Suppression of Androgen-Independent Prostate Cancer Cell Aggressiveness by FTY720: Validating Runx2 as a Potential Antimetastatic Drug Screening Platform

Chee-Wai Chua,1 Yung-Tuen Chiu,1 Hiu-Fung Yuen,2 Kwok-Wah Chan,2 Kwan Man,3 Xianghong Wang,1 Ming-Tat Ling,1 and Yong-Chuan Wong1

Abstract

**Purpose:** Previously, FTY720 was found to possess potent anticancer effects on various types of cancer. In the present study, we aimed to first verify the role of Runx2 in prostate cancer progression and metastasis, and, subsequently, assessed if FTY720 could modulate Runx2 expression, thus interfering downstream events regulated by this protein.

**Experimental Design:** First, the association between Runx2 and prostate cancer progression was assessed using localized prostate cancer specimens and mechanistic investigation of Runx2-induced cancer aggressiveness was then carried out. Subsequently, the effect of FTY720 on Runx2 expression and transcriptional activity was investigated using PC-3 cells, which highly expressed Runx2 protein. Last, the involvement of Runx2 in FTY720-induced anticancer effects was evaluated by modulating Runx2 expression in various prostate cancer cell lines.

**Results:** Runx2 nuclear expression was found to be up-regulated in prostate cancer and its expression could be used as a predictor of metastasis in prostate cancer. Further mechanistic studies indicated that Runx2 accelerated prostate cancer aggressiveness through promotion of cadherin switching, invasion toward collagen I, and Akt activation. Subsequently, we found that FTY720 treatment down-regulated Runx2 expression and its transcriptional activity, as well as inhibited its regulated downstream events. More importantly, silencing Runx2 in PC-3 enhanced FTY720-induced anticancer effects as well as cell viability inhibition, whereas overexpressing Runx2 in 22Rv1 that expressed very low endogenous Runx2 protein conferred resistance in the same events.

**Conclusion:** This study provided a novel mechanism for the anticancer effect of FTY720 on advanced prostate cancer, thus highlighting the therapeutic potential of this drug in treating this disease.

Based on the statistical figures from American Cancer Society, prostate cancer is again emerged as the most common cancer as well as the second leading cause of cancer-related death in American male (1). In recent years, an upward trend in prostate cancer incidences has also been observed in Asian countries, which include Hong Kong (2), possibly due to an increase in aged population. The establishment of serum prostate-specific antigen (PSA) in the past decades has enabled early detection of prostate cancer, although, in general, the figure of prostate cancer–related death has not decreased significantly. When diagnosed with advanced prostate cancer, androgen depletion therapy would initially be applied to these patients (3). The majority of tumors would respond to the treatment, resulting in shrinkage of tumors. Unfortunately, relapse of tumors would gradually occur in the majority of patients, leading to androgen-independent (AI) and chemotherapy-resistant metastatic prostate cancers (4). Novel therapeutic interventions are therefore urgently required.

Metastasis to bone is one of the hallmarks of advanced prostate cancer, which leads to bone pain and subsequently fractures in bone and spinal compression, thus contributing to high morbidity and mortality rates in patients with this disease (5). Previously, it has been proposed that prostate cancer cells must acquire bone cell–like properties, to reside, grow, and expand their colonies in the bone microenvironment (6). As shown in that study (6), parallelism of the gene expression profile between prostate cancer progression and osteoblast maturation is common and this may provide some insights into the involvement of bone-related factors in the development of.

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Received 12/5/08; revised 3/11/09; accepted 3/23/09; published Online First 6/9/09.

**Grant support:** RGC grants HKU7490/03M, HKU7470/04M, HKU Foundation Seed Funding/03, NSFC/RGC, NHKU738/03 (Y.C. Wong).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

**Note:** Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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doi:10.1158/1078-0432.CCR-08-3157
bone metastatic prostate cancer. Thus, bone-related factors seem to be an attractive therapeutic target in treating metastatic prostate cancer.

Runx2, a transcription factor involved in osteoblast differentiation (7), has been studied intensively for its role during carcinogenesis in several types of cancer (8–10). More importantly, previous studies have indicated that Runx2 was overexpressed in cancers that metastasized predominantly to bone, such as breast and prostate cancers (9, 10). It was believed that Runx2 could confer survival advantage to the cancer cells on the one hand, whereas, on the other hand, it could promote the metastatic ability of these cells by enhancing the secretion of various types of matrix metalloproteinases (9–11). However, the exact underlying mechanisms involved in Runx2-induced prostate cancer cell aggressiveness remained to be elucidated as not all of these studies were done using prostate cancer as a model.

FTY720, a fungus derivative, which was used initially as an immunomodulatory drug, was shown to possess anticancer properties in various types of cancer, including leukemia (12), lymphoma (13), glioma (14), liver (15), breast (16), bladder (17), and prostate carcinomas (18–20). Previous results from our laboratory showed that FTY720 treatment led to in vivo growth suppression on CWR22R, an AI prostate cancer xenograft, by inhibiting proliferation and angiogenesis, as well as promoting apoptosis (19). In addition, results from our laboratory also suggested an invasion inhibitory role of FTY720 on two AI prostate cancer cell lines, DU145 and PC-3, through inactivation of RhoA-GTPase (20). Recently, we also found that FTY720 could induce a reversal of epithelial-to-mesenchymal transition (EMT), a crucial event during carcinogenesis leading to metastasis, in AI prostate cancer cell lines as well as CWR22R xenograft.4 Moreover, a previous study has reported that FTY720 treatment could suppress the incidence of metastasis in an orthotopic breast cancer model (16). Taken together, these lines of evidence have highlighted the antimetastatic ability of this drug.

In the present study, we first validated the prognostic significance of Runx2 in prostate cancer progression and metastasis. Subsequently, we investigated the underlying mechanisms of Runx2 in promoting prostate cancer cell aggressiveness. Because various transcription factors, such as Twist and Snail, have been shown to negatively regulate E-cadherin expression, and consequently enhancing the aggressiveness of cancer cells (21, 22), we asked whether Runx2 would also exert its cancer-promoting effect through similar mechanism. In addition, it has been previously found that N-cadherin was a potential target of Runx2 in embryonic bone tissue of Runx2 knockout mice (23). Moreover, it has also been implicated that the interaction between osteoblastic factors would induce prostate cancer cell osteomimicity by suppressing E-cadherin gene expression while enhancing N-cadherin gene expression (24), indicating that a switch of E- to N-cadherin might play a very important role in promoting prostate cancer bone metastasis. These lines of evidence led us to postulate that Runx2, an important transcription factor in osteoblast differentiation (7) and prostate cancer progression (9, 10), might promote prostate cancer cell aggressiveness through induction of cadherin switching. Although Runx2 has been shown to cooperate with Akt pathway in regulating osteoblast and chondrocyte differentiation and cell migration (25), we were uncertain if Runx2 would also induce Akt activation in prostate cancer cells. Therefore, we examined the modulating effect of Runx2 expression on Akt phosphorylation status in prostate cancer cells. Finally, we tested the possibility of using Runx2 as a platform for antimetastatic drug screening using the drug FTY720. We hypothesized that FTY720 could induce downregulation of Runx2 expression, thus suppressing its regulated downstream events and consequently altering prostate cancer cell aggressiveness.

Materials and Methods

Patient cohort. A total of 128 localized prostate cancer specimens, dating from 1995 to 2004, were obtained from the archives at Queen Mary Hospital, The University of Hong Kong, with Ethics Committee approval. The specimens consisted of nodular hyperplasia or benign prostatic hyperplasia (BPH; n = 39), high-grade prostatic intraepithelial neoplasia (PIN; n = 8), and prostate cancer (n = 128) tissues. The cancer specimens were graded with scores ranging from 4 to 10 according to the Gleason criteria (26). Age distribution of the patients at the time of procurement of the tissue samples was 55 to 94 y, with a median of 73 y. This cohort of patients did not receive neoadjuvant therapy such as radiation or hormonal therapy.

Investigation of bone metastasis risks was assessed using a subset of Gleason score–matched patients (≥7). The patients were subdivided into nonmetastatic (no metastases detected, median follow-up 38 months, ranged from 12 to 90 months, n = 34) and metastatic

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4 Unpublished data.
(metastases detected within 6 mo after procurement of the specimens, \( n = 54 \)) groups. In addition, preoperative serum PSA values were available for 76% (97 of 128) of the patient cohort. Clinical and pathologic information of the patient cohort were summarized in Supplementary Table S1.

**Tissue microarray construction.** Tissue microarray was constructed using a Beecher Instruments tissue microarrayer. A total of six tissue microarrays, each containing ~80 cores, was made. Identification of representative areas of BPH, high-grade PIN, and prostate cancers from individual paraffin blocks and verification of the pathology of the newly built tissue microarrays was done by Dr. K.W. Chan (Department of Pathology, Faculty of Medicine, The University of Hong Kong).

**Immunohistochemical staining.** Immunostaining procedures were done as previously described (19). Deparaffinized and rehydrated sections were incubated with the primary antibody, anti-Runx2 rabbit serum, which was a generous gift from Professor Gerald Karsenty (Columbia University; 1:800), in a humidified chamber overnight at 4°C. Section treated with TBS alone served as a negative control. The following day, the sections were incubated with relevant secondary antibodies provided in the kits. The staining intensity was visualized using the diaminobenzidine substrate (Dako Corp.). Mayer’s hematoxylin was used for counterstaining the slides before further dehydration, clearing, and mounting steps.

**Evaluation of immunostaining results on prostate cancer patient sections.** All immunohistochemical staining results from 175 patient samples were scored by an individual observer to avoid interobserver variation in a blinded fashion. All three cores were examined and the hot core with the most intense immunostaining was selected and subjected to further quantification. In case of a needle biopsy, the whole section was examined and the hotspot area with the highest staining intensity was selected for further quantification. Under high-power (×400) magnification view, nuclear staining intensity was scored using a grading scale as 0 (negative), 1 (weak), 2 (moderate), or 3 (strong). The percentage (0-100%) of nuclei staining at each staining intensity level was determined. A nuclear scoring system was subsequently done as described previously (27). Nuclear score (ranging 0-300) was calculated by a formula as follows: \( \frac{a \times d + b \times c + c \times b + d \times a}{a + b + c + d} \), in which \( a, b, c, \) and \( d \) were percentages of nuclei stained at different staining intensity grades. In addition, cytoplasmic expression was also assessed separately according to an intensity scale as negative, weak, moderate or strong (0-3).

**Statistical analysis.** All statistical analyses were done with SPSS 11.1 software. Kolmogorov-Smirnov test was first used to examine distribution of the data. Multiple group comparison was done by either ANOVA or Kruskal-Wallis test, whereas the statistical difference between the two groups was determined by either the Student’s t test or the Mann-Whitney test. Correlation analysis was carried out by Spearman’s rank test procedure. Association of Runx2 and risk of metastasis in breast cancer patients was determined using the hazard ratio test. Differences were considered significant statistically if the 2-sided value of \( P < 0.05 \). All reported \( P \) values were two-sided.

**Drug.** FTY720 was kindly provided by Novartis Pharmaceuticals, Ltd., in a powder form. The drug was dissolved in autoclaved distilled water and stored at -20°C, with a final concentration of 1 mmol/L.

**Cell lines and culture conditions.** Cell lines used in the present study were the A1 prostate cancer cell lines PC-3 and 22Rv1. PC-3 was obtained from American Type Culture Collection, whereas 22Rv1 was a generous gift from Dr. Franky Chan (The Chinese University of Hong Kong). These cell lines were maintained in RPMI 1640 (Invitrogen) supplemented with either 5% (PC-3) or 10% (22Rv1) fetal bovine serum and penicillin/streptomycin at 37°C. A1 cells were maintained in RPMI 1640 containing 10 mmol/L sodium chloride, 10 mmol/L potassium chloride, 0.5 mmol/L dTT, 0.2% (v/v) NP40, 1 mmol/L sodium orthovanadate, 1 μg/ml leupeptin, 1 μg/ml aprotnin, and 1 μg/ml phenylmethylsulfonyl fluoride. The suspension was left on ice for 10 min followed by centrifugation at 3,000 rpm for another 10 min at 4°C. The supernatant was collected as cytosolic fraction. The remaining pellet was resuspended in ice-cold PBS and centrifuged at 3,000 rpm for 3 min at 4°C. This washing step was repeated twice to eliminate the remaining cytosolic residues. After the washing, the pellet was then lysed with another buffer containing 50 mmol/L Tris-HCl (pH 8.0), 500 mmol/L sodium chloride, 5 mmol/L EDTA, 0.5 mmol/L DTT, 1% (v/v) NP40, 1 mmol/L sodium orthovanadate, 1 μg/ml leupeptin, 1 μg/ml aprotnin, and 1 μg/ml phenylmethylsulfonyl fluoride. The suspension was left on ice for 30 min. Subsequently, the suspension was centrifuged at 13,000 rpm for 15 min at 4°C. The supernatant was collected as the nuclear fraction.

**Western blot analysis.** Experimental procedures were detailed as reported previously (21) with minor modifications. Briefly, equal amounts of protein for various samples were loaded and separated by electrophoresis on a SDS-polyacrylamide gel and blotted onto the nitrocellulose membranes (Amersham Biosciences). Blotting of unspecific binding of antibody was carried out with 10% nonfat dry milk in TBS-T for 1 h at room temperature and incubated with primary antibodies specific for Runx2, N-cadherin (Santa Cruz Biotechnology), E-cadherin (BD Biosciences), phospho-Akt (serine 473), and total Akt (Cell Signaling Biotechnology). Subsequently, relevant secondary antibodies were applied to the membranes followed by visualization using enhanced chemiluminescence Western blotting system (Amersham Biosciences). Internal loading controls used were β-actin (Santa Cruz Biotechnology) for total cell lysate and histone H1 (Santa Cruz Biotechnology) for nuclear lysate.

**Transient transfection procedures.** Cells were seeded on a six-well plate (IWAKI) 1 d before transfection. Plasmids used in the present study were full-length Runx2 plasmid, a generous gift from Professor Gerald Karsenty (Department of Genetics and Development, Columbia University; refs. 7, 28), and vector control, pCMV5, which was kindly provided by Dr. Zhenguo Wu (Hong Kong University of Science and Technology). The ratio of plasmid amount to volume of Fugene 6 (Roche Diagnostics) transfecting agent used in this study was 1:2.5, and all transfection procedures were carried out according to manufacturer’s recommendation. After incubating the cells with transfection mixture solution for 24 h, the existing culture medium was either replaced by fresh culture medium or further experimental procedures were done.

**Small interfering RNA duplexes treatment.** In this study, small interfering RNA (siRNA) duplexes specific for Runx2, \( \text{siRUNX2} \), were obtained from Qiagen, Inc., whereas the nontargeting siRNA (Dharmacon) was used to serve as control. Cells were seeded on a six-well plate (IWAKI) 1 d before transfection procedures. On the following day, siRNAs were transfected into the cells using Lipofectamine 2000 (Invitrogen). Briefly, 5 μL of Lipofectamine 2000 transfecting agent were added dropwise into 250 μL serum-free culture medium, and the mixture solution was incubated for 5 min at room temperature. In the meantime, 5 μL siRNA specific for Runx2 was added into another separate 250 μL serum-free culture medium. Both mixture solutions were then mixed together and incubated for a further 20 min. For each well of a six-well plate, only 1 mL of culture medium was left, whereas excessive medium was sucked off. The transfection mixture solution (500 μL) was subsequently added into the well and allowed to incubate for 24 h. After 24 h, old batches of culture medium were either replaced by fresh culture medium or further experimental procedures were carried out.

**Reverse transcription-PCR.** Reverse transcription-PCR (RT-PCR) was carried out as described previously (29). Total RNA was isolated using...
Trizol reagent (Invitrogen) according to the manufacturer’s recommendation. cDNAs of the cells were synthesized using the SuperScript First Strand Synthesis System (Invitrogen) and subsequently amplified with primers specific for Runx2 (forward: 5'-GGTGTTCCTGAGGCCGCTC-3' and reverse: 5'-CCAAGTTGAGGACACCTGA-3', 318 bp; ref. 30), N-cadherin (forward: 5'-CAAGTGCACTTGAAGCAGG-3' and reverse: 5'-TTAAGGGCTCAGTGTTGTC-3', 578 bp; ref. 31), and E-cadherin (forward: 5'-GTAACCCGCTAGAATGAC-3' and reverse: 5'-CCTGGTGGAATGTCAGAT-3', 380 bp; ref. 32). After an initial denaturation at 95°C of 10 min, amplification was done for 28 to 30 cycles, using the conditions as follows: 94°C/45 s; 61°C/45 s; 72°C/60 s. Glyceraldehyde 3-phosphate dehydrogenase was used as an internal control. The PCR products underwent electrophoresis on a 1.5% agarose gel and were then analyzed using a gel documentation system.

Luciferase reporter assay. To perform luciferase reported assay, cells were first seeded onto a 12-well plate. The following day, transient transfection was carried out as described previously by cotransfecting the cells with a plasmid containing Runx2-luciferase reporter, which carried a sequence of osteocalcin gene 2 promoter and designated as p6OSE2 (a gift from Professor Gerald Karsenty; refs. 7, 28) and pRL-TK-Luc, which served as an internal control for transfection efficiency. After 24 h, the existing culture medium was replaced by either fresh culture medium or FTY720. A further incubation period of 24 h was allowed. Subsequently, the cells were lysed and subjected to luciferase reporter assay using the dual-luciferase reporter assay system (Promega) as recommended by the manufacturer. All experiments were carried out in a triplicate manner and the whole experiment set up was repeated twice. Data obtained were presented as mean ± SD.

Immunofluorescent staining. Experimental procedures were carried out as detailed in the previous study (21) with minor modifications. Briefly, cleaned coverslips were laid onto the wells of a six-well plate and subsequently followed by seeding of cells. Further experimental procedures were carried out on the following day. At the end of the experiment, the cells were fixed with 4% paraformaldehyde in PBS for 10 min at room temperature. After washing with PBS, permeabilization of cells was carried out with 1% Triton X-100 for 15 min at room temperature. Blocking for unspecific binding was done by 1% bovine serum albumin for 30 min at room temperature. The fixed cells were then incubated with primary antibodies specific for E-cadherin (BD Transduction Laboratories) and N-cadherin (Santa Cruz Biotechnology) for 1 to 2 h at 37°C, and followed by incubation with relevant fluorescein-labeled secondary antibodies for 1 h at room temperature. Last, the cell-coated coverslips were mounted and fluorescent signals were captured by an epifluorescent microscope under ×400 magnification.

Type I collagen invasion assay. Type I collagen invasion assay was carried out as described by Bracke et al. (33) with minor modifications. Briefly, collagen solution was prepared by mixing all the precooled (4°C) components, including collagen type 1 (BD Biosciences), MEM (Invitrogen), PBS, sodium bicarbonate (NaHCO₃) solution, and sodium hydroxide (NaOH). The solution was mixed gently to avoid introduction of air bubbles. The outer wells of a six-well plate were poured with 1.25 mL of the mixture solution per well, whereas 1.35 mL of the mixture solution were added into each of the middle wells. The collagen solution was allowed to solidify for 2 h at 37°C in a cell culture incubator. Subsequently, cells were trypsinized and resuspended in culture medium with a total volume of 2 mL. The cell suspension was then poured onto the solidified gel and allowed to attach and grow on the gel for 24 h. Twenty-four hours later, cell morphology was observed under an inverted microscope. Solitary cells with rounded shape were considered to be invading cells / total number of cells

Results

Verification of the role of Runx2 during prostate cancer progression and metastasis

To investigate the role of Runx2 during prostate cancer progression and metastasis, an immunohistochemistry study was carried out on various sections of tissue microarrays and needle biopsies obtained from primary sites of the patients with known clinical and pathologic information (Supplementary Table S1). We used an antiserum against Runx2 (a generous gift from Professor G. Karsenty, Columbia University) that has been tested for its sensitivity and specificity in prostate cancer tissues previously (10). As shown in Fig. 1A, prostate cancer showed various staining intensities of Runx2 expression, whereas, by comparison, BPH and high-grade PIN, in general, showed undetectable to very low Runx2 expression. As various degrees of nuclear staining intensities were observed in these clinical samples, we then used a nuclear scoring system (27) to evaluate the nuclear expression of Runx2. When comparing with BPH and high-grade PIN, prostate cancer tissues exhibited a significant higher Runx2 nuclear score, with a P value of <0.0001 (Fig. 1B). By contrast, Runx2 cytoplasmic staining evaluation revealed that staining pattern distributions of high-grade PIN and prostate cancer were significantly different compared with BPH (high-grade PIN versus BPH, P < 0.01 and prostate cancer versus BPH, P < 0.0001; Table 1). Both groups showed more tissues with Runx2-positive staining (87% for high-grade PIN and 84.4% for prostate cancer) compared with BPH samples (36.8%) as shown in Table 1. Taken together, these results indicated that Runx2 protein might be involved in prostate carcinogenesis.

Subsequently, we compared the nuclear and cytoplasmic expressions of Runx2 with various clinical and pathologic indicators to further evaluate the prognostic significance of this protein. We first assessed if there was any correlation between nuclear or cytoplasmic expression of Runx2 and Gleason score, a well-established classification system, and known prognostic indicator of prostate cancer (26). As shown in Table 2, Runx2 nuclear score but not its cytoplasmic expression was associated with a higher Gleason score (correlation coefficient, r = 0.233, P < 0.01).

It was reported previously that the risk of metastasis increased with preoperative serum PSA levels higher than 20 ng/mL (34–36). Next, using the patient cohort with available preoperative serum PSA levels, we subdivided these patients into two groups, i.e., <20 and ≥20 ng/mL. We observed that patients with preoperative serum PSA ≥20 ng/mL possessed...
higher Runx2 nuclear score compared with those with PSA <20 ng/mL with a P value of <0.01 (Fig. 1C). Similar to the Gleason score correlation results, no difference was observed between these two groups for their Runx2 cytoplasmic expression pattern distribution (Supplementary Table S2). Taken together, these results indicated that nuclear expression of Runx2 but not its cytoplasmic expression possessed prognostic significance in prostate cancer.

Using Gleason score–matched (≥7) prostate cancer samples, we investigated whether Runx2 nuclear score possessed metastasis prediction ability in this cohort of patients. As shown in Fig. 1D (top), it was clearly evidenced that localized prostate cancer with diagnosed metastasis showed much higher expression of Runx2 in nuclei compared with cases without metastasis. Statistically, the metastatic group showed significantly higher Runx2 nuclear score compared with the nonmetastatic group with P < 0.01 (Fig. 1D, bottom). We then further confirmed the prognostic value of Runx2 nuclear score in these high Gleason score prostate cancer patients using Cox regression analysis. First, we used Runx2 nuclear score as a continuous variable in the univariate Cox regression model generated as shown in Table 2. We found that Runx2 nuclear score was associated with higher risk of metastasis in these patients (hazards ratio, 1.01; 95% confidence interval, 1.003-1.016;

![Fig. 1. Expression of Runx2 in prostate cancer. A, representative photos of Runx2 expression in BPH, high-grade PIN, and prostate cancer tissues. Tumor sections were immunostained with an antiserum specific for Runx2 protein. Photos were taken under ×100 (top) and ×400 (bottom) magnifications. B, representative results of Runx2 nuclear expression evaluation between BPH, high-grade PIN, and prostate cancer. Nuclear expression of Runx2 was evaluated and quantified as described in Materials and Methods. Note that Runx2 nuclear expression was significantly increased in prostate cancer compared with BPH and high-grade PIN (P < 0.0001). C, representative results of Runx2 nuclear score comparison between prostate cancer patients with serum PSA <20 and ≥20 ng/mL. A cohort of patients with available serum PSA information was subdivided into two groups, i.e., PSA <20 and ≥20 ng/mL, and the Runx2 nuclear scores of these two groups of patients were compared as detailed in Materials and Methods. Note that Runx2 nuclear expression was significantly higher in prostate cancer patients with PSA ≥20 ng/mL compared with those of with PSA <20 ng/mL counterparts (P < 0.001). D, representative photos (top) and results (bottom) comparing Runx2 nuclear expression between prostate cancer patients with and without metastasis. Gleason score–matched (≥7) patients were subdivided into nonmetastatic and metastatic groups. Photos were taken under ×400 magnification, whereas Runx2 nuclear score between these two groups was compared as described in Materials and Methods. Note that prostate cancer patients with metastasis incidences showed higher Runx2 nuclear score compared with those without metastasis with P < 0.01. All data are shown as mean ± SE. Scale bar, SE.]

Clin Cancer Res 2009;15(13) July 1, 2009 4326  www.aacrjournals.org

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Effect of FTY720 on Runx2 Nuclear Expression

Table 1. Comparison of Runx2 cytoplasmic expression between BPH, high-grade PIN, and prostate cancer

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<tr>
<th></th>
<th>Staining intensities (%)</th>
<th>No. samples</th>
<th>P</th>
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<tbody>
<tr>
<td></td>
<td>0</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>BPH</td>
<td>24 (63.2)</td>
<td>13 (34.2)</td>
<td>1 (2.6)</td>
</tr>
<tr>
<td>High-grade PIN</td>
<td>1 (12.5)</td>
<td>5 (62.5)</td>
<td>2 (25)</td>
</tr>
<tr>
<td>Prostate cancer</td>
<td>20 (15.6)</td>
<td>61 (47.7)</td>
<td>37 (28.9)</td>
</tr>
</tbody>
</table>

NOTE: Statistical comparison between various groups was carried out using χ² test. Note that Runx2 cytoplasmic stainings of both high-grade PIN and prostate cancer samples were significantly different when comparing with BPH (high-grade PIN versus BPH, P < 0.01, and prostate cancer versus BPH, P < 0.0001).

P = 0.005), meaning that with every increase in Runx2 nuclear score unit, there was a 1% increase in the risk of developing metastasis in this cohort of patients. We then further divided these patients according to the mean value (= 105 Runx2 nuclear score). Similarly, a significantly higher risk for these patients to develop metastasis (hazards ratio, 2.44; 95% confidence interval, 1.401-4.251; \( P = 0.002 \)) when higher expression of nuclear Runx2 (nuclear score ≥105) was observed (Table 3). These results suggested that nuclear expression of Runx2 could be used as a prognostic marker to predict the occurrence of metastasis in advanced prostate cancer.

Effect of Runx2 on cadherin switching status, cell invasion toward type I collagen, and Akt activation in AI prostate cancer

Establishment of Runx2 transfectants. To investigate the possible underlying mechanisms involved in Runx2-induced prostate cancer progression, we established several transfectants with various degrees of Runx2 expression. Using several AI prostate cancer cell lines available in our laboratory, namely 22Rv1, DU145, and PC-3, we first compared the expression of Runx2 in these prostate cancer cell lines. As Runx2 proteins are focally localized at the nuclear region and it has been reported that activation of Runx2 would consequently promote its nuclear localization (37, 38), we assessed primarily the nuclear expression of Runx2 when compared with the vector control counterpart treated with nonspecific targeting siControl (Fig. 2A, left). On the other hand, 22Rv1, an AI prostate cancer cell line that expressed very low endogenous Runx2 expression, was transiently transfected with either a plasmid containing full-length Runx2 gene (7, 28) or vector control pCMV5 (Fig. 2A, right). As shown in Fig. 2B (right), Runx2-overexpressing 22Rv1 cells exhibited a much higher expression of Runx2 when compared with the vector control–expressing cells. We have, therefore, successfully established Runx2-overexpressing 22Rv1 cells as well as Runx2-silencing PC-3 cells.

Runx2 and cadherin switching. Cadherin switching has been indicated to play a crucial role during prostate cancer progression. Low E-cadherin expression concomitant with high N-cadherin expression were shown in tissues of prostate cancer patients with aggressive disease phenotype, and cadherin switching was associated with higher Gleason score and poorer prognosis in these patients (39–41). Because Runx2 expression was correlated positively with Gleason score in the patient samples in the present study (Table 2), we next investigated if Runx2 would promote cadherin switching in AI prostate cancer using the established transfectants (Fig. 2A). We found that silencing of Runx2 expression in PC-3 cell line that expressed a high level of this protein resulted in a reverse cadherin switching status, in which E-cadherin expression was enhanced whereas N-cadherin expression was decreased in these cells (Fig. 2B, top left panels, and C). On the other hand, Runx2 overexpression led to down-regulation of E-cadherin expression but up-regulation of N-cadherin in 22Rv1 cells (Fig. 2B, top right panels, and D). Interestingly, silencing Runx2 in PC-3 cells resulted in up-regulation of E-cadherin mRNA expression levels whereas overexpressing Runx2 in 22Rv1 cells caused suppression on E-cadherin transcription (Fig. 2B, bottom). By comparison, N-cadherin mRNA expression levels were not affected by modulation of Runx2 expression in these cell lines (Fig. 2B, bottom). These results indicated that Runx2-induced suppression of E-cadherin expression might be mediated through transcriptional regulation, whereas up-regulation of N-cadherin expression induced by Runx2 might involve a posttranscriptional mechanism. Taken together, these results suggested that Runx2-induced cadherin switching during prostate cancer progression.

Runx2 and cell invasion toward type I collagen. To grow within the bone region, metastatic prostate cancer cells must acquire the ability to invade into extracellular matrix. Because type I collagen is the most abundant protein as well as the

Table 2. Correlation between Gleason score and Runx2 expression in prostate cancer

<table>
<thead>
<tr>
<th></th>
<th>Correlation coefficient, ( r )</th>
<th>P</th>
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<tr>
<td>Gleason score vs Runx2 nuclear score</td>
<td>0.233</td>
<td>0.008</td>
</tr>
<tr>
<td>Runx2 cytoplasmic expression</td>
<td>0.009</td>
<td>0.919</td>
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NOTE: Correlation between Gleason score and Runx2 expression was done using Spearman’s rank test procedure. Note that Runx2 nuclear score but not its cytoplasmic expression correlated positively with Gleason score, \( P < 0.01 \).
major component of bone matrix (42), we investigated the invasive capability of various transfectants (PC-3-siControl, PC-3-siRunx2, 22Rv1-pCMV5, and 22Rv1-Runx2) toward type I collagen. After treating PC-3 cells with siRunx2 duplexes, a lot of cells remained in rounded morphology even after being allowed to attach on collagen I surface for 24 hours, in comparison with PC-3 cells treated with siControl (Fig. 3A, left). When counting the number of invading cells, siRunx2-treated group was shown to comprise less invading cells (31.61 ± 5.11%) compared with the siControl-treated group (67.01 ± 4.55%; Fig. 3A, right). On the contrary, as shown in Fig. 3B, 22Rv1 cells transfected with Runx2 consisted of more invading cells (66.45 ± 3.19%), as exhibited by cytoplasmic extension that indicated the invasive behavior, when comparing with 22Rv1 cells transfected with vector control (37.28 ± 1.81%). These lines of evidence indicated that Runx2 promoted the aggressiveness of AI prostate cancer cells by enhancing the invasive capability of these cells toward type I collagen.

Runx2 and Akt activation. Akt activation has been shown to orchestrate multiple signaling pathways in prostate cancer (43). It was previously reported that Runx2 augmented migration of osteoblast and chondrocyte through activation of the PI3K-Akt pathway (25). However, to our knowledge, there is no report about the potential cross-talk between Runx2 and Akt pathway using prostate cancer as a model. Next, we evaluated the effect of Runx2 expression modulation on Akt phosphorylation status in AI prostate cancer cell lines. As shown in Fig. 3C (left), knocking down of Runx2 expression in PC-3 cells resulted in inhibition of Akt phosphorylation. On the other hand, over-expression of Runx2 in 22Rv1 cells led to increased level of phospho-Akt (Fig. 3C, right). This observation provided a novel linkage between Runx2 and Akt pathway, thus suggesting the involvement of Akt activation in Runx2-induced prostate cancer progression.

Effect of FTY720 treatment on Runx2 expression and transcriptional activity
To test the possibility of using Runx2 as a potential antimetastatic drug screening platform, we used an anticancer drug, FTY720, which have been previously found to suppress the growth as well as the aggressiveness of AI prostate cancer (19, 20). First, we investigated if FTY720 treatment would lead to down-regulation of Runx2 expression as well as its transcriptional activity using PC-3 cell line as a model. As shown in Fig. 4A, we observed that after treating PC-3 cells with FTY720 for 48 hours, Runx2 nuclear as well as mRNA expression levels were down-regulated in a dose-dependent manner. The FTY720-induced suppression effect on Runx2 expression was found to be not related to the apoptotic-inducing effect of FTY720 because the doses used in the present study did not result in any apoptosis-related events (Supplementary Fig. S2). Subsequently, we evaluated if down-regulation of Runx2 protein by FTY720 treatment would consequently contribute to an inhibition on its transcriptional activity using a reporter carrying the OSE2 sequence in the promoter of osteocalcin gene 2, a downstream effector of Runx2 (7, 28). Luciferase reporter assay revealed that 48 hours of FTY720 treatment reduced Runx2 transcriptional activity in PC-3 cells when comparing with the untreated counterparts (Fig. 4B). Taken together, these lines of evidence indicated that FTY720 could suppress Runx2 expression as well as its transcriptional activity levels, suggesting a novel mechanism of FTY720-induced anticancer effect.

Effect of FTY720 on cadherin switching status, cell invasion toward type I collagen, and phosphorylation status of Akt in PC-3 cells
Next, we investigated the effect of FTY720 on several Runx2-regulated downstream events identified (Figs. 2 and 3); that is, induction of cadherin switching, enhancement of cell invasiveness toward type I collagen, and activation of Akt. Western blot analysis revealed that FTY720 treatment promoted the reversal of cadherin switching in PC-3 cells, with an increase of E-cadherin expression concomitant with a reduction of N-cadherin expression in a dose-dependent manner (Fig. 4C). In addition, we also found that Akt phosphorylation was down-regulated with increasing doses of FTY720 at 48 hours (Fig. 4C). When evaluating the invasion ability toward type I collagen after FTY720 treatment, we observed that PC-3 cells treated with FTY720 exhibited less invasive behavior with rounded shape in morphology (Fig. 4D, top). As shown in Fig. 4D (bottom), manual counting of invading cells versus non-invading cells revealed that FTY720 treatment caused a decrease in the percentage of invading cells in a dose-dependent manner (untreated control versus 1.5 and 3 μmol/L FTY720: 58.86 ± 0.86% versus 30.69 ± 1.83% and 16.22 ± 1.47%). Taken together, these observations indicated that FTY720 treatment altered Runx2-regulated downstream events.

### Table 3. Univariate Cox regression analysis of Runx2 nuclear score on metastasis incidences in Gleason score-matched (≥7) prostate cancers

<table>
<thead>
<tr>
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<th>Hazards ratio (95% confidence interval)</th>
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<tbody>
<tr>
<td>Runx2 nuclear score*</td>
<td>1.01 (1.003-1.016)</td>
<td>0.005</td>
</tr>
<tr>
<td>Runx2 nuclear score &lt; vs ≥ mean = 105†</td>
<td>2.44 (1.401-4.251)</td>
<td>0.002</td>
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NOTE: Runx2 nuclear score could serve as a predictor of metastasis in this cohort of patients with matched Gleason score (≥7). *Continuous variable was used for calculation so that for each unit increase in Runx2 nuclear score, there is a 1% increase in the risk of developing metastasis in this cohort of patients. † Patients were subdivided into two groups according to the mean value (=105) of this cohort of patients.

FTY720 promotes cadherin switching reversal and suppression of cell invasion toward type I collagen through modulation of Runx2 expression
To further illustrate the involvement of Runx2 protein in FTY720-induced cadherin switching reversal, suppression of cell invasion toward type I collagen, and Akt dephosphorylation, we modulated the expression of Runx2 in PC-3 cells with
relatively higher expression of Runx2 and 22Rv1, the AI prostate cancer cell line with low endogenous Runx2 expression (Supplementary Fig. S1), and subsequently investigated the effect of FTY720 on cadherin switching, cell invasion, and Akt phosphorylation status in these transfectants. As shown in Fig. 5A (left), transfection of siRunx2 duplexes on PC-3 cells resulted in down-regulation of Runx2 when comparing with the cells treated with siControl. Interestingly, whereas siControl-treated PC-3 cells exhibited an increased level of E-cadherin expression upon FTY720 treatment, further enhancement of E-cadherin expression was observed in siRunx2-treated PC-3 cells, which already showed relatively higher expression of E-cadherin before FTY720 treatment (Fig. 5A, left; Supplementary Fig. S3A). By contrast, whereas FTY720 treatment led to a reduced level of N-cadherin in PC-3-siControl transfectants, N-cadherin expression level in PC-3-siRunx2 transfectants, which was already lower compared with FTY720-treated PC-3-siControl transfectants, remained at a relatively low level after

Fig. 2. Runx2 promotes cadherin switching in prostate cancer. A, representative results of Western blot analysis on Runx2 expression for PC-3 cells after treatment with either siControl or siRunx2 duplexes (left) as well as 22Rv1 cells transfected with either pCMV5 or full-length Runx2 (right). Transient transfection was carried out as described in Materials and Methods. Note that treatment of siRunx2 duplexes resulted in decreased levels of Runx2 expression in PC-3 cells whereas Runx2-overexpressing 22Rv1 cells exhibited a much higher Runx2 expression. B, representative Western blot (top) and RT-PCR (bottom) results of E-cadherin and N-cadherin expressions in PC-3 cells treated with either siRunx2 or nonspecific targeting duplexes, siControl (left), and in Runx2-overexpressing 22Rv1 cells and vector control, pCMV5 (right). Total cell lysates and RNAs were collected and subjected to Western blot and RT-PCR analysis. C and D, representative photos of immunofluorescent staining results of E-cadherin (left panels) and N-cadherin (right panels) for PC-3 cells treated with siRunx2 and siControl (C) and for 22Rv1 transfected with Runx2 and pCMV5 plasmids (D). Cells were seeded on coverslips in a six-well plate, followed by transient transfection, and subsequently fixed before immunostaining procedures as detailed in Materials and Methods. 4',6-Diamidino-2-phenylindole (DAPI) staining indicated the location of nucleus. Photos were taken under ×400 magnification. Note that silencing of Runx2 resulted in up-regulation of E-cadherin expression but down-regulation of N-cadherin expression in PC-3 cells whereas Runx2 overexpression caused reversion in the identical phenomenon in 22Rv1 cells. In addition, modulation of Runx2 expression resulted in alteration of E-cadherin but not N-cadherin expression at the transcription levels.
FTY720 treatment (Fig. 5A, left, and Supplementary Fig. S3B). On the other hand, whereas FTY720 treatment led to enhanced E-cadherin expression and reduced N-cadherin in 22Rv1-pCMV5 transfectants, the cadherin switching reversal induced by FTY720 was not observed in Runx2-overexpressing 22Rv1 transfectants (Fig. 5A, right). Runx2 expression was also evaluated in these transfectants at the end of the treatment period, in which much higher expression of Runx2 in 22Rv1-Runx2 transfectants was evident compared with the vector controls, indicating success of the transient transfection procedures (Fig. 5A, right). These results indicated that silencing of Runx2 expression in PC-3 cells led to sensitization of FTY720-induced cadherin switching reversal whereas overexpression of Runx2 in 22Rv1 cells conferred resistance in the similar event, thus suggesting the involvement of Runx2 in FTY720-induced reversal of cadherin switching.

In addition, we also investigated whether Runx2 protein was involved in FTY720-induced suppression of cell invasion toward type I collagen. After treating the transfectants with FTY720, we found that the percentage of invading cells was further decreased in PC-3-siRunx2 transfectants with an 85% reduction when comparing with the PC-3-siControl counterpart, which showed a 67% reduction in the percentage of invading cells after drug treatment (Fig. 5B, left). By comparison, Runx2-overexpressing 22Rv1 transfectants showed less invading cells after FTY720 treatment with a reduction of only 25% in the percentage of invading cells when comparing with vector control – expressing 22Rv1 cells, with a 59% reduction in
invading cells percentage after treatment with FTY720 (Fig. 5B, right). These results suggested that, on the one hand, with reducing level of Runx2 expression, the invasive ability of PC-3 cell was further suppressed when challenged with FTY720, whereas, on the other hand, overexpressing Runx2 in 22Rv1 cells that expressed very low endogenous Runx2 expression caused less inhibition of cell invasion toward type I collagen when challenged with FTY720.

Taken together, our results suggested that FTY720-induced cadherin switching reversal and suppression of cell invasion toward type I collagen might be mediated at least partially by modulating Runx2 expression, thus suggesting a key role of Runx2 in mediating FTY720-induced anticancer effects.

**Regulation of FTY720-induced down-regulation of Akt as well as cell viability inhibition by Runx2**

Moreover, we also evaluated the effect of Runx2 expression modulation on Akt phosphorylation status in AI prostate cancer cell lines after FTY720 treatment. It was shown that siRunx2 treatment led to a further down-regulation of phospho-Akt in PC-3 cells when comparing with that treated with siControl duplexes after FTY720 treatment (Fig. 5C, left).

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**Fig. 4.** Effect of FTY720 on Runx2 expression and its regulated downstream events. A, representative Western blot (top panels) and RT-PCR (bottom panels) results of Runx2 expression in PC-3 cells after FTY720 treatment. PC-3 cells were treated with various doses of FTY720 (0, 0.75, 1.5, and 3 μmol/L) for 48 h, and nuclear lysates as well as total RNAs were collected to evaluate the changes in Runx2 expression. Note that FTY720 treatment led to suppression of both Runx2 nuclear as well as mRNA expressions in a dose-dependent manner. B, luciferase reporter assay results for Runx2 transcriptional activity evaluation between FTY720-treated PC-3 cells (solid column) and untreated controls (open column). PC-3 cells were transfected with a plasmid containing a sequence of osteocalcin gene 2 promoter, which is a downstream target of Runx2, followed by treatment of FTY720 for 48 h. Subsequently, luciferase reporter assay was done as detailed in Materials and Methods. Data are mean ± SD of an individual experiment in a triplicate manner for each treatment group. C, representative results of E-cadherin and N-cadherin expression as well as phosphorylation status of Akt in PC-3 cells after treatment of FTY720 for 48 h. After treatment of FTY720 for 48 h, total lysates of PC-3 cells were collected and subjected to Western blot analysis as detailed in Materials and Methods. Note that FTY720 treatment increased E-cadherin expression but reduced N-cadherin expression as well as phosphorylation status of Akt in PC-3 cells after treatment of FTY720 for 48 h. After treatment of FTY720 for 48 h, total lysates of PC-3 cells were collected and subjected to Western blot analysis as detailed in Materials and Methods. Data are mean ± SD of an individual experiment in a triplicate manner for each treatment group. D, morphologic appearance of PC-3 cells treated with 0, 1.5, and 3 μmol/L FTY720 on type I collagen surface (top) and comparison of invading cell percentage between these groups (bottom). PC-3 cells were seeded on wells in a six-well plate that precoated with type I collagen, together with either medium alone or medium with various doses of FTY720 (1.5 and 3 μmol/L). Subsequently, manual counting was carried out as described in Materials and Methods. All photos were taken under ×400 magnification. Cytoplasmic extension was considered as an invasive behavior, and cells with such characteristic were counted as invading cells. Note that FTY720 treatment reduced the percentage of invading cells in a dose-dependent manner. All data are presented as mean ± SD of an individual set of experiment done in a triplicate manner. The experiment setting was repeated three times. Scale bar, SD.
Fig. 5.  FTY720 suppresses cell aggressiveness of prostate cancer cells through modulation of Runx2 expression.  

A.  comparison of E-cadherin and N-cadherin expression status between PC-3 cells treated with siRunx2 and siControl (left panels) as well as 22Rv1 cells overexpressing Runx2 and vector control, pCMV5 (right panels) after being challenged with or without FTY720.  Following the transfection procedures, cells were further challenged with either medium containing 3 μmol/L FTY720 or medium alone for 24 h; subsequently, cell lysates were collected and subjected to Western blot analysis as detailed in Materials and Methods.  Runx2 expression levels of various transfectants were also evaluated.  Note that siRunx2 duplexes treatment sensitized FTY720-induced reversal of cadherin switching in PC-3 cells, whereas by comparison, overexpressing Runx2 in 22Rv1 cells resulted in resistance on this event. 

B.  comparison of the percentage of invading cells between PC-3 cells treated with siRunx2 and siControl (left) as well as between 22Rv1 cells overexpressing Runx2 and vector control (right) after challenged with (solid columns) or without (open columns) FTY720 for 24 h.  After transient transfection procedure, cells were reseeded on type I collagen surface together with either medium containing FTY720 or medium alone, and allowed to grow for 24 h.  Cell with cytoplasmic extension was considered as an invading cell.  Quantification of invading cells was done as described in Materials and Methods.  Note that silencing of Runx2 in PC-3 cells caused further suppression of cell invasiveness when challenged with FTY720 whereas overexpression of Runx2 in 22Rv1 cells, by comparison, led to decrease of cell invasion to a lower extent after treatment of FTY720. 

C.  comparison of Akt phosphorylation status between PC-3 cells treated with siRunx2 and siControl (left panels) as well as 22Rv1 cells overexpressing Runx2 and vector control, pCMV5 (right panels) after challenged with or without FTY720.  After the transfection procedures, cells were treated with either medium containing FTY720 or medium alone for 24 h, and followed by cell lysates collection and western blot analysis as described in Materials and Methods.  Note that silencing of Runx2 in PC-3 cells caused further down-regulation of Akt phosphorylation when challenged with FTY720.  By comparison, Runx2-overexpressing 22Rv1 transfectants, which had an enhanced phospho-Akt expression level, did not show marked changes in phospho-Akt expression level after FTY720 treatment.  Whereas FTY720-treated and untreated vector control expressing 22Rv1 cells did not show any difference in their Akt phosphorylation levels due to very low to undetectable expression levels.

D.  representative results of cell viability assessment between PC-3 cells treated with either siRunx2 or siControl duplexes (left) as well as 22Rv1 cells transfected with either Runx2 or pCMV5 plasmids (right) after FTY720 challenge.  Transfected cells were treated with various doses (0, 1.5, 3, and 6 μmol/L) of FTY720 for 24 h, and subjected to evaluation of cell viability using 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay as detailed in Materials and Methods.  Note that knocking down of Runx2 sensitized FTY720-induced cell viability inhibition in PC-3 cells whereas overexpressing Runx2 in 22Rv1 cells resulted in resistance on FTY720-induced cell viability inhibition in 22Rv1 cells.  Data are shown as mean ± SD of three individual set of experiments in a triplicate manner.  Scale bar, SD.
Consistent with a previous observation,\textsuperscript{5} FTY720 treatment did not cause any alteration in Akt phosphorylation in 22Rv1-pCMV5 transfectants that expressed very low to undetectable phospho-Akt, whereas treatment of FTY720 on 22Rv1 transfectants did not markedly affect the levels of induced Akt phosphorylation in these transfectants (Fig. 5C, right).

We previously found that FTY720 treatment induced inhibition of cell viability in various AI prostate cancer cell lines.\textsuperscript{6} Next, we investigated if modulation of Runx2 expression would lead to different response toward FTY720 drug treatment using various doses (from 0 to 6 \textmu mol/L) of FTY720. As shown in Fig. 5D (left), PC-3 cells were resistant to FTY720 treatment even at 6 \textmu mol/L, but knocking down of Runx2 significantly sensitized the cells to the similar treatment. On the other hand, 22Rv1-overexpressing Runx2 cells were more resistant to FTY720 treatment compared with 22Rv1 cells transfected with vector control plasmid (pCMV5), in which FTY720 treatment did not attenuate cell viability of 22Rv1-Runx2 transfectants but induced a reduction of cell viability in the control transfectants in a dose-dependent manner (Fig. 5D, right).

In summary, these results suggested that FTY720-induced down-regulation of Akt phosphorylation might be regulated by Runx2. Whereas knocking down of Runx2 in PC-3 cells sensitized FTY720-induced cell viability suppression, over-expressing Runx2 in 22Rv1 cells conferred resistance in FTY720-induced cell viability inhibition, suggesting a pivotal role of Runx2 on prostate cancer cell survival.

**Discussion**

Bone metastasis remains the main obstacle of advanced prostate cancer patients, which contributes to the majority of cancer-related deaths in these patients (5). However, there is still no effective therapeutic option in curing this disease phenotype. In the present study, we provided a novel therapeutic potential of FTY720 in treating advanced and metastatic prostate cancer by which FTY720 could down-regulate nuclear expression as well as the transcriptional activity of Runx2, consequently leading to the reversal of cadherin switching, suppression of invasion toward type I collagen, and inactivation of Akt pathway in an aggressive bone metastatic AI prostate cancer cell line, PC-3.

Previously, it was reported that Runx2 was up-regulated in bone metastases in prostate cancer patients (10). In the current study, we showed for the first time that the expression of Runx2 was readily up-regulated in primary prostate cancers with a more aggressive disease phenotype. This was supported by the notion that Runx2 expression was correlated with Gleason score (Table 2), a well-established prognostic indicator of prostate cancer (26). In addition, higher expression of Runx2 was observed in patients with higher levels of PSA (\geq 20 ng/mL), a cutoff value that has been indicated with higher risk of metastasis (34 – 36), when comparing with patients with <20 ng/mL PSA levels (Fig. 1C). More importantly, in high Gleason score (\geq 7) patients, association of Runx2 expression in primary prostate cancer and metastasis was established as evaluated by hazard ratio assessment (Table 3). As the majority of the metastasis cases diagnosed in the present set of tissues were primarily found in the bone region (Supplementary Table S1), this supported the “osteomimetic hypothesis” that described the ability of prostate cancer cells in gaining bone-like properties before metastasizing to bone (6), and, in our case here, the enhancement of osteogenic protein Runx2 in prostate cancers that possessed high propensity to spread to bone.

Further mechanistic evaluation revealed that Runx2 partially promoted the EMT program, by which switching of E-cadherin to N-cadherin was observed (Fig. 2B-D) without obvious and consistent alteration in other EMT-related markers expression (data not shown). Although previous report has suggested that Runx2 promoted EMT via regulating various mesenchymal genes during tooth morphogenesis (44), our study was the first to report the possible involvement of Runx2 in EMT using prostate cancer as a model. Interestingly, in the present study, we have also identified Runx2 as a transcriptional repressor of E-cadherin (Fig. 2B), thus providing a novel mechanism for Runx2-induced enhancement of cell aggressiveness in AI prostate cancer. In addition, our results have suggested that Runx2-induced up-regulation of N-cadherin expression might be a posttranscriptional event because N-cadherin mRNA expression was unaltered when modulating Runx2 expression in these prostate cancer cells (Fig. 2B). It has been suggested that transcriptional control of N-cadherin might not be necessarily required for cadherin switching to occur, and one of the possible options that could determine the expression levels of N-cadherin was through modulation of p120 catenin, which acted as a cadherin stabilizer (45). It might be possible that by attenuating the interaction between p120 and N-cadherin, Runx2 could prevent degradation of N-cadherin, consequently maintaining high expression of N-cadherin proteins in prostate cancer cells. Nevertheless, more studies have to be carried out to verify such postulation. Moreover, it would also be interesting to investigate the role of Runx2-induced EMT in prostate cancer as well as the expression pattern of Runx2 in various stages of prostate carcinogenesis using available prostate cancer progression animal models such as TRAMP (46, 47) and Nkx3.1:PTEN (48, 49) transgenic mice.

Additionally, we found that Runx2 promoted invasiveness of AI prostate cancer cell toward type I collagen (Fig. 3A and B). As type I collagen constitutes extracellular matrices of bone (42), the ability of Runx2 to promote such invasion capability indicated that by expressing this protein, prostate cancer cells could obtain great advantage in overcoming these barriers of bone and eventually moving forward into the bone region. Of note, one of the possible mechanisms for supporting such invasiveness enhancement was the ability of Runx2 in promoting the secretion of various matrix metalloproteinases (9). However, it is unclear whether Runx2-expressing cells possessed the propensity to metastasize specifically to bone or, in other words, chemoattracted to bone, and this definitely warrants further investigation. Furthermore, we observed that Runx2 could induce activation of Akt, a molecule that modulates various signaling pathways during prostate carcinogenesis (43) as shown in Fig. 3C. It is possible that, on the one hand, Akt protein accelerates Runx2 function in promoting prostate cancer cell aggressiveness, whereas, on the other hand, it strengthens the survival ability of these cancer cells under various stresses during metastasis as well as in stringent bone environment. More studies are therefore urgently required to

\* Unpublished data.

\* Unpublished data.
obtain a clearer and more global picture about the role of Runx2 in this regard during prostate carcinogenesis. A previous report has found that Runx2 could up-regulate several upstream regulators for Akt activation, including the PI3K subunits p110β and p85β, and subsequently showed that Runx2-induced cell migration was diminished after treating the cells with the PI3K/Akt inhibitor LY294002 (25). Therefore, in the future study, it would also be interesting to investigate if Runx2 would adapt a similar mechanism in regulating the phosphorylation of Akt in prostate cancer cells. In addition, Javed and coworkers showed that impairment of intranuclear trafficking of Runx2 caused inability of breast cancer cells to form bone metastases in nude mice (50). Further mechanistic evaluation revealed that breast cancer cells carrying Runx2 mutant protein with defects in its intranuclear trafficking ability were less invasive and lacking of osteolytic properties and would also suppress the osteogenic properties of bone marrow stromal cells (50). It seemed that Runx2 protein plays multiple roles during carcinogenesis, from the very early stage when the cancer cells are still confined in the primary site as shown in our study until the cells successfully metastasize and reside in the bone. This was especially true in cancers that predisposed with high incidences of bone metastasis such as prostate and breast carcinomas, thus highlighting the possibility of using this protein as a therapeutic target.

Consistent with our previous results showing that FTY720 could promote the reversal of EMT in AI prostate cancer cell lines,7 our observation in this study indicated that Runx2 might be one of the factors that influenced the FTY720-induced EMT reversal. It was possible that by down-regulating Runx2 expression as well as its transcriptional activity in PC-3 cells (Fig. 4A and B), FTY720 could down-regulate N-cadherin and up-regulate E-cadherin expression (Fig. 4C), and to a greater extent when applying siRunx2 treatment in these cells (Fig. 5A, left). Further comparison of the effect of FTY720 on both 22Rv1 cells transfected with pCMV5 and Runx2 revealed that overexpression of Runx2 caused failure of FTY720 to induce cadherin switching reversal, in which the expression of N-cadherin remained at relatively higher level, concomitant with relatively lower E-cadherin expression level when comparing with the control counterpart (Fig. 5A, right). In addition, knocking down of Runx2 in PC-3 cells resulted in a further suppression of Akt phosphorylation and this might consequently lead to inhibition of cell viability in these PC-3 transfectants when challenged with FTY720 (Fig. 5C and D, left), as the survival promoting effect of Akt activation was well documented (43). In line with previous results showing that FTY720 did not cause dephosphorylation of Akt in 22Rv1 cells,8 no difference was observed in Akt phosphorylation between 22Rv1-pCMV5 transfectants treated and untreated with FTY720, possibly due to low to undetectable endogenous level of phospho-Akt in this cell line (Fig. 5C, right). By comparison, the enhanced phospho-Akt level in 22Rv1-Runx2 transfectants was not affected after treatment of FTY720 (Fig. 5C, right), and this possibly resulted in a resistant phenotype to FTY720-induced cell viability inhibition in these transfectants (Fig. 5D, right). These lines of evidence led us to postulate that early stage of EMT program did not require Runx2 or Akt activation; however, as prostate cancer progressed and prepared to invade and metastasize to secondary organs, or we termed it here as the late stage of EMT, the expression of Runx2 would eventually be enhanced and consequently followed by activation of Akt. Thus, when applying FTY720 on cells with various stages of carcinogenesis, distinct strategies might be used to inhibit the aggressiveness of prostate cancer cells, by which Runx2/Akt-independent pathways might be targeted during early carcinogenesis, whereas the anticancer effect of FTY720 would target on Runx2/Akt-dependent pathways at the later stage of carcinogenesis. More studies are therefore urgently required to draw a more convincing conclusion in this particular aspect.

Compelling evidences suggested that metastasis and colonization of prostate cancer cells at the secondary site necessitate multiple steps (5, 6). Before residing in the bone, the most common site of prostate cancer metastasis, cancer cells must overcome the stringent boundaries that block them from getting into the bone. We found that in the present study, FTY720 could inhibit the invasion capability of PC-3 cells toward type I collagen, possibly through down-regulation of Runx2 expression (Figs. 4D and 5B). It was unclear, however, what the consequences of such failure in prostate cancer cells are and whether the incompetence of prostate cancer cells to invade toward type I collagen after FTY720 treatment would lead to the inability of cancer cells to enter the bone region. Moreover, as bone metastases of prostate cancer also showed high expression of Runx2 (10), this indicated that Runx2 might contribute to the colonization of prostate cancer cells. It would be interesting to investigate if the suppression of Runx2 by FTY720 would abrogate the cancer cell to integrate into the vicious cycle formed between cancer cells and host cells in bone, thus leading to incapability of these cells to form metastases. More in vivo studies using various metastatic prostate cancer models have to be done to get further insights on the efficacy of FTY720 in treating metastatic prostate cancer.

Although we showed that FTY720 could down-regulate the expression and transcriptional activity of Runx2 in this study, we were inconclusive on whether this effect was specific to FTY720 drug treatment. Nevertheless, our observations suggested that FTY720 might be a potential therapeutic intervention in treating advanced prostate cancer. In conclusion, we have provided two major findings in the current investigation: We have first identified Runx2 as a potential therapeutic target of metastatic prostate cancer and subsequently discovered that FTY720 was able to suppress Runx2 expression, thus altering several Runx2-regulated downstream events; that is, reversing the cadherin switching, suppressing invasion toward type I collagen, and inactivating Akt.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Novartis Pharmaceuticals, Ltd., Basel, Switzerland, for their generosity in providing us the FTY720 used in the present study; Professor Gerald Karsenty for kindly providing us the anti-Runx2 rabbit serum as well as various plasmids including full-length Runx2 and Runx2-luciferase reporter, p60SE2; Dr. Zhenguo Wu for the pCMV5 empty vector; and Dr. Franky Chan for the 22Rv1 cell lines.

7 Unpublished data.
8 Unpublished data.
Effect of FTY720 on Runx2 Nuclear Expression

References

Suppression of Androgen-Independent Prostate Cancer Cell Aggressiveness by FTY720: Validating Runx2 as a Potential Antimetastatic Drug Screening Platform

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