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Role of Proprotein Convertases in Prostate Cancer Progression[1,2]

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Abstract

Better understanding of the distinct and redundant functions of the proprotein convertase (PC) enzyme family within pathophysiological states has a great importance for potential therapeutic strategies. In this study, we investigated the functional redundancy of PCs in prostate cancer in the commonly used androgen-sensitive LNCaP and the androgen-independent DU145 human cell lines. Using a lentiviral-based shRNA delivery system, we examined *in vitro* and *in vivo* cell proliferation characteristics of knockdown cell lines for the endogenous PCs furin, PACE4, and PC7 in both cell lines. Of the three PCs, only PACE4 was essential to maintain a high-proliferative status, as determined *in vitro* using XTT proliferation assays and *in vivo* using tumor xenografts in nude mice. Furin knockdowns in both cell lines had no effects on cell proliferation or tumor xenograft growth. Paradoxically, PC7 knockdowns reduced *in vitro* cellular proliferation but had no effect *in vivo*. Because PCs act within secretion pathways, we showed that conditioned media derived from PACE4 knockdown cells had very poor cell growth–stimulating effects *in vitro*. Immunohistochemistry of PACE4 knockdown tumors revealed reduced Ki67 and higher $p27^{KIP}$ levels (proliferation and cell cycle arrest markers, respectively). Interestingly, we determined that the epidermal growth factor receptor signaling pathway was activated in PC7 knockdown tumors only, providing some explanations of the paradoxical effects of PC7 silencing in prostate cancer cell lines. We conclude that PACE4 has a distinct role in maintaining proliferation and tumor progression in prostate cancer and this positions PACE4 as a relevant therapeutic target for this disease.

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Introduction

The mammalian proprotein convertases (PCs) form a family of enzymes that is responsible for the activation of numerous protein precursors within the secretory pathway. Nine different PCs have been identified, namely, furin, PACE4, PC1/3, PC2, PC4, PC5/6, PC7, PCSK9, and SKI-1/S1P [1,2]. Only the first seven of these serine proteases process substrates with an optimal PC recognition sequence R-X-K/R-R↓, whereas the minimal consensus sequence is R-X-X-R↓. A variety of substrates have been described such as precursors of hormones, enzymes, growth factors, receptors, cell membrane proteins, and plasma proteins but also a number of pathogenic proteins such as viral glycoproteins and bacterial toxins. There is growing evidence for the involvement of PCs in various cancer types, including roles in each of the biologic capabilities acquired during the multistep development of human tumors [3]. These biologic capabilities include proliferative signaling [4], evading growth suppressors [5], enabling replicative immortality [6], inducing angiogenesis [7], and activating invasion and metastasis [8].

Abbreviations: PC, proprotein convertase; RPMI, Roswell Park Memorial Institute medium; FBS, fetal bovine serum; EGF(R), epidermal growth factor (receptor); PSA, prostate-specific antigen; MVD, microvessel density; NT, non-target

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[2]This article refers to supplementary materials, which are designated by Tables W1 and W2 and Figures W1 and W2 and are available online at www.neoplasia.com.

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Prostate cancer is a worldwide health problem and is the second leading cause of cancer deaths behind lung cancer in North American men [9]. Prostate cancer threatens the life of patients by its ability to overcome initial hormone therapies as it becomes castration resistant, which then limits traditional therapeutic interventions and leads to metastasis and poor prognosis. Thus, the identification and validation of novel pharmacological targets suitable for the treatment of such aggressive tumors represents an unmet need.

In a recent study, we showed that PACE4 was highly overexpressed in prostate cancer tissues [10]. PACE4 silencing, based on an engineered delta-ribozyme approach, in the androgen-independent DU145 prostate cancer model cell line resulted in reduced proliferation and tumor xenograft progression in athymic nude mice. We thus hypothesized that PACE4 may be a potential druggable target for prostate cancer. If this is the case, then further information on the role of co-expressed PCs in prostate cancer becomes essential as redundancy among the PCs is well established. Thus, in prostate cancer, we raise the question as to whether the sole inhibition of PACE4 would suffice to produce beneficial effects or whether other PCs should also be considered for inhibition to attain this goal. Therefore, in the present study, we examined the role of other PCs in the cellular proliferation and tumor progression of prostate cancer cells using molecular silencing methods. We used lentivirus-delivered shRNAs to knockdown furin, PC7, and PACE4 in the androgen-sensitive LNCaP cell line and also in the androgen-independent DU145 cell line and then examined cell proliferation and tumor growth characteristics, *in vitro* and *in vivo*.

Materials and Methods

Cell Culture

All cell lines used were obtained from ATCC (Manassas, VA). Human prostate cancer cell lines DU145 and LNCaP were maintained in Roswell Park Memorial Institute medium (RPMI 1640) supplemented with either 5% fetal bovine serum (FBS; Wisent Bioproducts, St Bruno, Canada) for DU145 or 10% for LNCaP. Human embryonic kidney 293FT cells (HEK293FT; Invitrogen, Carlsbad, CA) were grown in Dulbecco's modified Eagle's medium and 10% FBS supplemented with 6 mM glutamine and 500 µg/ml G418. HT1080 cells were grown in Dulbecco's modified Eagle's medium with 10% FBS. Cells were grown at 37°C in a water-saturated atmosphere with 5% CO_2.

Lentivirus Production and shRNA Transduction

Production of lentiviral particles containing the MISSION RNAi pLKO.1-puro vector (see Table W1 for shRNA sequences) was carried out in HEK293FT cells following manufacturer's instructions (Sigma-Aldrich, St Louis, MO). Viral titers were calculated using a serial dilution approach with HT1080 cells. Lentivirus transductions were carried out in 6-well plates with 6×10^4 cells or in 12-well plates with 5×10^4 cells (for DU145 and LNCaP, respectively) with a multiplicity of infection of 3. After 2 days, the infected cells were selected using growth medium containing 1 or 2 µg/ml puromycin, respectively, for LNCaP and DU145 cells. Cells were further cultured under selective pressure using media with these puromycin concentrations for culture on passages. On characterization, the selected polyclonal cell populations were used for further studies to avoid any artifact associated with individual clone selection [11]. Cell populations were cultured for 10 to 12 passages before being discarded.

Real-time Quantitative Polymerase Chain Reaction

Total RNA was extracted using the Qiagen RNA Isolation Kit (Qiagen, Valentia, CA). RNA quality was assessed using an Agilent Bioanalyzer with RNA Nano Chips (Agilent Technologies, Palo Alto, CA). For tumor RNA isolation, Trizol-chloroform extractions (Invitrogen) were carried out on tissues crushed in liquid nitrogen. Real-time quantitative polymerase chain reaction (qPCR) reactions were performed as previously described [10]. Briefly, 1 µg of RNA was reverse transcribed and qPCR analysis reactions were done with a Stratagene Mx3005P instrument. All primers used are listed in Table W2. Relative expression levels were calculated using β-actin as a reference gene with the formula $(1 + \text{amplification efficiency})^{-\Delta(\Delta CT)}$. Experiments were done in duplicate in three independent experiments ($n = 3$).

XTT Proliferation Assay

The cellular proliferation was measured with the colorimetric XTT Cell Proliferation Kit II following the manufacturer's instructions (Roche Applied Science, Indianapolis, IN). Briefly, cells were seeded (1000 cells/well for DU145 and 3000 cells/well for LNCaP) in four 96-well plates in triplicate. For the next days, a plate was revealed after a 4-hour incubation with the reagent solution. Absorbance values were measured at a wavelength of 490 nm with a reference at 690 nm in a microplate reader (SpectraMax190; Molecular Devices, Sunnyvale, CA). For each time point, data were reported as percentage of mean values measured at 24 hours with corrections applied for the respective blanks (complete medium).

Conditioned Growth Medium Preparation and Proliferation Induction Measurements

As previously described, conditioned media were produced from four 10-cm plates seeded with 1.0×10^6 cells in complete growth media [10]. The next day, the cells were washed twice with phosphate-buffered saline (PBS) and the growth media were replaced with 5 ml of serum-free RPMI for 1 hour. The conditioned media were then collected, filtered through 0.45-µm filters, and concentrated to a final volume of 350 µl with Amicon Ultra centrifugal filter devices (Millipore, Billerica, MA) with a 3-kDa molecular weight cutoff. Fresh RPMI medium was treated the same way as a control.

The potential of these concentrated media to induce proliferation was measured as follows: for DU145, 50 µl of conditioned media was applied on control or shRNA-transfected cells seeded in 96-well plates (2000 cells/well) in triplicate. After a 48-hour incubation period, 12.5 µl of Thiazolyl Blue Tetrazolium Blue (MTT) compound (5 mg/ml; Sigma-Aldrich) was added to each well (25 µl for LNCaP). After a 4-hour incubation, the medium was carefully removed and cells were solubilized with a mixture of isopropanol/1N HCl (25:1). For LNCaP cells, 4500 cells/well were used and 50 µl of conditioned media was added to 50 µl of fresh 5% FBS medium.

The absorbance was measured at a wavelength of 550 nm with a reference at 650 nm in a microplate reader. MTT was used instead of XTT because the latter would have required a specific background subtraction for each conditioned medium generated.

Western Blot Analysis

Total proteins were extracted from excised tumors crushed in liquid nitrogen using RIPA buffer supplemented with protease inhibitors (complete Mini; Roche). Protein concentrations were determined using a BCA Protein Assay Kit (Pierce, Rockford, IL) and

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