

Characterization of the low pH/low nutrient-resistant LNCaP cell subline LNCaP-F10

KAZUHIRO IGUCHI¹, YURI HAYAKAWA¹, KENICHIRO ISHII², KAORU MATSUMOTO¹,
SHIGEYUKI USUI¹, YOSHIKI SUGIMURA³ and KAZUYUKI HIRANO¹

¹Laboratory of Pharmaceutics, Gifu Pharmaceutical University, Gifu; Departments of ²Oncologic Pathology,
³Nephro-Urologic Surgery and Andrology, Mie University Graduate School of Medicine, Edobashi, Tsu, Mie, Japan

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Abstract. Intratumoral regions of low extracellular pH and low nutrition are common features of solid tumors. Although cancer cells normally die when they are removed from their environment, a small population of cells survive. In the present study, the subline LNCaP-F10, of the prostate cancer cell line LNCaP, was isolated and its low pH/low nutrient-resistant properties were examined. LNCaP-F10 cells were grown under low-pH/low-nutrient conditions, which caused cell death of the LNCaP cells. The cell death was associated with oligonucleosomal DNA fragmentation and poly (ADP-ribose) polymerase (PARP) cleavage, indicating that low-pH/low-nutrient induced apoptosis in these cells. Significant differences in the expression of *BCL2*, *BIRC5* and *DAPK1* were detected between LNCaP-F10 and LNCaP cells. Tumor growth caused by implantation of LNCaP-F10 cells into the renal subcapsular space of nude mice in the absence or presence of prostate stromal cell stimulation was greater than that caused by implantation of LNCaP cells. LNCaP-F10 cells were resistant to apoptosis induced by an environment of low-pH/low-nutrient *in vitro*, and displayed malignant potential *in vivo*.

Introduction

The intratumor environment is characterized by low extracellular pH, low nutrition, and chronic hypoxia due to poor vascular development (1,2). The environment is unsuitable for the survival of cancer cells; however, a minor population of cancer cells becomes resistant to the environment and acquires the ability to survive (1). In prostate cancer, the characteristics of the intratumor environment have been demonstrated using *in vivo* electrode measurements of oxygen levels (3).

The cancer cells that survive in the environment have been considered potential therapeutic targets for the treatment of solid tumors, including prostate cancer.

The human prostate cancer cell line LNCaP is a widely used model of androgen-sensitive prostate cancer, and several sublines of LNCaP cells have been established and used to study the molecular and cellular biology of prostate cancer cells. The androgen-independent LNCaP subline LNCaP-C4-2 was established from LNCaP tumors in castrated mice (4). LNCaP-abl and AIDL cells are also androgen-independent sublines, generated *in vitro* by culturing LNCaP cells under androgen-depleted conditions (5,6). LNCaP-IL6⁺ and LNCaP-CR cells are resistant to cytokine-induced apoptosis (7,8). LNCaP-H1 cells were raised under chronic hypoxia, and exhibited androgen-independent growth *in vitro* and *in vivo* (9).

We previously established sublines from prostate cancer LNCaP cells by the limiting dilution method, i.e., LNCaP-E9 and LNCaP-G4, which are slightly and highly androgen-sensitive, respectively (10). In the present study, the LNCaP-F10 cell line, which was isolated in a similar manner, was further characterized by its ability to survive in an acidic environment under low-nutrient conditions. LNCaP-F10 cells were examined to determine the mechanisms underlying their adaptation to a low-pH/low-nutrient environment.

Materials and methods

Materials. 2',7'-Bis-(2-carboxyethyl)-5-(and-6)-carboxy-fluorescein, acetoxymethyl ester (BCECF-AM) was obtained from Dojin Laboratory (Kumamoto, Japan). The anti-androgen receptor (AR) rabbit polyclonal antibody (N-20) and anti-prostate-specific antigen (PSA) mouse monoclonal antibody (A67-B/E3) used for western blotting were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). A rabbit polyclonal anti-PSA antibody for immunohistochemistry was purchased from DakoCytomation, Inc. (Copenhagen, Denmark). Anti-Bcl-2 and anti-β-actin mouse monoclonal antibodies were purchased from Sigma (St. Louis, MO, USA). Anti-caspase-3 rabbit polyclonal and anti-poly (ADP ribose) polymerase (PARP) monoclonal antibodies were from BD Biosciences (Franklin Lakes, NJ, USA). Anti-bad and anti-bax rabbit polyclonal antibodies, and the anti-survivin (6E4) mouse monoclonal antibody were purchased from Cell Signaling Technology (Beverly, MA, USA).

Correspondence to: Professor Kazuyuki Hirano, Laboratory of Pharmaceutics, Gifu Pharmaceutical University, 1-25-4 Daigaku-nishi, Gifu 501-1196, Japan
E-mail: hirano@gifu-pu.ac.jp

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Mouse monoclonal anti-E-cadherin (clone 36) antibody was purchased from BD Transduction Laboratories, Inc. (Lexington, KY, USA). All other chemicals were of analytical grade.

Cell culture. Human prostatic carcinoma LNCaP cells were obtained from the American Type Culture Collection (Rockville, MD, USA). LNCaP-F10 cells were previously isolated as a subline of LNCaP cells by the limiting dilution method (10). LNCaP and LNCaP-F10 cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS) under a humidified atmosphere with 5% CO₂ at 37°C.

Cell growth assay. Cells were seeded onto 6-cm dishes at a density of 7x10⁵ cells/dish with normal medium (RPMI-1640 + 10% FBS, pH 7.4) or low-pH/low-nutrient medium (RPMI-1640 + 0.5% FBS and 10 mM PIPES, pH 6.3). The cells were incubated for the indicated times, and the detached and adherent cells were collected. The cells were stained with a 0.1% trypan blue solution and counted under a microscope using a hemocytometer.

Intracellular pH measurement. Cells were incubated in a normal medium or a low-pH/low-nutrient medium for 24 h. The intracellular pH was then measured using BCECF-AM, a pH-sensitive probe, as previously described (11). Cellular pH was analyzed by FACScan using CellQuest Pro software (BD Biosciences) with excitation at 488 nm and a ratio of emissions at 520 (pH sensitive) and 680 nm (pH insensitive). For each experiment, standard curves were established with a series of cells at pH 6.3, 6.7, 7.0 and 7.3.

DNA fragmentation assay. The DNA fragmentation assay was performed as previously described (11). Cells were seeded onto 10-cm dishes at a density of 2x10⁶ in a normal medium or a low-pH/low-nutrient medium for the indicated times. Detached cells were collected and lysed in buffer [10 mM Tris-HCl, 10 mM ethylenediaminetetraacetic acid (EDTA), and 1% Triton X-100] and treated with Proteinase K and RNase A. The DNA was separated by electrophoresis on 1.5% gels and visualized by staining with ethidium bromide.

Real-time reverse transcriptase-polymerase chain reaction (Real-time RT-PCR). Real-time RT-PCR was performed with a slightly modified version of a previously described protocol (10). Total RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and first-strand complementary DNA was synthesized from 1 µg of total RNA using PrimeScript reverse transcriptase (Takara, Otsu, Japan). Real-time monitoring of PCR reactions was performed using the Thermal Cycler Dice Real-Time system (Takara) with SYBR Premix Ex Taq (Takara). At the end of the reaction, a dissociation curve analysis was performed to examine the specificity of the product. PCR was performed using the following conditions: 35 cycles of 10 sec at 95°C and 30 sec at 60°C. Gene-specific primers were designed for each gene and used for RT-PCR (Table I). B2MG, a housekeeping gene, was used for normalizing the target mRNA expression.

Preparation of cell lysates and western blot analysis. Cell lysis and western blotting were performed as previously described

Table I. Primer sequences for real-time RT-PCR.

Gene name	Sequence
<i>APAF</i>	Sense: 5'-TCTGCTGATGGTGCAAGGAT-3' Antisense: 5'-CCGTGTGGATTCTCCCAAT-3'
<i>BAD</i>	Sense: 5'-CCAGCTGTGCCTTGACTACG-3' Antisense: 5'-TGCTCACTCGGCTCAAACCTC-3'
<i>BAX</i>	Sense: 5'-CAGCAAACCTGGTGTCTCAAGG-3' Antisense: 5'-CAACCACCTGGTCTTGGAT-3'
<i>BCL2</i>	Sense: 5'-TCCAGATGGCAAATGACCAG-3' Antisense: 5'-CTGGGTGGGTCTGTGTTGAA-3'
<i>BCL2L1</i>	Sense: 5'-GGGTTCCTTTCCCTTCCATC-3' Antisense: 5'-GGAGTCCTGGTCTTGCATC-3'
<i>BIK</i>	Sense: 5'-GTGCTGGAACACTGCTGAGG-3' Antisense: 5'-GGCAGGAGTGAATGGCTCTT-3'
<i>BIRC5</i>	Sense: 5'-CAGCTTCGCTGGAAACCTCT-3' Antisense: 5'-AGGCTAGGGACGACGATGAA-3'
<i>BIRC6</i>	Sense: 5'-GCAGTGATAAGCGGGTAGGC-3' Antisense: 5'-TTGTGGCTCTGCACACCTCT-3'
<i>DAPK1</i>	Sense: 5'-ATGGCAACATGCCTATCGTG-3' Antisense: 5'-TGGCTCCCATCAGACAGAGA-3'
<i>MCL1</i>	Sense: 5'-GGGGCAGGATTGTGACTCTC-3' Antisense: 5'-AAACCCATCCCAGCCTCTTT-3'
<i>XIAP</i>	Sense: 5'-GACTACCGCCCCAGCATTAG-3' Antisense: 5'-CCCAGCCCCATTTTATTTC-3'
<i>B2MG</i>	Sense: 5'-TGTGCTCGCGCTACTCTCTC-3' Antisense: 5'-CCATTCTCTGCTGGATGACG-3'

(12). In brief, cells were lysed in RIPA buffer [10 mM Tris-HCl (pH 7.5), 1% Nonidet P-40, 0.1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 150 mM NaCl and 1 mM EDTA] containing 200 µM phenylmethylsulfonyl fluoride, 5 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin, 2 mM sodium orthovanadate, 10 mM sodium fluoride and 2.5 mM β-glycerophosphate. A total of 20 µg of each cell lysate was subjected to SDS-polyacrylamide gel electrophoresis (10%), and transferred to a polyvinylidene fluoride (PVDF) membrane. After blocking with 1% bovine serum albumin, the membrane was incubated with primary antibodies overnight at 4°C. After repeated washing, the membrane was incubated with the indicated secondary horseradish peroxidase-conjugated antibody for 2 h at room temperature. Immunoreactive bands were detected using the ECL or ECL Plus reagent (GE Healthcare, Buckinghamshire, UK).

Xenografting. All animals were maintained in a specific pathogen-free environment. The Mie University's Committee on Animal Investigation approved the experimental protocol. As previously described, 50x10⁴ cells of the LNCaP and LNCaP-F10 lines were prepared in 50 µl of neutralized type 1

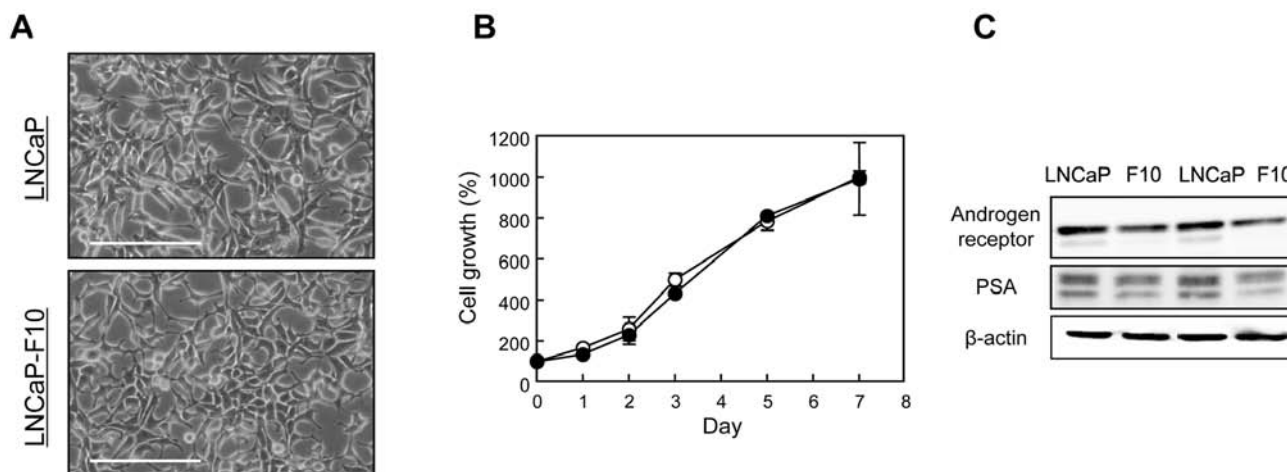


Figure 1. Cell morphology, growth, and AR status of LNCaP-F10 cells. LNCaP and LNCaP-F10 cells were cultured in a normal medium (RPMI-1640 + 10% FBS, pH 7.4) for 1 day (A and C) or for the indicated days (B). (A) Phase contrast microscopy images of LNCaP and LNCaP-F10 cells. Bar, 20 μ m. (B) Cell growth. The cell number was counted using a hemacytometer. ○, LNCaP cells; ●, LNCaP-F10 cells. (C) Western blotting. The cell extracts of LNCaP and LNCaP-F10 cells were subjected to SDS-PAGE, transferred onto a PVDF membrane and probed with anti-AR, PSA or β -actin antibodies. Duplicate samples are shown.

rat tail collagen gel (13). The cells were grafted into the subrenal capsule of 8-week-old adult homozygous athymic cluster of differentiation-1 male nude mice (CLEA Japan, Inc., Tokyo, Japan) (13). The mice were sacrificed and the grafts were harvested at 4 weeks post-grafting. The grafts were fixed in a 10% formalin neutral buffer solution (Wako Pure Chemical Industries, Osaka, Japan) for hematoxylin and eosin (H&E) and regular immunohistochemical staining.

Immunohistochemistry. Sections (3 μ m) were cut from the representative paraffin-embedded samples. For immunohistochemistry, the sections were deparaffinized in Histo-Clear (National Diagnostics, Atlanta, GA, USA) and rehydrated in a graded series of ethanol concentrations. Endogenous peroxidase activity was blocked by 0.3% hydrogen peroxide in methanol for 20 min. After extensive washing in tap water, antigen retrieval was performed using 10 mM sodium citrate buffer (pH 6.0) for AR and antigen unmasking solution (Vector Laboratories, Burlingame, CA, USA) for PSA and E-cadherin immunostaining. After rinsing in phosphate-buffered saline (PBS), the sections were incubated with the appropriate normal serum for at least 3 h at room temperature to block nonspecific binding. The sections were then incubated with anti-AR, anti-PSA, and anti-E-cadherin antibodies at 4°C overnight. After incubation with the primary antibody, sections were incubated with the appropriate biotinylated secondary anti-mouse, rabbit, or rat immunoglobulin diluted with PBS for 30 min at room temperature. The antigen-antibody reaction was visualized with the Vectastain avidin-biotin complex (ABC) kit (Vector Laboratories) using 3,3'-diaminobenzidine tetrahydrochloride (DAB) as a substrate. The sections were counterstained with hematoxylin and examined by light microscopy.

Statistical analysis. Statistical significance was assessed by two-way ANOVA followed by Bonferroni test or Student's t-test using the PRISM4 software (Graphpad Software, San Diego, CA, USA). $P < 0.05$ was considered to indicate a statistically significant result.

Results

Growth of LNCaP and LNCaP-F10 cells in a normal or low-pH/low-nutrient environment. LNCaP-F10 and LNCaP cells grown in a normal medium (RPMI-1640 + 10% FBS, pH 7.4) showed similar morphology and growth rates (Fig. 1A and B). AR and PSA were expressed at lower levels in LNCaP-F10 cells than the levels in LNCaP cells (Fig. 1C). Incubation in a low-pH/low-nutrient medium (RPMI-1640 + 0.5% FBS + 10 mM PIPES, pH 6.3) caused swelling and cell death in LNCaP cells, whereas LNCaP-F10 cells showed reduced growth rates but no cell death (Fig. 2A and B).

Intracellular pH of LNCaP and LNCaP-F10 cells in the low-pH/low-nutrient environment. To determine whether LNCaP-F10 cells maintain a normal intracellular pH in an acidic extracellular environment, the intracellular pH of cells incubated in the low-pH/low-nutrient medium was measured for 1 day. No significant differences in the intracellular pH were found between the 2 cell lines; cells reached an intracellular pH of 6.6 when incubated in the low-pH/low-nutrient medium (data not shown).

Death of LNCaP cells in the low-pH/low-nutrient environment. We analyzed DNA fragmentation, which is a hallmark of apoptosis, to determine whether the death of LNCaP cells in the low-pH/low-nutrient medium was due to apoptosis. As shown in Fig. 3A, DNA fragmentation was detected in LNCaP cells detached after incubation in the low-pH/low-nutrient medium for 3 days. Furthermore, cleavage of procaspase-3 and PARP was observed in these cells, confirming that cell death was caused by apoptosis (Fig. 3B). These results suggested that LNCaP-F10 cells may be resistant to apoptosis induced by extracellular environmental stress, i.e., low pH and low nutrition.

We then determined the expression levels of apoptosis-related genes in LNCaP and LNCaP-F10 cells incubated in normal or low-pH/low-nutrient media. When grown in a

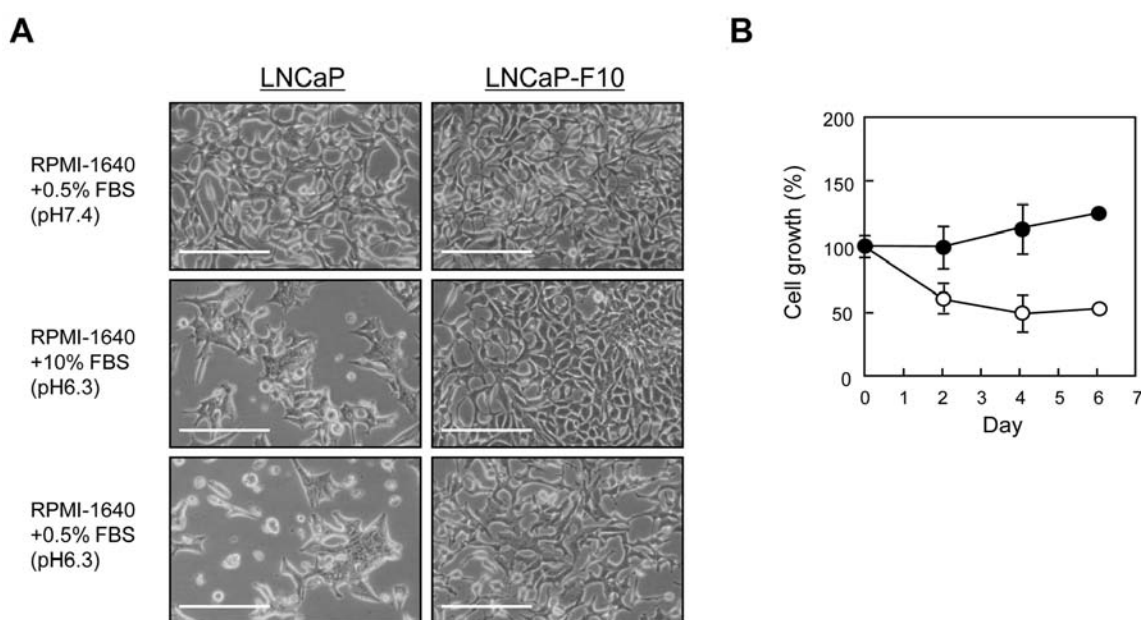


Figure 2. Growth of LNCaP and LNCaP-F10 cells under low-pH/low-nutrient conditions. (A) Phase contrast microscopy images of LNCaP and LNCaP-F10 cells. LNCaP and LNCaP-F10 cells were cultured in a neutral-pH/low-nutrient medium (pH 7.4/0.5% FBS), a low-pH/high-nutrient medium (pH 6.3/10% FBS) or a low-pH/low-nutrient medium (pH 6.3/0.5% FBS) for 3 days. Bar=20 μ m. (B) LNCaP and LNCaP-F10 cells were incubated in a low-pH/low-nutrient medium for the indicated days. The cell number was counted using a hemocytometer. \circ , LNCaP cells; \bullet , LNCaP-F10 cells.

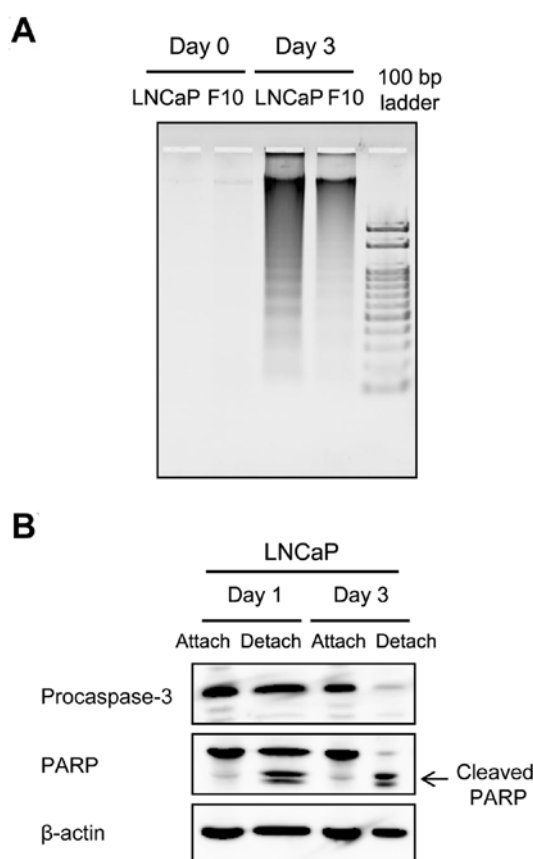


Figure 3. Induction of apoptosis in LNCaP cells by low-pH/low-nutrient. (A) LNCaP and LNCaP-F10 cells were incubated in the low-pH/low-nutrient medium for 0 or 3 days. The detached cells were collected, and cellular DNA was extracted and electrophoresed on 1.5% agarose gels. (B) LNCaP cells were incubated in the low-pH/low-nutrient medium for 1 and 3 days. After incubation, the detached and attached cells were collected separately, and cell lysates were prepared. The cell lysates (2.4×10^4 cells) were subjected to western blotting.

normal medium, the expression levels of *BCL2* and *BIRC5* were significantly higher in the LNCaP-F10 cells compared to LNCaP cells (Fig. 4A). *DAPK1* expression in LNCaP-F10 cells was markedly lower compared to LNCaP cells. *BIRC5* expression was decreased in cells incubated in the low-pH/low-nutrient medium in the 2 cell lines. As shown in Fig. 4B, the expression of the bcl-2 and survivin proteins (encoded by *BCL2* and *BIRC5*, respectively) was also markedly higher in LNCaP-F10 cells compared to that in the LNCaP cells.

Tumorigenic characteristics of LNCaP and LNCaP-F10 cells in vivo. The tumorigenicity of LNCaP-F10 cells was compared to that of the parental LNCaP cell line. When the cells were implanted into the renal subcapsular space, the weight of LNCaP-F10 tumors with or without prostate stromal cell (PrSC) stimulation was significantly greater compared to that of the parental LNCaP tumors (Fig. 5A and B). Cell proliferation (Ki-67 labeling index) in LNCaP-F10-derived tumors was also increased compared to that of the parental LNCaP tumors (Fig. 5C).

H&E staining showed that LNCaP-F10 + PrSC tumors had fewer blood spaces than LNCaP + PrSC tumors (Fig. 6i and m). No significant differences in AR, PSA, and E-cadherin expression levels were found between the 2 tumors without PrSC (Fig. 6b-d and f-h). Of note, the number of AR-positive cells was significantly decreased and PSA was undetectable in the LNCaP-F10 + PrSC tumors (Fig. 6n and o). In addition, the membrane localization of E-cadherin was decreased in the LNCaP-F10 + PrSC tumors (Fig. 6p).

Discussion

In the present study, LNCaP-F10 cells, isolated from prostate cancer LNCaP cells by limiting dilution, were observed to

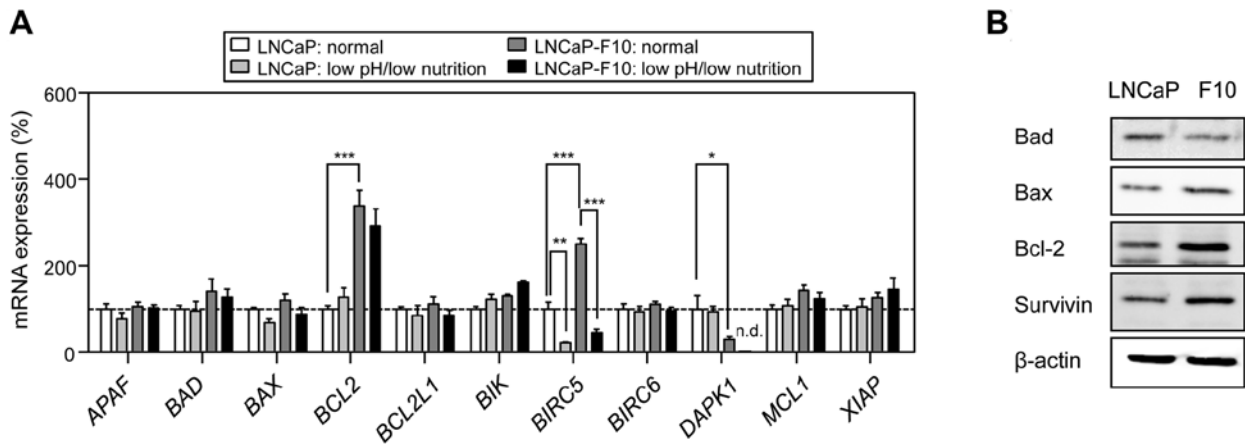


Figure 4. Expression of apoptosis-related factors in LNCaP and LNCaP-F10 cells. (A) LNCaP and LNCaP-F10 cells were incubated in the normal medium or the low-pH/low-nutrient medium for 1 day. After incubation, total RNA was isolated and subjected to quantitative real-time RT-PCR analysis. The values were normalized to the expression of *B2M*, and the average (\pm SD) of 3 values are shown. The values obtained from LNCaP cells incubated in the normal medium were set to 100%. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. n.d., not detected. (B) LNCaP and LNCaP-F10 cells were cultured in the normal medium for 1 day. After incubation, the cells were lysed in RIPA buffer, and 15 μ g of the lysates were subjected to western blotting.

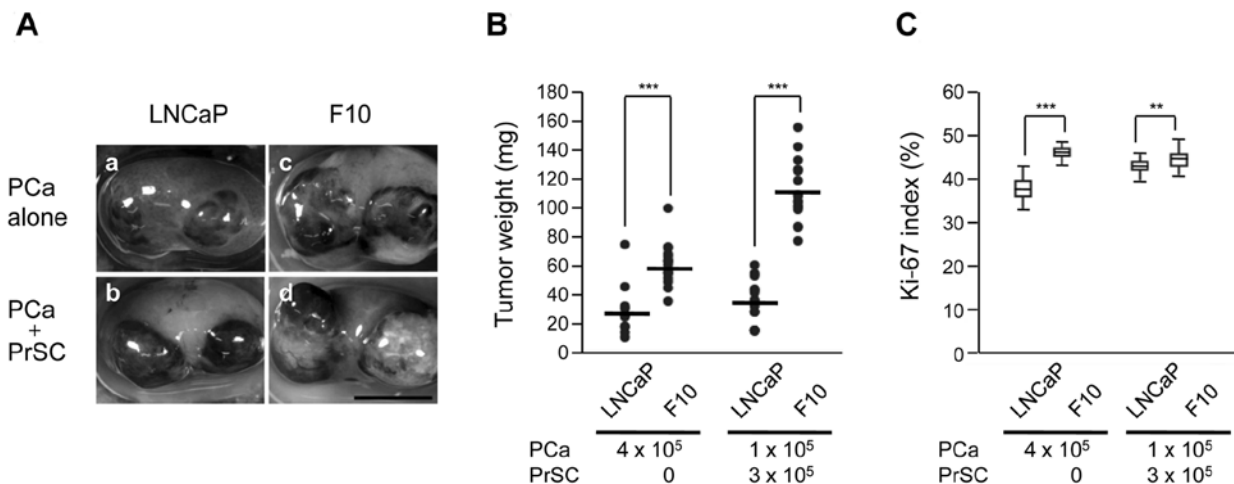


Figure 5. Tumorigenesis of LNCaP and LNCaP-F10 cells *in vivo*. Recombinants composed of human prostate cancer cells and human normal prostate stromal cells (PrSC) were grown beneath the renal capsule of nude mice for 4 weeks. (A) LNCaP (a and b) and LNCaP-F10 cells (c and d) were grafted without PrSC (PCa cells alone) or recombined with PrSC (PCa + PrSC). (A) Gross appearance. (B) Tumor weight. (C) Cell proliferation. Values represent the mean \pm SD. ** $P < 0.01$, *** $P < 0.001$. Bar, 5 mm.

survive in a low-pH/low-nutrient condition, whereas parental LNCaP cells demonstrated significant cell death. Cell death of LNCaP cells occurred in a low-pH/high-nutrient medium (pH 6.3/10% FBS), but not in a neutral-pH/low-nutrient medium (pH 7.4/0.5% FBS), suggesting that an acidic extracellular environment was the main factor inducing cell death (Fig. 2A). Moreover, dead cells were not observed after incubation in a medium containing 10 mM PIPES at pH 7.4 compared to pH 6.3, indicating that the cell death was not caused by PIPES toxicity (data not shown). Previous studies have demonstrated that an acidic environment leads to p53-dependent and caspase-mediated apoptosis (14,15). Since LNCaP cells express wild-type p53, the apoptosis observed in this study may be mediated by p53 activation under an acidic environment. Although the detailed mechanisms underlying the resistance of LNCaP-F10 cells to apoptosis remain unclear, the possibility of p53 dysfunction in LNCaP-F10 cells should

be considered. We are currently investigating the differences in the p53 function between parental LNCaP cells and the LNCaP-F10 subline.

Our results showed differentially expressed genes between LNCaP and LNCaP-F10 cells, including *BCL2*, *BIRC5* and *DAPK1*, which are involved in apoptosis. Bcl-2 is a key anti-apoptotic factor and its mRNA and protein expression levels were significantly higher in the LNCaP-F10 cells compared to that of the LNCaP cells. The high level of bcl-2 expression in LNCaP-F10 cells suggested that this cell line may be resistant to cell death induction. However, no significant differences in cell viability were observed between LNCaP and LNCaP-F10 cells treated with chemotherapeutic drugs such as etoposide, paclitaxel or docetaxel (data not shown). These results indicate that LNCaP-F10 cells exhibit specific resistance to the low pH/low nutrient stress rather than nonspecific resistance. Survivin (*BIRC5*), an anti-apoptosis

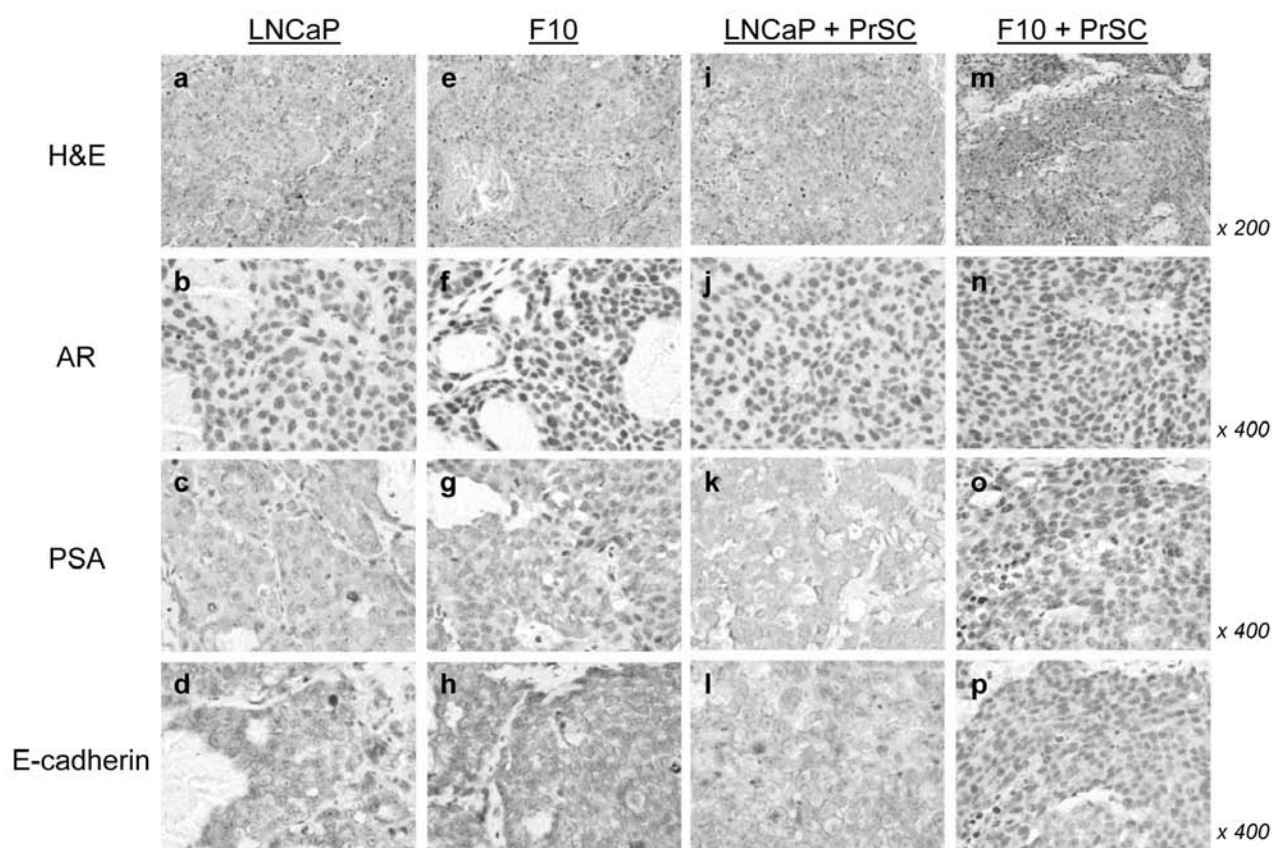


Figure 6. Histopathological characteristics of LNCaP and LNCaP-F10 tumors. Histopathological comparisons between LNCaP (a-d and i-l) and LNCaP-F10 (e-h and m-p) tumors with or without PrSC grown beneath the renal capsule of male SCID mice. (a, e, i and m) H&E staining; (b, f, j and n) AR staining; (c, g, k and o) PSA staining; (d, h, l and p) E-cadherin staining.

regulator, was upregulated in the LNCaP-F10 cells compared to LNCaP cells, and the expression was suppressed by the acidic environment. The mechanisms underlying the upregulation of survivin and its physiological implications remain unclear. However, p53 has been shown to regulate survivin expression negatively in human cancer cells, suggesting the involvement of p53 in the regulation of apoptosis by the acidic environment (16).

In our xenograft model, LNCaP-F10 tumors were significantly larger compared to those of the parental LNCaP cell line even without stromal stimulation. We previously showed that tumors derived from the LNCaP sublines, i.e., LNCaP-E9 and AIDL, induced by stromal cells were larger than LNCaP tumors, whereas in the absence of stromal cell stimulation, LNCaP and LNCaP subline tumors did not show a significant size difference (13,17). These results suggest that LNCaP-F10 cells have a more aggressive potential than LNCaP cells. In the present study, the pro-apoptotic factor *DAPK1* was expressed at significantly lower levels in LNCaP-F10 cells compared to LNCaP cells. *DAPK1* is a known tumor suppressor; its down-regulation is strongly correlated with tumor recurrence and metastasis, and its expression is often absent in cancer tissues (18-20). In addition, bcl-2 and survivin have been reported to be critical for the tumorigenicity of cancer cells, including prostate cancer cells (21,22). The differential expression of these genes in LNCaP-F10 cells may be associated with the differences in the tumorigenicity of these cells observed in

our *in vivo* experiments, and in the sensitivity to low-pH/low-nutrient-induced apoptosis determined *in vitro*.

In summary, LNCaP-F10 cells were found to be resistant to a low-pH/low-nutrient environment *in vitro* and to exhibit a more malignant phenotype compared to LNCaP cells *in vivo*. *BCL2*, *BIRC5* and *DAPK1* were differentially expressed in LNCaP-F10 cells compared to LNCaP cells, suggesting that these factors may be involved in the unique features of LNCaP-F10 cells. A tumor-aggressive phenotype has been associated with the overexpression, mutation or deletion of specific oncogenic products (23,24); therefore, further identification of genes differentially expressed in LNCaP and LNCaP-F10 cells would be beneficial in designing efficient therapeutic strategies.

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