N-Myc induces the tumor progression of prostate cancer by regulating FSCN1

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Abstract. The oncoprotein N-Myc has a carcinogenic effect in numerous types of cancer, and it can cause castration resistance in prostate cancer (PCa), and leads to the development of small cell neuroendocrine cancer by regulating multiple target genes. Immunohistochemical staining, RT-qPCR, western blotting, wound healing and CCK-8 assays were used to detect the expression of N-Myc and FSCN1 as well as AR and CgA at the human level and cell level. The immunohistochemical results revealed that the protein levels of N-Myc proto-oncogene protein (N-Myc) and fascin (FSCN1) in PCa were significantly higher than that of hyperplastic tissues ($P<0.05$), and there was a weak correlation between them ($P=0.002$). *In vitro*, N-Myc and FSCN1 were overexpressed in LNCaP and C4-2 cell lines. The results revealed the promoting effect of N-Myc and FSCN1 on malignant progression of PCa. In addition, the endogenous FSCN1 was knocked down in the C4-2 cell line, and the results revealed that the silencing of FSCN1 enhanced the expression of N-Myc and weakened the expression of the neuroendocrine marker CgA. Therefore, the present findings indicated that N-Myc may promote the malignant process of PCa by regulating FSCN1 and FSCN1 may have a reverse regulatory effect on N-Myc.

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Introduction

Prostate cancer (PCa) is one of the most common malignancies of the urinary system among men. According to estimates in the literature, the incidence of prostate cancer accounts for 20% of all male cases, and the mortality rate accounts for 10%, second only to lung cancer (1). Although the incidence and mortality rates of PCa in China are considerably lower than those in Western countries, the incidence and mortality rate of early-stage PCa in Chinese males are increasing annually, due to the gradual westernization of the lifestyles of Chinese people (2). The standard treatment for PCa is hormone therapy, however, castration resistance is often developed following androgen deprivation therapy (ADT). At present, the molecular mechanism of PCa resistance remains elusive.

The most effective treatment for early localized non-metastatic PCa, known as androgen-dependent PCa (ADPC), is surgical castration with ADT, which inhibits androgen production or blocks the function of androgen receptor (AR) (3). However, after 18-24 months of treatment (4), the therapeutic effect of ADT gradually declines, with almost all patients eventually progressing and developing hormone resistance or castration-resistant PCa (CRPC) (5), followed by advanced fatal PCa. Prostate neuroendocrine carcinoma is a subtype of invasive CRPC with a high degree of malignancy and low survival rates (6). Most evidence has revealed that neuroendocrine PCa (NEPC) is becoming resistant to ADT treatment.

NEPC exhibits 50% ERG rearrangement (7) and 63% PTEN (8), similar to CRPC (9). In addition, the cellular variability of NEPC is associated with the reduced or absent expression of AR and AR downstream genes, such as prostate-specific antigen (PSA). NEPC can express NSE, chromogranin A (CgA), SYP and other neuroendocrine markers associated with certain genomic alterations, including RB1 (8) and TP53 deletions and mutations, and certain specific pathway disorders involving neurons, stem cell programs, and EMT (10).

N-Myc proto-oncogene protein (N-Myc) is a member of the Myc family of transcription factors. The high expression of N-Myc can lead to uncontrolled proliferation, affect cell metabolism, and promote cell invasion, apoptosis, and differentiation (11). N-Myc expression disorders are associated with the development of a variety of tumors, including central

nervous system tumors, such as neuroblastoma (12), medulloblastoma (13,14) and pleomorphic glioma (15,16), as well as pancreatic (17) and other types of cancer. N-Myc overexpression has been revealed to drive NEPC tumorigenesis and we most recently demonstrated that N-Myc can regulate an miRNA/ATM pathway to promote the progression of PCa (18).

Fascin (FSCN1) is an actin-binding protein that participates in cytoskeleton regulation and forms filamentous pseudopods to initiate cell movement and migration (19). The absence of FSCN1 protein expression in most adult epithelial cells, including lung epithelial cells (20), suggests that it is optional for normal physiology and metabolism in untransformed epithelia. However, FSCN1 overexpression has been revealed to play an important role in tumorigenesis (21), metastasis (22) and cancer stemness (23). Notably, metastatic cancer cells markedly increase the expression of FSCN1 (24-26).

In the present study, it was revealed that the expression of N-Myc and FSCN1 was considerably higher in PCa tissues than that in benign prostatic hyperplasia (BPH) tissues. The expression of N-Myc and FSCN1 in PCa cells was also examined, and it was found that N-Myc can upregulate FSCN1 expression in PCa cells. Furthermore, it was demonstrated that FSCN1 mediated, at least partially, N-Myc-induced aggressive phenotypes of PCa, including proliferation, migration and neuroendocrine differentiation.

Materials and methods

Collection of clinical samples. A total of 95 PCa tissue samples and 64 BPH tissue samples were collected from patients who had not received endocrine therapy between January 2015 and December 2016 at the First Affiliated Hospital of Anhui Medical University with the consent of all participants and approval by the Biomedical Ethics Committee of Anhui Medical University (approval no. 20170209). This study retrospectively collected clinical data, such as age, PSA levels, TNM clinical staging, Gleason scores, and metastatic status from the medical records of patients. PCa samples can be divided into three groups in accordance with the Gleason score. To determine the variation in adenocarcinoma structure in different regions of the same tumor, the scores of primary and secondary differentiation were scored separately, and the total values of the two parts were calculated. By defining different output criteria, we classified Gleason scores of >7 as poorly differentiated. Other scores were divided into middle and high differentiation. TNM clinical staging was performed in accordance with the AJCC clinical staging method (8th edition). The present study was approved by the institutional review board of Anhui Medical University.

Cell culture and cell lines. Human LNCaP cells with ADPC characteristics were obtained from the Cell Bank of the Chinese Academy of Sciences. C4-2 cells with CRPC characteristics and PC3 cells with NEPC characteristics were provided by the Institute of Urology of the First Affiliated Hospital of Anhui Medical University. All cells were cultured in RPMI-1640 medium (cat. no. SH30809.01; HyClone; Cytiva) supplemented with 10% fetal bovine serum (cat. no. 04-001-01A; Biological Industries) and 1% penicillin/streptomycin (cat. no. C0222; Beyotime Institute of Biotechnology). All cells were incubated at 37˚C in a 5% CO_2 and 95% air-humidified atmosphere. N-Myc and FSCN1 were overexpressed in LNCaP and C4-2 cell lines. In addition, FSCN1 was knocked down using FSCN1/short hairpin RNA (shRNA)s in cell lines with N-Myc overexpression.

Overexpression with lentiviral transfection. The CONSITE database was used for transcription factor binding site analysis (27). The FSCN1 promoter gene sequence was used as the analyzing template, and the cutoff value of the transcription factor was set to 88%, and it was revealed that the N-Myc binding site was indeed present in the promoter region of FSCN1. Thus, we further explored the relationship between the two genes. LNcap and C4-2 cell lines stably expressing N-Myc, FSCN1, no-load control and blank control were constructed with lentiviral transfection in both LNCap and C4-2 cell lines. LNCap cells were cultured in a 6-cm dish to 80-90% confluence, then the cells were diluted to $1x10^5$ cells/ml. Subsequently, $5x10^4$ cells/well were inoculated into a 96-well plate, mixed and placed at 37˚C and 5% CO_2 culture for 24 h. The lentiviral stock solution was diluted 1:10, the culture solution was discarded in each well, 100 μl of diluted virus solution was added, and a blank control group was set concurrently, and the culture was continued for 24 h. Then, the virus solution was removed from each well and 100 μl RPMI-1640 medium was added to continue culturing for 72 or 96 h. The lentiviral infection efficiency was observed under a microscope (white light and fluorescence; magnification, x100).

Design short hairpin RNA to interfere with FSCN1 expression. Plasmid-encoded short hairpin RNA (shRNA-FSCN1) and shRNA-control were provided by Shanghai GenePharma Co., Ltd. When these cells reached 50% confluence in 6-multiwell plates, they were transfected using a shRNA targeting the FSCN1 (Shanghai GenePharma Co., Ltd.) and 8 μl Lipofectamine 2000 (Thermo Fisher Scientific, Inc.) in Opti-MEM medium (Thermo Fisher Scientific, Inc.), and the serum medium was replaced after 4-6 h. The sequences of shRNA-FSCN1 and shRNA-control were designed as follows: 5'-CTCAGAGCTCTTCCTCATGAA-3' and 5'-TTCTCCGAA CGTGTCACGT-3'. The efficiency of FSCN1-knockdown was analyzed by RT-qPCR and western blotting.

Immunohistochemistry (IHC). Paraffin tissues from benign prostate hyperplasic and PCa tissues were processed into 4 μm-thick tissue sections. Following deparaffinization and rehydration with xylene through graded ethanol, the sections were boiled in 10 mM citrate buffer (pH 6.0) for 10 min for heat-induced antigen retrieval and blocked with 3% H_2O_2 for 10 min to block endogenous peroxidase activity. Sections were incubated overnight with antibodies against the intracellular domain of N-Myc (cat. no. 51705S; 1:640; Cell Signaling Technology, Inc.) or FSCN1 (cat. no. ab126772; 1:200; Abcam). N-Myc-positive staining was localized to the nucleus and/or cytoplasm, and FSCN1-positive staining was localized to the cytoplasm and was positive for pale yellow to tan particles with diffuse homogeneity. Neuroblastoma tissue was used as a positive control of N-Myc, Hodgkin lymphoma tissue was used as a positive control of FSCN1, PBS was used as a negative control

instead of a primary antibody, and tissues were visualized by DAB staining. The paraffin sections were observed under the microscope by two pathologists, who have worked at The First Affiliated Hospital of Anhui Medical University for numerous years, to be scored and graded. N-Myc staining was located in the nucleus or nucleus/cytoplasm, and FSCN1 staining was located in the cytoplasm, which had brown particles. The evaluation of immunohistochemical staining results was based on the proportion of positive cells in the total number of cells and the staining intensity score. The definition of the immunohistochemical positive expression of N-Myc and FSCN-1 was as follows: i) According to the score of dyeing intensity, 0 points for no coloring, 1 point for light yellow, 2 points for brown and 3 points for tan; ii) according to the percentage of positive cells, a score of ≤25% was 1 point, 26-50% 2 points and >50% 3 points; iii) after multiplying the two results into the final result score, scores of ≤6 were placed into the low-expression group and scores of >6 into the high-expression group.

Cell proliferation assay. After C4-2/Vector, C4-2/N-Myc, C4-2/N-Myc/shFSCN1 and C4-2/FSCN1 cells were grown to the logarithmic growth phase, 3,000 cells were isolated and plated into three 96-well plates. Concurrently, duplicate wells and blank control wells were set, and culture plates were placed at 37˚C and 5% CO_2 culture for 24, 48 and 72 h, and then 10 μl CCK-8 (cat. no. BB-4202-500T; BestBio) solution was added to each well, and incubation was continued in the incubator for 1 h. Finally, the absorbance of each well was measured at a wavelength of 450 nm using a microplate reader, and the OD value was read. All cell proliferation assays were repeated in triplicate.

Wound healing assay. In total, $5x10^5$ C4-2/Vector, C4-2/N-Myc and C4-2/FSCN1 cells were inoculated into 6-well plates, and cultured to 80-90% fusion in a 37˚C incubator. The following day, two parallel lines were drawn in each cell-containing well with a 10-μl pipette tip. The cells were rinsed with PBS solution to remove the non-migrated cells, serum-free medium was added, and incubation continued in a 37˚C and 5% CO_2 incubator. Finally, the cells were observed and images were captured with an inverted microscope (magnification, x10) at 0, 24, 48 and 72 h.

RT-qPCR. Total RNA was extracted using TRIzol reagent (Thermo Fisher Scientific, Inc.). Next, RNA concentration and purity were determined using an ultramicronucleic acid protein detector. The amount of RNA required in the reverse transcription reaction system using cDNA Synthesis Kit (Takara Bio, Inc.) was calculated on the basis of the measured RNA concentration, and RT was performed to obtain cDNA in a final volume of 20 μl. An aliquot of the resulting cDNA (1 μl) was diluted at 1:5 and used for qPCR assays performed in 20-μl reactions containing 10 μl 2X SYBR Premix Ex Taq II (Takara Bio, Inc.). Assays were performed in triplicate, and control qPCR reactions with GAPDH as a reference for normalization were included. Default amplification conditions of the ABI 7500 Fast Real-Time PCR System were used. The comparative Cq method ($2^{\Delta\Delta Cq}$) method was used for expression analysis (28). The experiment was repeated twice (n=2). The RT-qPCR thermocycling conditions were as follows: Initial denaturation (95˚C for 30 sec), 40 cycles of denaturation (95˚C for 5 sec) and annealing (60˚C for 34 sec). The primers used in the present study were as follows: GAPDH forward, 5'-CATGAGAAGTATGACAACAGCCT-3' and reverse, 5'-AGTCCTTCCACGATACCAAAGT-3'; N-Myc forward, 5'CACGTCCGCTCAAGAGTGTC-3' and reverse, 5'-GTTTCTGCGACGCTCACTGT-3'; and FSCN1 forward, 5'-GGGGAGCATGGCTTCATC-3' and reverse, 5'-TGCCCACCGTCCAGTATTT-3'.

Western blot analysis. Total protein was extracted from PCa cell lines using RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA) and 1% phenylmethanesulfonyl fluoride, and the protein concentration was determined by BCA assay (Beyotime Institute of Biotechnology). Equal amounts of protein lysate (20 μg per lane) were loaded onto 10% polyacrylamide gels and subsequently transferred to nitrocellulose membranes (GE Healthcare; Cytiva). Then, the membranes were blocked with 5% skimmed milk powder and sealed at room temperature for 1 h. Subsequently, the membranes were incubated with primary antibodies at 4˚C overnight and with the secondary antibodies at room temperature for 1 h. Finally, the results were visualized using an ECL blot analysis system (Bioshine ChemiQ series; Bioshine). Immunoblotting was performed using the following antibodies: Anti-N-Myc (product no. 51705S; 1:1,000) and anti-AR (product no. 5153S; 1:2,000) from CST Signaling Technology, Inc., anti-GAPDH (cat. no. 10494-1-AP; 1:1,000) and anti-CgA (cat. no. 60135-1-Ig; 1:2,000) both from ProteinTech Group Inc., and anti-FSCN1 (cat. no. ab126772; 1:10,000) from Abcam. Goat anti-mouse secondary antibody (cat. no. A0216; 1:5,000) and goat anti-rabbit secondary antibody (cat. no. A0208; 1:5,000) were purchased from Beyotime Institute of Biotechnology.

Statistical analysis. Data were subjected to statistical analysis using SPSS 16.0 software (SPSS Inc.). The difference between the two groups of qualitative data was compared through χ^2 test. Quantitative data were compared using paired t-test. Spearman's correlation was performed to assess the correlation between N-Myc and FSCN1 expression in PCa tissues. ANOVA was used to compare multiple groups. Dunnett's post hoc test was used for variance analysis when the variance was uneven, and Bonferroni was used for variance analysis when the variance was uniform. In all cases, $P<0.05$ was considered to indicate a statistically significant difference (*, **, *** and **** symbols indicated the significance of 0.05, 0.01, 0.001 and 0,0001 levels, respectively, as indicated in the figures and legends.

Results

N-Myc and FSCN1 expression is positively correlated and associated with tissue type and cancer progression. Tissues were divided into benign prostatic hyperplasia, PCa without bone metastasis and PCa with bone metastasis, and the differences and clinical significance of the three groups were analyzed through IHC analysis of the FFPE sections. For the first time, we focused on the correlation between N-Myc and FSCN1 in clinical prostate samples. IHC results indicated that N-Myc and FSCN1 expression levels were higher in PCa than

Table I. The expression of N-Myc, FSCN1 in prostate clinical samples.

		N-Myc			FSCN1		
Group	n	Low, n (%)	High, n (%)	P-value	Low, n (%)	High, n (%)	P-value
Benign prostate	64	56 (78.5)	8 (12.5)	0.035[a]	57 (89.06)	7 (10.94)	<0.001[b]
PCa	95	70 (73.68)	25 (26.32)		58 (61.05)	37 (38.95)	

[a]P<0.05, [b]P<0.001. N-Myc, N-Myc proto-oncogene protein; FSCN1, fascin.

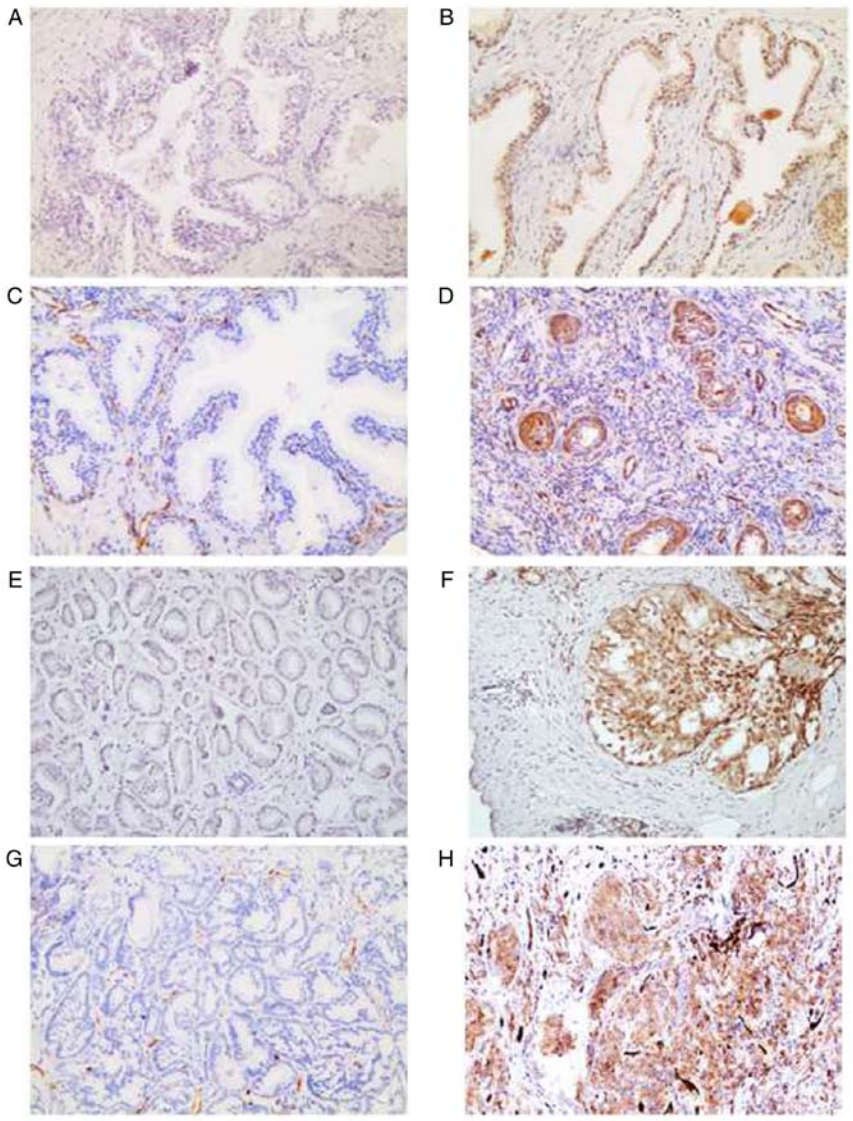

Figure 1. Expression of N-Myc and FSCN1 in prostate tissues. (A) N-Myc is negative in prostatic hyperplasia tissues. (B) N-Myc is positive in prostatic hyperplasia tissues. (C) FSCN1 is negative in prostatic hyperplasia tissues. (D) FSCN1 is positive in prostatic hyperplasia tissues. (E) N-Myc is negative in PCa tissues. (F) N-Myc is positive in PCa tissues. (G) FSCN1 is negative in PCa tissues. (H) FSCN1 is positive in PCa tissues. N-Myc, N-Myc proto-oncogene protein; FSCN1, fascin; PCa, prostate cancer.

in benign tissues (Fig. 1, Table I). IHC was used to analyze the expression of clinical samples in detail. It was found that the high expression of N-Myc was associated with Gleason score, TNM stage and bone metastasis. The high expression of FSCN1 was associated with Gleason score and bone metastasis (Table II).

Notably, the expression of N-Myc and FSCN1 in PCa has been reported separately, however the link between the two has not been reported. The present findings suggested that the expression of N-Myc and FSCN1 were weakly positively correlated (Table III), and had a mutual regulation and interact to promote the clinical progression of PCa. The specific

Table II. Association between the expression of N-Myc, FSCN1 and the clinicopathological features of prostate cancer patients.

Characteristics	n	N-Myc expression			FSCN1 expression		
		Low, n (%)	High, n (%)	P-value	Low, n (%)	High, n (%)	P-value
Age, years							
≤69	48	33 (68.75)	15 (31.25)	0.270	33 (68.75)	15 (31.25)	0.120
>69	47	37 (78.72)	10 (21.28)		25 (53.19)	22 (46.81)	
PSA at initial diagnosis (mg/l)							
<20	31	23 (74.19)	8 (25.80)	0.937	20 (64.52)	11 (35.48)	0.630
≥20	64	47 (73.43)	17 (26.56)		38 (59.38)	26 (40.63)	
Gleason score							
≤7	42	36 (85.71)	6 (14.29)	0.018[a]	33 (78.57)	9 (21.43)	0.002[b]
>7	53	34 (64.15)	19 (35.85)		25 (47.17)	28 (52.83)	
TNM stage							
I-II	43	37 (86.05)	6 (13.95)	0.013[a]	30 (69.77)	13 (30.23)	0.113
III-IV	52	33 (63.46)	19 (36.54)		28 (53.85)	24 (46.15)	
Osseous metastasis							
No	74	62 (83.78)	12 (16.22)	<0.001[c]	51 (68.92)	23 (31.08)	0.003[b]
Yes	21	8 (38.10)	13 (61.90)		7 (33.33)	14 (66.67)	

[a]P<0.05, [b]P<0.01, [c]P<0.001. N-Myc, N-Myc proto-oncogene protein; FSCN1, fascin.

Table III. Correlation between the expression of N-Myc and FSCN1 in prostate cancer tissues.

N-Myc	FSCN1		n	r_s	P-value
	High	Low			
High	16	9	25	0.307	0.002[a]
Low	21	49	70		
n	37	58	95		

[a]P<0.01. N-Myc, N-Myc proto-oncogene protein; FSCN1, fascin.

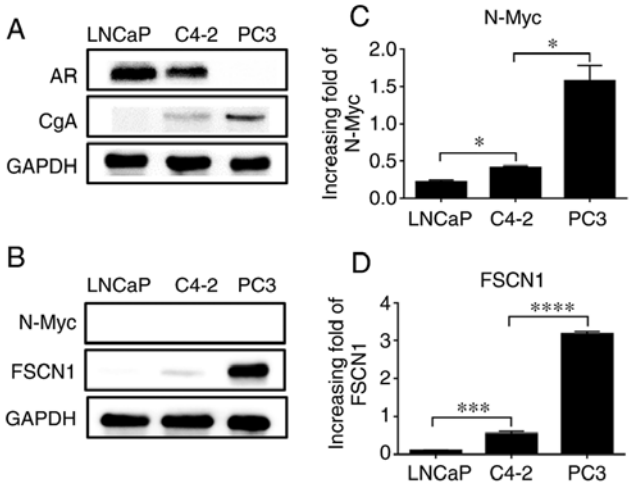

Figure 2. Expression of neuroendocrine phenotype, and N-Myc and FSCN1 in PCa cells. (A) Protein expression of AR and CgA in PCa cells. (B) Protein expression of N-Myc and FSCN1 in PCa cells. (C) mRNA expression of N-Myc in PCa cells. (D) mRNA expression of FSCN1 in PCa cells. N-Myc, N-Myc proto-oncogene protein; FSCN1, fascin; PCa, prostate cancer; AR, androgen receptor; CgA, chromogranin A.

mechanism, however, requires further study. In addition, we also attempted to study the binding site of N-Myc and FSCN1, but suitable results were not obtained, and therefore the data is not shown. In addition, the weak correlation between N-Myc and FSCN1 may be related to insufficient sample size.

Expression of AR, CgA, N-Myc and FSCN1 in PCa cell lines. The development of PCa can usually be divided into three stages: ADPC, CRPC and neuroendocrine PCa NEPC. The LNCaP, C4-2 and PC3 cells studied herein were from these three stages, respectively.

For *in vitro* studies, the protein expression of AR and CgA was measured in LNCaP, C4-2 and PC3 cell lines, and different hormone levels were revealed among them. Western blot analysis revealed that the expression of AR in LNCaP and C4-2 cells was significantly higher than that in PC3 cells, whereas the expression of neuroendocrine marker CgA was lower than that in PC3 cells (Fig. 2A); (the effect on AR and CgA expression at the mRNA level is not shown). This result indicated that PC3 cells have higher neuroendocrine characteristics and a higher malignancy. Next, we assessed the protein expression of N-Myc, which was not detected, but FSCN1 protein expression was detected (Fig. 2B). The reason that N-Myc was not detected may be that the expression of N-Myc in PCa cells is relatively low. At the mRNA level, the expression of N-Myc and FSCN1 were detected in all three cell lines, and it was found to gradually increase with the increase in cell malignancy; the difference was statistically significant (Fig. 2C and D).

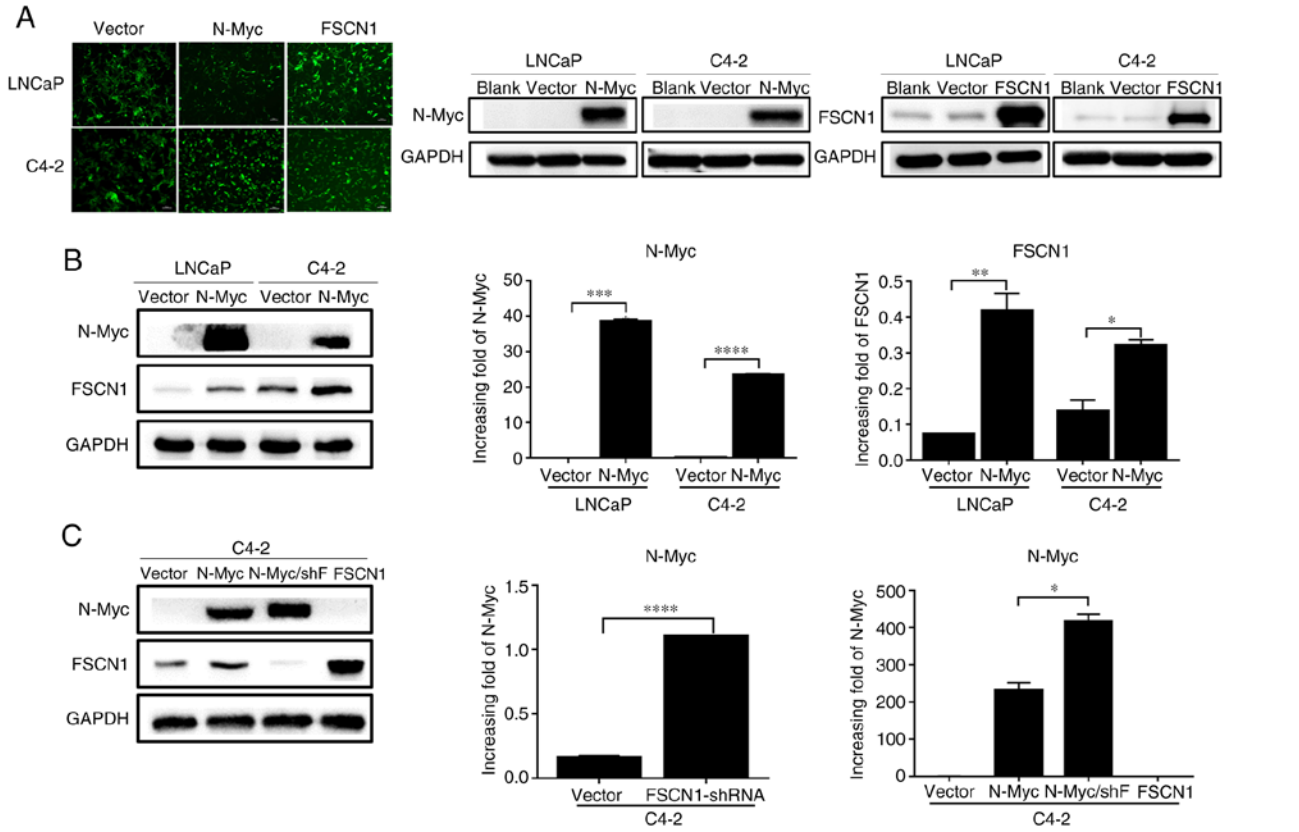

Figure 3. Detection of PCa cells following lentivirus overexpression of N-Myc and FSCN1. (A) Images of the stable LNCaP/Vector, LNCaP/N-Myc, LNCaP/FSCN1, C4-2/Vector, C4-2/N-Myc and C4-2/FSCN1 cell strains under a fluorescence microscope (x100), accompanied by western blot analysis. (B) Protein and mRNA expression of N-Myc and FSCN1 in LNCaP and C4-2 cell lines following N-Myc-overexpression. (C) Protein and mRNA expression of N-Myc and in stable C4-2 transfectants. All experiments were repeated in triplicate. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001. PCa, prostate cancer; N-Myc proto-oncogene protein; FSCN1, fascin; shF, short hairpin RNA FSCN1.

N-Myc overexpression upregulates FSCN1 expression in PCa cells. LNCaP and C4-2 cell lines were selected to construct 4 stable cell lines. Cell lines stably expressing N-Myc, FSCN1 and no-load control were constructed with lentiviral transfection in LNCaP, and C4-2 cell lines with green fluorescence accompanied by western blot analysis (Fig. 3A). Following N-Myc overexpression in PCa cells, increased expression of FSCN1 in the overexpressed group was detected at the protein and mRNA levels (Fig. 3B). The expression of FSCN1 was knocked down with an interference plasmid, and the expression of N-Myc was revealed to be increased in C4-2 cells (Fig. 3C). These results indicated that there was a direct or indirect regulatory relationship between N-Myc and FSCN1, and the binding site of N-Myc to FSCN1 was predicted through the NCBI website, and it was revealed that the N-Myc binding site was indeed present in the promoter region of FSCN1. These mechanisms require further study and discussion (the binding site of N-Myc and FSCN1 is not presented).

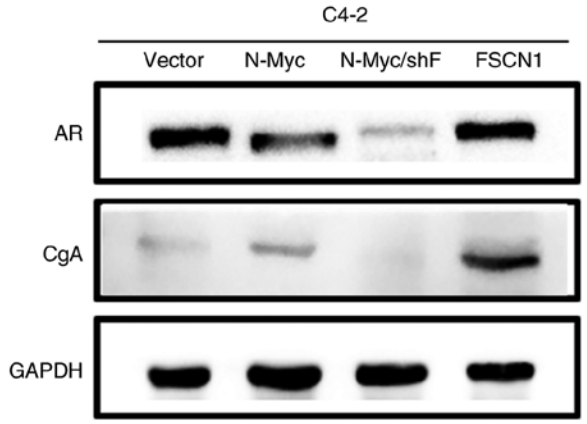

Figure 4. Expression of neuroendocrine phenotype AR and CgA in C4-2 cell lines. All experiments were repeated in triplicate. AR, androgen receptor; CgA, chromogranin A; N-Myc proto-oncogene protein; FSCN1, fascin; shF, short hairpin RNA FSCN1.

Effects of N-Myc and FSCN1 on neuroendocrine markers in PCa cells. The overexpression of N-Myc in C4-2 cells could reduce the expression of AR in PCa cells and promote that of CgA. The overexpression of FSCN1 could increase the expression of neuroendocrine marker CgA. The expression of AR and CgA was reduced following FSCN1 knockdown in C4-2/N-Myc cells (Fig. 4).

FSCN1 mediates N-Myc-induced proliferation and migration in C4-2 cells. The present study revealed that, over time, the proliferation rate of C4-2/N-Myc, C4-2/N-Myc/shFSCN1 and C4-2/FSCN1 was significantly faster than that of the C4-2/Vector control group, and the statistical results revealed that the difference in the proliferation rate was statistically significant over time (Fig. 5A).

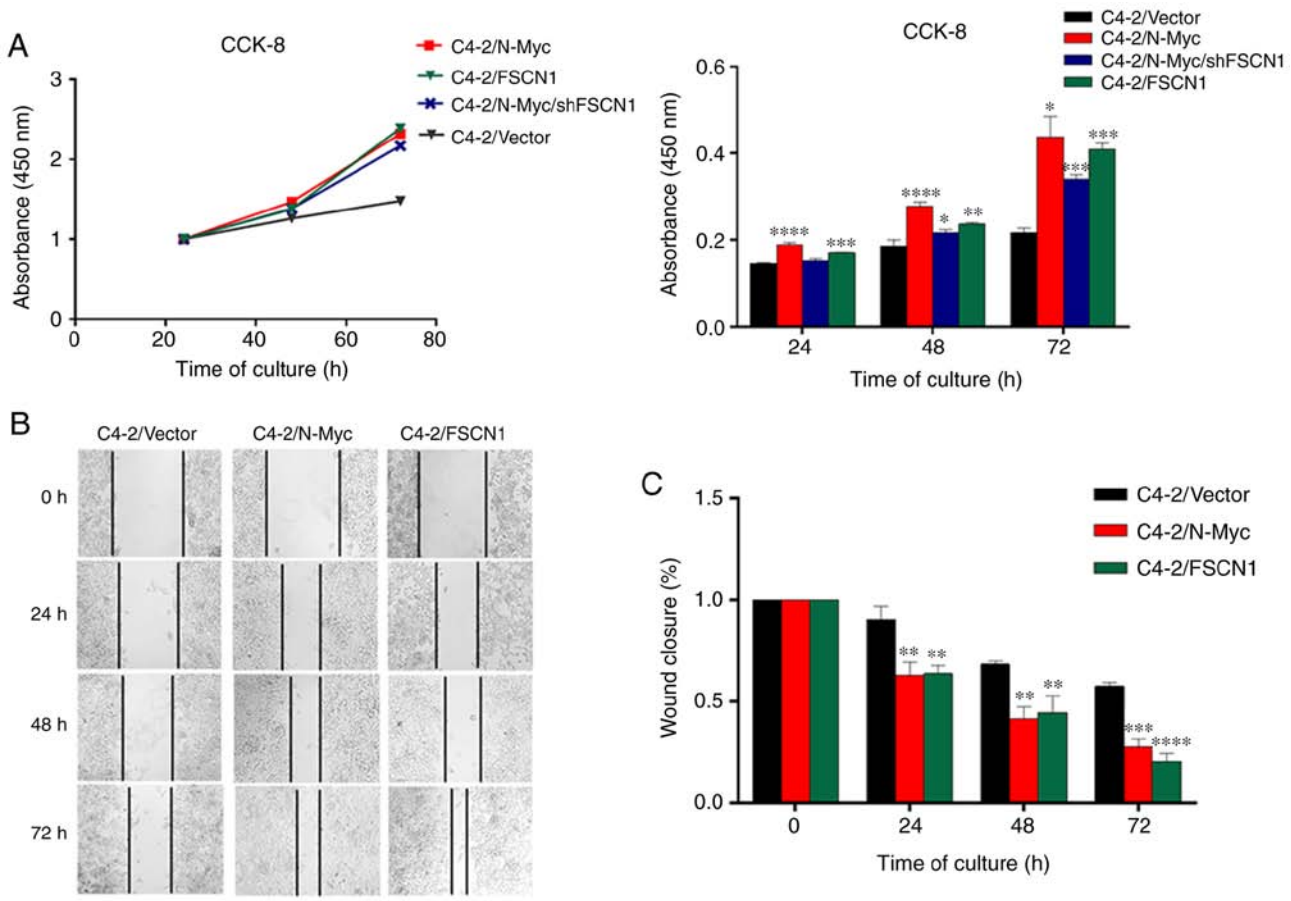

Figure 5. Effects of N-Myc and FSCN1 on the proliferation and migration ability of PCa cells. (A) Effect of N-Myc and FSCN1 on the proliferation of LNCaP and C4-2 cells, as compared with that of the C4-2/Vector control group. (B and C) Effect of N-Myc and FSCN1 on the migration ability of LNCaP and C4-2 cells, as compared with that of the control group. All experiments were repeated in triplicate. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001. PCa, prostate cancer; N-Myc, proto-oncogene protein; FSCN1, fascin; shF, short hairpin RNA FSCN1.

Effects of N-Myc and FSCN1 on the migration ability of PCa cells. C4-2/Vector, C4-2/N-Myc and C4-2/FSCN1 were inoculated in a 6-well plate to assess the migration ability of the 3 cell lines through a cell scratch test. The results revealed that the migration ability of C4-2 cells overexpressing N-Myc and FSCN1 was stronger than that of the control group (Fig. 5B), suggesting that N-Myc and FSCN1 can enhance the migration ability of C4-2 cells. Statistical analysis revealed that the difference in the migration ability was statistically significant (Fig. 5C). There was a limitation to this experiment. We also interfered with the expression of FSCN1 in C4-2/N-Myc cells, but these cells underwent apoptosis during migration and no available experimental results were obtained.

Discussion

N-Myc is found in ~40% of NEPC (29) and up to 20% of CRPC (30); however, studies on PCa are scarce, and its specific mechanism of action remains unclear. The results obtained by Dardenne *et al* (30) and Lee *et al* (31) in 2016 suggested that N-Myc is a driving gene for neuroendocrine PCa. The overexpression of N-Myc can lead to the neuroendocrine phenotype while lacking AR expression and castration resistance in PCa. N-Myc may be a potential therapeutic target for PCa. Yu *et al* confirmed that N-Myc promotes the progression of PCa and resistance to hormone therapy through the N-Myc/miR-421/ATM pathway (18).

Few studies have reported that N-Myc and FSCN1 are highly expressed in a variety of malignancies (32,33) and closely associated with the occurrence and development of tumors. N-Myc is amplified in NEPC (29) and CRPC (30). Lee *et al* (31) demonstrated that N-Myc can drive prostate cell carcinogenesis and lead to neuroendocrine transformation. Therefore, the amplification of N-Myc can affect the metabolic function of tumor cells and promote tumor cell proliferation and further tumor development.

Tumor cell movement is an important marker of invasion and metastasis, and FSCN1 is a factor that promotes tumor cell adhesion, as well as invasion and migration (19). FSCN1 plays a metabolic role in the metastatic settlement of non-small cell lung cancer (34).

Dardenne *et al* (30) revealed that in the PCa LNCaP and 22RV1 cell lines, the upregulation of N-Myc could also promote FSCN1 upregulation. The mechanism through which N-Myc causes PCa progression is unclear. The aforementioned studies revealed that N-Myc has a regulatory effect on FSCN1. The results of testing were further verified, and it was found that N-Myc may promote the malignant progression of PCa by regulating FSCN1.

Notably, it was revealed that N-Myc and FSCN1 were expressed in PCa tissues and cells, and their expression was associated with tissue type and cancer progression. In particular, the positive rate of N-Myc and FSCN1 in the tumor tissues of patients with Gleason scores of >7 was higher than that in tissues from patients with scores of <7 points. The positive rate of N-Myc and FSCN1 in the tumor tissues of patients with stage III-IV disease was higher than that in the tumor tissues of patients with stage I-II disease. Moreover, the positive rate of N-Myc and FSCN1 in tumor tissues with bone metastasis was higher than that in tissues without bone metastasis. The aforementioned findings indicated that N-Myc expression is a late event in the history of tumor development. N-Myc and FSCN1 positive rates were unrelated to age and preoperative PSA levels. The present results were consistent with current findings, and N-Myc and FSCN1 were revealed to exist in PCa tissues. Moreover, through correlation analysis of the expression of N-Myc and FSCN1 in clinical samples of PCa, a weak positive correlation was identified between the expression of N-Myc and FSCN1 in PCa tissues. This weak correlation may be related to the number of tissue samples, but this result was consistent with the results of Dardenne *et al* (30) in cell lines. Therefore, it was surmised that a direct or indirect regulatory pathway or mutual regulation exists between N-Myc and FSCN1, thus promoting further tumor development. However, the specific mechanism of action of N-Myc and FSCN1 in PCa remains unclear and requires further study.

The binding site of N-Myc to FSCN1 was predicted through the NCBI website and CONSITE database, and it was determined that the N-Myc binding site was indeed present in the promoter region of FSCN1. Next, consistent with the results reported in the literature, the present results revealed that, at the cellular level, the expression of N-Myc and FSCN1 increased gradually at the protein and mRNA levels with the progression of PCa, indicating that N-Myc and FSCN1 were expressed in PCa cells, in which they played a crucial role. However, following repeated assessment of the protein expression level of N-Myc in LNCaP and C4-2 cells, which was always undetectable, we conclude that N-Myc is relatively low in protein levels in LNCaP and C4-2 cells.

Following the overexpression of N-Myc in LNCaP and C4-2 cells, the expression of FSCN1 was significantly higher than that in the control group, indicating that N-Myc could promote the expression of FSCN1 in PCa cells. As predicted, a regulatory role was observed between the two genes. After treating C4-2 and C4-2/N-Myc cells with FSCN1-shRNA, the expression of N-Myc increased. This suggested that a bidirectional regulation between N-Myc and FSCN1 may exist, and that the reduction of FSCN1 may reversely regulate the expression of N-Myc. In addition, the results of this experiment revealed that the expression levels of AR in LNCaP and C4-2 were higher than those in PC3, whereas the expression of CgA in PC3 was significantly increased and a small amount of the neuroendocrine marker CgA was expressed in C4-2 cells. These results indicated that C4-2 cells had begun to exhibit neuroendocrine properties, and that PC3 cells have a high degree of malignancy.

A number of studies (29,32) have suggested that N-Myc is a key cancer protein required for the development of the nervous system and neuroendocrine tumors. A previous study (35) has also suggested that FSCN1 is involved in the invasion and metastasis of PCa. N-Myc was overexpressed in LNCaP and C4-2 cell lines, and the AR expression was found to be decreased, indicating that N-Myc can inhibit the expression of AR in the original PCa cells, consequently rendering ADT ineffective. The expression of CgA was slightly increased following the overexpression of N-Myc, whereas that of CgA was significantly increased in the overexpressed FSCN1 group, and was significantly higher than that in the overexpressed N-Myc group. The expression of AR and CgA was significantly decreased following FSCN1-knockdown and was even lower than that in the control group. This result indicated that, although N-Myc is involved in neuroendocrine transformation, the role of FSCN1 in neuroendocrine transformation may be prominent, and N-Myc may be a neuroendocrine transformer promoted by the regulation of FSCN1. This phenomenon accounts for the increased expression of N-Myc, but not for the lack of increase in the expression of neuroendocrine marker CgA following FSCN1-knockdown.

In addition, this experiment also examined the effect of N-Myc and FSCN1 on the cell proliferation and migration ability of the C4-2 cell line. The results revealed that the proliferation rate of the overexpression group was significantly higher than that of the control group over time, indicating that N-Myc and FSCN1 can promote C4-2 cell proliferation and migration. However, following FSCN1 knockdown, the cell proliferation ability was not significantly affected and may be the same as the previously detected level. Although FSCN1 was knocked down, the expression of N-Myc was not decreased; thus, the proliferation ability was not significantly inhibited.

The present study has several limitations. We initially designed experiments to study the regulation of N-Myc on FSCN1. However, we did not expect that FSCN1 would reverse the regulation of N-Myc. Therefore, we did not study the effect of FSCN1 silencing on the endogenous expression of N-Myc. In addition, the present study also lacks investigation of more phenotypes/stages or neuroendocrine signals. We interfered with the expression of FSCN1 in C4-2/N-Myc cells, however these cells underwent apoptosis during migration and no available experimental results were obtained. Thus, the present experiment of observing the invasion and migration ability of PCa cells was not rigorous enough, however future studies may investigate these abilities.

In conclusion, in the present study, N-Myc and FSCN1 were revealed to be expressed in PCa. The positive correlation between the two may promote the clinical progression of PCa. At the cellular level, the expression of N-Myc and FSCN1 gradually increased with the malignant progression of PCa cells, and N-Myc could promote the expression of FSCN1. Conversely, the reduction of FSCN1 could reverse the expression of N-Myc, further suggesting a bidirectional regulation between N-Myc and FSCN1. N-Myc may promote the expression of FSCN1 to promote the phenotypic changes of PCa cells and CgA expression. N-Myc may promote the proliferation and migration of PCa by regulating the expression of FSCN1, leading to further tumor development. Thus,

the role of the N-Myc/FSCN1 pathway in the treatment of PCa should be investigated. However, N-Myc has numerous downstream regulatory factors, and its regulatory mechanism is markedly complex, thus the regulation of N-Myc on FSCN1 may be direct or indirect. These mechanisms require further study and discussion.

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Availability of data and materials

The datasets used in this study are available from the first author upon reasonable request.

Authors' contributions

YY and CL conceived and designed the study and the experiments. GH, ML, LF, LZ, LY XH and LX performed the experiments. GH, YC and ML analyzed the data. GH, WM, WL and LY collected the data. GH wrote the manuscript. YY, YC and WM reviewed and revised the manuscript. YY acquired funding. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

The present study was conducted with the consent of all participants and was approved by the Biomedical Ethics Committee of Anhui Medical University (approval no. 20170209).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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