Androgen receptor regulates CD168 expression and signaling in prostate cancer

Shi-Lung Lin, Donald Chang, Angela Chiang and Shao-Yao Ying

Department of Cell and Neurobiology, Keck School of Medicine, BMT-403, University of Southern California, 1333 San Pablo Street, Los Angeles, CA 90033, USA

To whom correspondence should be addressed. Tel: +1 323 442 1856; Fax: +1 323 442 3466; Email: sying@usc.edu

Introduction

Hormone-refractory prostate cancer (HRPC) is a leading cause of aging-related cancer death in men. Normal prostatic cell growth is controlled by androgens. Prostate cancer (CaP) often occurs when this control is disturbed as one ages. The mainstream treatment of androgen-dependent (AD) CaP is to remove androgen stimulation by surgery or hormonal therapy which, although temporarily effective, eventually results in a deadly androgen-independent (AI) CaP transformation (1).

The extracellular matrix, e.g. hyaluronan (HA), plays an important role in AI CaP transformation. The content of HA was elevated >100 μg/ml in many solid tumors (2). Recent immunohistochemical (IHC) studies in human CaP tissue arrays taken from 50 patients have shown that CD168, the receptor for hyaluronan-mediated motility (RHAMM), was overexpressed and highly activated in HRPC. HA deposited in the stromal tissues surrounding the tumor often provide insight into the mechanism underlying HRPC relapse.

HA-mediated CD168 signal transduction in HRPC reflected a strong stromal–epithelial interaction during AI CaP transformation. CD168, abundantly present in HRPC (particularly in AI CaP cells), consisted of two major isoforms: intracellular CD168 (85–95 kDa), which mainly existed in adherent cells, whereas cell-surface CD168 (72 kDa) presumably promoted motility of Ras-transformed cells and acted as an oncogene to cause metastatic transformation of immortalized cells (15,22). Malignant cancer transformation was frequently characterized by active interactions between extracellular matrix and tumor cells, resulting in alterations of the signal transduction pathways essential for stromal–epithelial regulation. In HRPC, high HA deposited in the stromal tissues surrounding the tumor often stimulated AI CaP transformation along with associated cell invasion and metastasis (3). Our previous studies have shown that ROCK1, the key kinase in the CD168-signaling pathway, was overexpressed in patients, who in turn represented >90% of hRPC and metastatic CaP patients (3). HRPC cells often lost androgen regulation, transforming into more malignant AI CaP (4,5). Using human macroarray and northern blot analyses of gene expression profiles in the prostatic epithelium obtained from 153 CaP patients, we observed that major components of the CD168-signaling pathway were consistently overexpressed in AI CaP (3.6). This finding has been recently confirmed by the National Institutes of Health (NIH)/National Center for Biotechnology Information (NCBI) gene expression omnibus (GEO) profiles GDS1390 record #GPL96.207165 (http://www.ncbi.nlm.nih.gov/projects/geo/gds/gds_browse.cgi?gds=1390), which showed that CD168 was overexpressed in >70% of AI CaP whereas only moderately elevated in <20% of AD CaP. Similar microarray results were presented in the NIH/NCBI GEO profiles GDS1439 record #GPL570.209709 (http://www.ncbi.nlm.nih.gov/projects/geo/gds/gds_browse.cgi?gds=1439). The finding in both NIH/NCBI GEO profiles and our tissue array studies has revealed the significance of HA-mediated CD168 signaling in AI CaP transformation after androgen ablation.

HA, an extracellular matrix polymer composed of the repeating disaccharide unit 2-deoxy, 2-acetamido- D-glucopyranosyl-(1,4)-D-glucuronopyranosyl-(1,3), was frequently localized in the stroma of solid tumors, facilitating cell migration, tumor invasion and metastasis (7–9). HA was synthesized by stromal fibroblasts in response to paracrine factors produced by tumor cells (10) and such a tumor–stromal interaction was crucial to the development and progression of HRPC (11). HA bound to HA receptors, such as CD168 and CD44, and stimulated the Rho-activated protein kinase (ROCK/ROK) signal transduction pathway in various cancers (12–14). The cytoplasmic domains of HA receptor isoforms coupled with RhoA, a Rho-GTPase of the Ras superfamily, form a complex that activated ROCK, which was found to increase myosin light chain phosphorylation and actin/myosin-coupled contraction for cancer migration and metastasis (13,14). Activated ROCK also phosphorylated its linker molecule, Gab1, and promoted the localization of both Gab1 and HA receptors for activation of phosphatidylinositol-(3,4,5)P3 kinase (PI3K), which then converted phosphatidylinositol-(4,5)P2 to phosphatidylinositol-(3,4,5)P3 (inositol-1,4,5-trisphosphate). This conversion resulted in the activation of the Akt/mTOR/eIF4E signal transduction (3), which increased malignant transformation and drug resistance in AI CaP (15,16). Nevertheless, the expression patterns of CD44 and its variants were not correlated with the HA level, Gleason’s score, pathological T classification, prostate-specific antigen recurrence, clinical invasion or metastasis of CaP in patients (17,18). Moreover, CD44v3,4,8–10, the major variants that interact with Rho-ROCK, was not expressed in either AI CaP cell lines or CaP patients (19,20). Again, the transduction of CD168 in CD44 knockout mice was found to compensate for CD44 (21). Thus, CD168 could be the predominant receptor for HA-mediated AI CaP transformation. The elevated activation of CD168–PI3K–Akt/mTOR/eIF4E signaling by extracellular HA may provide insight into the mechanism underlying HRPC relapse.

HA-mediated CD168 signaling in HRPC reflected a strong stromal–epithelial interaction during AI CaP transformation. CD168, abundantly present in HRPC (particularly in AI CaP cells), consisted of two major isoforms: intracellular CD168 (85–95 kDa), which mainly existed in adherent cells, whereas cell-surface CD168 (72 kDa) presumably promoted motility of Ras-transformed cells and acted as an oncogene to cause metastatic transformation of immortalized cells (15,22). Malignant cancer transformation was frequently characterized by active interactions between extracellular matrix and tumor cells, resulting in alterations of the signal transduction pathways essential for stromal–epithelial regulation. In HRPC, high HA deposited in the stromal tissues surrounding the tumor often stimulated AI CaP transformation along with associated cell invasion and metastasis (3). Our previous studies have shown that ROCK1, the key kinase in the CD168-signaling pathway, was overexpressed in...
human AI CaP PC3 and DU145 cells, but not in AD CaP LNCaP cells (3.6). Using western blotting and in vitro tumorigenicity assays, we further observed that the activation (phosphorylation) of the major CD168 signal proteins, including ROCK1, Gab1, PI3K,p110β, Akt1/2 and eIF4E, was concurrently and proportionally increased in response to the HA concentrations in AI CaP PC3 and DU145 cells. This led to more aggressive and malignant tumorigenesis with respect to elevated drug resistance, cancerous cell proliferation, invasion and metastasis into human bone marrow endothelial cell (hBMEC) layers (3). The PI3K-dependent Akt/mTOR/eIF4E signaling was an important mediator involved in multiple cellular functions, such as cell proliferation, antiapoptosis, migration and angiogenesis (16, 32). These findings indicate that HA deposition has a significant role in the activation of the complete CD168-signaling cascade and thus lead to the Akt/mTOR/eIF4E-associated tumorigenesis in AI CaP. However, how the loss of androgen sensitivity potentiated HA-mediated CD168 signaling in AI CaP cells is still unclear.

Recent studies of the NIH/NCBI GEO profiles GDS2057 record #GPL96 207165 (http://www.ncbi.nlm.nih.gov/projects/geo/gds/gds_browser.cgi?gds=2057) have shown that a time-dependent increase in CD168 expression levels could be stimulated by treatments of either the native androgen, dihydrotestosterone (DHT) or a synthetic androgen agonist RTI-018 in LNCaP cells. In addition, the NIH/NCBI GEO profiles GDS536 record #GPL96 209709 (http://www.ncbi.nlm.nih.gov/projects/geo/gds/gds_browser.cgi?gds = 536) further showed the dose-dependent increase of CD168 expression stimulated by treatments of another androgen agonist R1881 in LNCaP cells, suggesting that androgen receptor (AR) occupation plays a vital role in regulation of CD168 expression. We postulated that appropriate AR occupation was essential for normal function: deviation toward either elevated or depressed AR occupation may be tumorigenic. Loss of AR regulation in AI CaP cells may cause CD168 overexpression and subsequently increased HA-mediated AI CaP tumorigenecity. Based on this concept, we investigated the interaction of AR and the CD168 signaling in various AR-expressing CaP cells, including AD CaP LNCaP and PC3-AR9 cells as well as AI CaP C4-2B cells, and provided the first insight into the regulatory effects of AR on HA-mediated CD168 signaling in AI CaP versus AD CaP progression.

Materials and methods

Cell culture and treatments

Human CaP cell lines, LNCaP and PC3 cells were obtained from the American Type Culture Collection (Rockville, MD). The C4-2B and PC3-AR9 cell lines were obtained from Dr Gerhard Coetze’s laboratory at the University of Southern California and Dr Chawshang Chang’s laboratory at the University of Rochester Medical Center, respectively. All cell lines were grown in phenol red-free Dulbecco’s modified Eagle’s medium with 10% charcoal-stripped fetal bovine serum and 1% gentamycin as reported (3). For HA stimulation, HA (1000–5000 unit small molecular weight HA at 200 or 400 μg/ml) was added to cultured cells when they were ~40% confluence. For androgen stimulation, DHT (3, 12 or 48 ng/ml) was added with HA concurrently. To block ROCK function, ROCK inhibitor Y-27632 (2.5, 10 or 40 μg/ml) was added to the culture cells at the same time HA (400 μg/ml) and DHT (12 ng/ml) were added. To inhibit AR–DHT binding, an androgen antagonist, bicalutamide (1 μM) was added to the culture cells at the same time (400 μg/ml) and DHT (48 ng/ml) was added. These doses were selected because they produced optimal effects and showed no detectable cytotoxicity as reported previously (3).

AR responsiveness

To determine the relationship between the activation of AR and the CD168 signaling in CaP, we used four human CaP cell lines with different AR responsiveness, LNCaP and PC3-AR9 cell lines were sensitive to androgen stimulation whereas PC3 and C4-2B cell lines were androgen insensitive. Different AR responsiveness provided insight into the mechanism of the interaction of AR and CD168 in AD CaP versus AI CaP. For instance, the mutant-type T877A AR expressed in LNCaP cells exhibited sensitivity to androgen stimulation several fold higher than that of the wild-type AR (24). In contrast, C4-2B cells, a cell line derived from LNCaP, possessed the same T877A AR but lost some components of the AR-signaling pathway and became AI CaP (25). Again, PC3 cells possessed all the signal transduction components distal to the AR signaling but lost the AR itself (26). Transgenic expression of wild-type AR in PC3 cells, namely the PC3-AR9 line, restored AR responses to androgen (26).

Generation of ROCK1-depleted cell lines

MicroRNA (miRNA)-mediated, ROCK1-depleted LNCaP and PC3 cell lines were generated using cytomegalovirus promoter-driven vectors that expressed miRNA-based small hairpin RNA as reported previously (3,27). The miRNA was designed directed against the ROCK1 gene nucleotide 1504–1525 region (accession number NM005406), which contained a partial Rho-binding domain required for ROCK activity. For transfection, the vectors were liposomally encapsulated in FuGENE reagent (Roche, Indianapolis, IN) and added to cultured cells at 40% confluence. Positively transfected cells were isolated 24 h later using flow cytometry with anti-rGFP monoclonal antibody (Clonetech, Palo Alto, CA), and the ROCK1 knockdown efficacy was determined by northern and western blot analyses as reported previously (3,27).

IHC staining

Primary antibodies and IHC staining kits were obtained from Imgenex (San Diego, CA), EMD Biosciences (San Diego, CA), Chemicon (Temecula, CA), Santa Cruz Biotechnology (Santa Cruz, CA) and Upstate LLC (Charlottesville, VA). The active (phosphorylated) forms of Gab1, PI3K,p110β, Akt1/2, eIF4E and AR proteins were immunostained according to the manufacturers’ procedures (3). The antibody against active ROCK1, ROCK1(thr286), was generated in mice immunized with a synthetic peptide containing 14 amino acids adjacent to Thr-286 in the N-terminal kinase domain of the ROCK1 protein. The Thr-286 was masked by an inhibitory Cys/His-rich pleckstrin homology domain located in the C-terminus of the ROCK1. Rho, a small GTPase bound to the pleckstrin homology domain and removed the mask of the ROCK1 domain, which detected the active ROCK1 by the ROCK1(thr286) antibody. Biotinylated goat anti-rabbit or horse anti-mouse antibody was added as the secondary antibody (Chemicon) and streptavidin–horseradish peroxidase the tertiary antibody. Then, the bound antibody was detected with dianminobenzidene substrates for 2 min. Positive results were observed under a × 100 microscope with whole-field scanning and quantitative analyses were conducted with the aid of a Metamorph Imaging program (Nikon 80i and TE2000 microscopic quantitation systems).

Immunoprecipitation and western blot analysis

Cells were washed with ice-cold phosphate-buffered saline twice and lysed in the Celllytic-M lysis/extraction reagent (Sigma Chemicals, St Louis, MO) 1 day after treatments. Cell lysates were then used for immunoprecipitation and western blot analyses as reported previously (3,6). Proteins of ROCK1, Gab1, PI3K,p110β, Akt1/2, eIF4E and AR were directly detected in western blotting, whereas CD168 and PI3K,p110α required purification by immunoprecipitation prior to western blotting.

In vitro tumorigenicity assay

Assays of DNA-staging flow cytometry, cell invasion and adhesion to hBMEC layers were performed and analyzed as reported previously (3). The DNA-stage flow cytometry analysis assessed both mitotic cell proliferation in the cell cycle stage and cytotoxic apoptosis caused by the treatments.

Ex vivo tumorigenicity assay

We xenografted PC3-AR9 cells, with or without DHT (12 ng/ml) and/or HA (400 μg/ml) treatments, in 8-week-old castrated male mice (BALB/c nu/nu strain) to examine changes in cancer cell invasion and metastasis. Cells (2 × 106 cells in a total volume of 100 μl phosphate-buffered saline) were injected subcutaneously into the flanks (× 100 microscope with whole-field scanning and quantitative experiments were either conducted 2 months after xenograft or when tumors reached an average size of 100 mm3. The sizes of the tumors were calculated according to Rockwell et al. (28). We sampled the treated mice at different time intervals (e.g. 2, 4, 6 and 8 weeks after xenograft). At each time point, four animals per each group were examined. Animals showed signs of sickness or cachexia were dispatched and necropsies were performed. Prostate and other major tissues such as the blood, brain, lung, kidney, liver, intestine and spleen were removed for histological evaluation for the presence of tumors and any immunoreactive cytotoxicity. Terminal transferase-mediated dUTP nick-end labelling staining, IHC of interferons (INF-type I β/α and type-II γ) and pathologic- microscopic examination of various organs were performed to identify any immunogenic lesion in the mice. Tumor formation was monitored by palpating the mice and tumor volumes were calculated using the formula (length × width2)/2. Tumor lesions were counted, dissected, weighed and subjected to histological analyses. Tumor sections were prepared and examined by hematoxylin and eosin and IHC staining of the active forms of ROCK1, Gab1, PI3K,p110β, Akt1/2, eIF4E and AR proteins.

AR regulates CD168 expression and signaling in prostate cancer
Results

Stimulation of CD168 messenger RNA expression by androgen (DHT)

CD168 messenger RNA (mRNA) expression increased significantly in AD LNCaP and PC3-AR9 cells treated with DHT, but not in AI C4-2B cells as determined by northern blot analysis (Figure 1A). Similar results were reported in LNCaP cells by the NIH/NCBI GEO profiles GDS536 and GDS2057. Increased expression of CD168 mRNA and protein was also previously observed in AI PC3 cells treated with HA (3). However, CD168 proteins, but not mRNA, were moderately increased in AR-positive C4-2B cells, probably due to a defective AR-signaling pathway in C4-2B cells. Although both LNCaP and C4-2B cell lines possessed the same mutant-type AR that interacted with DHT, the C4-2B cells lost some functional AR-signaling components required for activating CD168 transcription. Thus, DHT stimulated AR signaling and increased CD168 mRNA expression in androgen-sensitive LNCaP and PC3-AR9 cells.

Correlation between DHT-induced AR signaling and CD168-associated signal activation

CD168 protein levels were markedly increased in AD LNCaP, PC3-AR9 and AI C4-2B cells treated with DHT (Figure 2A) as determined by immunoprecipitation–western blot analysis (Figure 1B). Both 85–95 and 72 kDa CD168 isoforms were increased; however, PC3-AR9 cells expressed more 72 kDa CD168 whereas LNCaP and C4-2B cells more 85–95 kDa isoforms. The 72 kDa CD168 was the isoform often found in metastatic cancer cells (15, 22). The level of DHT-induced 85–95 kDa CD168 was higher in LNCaP than C4-2B cells. The results were presented in Figure 2A and B and supplementary Figure 1 (available at Carcinogenesis Online). Because C4-2B cells possessed no functional AR signaling for activation of CD168 transcription, these findings suggest the lack of CD168 translation in C4-2B cells. It is highly likely that the increased level of CD168 proteins in C4-2B cells resulted from a posttranslational reservoir, which was regulated by androgen–AR interaction and consequently prevented the degradation of CD168 proteins.

The active forms of ROCK1, Gab1, P13Kζ110β, Akt1 and eIF4E proteins in the CD168-signaling transduction cascade treated with HA and/or androgen in the four CaP cell lines mentioned above were examined by using western blot analyses. HA-activated CD168 signal components were increased in response to the DHT-induced CD168 elevation in AD LNCaP and PC3-AR9 cells (Figure 2A and supplementary Figure 1, available at Carcinogenesis Online), which was similar to the full response of HA-mediated CD168 signal levels in AI PC3 cells reported previously (3). However, the CD168 signals were not activated in the absence of HA in all four cell lines (Figure 2B). These findings suggest that DHT-induced CD168 levels go hand in hand with the HA-activated CD168 signaling. Strong HA-induced CD168 signaling was previously observed in PC3 cells (3), which markedly promotes tumorigenicity in vitro, therefore, PC3 cells were included as positive controls. In contrast, some of the CD168 downstream signals, including ROCK1, Gab1 and P13Kζ110β, were lost in AI C4-2B cells, thus leading to negative responses to the HA stimulation. Given that androgen increased HA-mediated CD168 signal activation in AD CaP cells, high tumorigenicity occurred in DHT-treated AD LNCaP and PC3-AR9 cells.

Direct interaction between AR and CD168 proteins

Coimmunoprecipitations of AR and CD168 proteins were prepared for western blot analysis to measure the direct interaction between AR and CD168. Four antibodies, AR(C-19) targeting the C-terminus of AR, AR(N-20) targeting the N-terminus of AR, RHAMM(H-90)/CD168-N targeting the N-terminus of CD168 and RHAMM/E-19/CD168-C targeting the C-terminus of CD168 proteins, were used to precipitate the AR–CD168 complex. Both AR(C-19) and AR(N-20) antibodies successfully coprecipitated CD168 proteins in AD LNCaP and PC3-AR9 cells not treated with DHT (Figure 2C). However, the AR–CD168 complex was detected with the RHAMM(H-90)/CD168-N antibody, but not with the RHAMM(E-19)/CD168-C antibody, suggesting that AR may mask the CD168 recognition site for the antibodies. Given that the interacting domain of CD168 to Rho is also located in the C-terminal region, which can be detected by the RHAMM(E-19)/CD168-C antibody, the binding of AR to CD168 may block the CD168–Rho interaction, consequently preventing the downstream ROCK signal after the HA activation. These findings demonstrated that both mutant- and wild-type AR blocked the activation of 85–95 and 72 kDa CD168 proteins. This type of AR–CD168 interaction was disrupted by the binding of androgen (i.e. DHT) to AR in a dose-dependent manner (Figure 2D). Bicalutamide, an androindrogen drug combating with DHT, not only suppressed DHT-stimulated CD168 transcription (IP:CD168-C>C=N; Figure 2D, the most right lane) but also increased the release of AR-bound CD168 proteins (IP:AR>C>CD168; Figure 2D, the most right lane), resulting in partial inhibition of HA-mediated CD168/ROCK activation in LNCaP and PC3-AR9 cells (ROCK1; Figure 2D, the most right lane). These data suggest that AR bound to androgen and then released CD168 proteins from the AR–CD168 complex, consequently leading to the activation of HA-mediated CD168/ROCK signaling in AD CaP cells. Given that the AR–CD168 complex served as a potential means for preserving inactive
CD168 proteins, this may explain why DHT stimulated a moderate increase in CD168 protein but not mRNA expression in C4-2B cells (Figure 2A).

Effects of AR regulation on HA-stimulated tumorigenicity in AD and AI CaP cells

Having examined molecular interaction and signal transduction between the AR and CD168 pathways, the potential tumorigenicity resulting from the AR–CD168 interaction was investigated. Previously, HA-mediated CD168/ROCK1 signaling was demonstrated as an important mediator of AI CaP tumorigenicity in PC3 cells, promoting cancerous cell proliferation, antiapoptosis, invasion and metastasis to hBMEC layers (3). The tumorigenic effects of HA-mediated CD168/ROCK signaling in AD LNCaP, PC3-AR9 and AI C4-2B cells were investigated. Due to the lack of some CD168 signal components, the C4-2B cells were used as negative controls in the evaluation of HA-mediated tumorigenicity. In the absence of DHT, LNCaP cells with or without HA treatments presented limited tumorigenicity in cell proliferation (20.4 ± 2.9 versus 14.7 ± 3.4%; Figure 3A, upper panel), invasion (4.1 ± 1.1 versus 3.4 ± 0.8%; Figure 3B, gray bar) and metastasis to the hBMEC layer (7.6 ± 1.3%), which indicated that androgen-depleted LNCaP cells did possess sufficient responsiveness to HA stimulation (Figure 3). Nevertheless, in the presence of DHT stimulation, the same measurements of HA-mediated LNCaP tumorigenicity in cell proliferation, invasion and metastasis were significantly elevated from 31.6 ± 3.1 to 47.7 ± 3.7% (1.5-fold increase; Figure 3A, lower panel), 4.1 ± 0.9 to 13.8 ± 1.7% (3.2-fold increase; Figure 3B, gray bar) and ~7.6 ± 1.3% (Figure 3C), respectively. Inhibition of ROCK, the key kinase for HA-mediated CD168 signaling in the prostate cells, by treatments with either the ROCK inhibitor Y-27632 or anti-ROCK1 miRNA reversed most of the tumorigenic effects caused by the concurrent HA and DHT stimulations (Figure 3A–C, sets 5 and 6), suggesting that these elevated tumorigenic effects indeed resulted from the activation of the CD168/ROCK-signaling pathway.

![Fig. 2.](https://academic.oup.com/carcin/article-abstract/29/2/282/2526903)
pathway. Although the use of Y-27632 showed some cytotoxicity in LNCaP cells (Figure 3A, set 6, arrow), the results obtained were further substantiated by the miRNA-mediated ROCK1 depletion. In contrast to the results in LNCaP, HA did not produce any difference in the tumorigenicity between DHT-treated and untreated C4-2B cells (Figure 4). The rates of cell proliferation (36.1–38.4 versus 37.9–41.9%; Figure 4A), invasion (6.2–8.1 versus 7–7.9%; Figure 4B) and metastasis to the hBMEC layer (14–17.4 versus 13.7–18.9%; Figure 4C) in these cells are similar. LNCaP-derived C4-2B cells have been previously reported to exhibit high tumorigenicity in all three tested parameters even without HA stimulation (3). Inhibition of ROCK by Y-27632 slightly reduced cell proliferation (decrease from 42 to 32.2%; Figure 4A, set 4) of DHT-treated C4-2B cells. Based on the results of Figure 2B and the fact that C4-2B cells lack some active components of the CD168-signaling pathway, such as ROCK1, Gab1 and PI3Kp110α, their high tumorigenicity of this cell line may be associated with HA-independent signal transduction pathway. Our previous data have shown that HA-mediated CD168 signaling occurred in ~70% of advanced CaP patients with Gleason’s scores above 7, implicating that C4-2B cells may not representative of the majority of CaP patients (3).

Results obtained in PC3-AR9 cells were similar to those of LNCaP cells (Figure 5), using the same tumorigenicity assays described above. Androgen-depleted PC3-AR9 cells showed a high rate of apoptosis (20 ± 1%; Figure 5A, upper panel), which was similar to the response of prostatic epithelial cells after androgen ablation. This apoptotic effect could be prevented by DHT treatments, indicating that PC3-AR9 cells are highly AD (Figure 5A, lower panel). PC3-AR9 cells treated with no DHT, in the absence or presence of HA, showed no changes in cell proliferation (26 ± 1%; Figure 5A, upper panel), invasion (4.1 versus 4.9%; Figure 5B, black bar) and metastasis (1.8 versus 2.3%; Figure 5C, black line). On the contrary,

Fig. 3. DHT- and/or HA-mediated in vitro tumorigenicity in AD LNCaP cells. (A) Flow cytometry analysis of HA- and/or DHT-stimulated cell proliferation (P < 0.005; n = 3), showing assignment of cell populations with different DNA contents (y-axis) to different cell cycle stages (x-axis); from left to right, C (G0/G1 phase), D (S phase) and E (G2/M phase). Bar charts indicate the ratios of different cell populations (x-axis) versus different treatments (y-axis), including cells treated with no treatment (set 1), 200 μg/ml HA (set 2), 400 μg/ml HA (set 3), 400 μg/ml HA and antisense miRNA (miR) (set 4), 400 μg/ml HA and anti-ROCK1 miRNA (miR-ROK) (set 5) or with 400 μg/ml HA and 10 μg/ml Y-27632 (set 6). Gray bars refer to the resting cell population (G0/G1), whereas the black bars represent the mitotic cell population (M). DHT (12 ng/ml) significantly increased the response levels of HA-stimulated cell proliferation (labeled by *). (B) Functional analysis of HA-stimulated tumor invasion in matrigel chambers (P < 0.005; n = 4). The invasive tumor cell population (y-axis) of DHT/HA-treated LNCaP cells (gray bar, labeled by *) was significantly increased, whereas other cell sets not treated with DHT showed no change (black bar). Inhibition of the CD168/ROCK signaling using either Y-27632 or miR-ROK markedly suppressed the DHT/HA-stimulated cancer cell invasion. (C) Comparison of cell adhesion to the hBMEC monolayer in LNCaP cells treated with 12 ng/ml DHT (squares), 12 ng/ml DHT and 400 μg/ml HA (triangles), 12 ng/ml DHT or 400 μg/ml HA and 10 μg/ml Y-27632 (inverted triangle) (P < 0.005; n = 6). The cell adhesion rates (y-axis) were measured every 10 min for 50 min (x-axis).
HA-mediated tumorigenicity was significantly elevated as determined in cell proliferation, invasion and metastasis from 18.6 to 28.8% (1.6-fold increase; Figure 5A, lower panel), 3.2 to 13.7% (4.3-fold increase; Figure 5B, gray bar) and 6.2 to 21.1% (3.4-fold increase; Figure 5C, gray line), respectively. Inhibition of ROCK by Y-27632 completely reversed the tumorigenic effects caused by the concurrent stimulations of HA and DHT (Figure 5A–C, set 4). Y-27632 did not increase androgen depletion-induced apoptosis or induce any overt cytotoxicity in PC3-AR9 cells. Therefore, the consistent findings of HA-enhanced tumorigenicity in both androgen-stimulated LNCaP and PC3-AR9 cells may suggest that the interaction of androgen and AR was required for activating HA-mediated cancer progression via the CD168/ROCK-signaling pathway in AD CaP. Thus, the androgen–AR interaction may function as a regulatory mechanism to switch on and off the HA-mediated tumorigenic effects depending on the availability of androgen.

Ex vivo tumorigenicity in HA-stimulated PC3-AR9 cells under normal AR regulation

To further evaluate the tumorigenic effects of the AR–CD168 interaction in vivo, PC3-AR9 cells were injected subcutaneously into the right hindlimb of 6-week-old male BALB/c athymic nude mice and the tumor growth was monitored weekly. Three treatments, PC3-AR9 cells treated with 12 ng/ml DHT (AR9 + DHT), 400 μg/ml small molecular weight HA (AR9 + HA) or both (AR9 + DHT + HA) were given (Figure 6A). All tumors measured at the 4th week after injection (post-sc) were >100 mm³; an average size of 176 mm³ for AR9 + DHT, 108 mm³ for AR9 + HA and 422 mm³ for AR9 + DHT + HA tumors (supplementary Figure 2). Untreated PC3-AR9 cells failed to grow in the mice (26). AR9 + DHT + HA cells produced >2.4-fold tumors in mice than AR9 + HDT or AR9 + HA cells. Moreover, IHC staining (Figure 6B) of active ROCK1 and its downstream signal proteins, such as Gab1, P3Kp110α, Akt1/2 and eIF4E, further demonstrated that excessive activation of the complete CD168/ROCK1 signal transduction pathway only occurred in AR9 + DHT + HA tumors. Given that neither the AR9 + HA nor the AR9 + DHT tumors could activate the full CD168-signaling cascade, the interaction between AR and CD168 may play an important role in controlling HA-mediated AD CaP tumorigenicity in vivo. These findings suggest that concurrent androgen and HA stimulations can significantly increase the tumorigenicity of the native AR-expressing AD CaP cells in vivo, most probably through the activation of the CD168/ROCK1 signal transduction pathway.

Discussion

Our previous findings (3) and the NIH/NCBI GEO profiles relating to HA/CD168-mediated CaP progression implied that AR may regulate normal CD168 expression in the prostate. Therefore, deregulation of AR in AD CaP cells not only caused CD168 overexpression but also excessive HA-stimulated CD168 signaling, consequently resulting in the malignant transformation and metastasis of HRPC (3). The present study further confirmed the role of this AR-dependent regulatory mechanism in AD CaP cells (Figure 6C), indicating a direct interaction between AR and CD168 proteins. We observed that AR not only responded to the AD AR signals required for activating CD168
Fig. 5. DHT- and/or HA-mediated *in vitro* tumorigenicity in AD PC3-AR9 cells. (A) Flow cytometry analysis of HA- and/or DHT-stimulated cell proliferation (*P* < 0.005; *n* = 4), showing assignment of cell populations with different DNA contents (*y*-axis) to different cell cycle stages (*x*-axis); from left to right, C (cell death), D (G2/M phase) and E (G2/M phase). Bar charts (right) indicate the ratios of different cell populations (*x*-axis versus different treatments (*y*-axis), including cells treated with no treatment (set 1), 200 μg/ml HA (set 2), 400 μg/ml HA (set 3) and 400 μg/ml HA and 10 μg/ml Y-27632 (set 4). Gray bars refer to the resting cell population (G0/G1), whereas the black bars represent the mitotic cell population (M). DHT (12 ng/ml) significantly increased the HA-stimulated cell proliferation rates (labeled by *). (B) Functional analysis of HA-stimulated tumor invasion in matrigel chambers after cells treated with androgen (*P* < 0.001; *n* = 3). DHT/HA-treated PC3-AR9 cells (gray bar, labeled by *) significantly increased the invasive tumor cell population (*y*-axis), whereas all cell sets with no DHT showed no change (black bar). (C) Comparison of cell adhesion to the hBMEC monolayer among the set 1 (circles), set 2 (inverted triangles), set 3 (squares) and set 4 (triangles) PC3-AR9 cells (*P* < 0.005; *n* = 4). DHT markedly stimulated the adhesion rates of HA-treated cells to the hBMEC layer (*y*-axis; up to 22%). Inhibition of CD168/ROCK signaling with 10 μg/ml Y-27632 completely suppressed the DHT/HA-stimulated PC3-AR9 cell invasion and metastasis.

### Summary

Data presented indicate that CD168 signaling was the predominant stimulus for HA-mediated AI CaP transformation via the ROCK–PI3K–Akt/mTOR/IF-4E signal transduction pathway. Activation of this signaling pathway was directly related to the clinical staging, cell proliferation, cell invasion and metastasis of HRPC. AR, however, regulated the CD168 expression as well as its signal activation in preventing the occurrence of HA-stimulated tumorigenicity in CaP cells. A better understanding of the interaction between AR and CD168 signaling may provide significant insight into the mechanism underlying the HRPC relapse for advanced cancer therapy.

### Supplementary material

Supplementary Figures 1 and 2 can be found at http://carcin.oxfordjournals.org/
Fig. 6. DHT- and/or HA-mediated ex vivo tumorigenicity in AD PC3-AR9 cells ($P < 0.005; n = 3$). (A) Tumor sizes in AR9 + DHT + HA-treated cells were more sizes up to 3.9 fold larger than those of AR9 + HA-treated cells 4 weeks after the subcutaneous injection (4-week post-sc), indicating the stimulatory role of DHT in HA-mediated tumorigenicity. All tumors were localized in the injection sites (black arrows). No signs of cachexia or tumor metastasis were observed in all tested mice. (B) IHC staining analysis of active components of the CD168/ROCK signal transduction pathway in the tumors described in (A). The complete CD168/ROCK-signaling cascade, including ROCK1, Gab1, PI3Kp110α, Akt1/2 and eIF4E, was concurrently activated in the AR9 + DHT + HA tumors in the presence of DHT and HA, whereas treatments with either DHT or HA showed limited effects on the ROCK1, Gab1 and PI3Kp110α activation. (C) Proposed model for the mechanism by which AR interacts with CD168 in the AD prostatic epithelial cells.
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