Evidence that androgen-independent stromal growth factor signals promote androgen-insensitive prostate cancer cell growth *in vivo*

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Abstract

Activation of tumor–stromal interactions is considered to play a critical role in the promotion of tumorigenesis. To discover new therapeutic targets for hormone-refractory prostate tumor growth under androgen ablation therapy, androgen-sensitive LNCaP cells and the derived sublines, E9 (androgen-low-sensitive), and AIDL (androgen-insensitive), were recombined with androgen-dependent embryonic rat urogenital sinus mesenchyme (UGM). Tumors of E9+UGM and AIDL+UGM were approximately three times as large as those of LNCaP+UGM. Tumors grown in castrated hosts exhibited reduced growth as compared with those in intact hosts. However, in castrated hosts, E9+UGM and AIDL+UGM tumors were still approximately twice as large as those of LNCaP+UGM. Cell proliferation in tumors of E9+UGM and AIDL+UGM grown in castrated host, was significantly higher than that in tumors of LNCaP+UGM. *In vitro*, expression of fibroblast growth factor (FGF)-2 and IGF-I, but not FGF-7 mRNA, was significantly reduced in UGM under androgen starvation. In cell culture, E9 cells were responsive to FGF-2 and FGF-7 stimulation, while AIDL responded to FGF-7 and IGF-1. Expression of FGFR1 and FGFR2 was considerably higher in E9 than those in LNCaP, similarly expression of FGFR2 and IGF-IR were elevated in AIDL. These data suggest that activation of prostate cancer cell growth through growth factor receptor expression may result in the activity of otherwise androgen-independent stromal growth factor signals such as FGF-7 under conditions of androgen ablation.

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Introduction

Hormone-refractory prostate cancer (PCa), a heterogeneous disease, has varying degrees of androgen sensitivity. Androgen-ablation therapy for advanced PCa patients initially results in a good clinical response. However, most patients become resistant to androgen deprivation within a few years (Huggins & Hodges 2002). Once PCa cells become insensitive to androgen ablation, effective therapy is restricted. The progression of PCa cells from local invasion to distant metastasis and androgen insensitivity could be influenced by alterations in the tumor microenvironment, mutation of androgen receptor

(AR), and overexpression of growth factors and their receptors, leading to selection of cells with higher aggressive potential (Baldi *et al.* 2003).

In the tumor microenvironment, activation of tumor–stromal interactions is considered to play a critical role in the promotion of tumorigenesis (Tuxhorn *et al.* 2002a, Orimo *et al.* 2005). Solid tumors are composed of epithelial cancer cells infiltrating into a surrounding tumor stroma. Cells within the stroma resemble the ‘reactive stroma’ seen in wound healing and contain ‘carcinoma-associated fibroblasts’ (CAF; Tuxhorn *et al.* 2002b, Orimo & Weinberg 2006). Reactive stroma or CAF adjacent to

cancer cells secrete a number of growth factors such as members of the epidermal growth factor (EGF) family, fibroblast growth factors (FGFs), hepatocyte growth factor (HGF), insulin-like growth factors (IGFs), and nerve growth factor (Bhowmick *et al.* 2004), which are also produced by embryonic urogenital sinus mesenchyme (UGM; Cunha *et al.* 1992, 1995). UGM is composed of undifferentiated fibroblasts that instruct differentiation in the developing prostate. Molecules such as growth factors are involved in prostatic development and also in PCa progression, invasion, metastasis, and angiogenesis (Chung *et al.* 2005). Aberrant expression of peptide growth factors and activated signaling through their receptors in tumors are important in the development and progression of various cancers. In particular, activated signaling of FGFs, IGFs, and EGF has been implicated in PCa (Russell *et al.* 1998, El Sheikh *et al.* 2004, Gowardhan *et al.* 2005, Kawada *et al.* 2006).

Several studies have demonstrated the presence of myofibroblastic cells that have the capacity to contribute extracellular matrix and collagenous components to the reactive stroma around tumors (Ronnov-Jessen *et al.* 1995, Tuxhorn *et al.* 2002b, Eyden 2008). Myofibroblasts are mesenchymal cells sharing characteristics with fibroblasts and smooth muscle cells, and are activated fibroblasts typically found at sites of pathologic tissue remodeling, such as wound healing (Kalluri & Zeisberg 2006). In cancers, activated myofibroblasts in reactive stroma show irregularities in length and thickness as compared with fibroblasts. Recently, it has been reported that reactive stromal grading in radical prostatectomies or biopsies is a predictor of recurrence, and that high reactive stromal grading is associated with lower biochemical recurrence-free survival rates than low reactive stromal grading (Ayala *et al.* 2003, Yanagisawa *et al.* 2007).

In many studies on hormone-refractory PCa, androgen-insensitive AR-negative PCa cell lines PC-3 and DU145 have been used. However, these cells do not express functional AR in contrast to most hormone-refractory clinical PCas. As a result comparisons between the androgen-sensitive AR-positive PCa cell line LNCaP and these androgen-insensitive AR-negative PCa cell lines may not be relevant to acquisition of androgen insensitivity in clinical PCas (Tso *et al.* 2000). To overcome this problem, we derived two sublines from androgen-sensitive LNCaP – androgen-low-sensitive E9 (Iguchi *et al.* 2007) and androgen-insensitive AIDL (Onishi *et al.* 2001, Iguchi *et al.* 2004, Shibahara *et al.* 2005). These cells allow us to study the transition from androgen-sensitive PCa

to the androgen-insensitive state. The parental LNCaP cells and the E9 and AIDL derivative cells equally express AR protein, but androgen-dependent prostate-specific antigen (PSA) secretion is detected only in LNCaP cells. We have recently demonstrated that recombination of androgen-insensitive AIDL cells with embryonic rat UGM *in vivo* as compared with grafting the tumor cells alone emphasized the relatively aggressive tumorigenic phenotype of the androgen-insensitive subline of LNCaP cells (Kanai *et al.* 2008). In this study, we recombined LNCaP and its sublines E9 and AIDL with embryonic rat UGM to simulate the tumor microenvironment *in vivo*, and then evaluated the tumorigenesis between intact and castrated host. Finally, we confirmed that the androgen-independent stromal growth factor FGF-7 promoted cell growth, especially of androgen-insensitive PCa cells, *in vitro*.

Materials and methods

Growth factors

Recombinant rat EGF, rat FGF-2, and rat FGF-10 were purchased from R&D Systems, Inc. (Minneapolis, MN, USA). Recombinant rat FGF-7, rat HGF, rat IGF-I, and human transforming growth factor α (TGF- α) were purchased from QED Bioscience, Inc. (San Diego, CA, USA), Institute of Immunology Co., Ltd (Tokyo, Japan), ProSpec-Tany TechnoGene Ltd (Rehovot, Israel), and PeproTech, Inc. (Rocky Hill, NJ, USA) respectively. Synthetic androgen R1881 and dihydrotestosterone (DHT) were purchased from NEN Life Science (Boston, MA, USA) and Sigma-Aldrich, Inc. respectively.

Antibodies

Rabbit polyclonal anti-AR (N-20), rabbit polyclonal anti-EGFR (1005), rabbit polyclonal anti-FGFR1 (C-15), rabbit polyclonal anti-FGFR2 (C-17), and rabbit polyclonal anti-IGF-IR β (C-20) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Rabbit polyclonal anti-PSA, mouse monoclonal anti-neuron-specific enolase (NSE; Clone BBS/NC/VI-H14), and mouse monoclonal anti-human Ki-67 antigen (clone MIB-1) antibodies were purchased from DakoCytomation, Inc. (Copenhagen, Denmark). Mouse monoclonal anti-E-cadherin (clone 36) and rat monoclonal anti-mouse CD31/PECAM-1 (MEC13.3) antibodies were purchased from BD Transduction Laboratories, Inc. (Lexington, KY, USA). Rabbit monoclonal anti-VEGF receptor 2 (VEGFR; 55B11), mouse monoclonal anti-phospho-p44/42 MAPK (Thr202/Tyr204; E10); pErk1/2,

rabbit polyclonal anti-p44/42 MAP kinase; Erk1/2, rabbit polyclonal anti-phospho-Akt (Ser473); pAkt, and rabbit polyclonal anti-Akt antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Mouse monoclonal anti-actin (AC-15) antibody was purchased from Sigma-Aldrich, Inc. Rabbit polyclonal anti-human aminopeptidase-N (AP-N) antibody was established and characterized as previously reported (Ishii *et al.* 2001).

Cell culture

The androgen-sensitive AR-positive human PCa cell line LNCaP, and androgen-insensitive AR-negative human PCa cell lines PC-3 and DU145 were obtained from American Type Culture Collection (Rockville, MD, USA). Benign human prostate epithelial cell line BPH-1 was kindly gifted by Dr Simon W Hayward (Vanderbilt University Medical Center, Nashville, TN, USA). The androgen-low-sensitive E9 cells were obtained from parental LNCaP cell population by limiting dilution method in regular culture condition (Iguchi *et al.* 2007). By contrast, the androgen-insensitive AIDL cells were established from parental LNCaP cells by continuous passaging in hormone-depleted condition (Onishi *et al.* 2001). LNCaP and E9 cells were cultured in phenol red (+) RPMI-1640 supplemented with 10% fetal bovine serum (FBS). AIDL cells were cultured in phenol red (–) RPMI-1640 supplemented with 10% charcoal-stripped (CS)-FBS. PC-3 and DU145 cells were cultured in phenol red (+) DMEM with 10% FBS. BPH-1 cells were cultured in phenol red (+) RPMI-1640 supplemented with 5% FBS.

Hormonal treatment of cells

The cells, parental LNCaP, E9, and AIDL, were cultured in phenol red (–) RPMI-1640 medium supplemented with 0.5% CS-FBS for 2 days, and then the culture medium was replaced with medium containing various concentrations of the synthetic androgen R1881 for 2 days.

Growth factor stimulation of cell growth

Examination of growth factor stimulation was performed by the modified protocol as previously described (El Sheikh *et al.* 2004). The cells, parental LNCaP, E9, and AIDL, were cultured in phenol red (–) RPMI-1640 medium supplemented with 0.5% CS-FBS for 1 day, and then the culture medium was replaced with medium containing 10 ng/ml each of recombinant proteins EGF, FGF-2, FGF-7, FGF-10, HGF, IGF-I, and TGF- α for 4 days.

Preparation of cell lysate and western blot analysis

Sub-confluent cultured human PCa cells and BPH-1 cells were harvested by scraping, and a whole cell lysate was prepared as previously described (Iguchi *et al.* 2007). Briefly, all cells were cultured until 70–80% confluent in 100 mm dishes. The cell surface was washed with ice-cold PBS and then lysed with the buffer containing PBS, 1% Nonidet P-40, 10 μ M 4-(2-aminoethyl) benzensulfonyl fluoride, 0.8 μ M aprotinin, 50 μ M bestatin, 15 μ M E-64, 20 μ M leupeptin, and 10 μ M pepstatin A for 60 min on ice. The lysates were centrifuged at 10 000 *g* for 10 min, and then the supernatants were collected. The protein concentration was measured using a Bio-Rad Protein Assay Kit (Bio-Rad Laboratories). Equal amounts of protein (40 μ g/lane) electrophoresed in a 12.5% SDS-polyacrylamide gel were transferred to Immobilon polyvinylidene difluoride (PVDF) membranes (Millipore Corporate, Billerica, MA, USA). Ponceau S staining was used to monitor protein loading and transfer efficiency. After blocking, the membranes were incubated with primary antibodies at 4 °C overnight. After washing with Tris-buffered saline/Tween 20 at least three times, the membranes were incubated with the appropriate HRP-conjugated secondary antibodies for 1 h at room temperature. The specific protein bands were detected using a Super Signal West Pico reagent kit (Pierce Biotechnology, Rockford, IL, USA). The relative level of protein expression was semiquantified with an LAS-1000 plus image analyzer (Fuji Photo Film, Tokyo, Japan). Anti-AR, PSA, E-cadherin, NSE, VEGFR, AP-N, pAkt, Akt, pErk1/2, Erk1/2, EGFR, FGFR1, FGFR2, IGF-IR β , and actin were used at dilutions of 1:2500, 1:5000, 1:5000, 1:5000, 1:1000, 1:1000, 1:1000, 1:1000, 1:2000, 1:1000, 1:200, 1:200, 1:400, 1:200, and 1:5000 respectively.

RNA extraction and cDNA preparation

Total RNA was extracted using the Qiagen mini RNA Easy kit in accordance with the manufacturer's instructions (Qiagen, Inc). The RNA concentration was then determined spectrophotometrically.

From 50 ng total RNA, cDNA was reverse transcribed using oligo(dT) and Superscript II RNase H[–] reverse transcriptase (Invitrogen Co.) as previously described (Ogura *et al.* 2007).

Reverse transcriptase-PCR and real-time PCR analyses

PCR was performed with specific primers and annealing temperatures as shown in Table 1. The optimal PCR conditions were determined as

Table 1 Sequences of oligonucleotide primers for reverse transcriptase (RT)-PCR and real-time PCR

Gene name	Sequence	Annealing temperature (°C)	
		RT-PCR	Real-time PCR
Human			
PSA			
Sense	5'-GAGGTCCACACACTGAAGTT-3'	—	55
Antisense	5'-CCTCCTGAAGAATCGATTCT-3'		
GAPDH			
Sense	5'-CCAGCAAGAGCACAAGAGGA-3'	—	60
Antisense	5'-GCAACTGTGAGGAGGGGAGA-3'		
Rat			
AR			
Sense	5'-CAGCCCCAGCCAGCGACAGC-3'	65	—
Antisense	5'-CAGGGTGAGGGGCGGGCAGTAGGA-3'		
ER α			
Sense	5'-GCCTTATTGGATGCTGAACC-3'	55	—
Antisense	5'-TTGTAGAGATGCTCCATGCC-3'		
TGF β RII			
Sense	5'-CAACAACATCAACCACAATACG-3'	55	—
Antisense	5'-ATCTTTCACCTTCTCCCACAGC-3'		
Vimentin			
Sense	5'-CTCCTACCGCAGGATGTTTCG-3'	55	—
Antisense	5'-TAGTTGGCGAAGCGGTCATT-3'		
TN-C			
Sense	5'-GTCTCGGGGTCTCTAATCAC-3'	55	—
Antisense	5'-CCACAGACTCATAGACCAGG-3'		
α SMA			
Sense	5'-TCAATGTCCCTGCCATGTATG-3'	55	—
Antisense	5'-TAGAGGTCTTCCTGATGTCA-3'		
Desmin			
Sense	5'-TCTCAAGGGCACCAACGACT-3'	55	—
Antisense	5'-ATCCCGGGTCTCAATGGTCT-3'		
EGF			
Sense	5'-CCGCAGACTTACCCAGAACGA-3'	60	60
Antisense	5'-TGGGATAGCCCAATCCGAGA-3'		
FGF-2			
Sense	5'-CGAACCGGTACCTGGCTATGA-3'	60	60
Antisense	5'-GTATTTCGTGACCGGTAAGTGTG-3'		
FGF-7			
Sense	5'-CCCAGTGGTACCTGAGGATTGA-3'	60	60
Antisense	5'-CCTTTGATTGCCACAATTCCAAC-3'		
FGF-10			
Sense	5'-CGGAGTTGTTGCCGTCAAAG-3'	60	60
Antisense	5'-GCCGTTGTGCTGCCAGTTA-3'		
HGF			
Sense	5'-TGGTGTTCACAAGCAATCCAGA-3'	60	60
Antisense	5'-CCGTTGCAGGTCATGCATTC-3'		
IGF-I			
Sense	5'-TTCAGTTCGTGTGTGGACCAAG-3'	60	60
Antisense	5'-CAGCTCCGGAAGCAACTC-3'		
TGF α			
Sense	5'-CCCAGTACCCATACACAGATGCAG-3'	60	60
Antisense	5'-GCAGGACGATGTCCAACCAG-3'		
β -actin			
Sense	5'-GGCTACAGCTTCACCAACCAC-3'	58	—
Antisense	5'-TACTCCTGCTTGCTGATCCAC-3'		
GAPDH			
Sense	5'-GGCACAGTCAAGGCTGAGAATG-3'	55	60
Antisense	5'-ATGGTGGTGAAGACGCCAGTA-3'		

the amount of amplification product in proportion to that of input RNA. After PCR, the products were resolved on 2% agarose gels and visualized with ethidium bromide.

Real-time PCR was carried out in an iCycler iQ Detection System (Bio-Rad Laboratories) with iQ SYBR-Green Supermix reagents (Bio-Rad Laboratories) as previously described (Ogura *et al.* 2007). PCR amplification reaction was performed with specific primers and annealing temperatures as shown in Table 1. After PCR, melting curve analysis was performed to verify specificity and identity of the PCR products. All data were analyzed with the iCycler iQ Optical System Software Version 3.0A (Bio-Rad Laboratories).

Preparation of recombinants composed of human prostate cancer cells and embryonic rat UGM

Embryonic rat UGM was prepared from 18-day rat fetuses (plug date denoted as day 0) as previously described (Kanai *et al.* 2008). Briefly, urogenital sinuses were dissected from the fetuses and separated into epithelial and mesenchymal components by tryptic digestion and mechanical separation. UGM was then further digested to a single-cell suspension by 90 min digestion at 37 °C with 187 U/ml collagenase (Gibco-BRL). Following digestion, the cells were washed extensively with RPMI-1640 medium. Viable cells were then counted after trypan blue staining using a hemocytometer. For *in vitro* cell culture, the cells were cultured in RPMI-1640 medium supplemented with 5% FBS.

Sub-confluent cultures of parental LNCaP, E9, and AIDL cells were trypsinized and counted. Recombinants with UGM were prepared by mixing 1×10^5 cancer cells and 3×10^5 UGM in suspension. Grafts without UGM contained only 4×10^5 cancer cells. Pelleted cells were resuspended in 50 μ l neutralized type I rat tail collagen gels, and then grafted beneath the renal capsule of 8-week-old adult homozygous athymic cluster of differentiation-1 male nude mice (CLEA Japan, Inc., Tokyo, Japan). All animals were maintained in a specific pathogen-free environment under controlled conditions of light and humidity. Food and tap water were provided *ad libitum*. The Mie University's Committee on Animal Investigation approved the experimental protocol.

Processing of tumors

Mice were killed at 4 weeks, and grafts were harvested. Tumor volume (mm^3) was estimated by the formula $v = 0.5236 \times (\text{short axis})^2 \times \text{long axis}$ (Long *et al.* 2000).

Half of the tumors were fixed in IHC zinc fixative (BD Biosciences Pharmingen, San Diego, CA, USA) to detect mouse-specific CD31, and the others were fixed in 10% neutral-buffered formalin (Wako Pure Chemical Industries, Osaka, Japan) at room temperature overnight for H and E and immunohistochemical staining, and then processed and embedded in paraffin in accordance with standard procedures.

Histopathology and immunohistochemistry

Serial sections (3 μ m thick) were cut on a Leica RM2125 rotary microtome (Leica Microsystems, Wetzlar, Germany) and mounted on glass slides. Sections were deparaffinized in Histoclear (National Diagnostic, Atlanta, GA, USA) and hydrated in graded alcoholic solutions and running tap water. For histopathology, standard H and E staining was carried out. Next, immunohistochemical staining was performed with a Vectastain ABC *elite* kit (Vector Laboratories, Inc., Burlingame, CA, USA) following our protocol. After deparaffinization and hydration, endogenous peroxidase activity was blocked with 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 of pH 6.0 for Ki-67. For mouse-specific CD31, antigen retrieval was not performed. Following a period of cooling and then rinsing in PBS, the sections were incubated in blocking solution for at least 30 min at room temperature. The sections were then incubated with primary antibodies at 4 °C overnight. After incubation with primary antibodies, sections were incubated with appropriate biotinylated secondary anti-mouse or anti-rat immunoglobulin included in the ABC *elite* kit for 30 min at room temperature. The antigen–antibody reaction was visualized using 3,3'-diaminobenzidine tetrahydrochloride as a substrate. Sections were counterstained with hematoxylin and examined by light microscopy. Anti-Ki-67 and anti-CD31 antibodies were used at dilutions of 1:300 and 1:200 respectively. For examination, four sections per tumor specimen were used. This gave a total number of at least 32 sections (four sections $\times$ at least eight tumor specimens from each recombinant) for analyzing each stain. Representative pictures were taken from ten separate microscopic fields from each tumor specimen.

Quantification of microvessels in tumors

A 'microvessel' was defined as mouse-specific CD31-positive endothelial cells that formed a vascular lumen. The number of microvessels was counted

under an optical microscope at $\times 200$ magnification in ten fields of representative areas in tumors. The numbers of CD31-positive lumens were determined in a double-blinded fashion by two separate investigators, and then microvessel density (MVD) was calculated.

Statistical analysis

The results were expressed as the means $\pm$ s.d. Differences between the two groups were determined using Student's *t*-test. Values of $P < 0.05$ were considered statistically significant.

Results

Characteristics of human prostate cancer cell LNCaP sublines *in vitro*

To understand the physiological changes occurring in the hormone-refractory state, we generated new sublines derived from androgen-sensitive LNCaP cells. The parental LNCaP, as well as the E9 and AIDL cell sublines grew in culture with or without additional androgen stimulation. The androgen sensitivity of parental LNCaP, E9, and AIDL cells was confirmed by the induction of PSA mRNA during treatment with the synthetic androgen R1881 in cell culture (Fig. 1A). The induction of PSA mRNA in R1881-treated LNCaP cells was the highest among the three cell lines. R1881-treated E9 cells resulted in weak PSA mRNA induction, while no induction of PSA mRNA was observed in R1881-treated AIDL cells.

The parental LNCaP cells and the E9 and AIDL cell sublines expressed similar levels of AR and E-cadherin protein in culture. However, PSA protein was detected only in parental LNCaP cells (Fig. 1B). The expression of NSE in E9 cells was higher than in parental LNCaP and AIDL cells. VEGFR and AP-N were not detected in any of the cells. Under basal culture conditions without androgenic stimulation, phosphorylation of Akt was inhibited strongly in E9 cells but not in AIDL cells. Under the same basal conditions activation of Erk1/2 was not observed in any of the three cell lines. The differences between androgen insensitive but AR-expressing cells (AIDL) and AR-negative androgen-insensitive cells (PC3 and DU145) are a central theme of this communication. The presence and absence of AR in these cells were confirmed by western blotting, while PSA was used as a reporter of AR activity (Fig. 1B).

Figure 1 Characteristics of human prostate cancer cell LNCaP sublines *in vitro*. (A) The androgen sensitivity of LNCaP sublines was confirmed by induction of PSA mRNA during treatment with synthetic androgen R1881 in cell culture. Parental LNCaP, E9, and AIDL cells were treated with the indicated concentrations of R1881 for 2 days. Total RNA was isolated from the cells, and then subjected to real-time PCR analysis. (B) Forty micrograms of cell lysates from growing cultures of parental LNCaP, E9, AIDL, PC-3, DU145, and BPH-1 cells were separated by electrophoresis with 12.5% SDS-polyacrylamide gel. After proteins in the gel were transferred to a PVDF membrane by electroblotting, blots were probed with antibody against each protein. Adequate loading among the three cell lines was confirmed by blotting for actin.

Gene expression in embryonic rat UGM

Embryonic rat UGM was prepared from 18-day rat fetuses. Reverse transcriptase (RT)-PCR showed that UGM expressed mRNA of receptors AR, ER α , and TGF β RII; stromal differentiation markers

vimentin, TN-C, α SMA, and desmin; and growth factors EGF, FGF-2, FGF-7, FGF-10, HGF, IGF-I, and TGF- α (Fig. 2).

Effects of recombination with embryonic rat UGM on tumorigenesis of LNCaP sublines *in vivo*

All recombinants composed of cancer cells and embryonic rat UGM grew well when grafted beneath renal capsules. 100% of grafts were recovered ($n=8$ for each condition).

Gross inspection of parental LNCaP, E9, and AIDL recombinants with embryonic rat UGM showed that they formed mottled solid tumors (partially white and red; Fig. 3A). By contrast, all tumors of parental LNCaP and its sublines without UGM formed reddish tumors of spheroidal or irregular shapes filled with blood (Fig. 3A). Tumors of E9+UGM and AIDL+UGM were approximately three times as large as those of parental LNCaP+UGM ($P<0.0001$), while tumors of E9, AIDL, and parental LNCaP without UGM did not differ significantly in size (Fig. 3B). All tumors grown in castrated hosts showed reduced tumorigenesis as compared with those in intact hosts. However, the tumors of E9+UGM and AIDL+UGM grown in castrated hosts maintained the same size differential when compared with those of parental LNCaP+UGM ($P<0.01$; Fig. 3B). In addition to the tumor size grown in castrated host, cell proliferation

(Ki-67 labeling index) in tumors of E9+UGM and AIDL+UGM was significantly higher than that in tumors of parental LNCaP+UGM ($P<0.001$; Fig. 3C).

Effects of castration on histopathological characteristics among the tumors of LNCaP sublines

Regardless of the presence or absence of UGM, H and E staining showed no apparent histopathological difference among tumors derived from the three cell lines (Fig. 4). All tumors of parental LNCaP and its sublines without UGM consisted sheets of anaplastic cells and large blood-filled spaces. By contrast, all tumors of parental LNCaP and its sublines with UGM showed fewer blood spaces and fewer large dilated-vessels. In tumors that contained UGM, the number of CD31-positive vessels (=MVD) in all tumors grown in castrated hosts was significantly lower than those in intact hosts ($P<0.01$; Table 2). Among tumors of parental LNCaP, E9, and AIDL+UGM, there was no significant difference of MVD in intact versus castrated host. In tumors without UGM, however, MVD in AIDL tumors grown in both intact and castrated host was significantly higher than that in parental LNCaP tumors ($P<0.05$).

Effects of androgen ablation on cell proliferation and growth factor mRNA level in embryonic rat UGM culture *in vitro*

In the absence of DHT, cell proliferation of UGM was significantly suppressed ($P<0.01$; Fig. 5A). However, the cells did not appear to be androgen-dependent for survival since no significant elevation in cell death was detected in the absence of DHT, and the original cell number was maintained for 48 h with no significant increase of trypan blue-incorporation. Among stromal growth factors detected by RT-PCR (Fig. 2), mRNA expression of FGF-2, FGF-10, and IGF-I, but not FGF-7, was significantly reduced in androgen-starved culture condition of UGM ($P<0.05$; Fig. 5B).

Effect of growth factor stimulation on cell proliferation of LNCaP sublines *in vitro*

In *in vitro* cell culture, each cell line had unique responsiveness to the seven different growth factors EGF, FGF-2, FGF-7, FGF-10, HGF, IGF-I, and TGF- α (Fig. 6). Of note, purified proteins of EGF, FGF-2, FGF-7, FGF-10, HGF, and IGF-I are derived from rat, but only TGF- α protein is derived from human. Parental LNCaP cells showed responsiveness to EGF and TGF- α ($P<0.05$). In addition to EGF and TGF- α ,

Figure 2 Gene expression in embryonic rat UGM. Total RNA was isolated from the cells, and then subjected to RT-PCR analysis. The products were resolved on 2% agarose gels and visualized with ethidium bromide.

Figure 3 Effects of recombination with embryonic rat UGM on tumorigenesis of LNCaP sublines *in vivo*. Tissue recombinants composed of human prostate cancer cells and embryonic rat UGM were grown beneath renal capsule for 4 weeks. Parental LNCaP, E9, and AIDL cells were grafted without embryonic rat UGM (4×10^5 PCa cells alone) or recombined with embryonic rat UGM (PCa/UGM: $1 \times 10^5/3 \times 10^5$ cells). Gross appearances of parental LNCaP (a and b), E9 (c and d), and AIDL (e and f) recombinants with or without embryonic rat UGM are shown in (A). Among the three cell lines, tumor volume (B) and cell proliferation (C) were compared with focus on embryonic rat UGM (presence or absence) and androgen level (intact or castrated) in the host. Values represent the means $\pm$ s.d. * $P < 0.0001$ versus LNCaP + UGM in intact mice, † $P < 0.01$, †† $P < 0.001$ versus LNCaP + UGM in castrated mice.

FGF-2, and FGF-7 significantly increased proliferation of E9 cells ($P < 0.05$). Proliferation of AIDL cells was significantly increased by FGF-7 and IGF-I, but not by EGF, FGF-2 or TGF- α ($P < 0.01$).

Expression of growth factor receptors in LNCaP sublines

Each cell line showed varied expression of growth factor receptor. In E9 cells, expression of EGFR, FGFR1, and FGFR2 was considerably higher than in parental LNCaP cells (Fig. 7). The expression of EGFR and FGFR1 in AIDL cells was slightly lower than those in parental LNCaP cells, while the expression of FGFR2 and IGF-IR were considerably higher than those in parental LNCaP cells. PC-3 and DU145, and benign human prostate epithelial cell line BPH-1 was used as positive or negative control for western blot analyses.

Discussion

Decrease or loss of androgen sensitivity in PCa is a clinical concern. Although androgen ablation therapy is beneficial for advanced PCa patients, PCa usually becomes hormone refractory. Halin et al. (2007) have recently demonstrated that androgen-insensitive PCa cells can respond to castration when growing in an

androgen-dependent prostate environment. When androgen-insensitive AT-1 PCa cells were injected into the ventral prostate of Copenhagen rats, an androgen-dependent environment, castration reduced AT-1 tumor growth and vascular density in the tissue surrounding the tumor. These data demonstrated the importance of cancer cell microenvironment for the action of androgens, i.e. tumor growth of androgen-insensitive PCa cells was regulated by androgen-dependent stromal signals including growth factors and cytokines. However, the mechanisms by which androgen-insensitive PCa cell response to stromal signals under low-androgen conditions are not yet fully understood.

Tumor–stromal interactions contribute significantly to the development and progression of PCa (Camps et al. 1990). As an *in vivo* experimental model, several groups have demonstrated that coinoculation of commercialized human prostatic stromal cells (PrSC) with human PCa cells increased tumorigenicity in terms of tumor incidence and tumor growth (Uemura et al. 2005, Verona et al. 2007). In addition, Kawada et al. (2006) have shown that the PrSC-conditioned medium increased the cell proliferation of human PCa cells *in vitro*, suggesting that the secreted factors from PrSC-regulated tumor growth *in vivo*. In a tissue recombination model, however, PrSC cells showed

Figure 4 Effects of castration on histopathological characteristics among the tumors of LNCaP sublines. Without embryonic rat UGM, i.e. PCa alone, histopathology of LNCaP (a and d), E9 (b and e), and AIDL (c and f) tumors grown in both intact (a–c) and castrated (d–f) host are shown in the upper panel. With embryonic rat UGM, i.e. PCa + UGM, histopathology of LNCaP (g and j), E9 (h and k), and AIDL (i and l) tumors grown in both intact (g–i) and castrated (j–l) host are shown in the lower panel. Scale bar = 100 μ m, magnification $\times 200$.

considerably smaller effects on growth of LNCaP sublines than embryonic rat UGM (data not shown). Tumors of E9 + PrSC and AIDL + PrSC were approximately one-fifth as large as those of E9 + UGM and AIDL + UGM. This result suggests that embryonic rat UGM may be a more effective inducer of growth of anaplastic LNCaP sublines. Thus, we chose embryonic rat UGM to investigate the role of stromal growth factor signals on tumor growth of LNCaP sublines.

In regard to the effects of growth factors or cytokines in tumor growth, we needed to consider both autocrine

and paracrine mechanisms. Our results showed no significant difference in tumorigenicity among the three cell lines when grown without UGM (Fig. 3). It is well known that the stromal cells surrounding PCa are involved in the development and progression of hormone-refractory PCa and secrete several growth factors and cytokines involved in this process (Scher *et al.* 1995, Matsubara *et al.* 1999, Uemura *et al.* 2005, Halin *et al.* 2007).

FGF-7/keratinocyte growth factor (KGF) was identified as an androgen-induced growth factor derived exclusively from stromal cells (Finch *et al.* 1989, Yan *et al.* 1992, Sugimura *et al.* 1996). Sugimura *et al.* (1996) have demonstrated that FGF-7/KGF in cooperation with androgen plays an important role as a mesenchymal paracrine mediator during epithelial growth and ductal branching morphogenesis in the rat ventral prostate. In the absence of androgen, however, FGF-7/KGF partially stimulated prostatic growth and ductal branching morphogenesis, suggesting the possibility that FGF-7/KGF could replace some aspects of the action of androgens on epithelial growth. In human prostate, Planz *et al.* (1999) have reported that FGF-7/KGF was detected in both fibroblasts and smooth muscle cells in prostate of normal, BPH, and PCa tissues. Interestingly, Leung *et al.* (1997) have reported that upregulation of FGF-7/KGF and its receptor FGFR2 protein were related to hormone-refractory prostate tumors in human prostate specimens.

There are numerous mechanisms by which PCa cells may become androgen insensitive, these include AR gene mutation, AR amplification or activation of AR signaling by growth factors and cytokines in tumors (Culig *et al.* 1994, Koivisto *et al.* 1998). Planz *et al.* (2004) have reported that FGF-7/KGF stimulated cell proliferation of LNCaP cells in the presence of the anti-androgenic agent flutamide, showing that FGF-7/KGF-induced cell proliferation in LNCaP cells is independent of cellular AR signaling. Our results support the idea that androgen-independent stromal

Table 2 Effect of castration on microvessel density in tumors of LNCaP sublines with or without embryonic rat urogenital sinus mesenchyme (UGM)

Cell line	Without UGM		With UGM	
	Intact	Castrated	Intact	Castrated
Parent	2.9 $\pm$ 1.5	1.9 $\pm$ 1.9	10.0 $\pm$ 3.9	3.4 $\pm$ 2.4 ^{\$}
E9	4.8 $\pm$ 2.9	3.5 $\pm$ 2.2	11.6 $\pm$ 3.1	3.2 $\pm$ 1.9 ^{\$}
AIDL	7.5 $\pm$ 2.1*	5.1 $\pm$ 3.3 [†]	13.0 $\pm$ 5.4	4.6 $\pm$ 3.6 [‡]

Mouse-specific CD31-positive vessels with lumen were counted in ten different areas at $\times 200$ magnification. Values represent the means $\pm$ s.d. * $P < 0.001$ versus parental tumors without UGM grown in intact host, [†] $P < 0.05$ versus parental tumors without UGM grown in castrated host, [‡] $P < 0.01$, ^{\$} $P < 0.001$ versus tumors of each cell line with UGM grown in intact host.

Figure 5 Effects of androgen ablation on cell proliferation and growth factor mRNA expression in embryonic rat UGM culture *in vitro*. UGM was cultured in the presence or absence of 1 nM DHT for 48 h. (A) Cell proliferation. The cell number was calculated by hemocytometer. (B) Growth factor mRNA expression. Total RNA was isolated from the cells, and then subjected to real-time PCR analysis. Values represent the means $\pm$ s.d. in triplicate experiment. * P <0.05, ** P <0.01 versus 1 nM DHT treatment.

growth factors such as FGF-7/KGF may bypass the functionally inactive AR and promote cell proliferation of androgen-insensitive PCa cells during androgen ablation therapy.

In a solid tumor, stromal paracrine factors, mainly growth factors and cytokines, regulate proliferation, cell death, and differentiation of cancer cells. Although prostatic stromal cells, even in tumors, express functional AR protein, expression level of AR is

heterogeneous and varies during cancer progression (Singh & Figg 2004). A number of growth factors and cytokines in prostate are considered to be dependent on androgen status (Chang & Chung 1989, Niu *et al.* 2001, Ohlson *et al.* 2007). In this study, we observed that the expression of FGF-2 and IGF-I mRNA was dramatically decreased in UGM cultured under conditions of androgen starvation, while FGF-7 mRNA was not influenced by androgen status. This result may reflect those of previous reports. Niu *et al.* (2001) have shown that expression of FGF-2 mRNA in dog prostate was dramatically decreased at day 14 after castration. In addition, expression of IGF-I mRNA in benign human prostatic stroma was significantly decreased after surgical castration therapy (Ohlson *et al.* 2007). In contrast to FGF-2 and IGF-I, expression of FGF-7 mRNA in rat prostate was not significantly influenced by castration (Nemeth *et al.* 1998). Therefore, androgen-independent expression of certain stromal growth factors such as FGF-7 could be given consideration as a concept to treat hormone-refractory PCa in combination with androgen ablation.

Cano *et al.* (2007) have recently reported that responsiveness of AR-mediated transcriptional activity to androgen in cancer stromal cells was lower than that in benign prostatic stromal cells. Our results suggest that activated signaling of FGFs, even under low-androgen conditions, could be targeted for treatment of hormone-refractory PCa. Udayakumar *et al.* (2003) demonstrated that pharmacological inhibition of FGFR1 signaling by SU5402 suppressed

Figure 6 Effect of growth factor stimulation on cell proliferation of LNCaP sublines *in vitro*. Cells were treated with 10 ng/ml each growth factor for 4 days under serum-starved conditions. Values represent the means $\pm$ s.d. in triplicate experiment. * P <0.05, ** P <0.01 versus untreated parental LNCaP, † P <0.05, †† P <0.001 versus untreated E9, ‡ P <0.01 versus untreated AIDL.

Figure 7 Expression of growth factor receptors in LNCaP sublines. Forty micrograms of cell lysates from growing cultures of parental LNCaP, E9, AIDL, PC-3, DU145, and BPH-1 cells were separated by electrophoresis with 12.5% SDS-polyacrylamide gel. After proteins in the gel were transferred to a PVDF membrane by electroblotting, blots were probed with antibody against each protein. Adequate loading among the three cell lines was confirmed by blotting for actin.

tumor growth of LNCaP cells coinjected subcutaneously with human prostate fibroblasts into athymic nude mice. [Gowardhan *et al.* \(2004\)](#) have also reported that expression of soluble FGFR1, blocking ligand-receptor binding by adenoviral vector, suppressed *in vitro* FGF (FGF-1, FGF-2 or FGF-7)-induced signaling and cell functions such as proliferation and invasion in human PCa DU145 cells. For targeting activated signaling of FGFR2, [Takeda *et al.* \(2007\)](#) have recently demonstrated that AZD2171, a selective VEGF signaling inhibitor that inhibits all VEGF receptor tyrosine kinases, completely inhibited the phosphorylation of FGFR2 and downstream signaling proteins in gastric cancer cell lines. They have suggested that AZD2171 can provide an additional therapeutic benefit for treatment of FGFR2 signaling-dependent cancer cells.

Normal prostatic stroma is predominantly composed of smooth muscle cells with very few fibroblasts, myofibroblasts or collagen fibers. However, stromal changes during cancer progression result in a decrease in the prevalence of smooth muscle cells. Tumor stroma surrounding cancer cells is enriched in fibroblasts and myofibroblasts, and called 'reactive stroma' or 'CAF' ([Micke & Ostman 2004](#)). Stromal components in tumors play very important roles in the

enhancement of tumor progression by stimulating angiogenesis and promoting cancer cell survival, proliferation, and invasion ([Kalluri & Zeisberg 2006](#)). Thus, tumor–stromal interactions must be considered as an important biological component of the cancer.

In various solid tumors, including those of breast, colon, lung, and prostate, tumor stromal cells including CAF have been implicated in tumor growth, progression, angiogenesis, and metastasis ([Micke & Ostman 2004](#), [Orimo *et al.* 2005](#)). The origins of CAFs have not been well defined, whereas [Mishra *et al.* \(2008\)](#) have recently reported that bone marrow-derived mesenchymal stem cells (MSCs) could be a candidate for the origin of CAFs in solid tumor. They showed that MSCs became activated and resembled a CAF-like myofibroblastic phenotype on exposure to conditioned medium from human breast cancer MDAMB231 cells. Several reports have already demonstrated that circulating MSCs in the bloodstream have been recruited and localized to develop tumors ([Hall *et al.* 2007](#), [Karnoub *et al.* 2007](#)), suggesting that certain factors secreted from tumors may recruit MSCs into solid tumors. Although tumor-derived specific factors to recruit MSCs have not been identified, [Wang *et al.* \(2004\)](#) have reported that differentiation of MSCs into myofibroblasts could be regulated in response to cytokine TGF-β. TGF-β participates in cellular proliferation and differentiation not only during normal processes such as embryonic development and wound healing, but also during abnormal processes such as cancer progression and angiogenesis. [Verona *et al.* \(2007\)](#) have recently reported that TGF-β stimulated prostatic stromal cells to express a number of genes related to myofibroblastic differentiation and promoted reactive stroma formation and carcinoma growth *in vivo*. These results suggest that MSCs, recruited by tumors and activated by TGF-β in a solid tumor, may be a possible candidate as source of CAFs.

In conclusion, we have presented evidence that activation of PCa cell growth through paracrine activation of growth factor receptors may contribute to androgen-independent PCa growth. Our results warrant further investigations into the role of androgen-independent stromal growth factor signals in the progression of androgen-insensitive PCa cells under low-androgen conditions. In addition, our *in vivo* recombination model of androgen-insensitive AR-positive PCa cells with embryonic rat UGM may be useful in developing new therapeutic strategies such as a tumor stroma targeted therapy, under androgen-manipulated status.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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