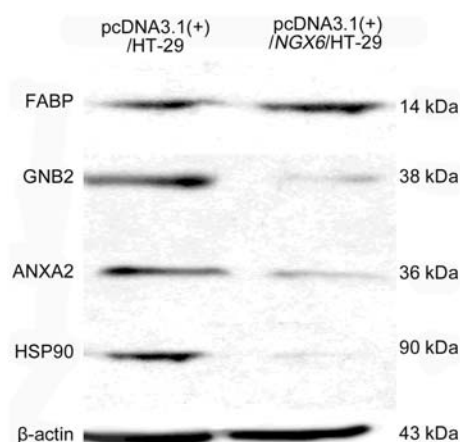


Figure 5 Western blot analysis of proteins differentially expressed in pcDNA3.1(+)/HT-29 and pcDNA3.1(+)/NGX6/HT-29 cells. Western blot showed the down-regulation of HSP90, GNB2, and ANXA2 and the up-regulation of FABP in pcDNA3.1(+)/NGX6/HT-29 cells. β-Actin served as a loading control. Shown were representative blots from three independent experiments with similar results.

NGX6 inhibits the activity of the PI3K/AKT and MAPK pathways in colon cancer cells through HSP90. In our previous studies we used cDNA chips and western blot to demonstrate that NGX6 negatively regulated the EGFR-mediated JNK pathway [17–19]. Combined with the results of the proteomic analysis, these results strongly suggest that negative regulation of the JNK pathway through the activity of the NGX6 gene is the main inhibitory mechanism.

ANXA2 is a calcium-dependent phospholipid-binding protein, which is overexpressed in a variety of human tumor tissues [6]. ANXA2 has been shown to promote colon cancer cell proliferation and invasion by regulation of the MAPK, PI3K/AKT, and NF-κB pathways [20–22]. In the present study, we found that the expression of ANXA2 was low in NGX6-overexpressing colon cancer cells, suggesting that NGX6 may play a role in tumor suppression via the regulation of the MAPK, PI3K/AKT, and NF-κB pathways through ANXA2.

The GNB2 belongs to the G family of proteins. Previous studies demonstrated that GNB2 affected the proliferation and metastasis of cancer cells by activating the MAPK pathway through K-Ras [23,24]. In this study, it was found that GNB2 was down-regulated in NGX6-overexpressing colon cancer, which suggests that NGX6 may inhibit the MAPK pathway by regulating GNB2.

FABP5 is associated with the development and metastasis of a variety of tumors [8–10], and is a peroxisome proliferator-activated receptor (PPAR-γ) ligand [25]. The activation of PPAR-γ can induce increases in protein phosphatase 2 (PP2), and then negatively regulate MAPK, ERK, and other signaling pathways in controlling the proliferation and metastasis of tumor cells. Reduced expression of FABP5 has been reported in colon cancer [26]. In this

study, we found that *NGX6* increased FABP5 expression in pcDNA3.1(+)/HT-29 cells, indicating that *NGX6* may inhibit the proliferation and metastasis of colon cancer by up-regulating FABP5 and the MPAK and NF- κ B pathways.

ALDH1 is a cytosolic enzyme catalyzing intracellular oxidation of acetaldehyde to acetic acid, and has been proposed as one of the general markers of normal stem cells and cancer stem cells [27]. A recent proteomics study showed that ALDH1 was down-regulated in lymph node metastasis of colon carcinoma cells [28]. Our experiments showed that ALDH1 is significantly up-regulated in colon cancer cells transfected with *NGX6*, suggesting that *NGX6* may up-regulate ALDH1 expression to inhibit oncogenesis and metastasis.

The hnRPH1 is a group of RNA-binding proteins with a range of key cellular functions, which are dysregulated in tumorigenesis including regulation of translational and RNA processing. A recent study demonstrated the significant difference in hnRPH1 immunoreactivity between primary colon tumors and matched lymph node metastasis [29], suggesting that hnRPH1 is closely related to colon cancer development and metastasis. In our experiment, it was found that *NGX6* could down-regulate the expression of hnRPH1, which may inhibit colon cancer cell proliferation, invasion and metastasis.

Tumorigenesis often involves the altered activation of multiple signal pathways and these pathways form a gene regulation network of crosstalk between them. In this study, we identified a diversity of differentially expressed proteins by a proteomic approach in *NGX6*-overexpressing colon cancer cells. This suggests that *NGX6* modulates multiple signaling pathways, such as MAPK, PI3K/AKT, and NF- κ B, to execute its tumor suppressing function (Fig. 6). Although further studies, as well as a survey of a large volume of

clinical colon cancer samples, are needed to verify the clinical relevance of the proteins we identified, this study provides significant clues toward singling out new targets for the diagnosis, therapy, and prognosis of colon cancer.

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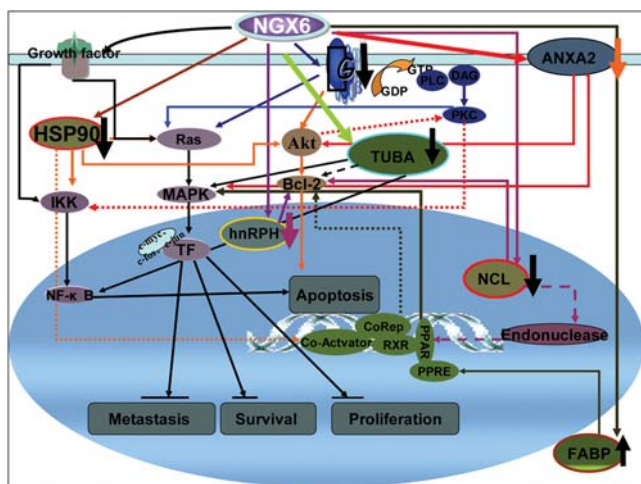


Figure 6 Proposed signaling model downstream of *NGX6* Solid lines represent the downstream molecules that are regulated directly by the upstream molecules. The dashed lines represent indirect regulation.

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