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Stromal Hyaluronan Interaction with Epithelial CD44 Variants Promotes Prostate Cancer Invasiveness by Augmenting Expression and Function of Hepatocyte Growth Factor and Androgen Receptor*

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The main aim of our study is to determine the significance of the stromal microenvironment in the malignant behavior of prostate cancer. The stroma-derived growth factors/cytokines and hyaluronan act in autocrine/paracrine ways with their receptors, including receptor-tyrosine kinases and CD44 variants (CD44v), to potentiate and support tumor epithelial cell survival. Overexpression of hyaluronan, CD44v9 variants, and stroma-derived growth factors/cytokines are specific features in many cancers, including prostate cancer. Androgen/androgen receptor interaction has a critical role in regulating prostate cancer growth. Our previous study showed that 1) that increased synthesis of hyaluronan in normal epithelial cells promotes expression of CD44 variants; 2) hyaluronan interaction with CD44v6-v9 promotes activation of receptor-tyrosine kinase, which stimulates phosphatidylinositol 3-kinase-induced cell survival pathways; and 3) CD44v6/short hairpin RNA reduces colon tumor growth *in vivo* (Misra, S., Hascall, V. C., De Giovanni, C., Markwald, R. R., and Ghatak, S. (2009) *J. Biol. Chem.* 284, 12432–12446). Our results now show that hepatocyte growth factor synthesized by myofibroblasts associated with prostate cancer cells induces activation of HGF-receptor/cMet and stimulates hyaluronan/CD44v9 signaling. This, in turn, stabilizes the androgen receptor functions in prostate cancer cells. The stroma-derived HGF induces a lipid raft-associated signaling complex that contains CD44v9, cMet/phosphatidylinositol 3-kinase, HSP90 and androgen receptor. CD44v9/short hairpin RNA reverses the assembly of these components in the complex and inhibits androgen receptor function. Our results provide new insight into the hyaluronan/CD44v9-regulated androgen receptor function and the consequent malignant activities in prostate cancer cells. The present study describes a physiologically relevant *in vitro* model for studying the molecular mechanisms by which stroma-derived HGF and hyaluronan influence androgen receptor and CD44 functions in the secretory epithelia during prostate carcinogenesis.

Prostate cancer is the second leading cause of death in American men (1). High levels of the androgen, dihydrotestosterone (DT),[3] that bind to nuclear androgen receptor correlate with prostate cancer (2). High levels of hyaluronan in the prostate tumor microenvironment correlate with high prostate-specific antigen (PSA) levels in prostate cancer (3–5). Elevated levels of hyaluronan stimulate transformation of androgen-independent prostate cancer (6, 7). Recent evidence links hyaluronan with malignant cell activities and tumor cell invasiveness (8–12). Manipulating hyaluronan synthase expression in cell culture can alter cell survival pathways (8, 13–15), anchorage-independent growth (8, 15, 16), invasiveness (15, 17–20), epithelial-mesenchymal transformation (19, 21), activation of receptor tyrosine kinases such as ErbB2 (17, 20, 22–24) and epithelial growth factor receptor (25), and drug sensitivity (15, 17, 22). In animal models, suppression of hyaluronan synthase activity and inhibition of hyaluronan/CD44 interaction reduce ectopic tumor growth *in vivo* and tumor progression (8, 12, 26, 27).

Numerous studies have provided evidence that hyaluronan receptor CD44 may have an important role in promoting tumor invasion and metastasis (11, 12, 28–31). The standard CD44 (CD44s) of normal cells lacks variant exons and is distinct from the variant forms found in cancer cells. Alternate splicing and post-translational modification of CD44 variants in pathological conditions enhances hyaluronan binding and increases

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[3] The abbreviations used are: DT, dihydrotestosterone (an androgen); HA, hyaluronan; PSA, prostate-specific antigen; PCa, prostate cancer; PCMF, prostate cancer-associated myofibroblast; shRNA, short hairpin RNA; siRNA, small interfering RNA; AR, androgen receptor; CM, conditioned medium; SDM, step down medium; PI3K, phosphatidylinositol 3-kinase; HGF, hepatocyte growth factor; TNF, tumor necrosis factor; scrshRNA, scrambled shRNA; p-cMet, pIGF1R, and p-AKT, phosphorylated cMet, IGF1R, and AKT, respectively; ELISA, enzyme-linked immunosorbent assay; RT, reverse transcription; Ab, antibody.

tumorigenicity (32). Elevated expression of CD44v9 (variant 9) promotes the malignant behavior of prostate cancer cells (33). In contrast, up-regulation of the CD44s reduces the tumorigenicity of prostate tumor cells (34, 35), highlighting the importance of CD44 variants in prostate cancer. Recent studies also indicate important roles of stroma-derived HGF in prostate cancer growth and metastasis (36). HGF modifies the phenotype of the epithelial cells, thereby promoting tumor cell invasiveness, and induces up-regulation of CD44 variants in cancer cells (37).

In a recent study, we showed that experimental up-regulation of hyaluronan synthesis in phenotypically normal intestinal epithelial cells stimulated MMP1/2/9 production as well as CD44v6 (variant 6) expression (38). We also demonstrated that increased hyaluronan stimulates CD44 variants in normal epithelial cells (18) and that hyaluronan/CD44 variant interactions activate multiple receptor tyrosine kinases in prostate and other cancers (20). Thus, in addition to facilitating cell invasion, hyaluronan interacting with CD44 variants acts as a physiological stimulus for macrophage cytokine and growth factor production (21, 39, 40). These cytokines/growth factors in turn can stimulate CD44 variants formation and increased hyaluronan binding (40, 41). Hyaluronan binding to "constitutively activated" CD44 variants (CD44v) induces intracellular signaling during cell dynamic processes but does not do so under conditions of adult tissue homeostasis (40, 42). However, the cellular processes and the associated molecular mechanisms by which this important HGF-induced hyaluronan/CD44v9 signaling cascade mediates invasiveness through PI3K-androgen receptor activity remain largely unknown.

To understand the tumor biology underlying prostate cancer growth through the regulatory effects of the hyaluronan/CD44v9 interaction on androgen receptor-mediated malignant activities, we used human prostatic epithelial cells of normal and malignant prostate tumor origin and associated myofibroblasts in a coculture system to recreate possible interactions between prostate cancer cells and surrounding stromal myofibroblasts. The results suggest that prostate tumor epithelial cells up-regulate HGF production in prostate cancer-associated myofibroblasts, whereas myofibroblast-derived HGF and hyaluronan lead to invasive growth in prostate cancer cells by inducing cMet activation and by augmenting hyaluronan/CD44 variant-induced signaling, which, in turn, stabilizes the androgen receptor functions in these cells.

We provide evidence that 1) IGF1 and TNF-α produced by the tumor cells induce HGF production in the prostate cancer-associated myofibroblasts; 2) stromal HGF induces hyaluronan, which then interacts with CD44v9 to activate cMet to form a lipid raft-associated multimeric signaling complex that contains CD44v9, phosphorylated cMet (p-cMet), androgen receptor, HSP90, and p110α/PI3K (catalytic subunit of PI3K), and stability of this signaling complex is necessary for androgen receptor function and consequent cell invasiveness; 3) androgen receptor directly interacts with CD44v9, and blocking androgen/receptor inhibits HGF-induced CD44v9 expression and function; 4) the androgen (DT) stimulates hyaluronan-induced CD44v9 signaling in DT-dependent LNCaP cells, which correlates with endogenously activated hyaluronan/CD44v9 signaling of DT-independent C4-2 cells; and 5) CD44v9/short hairpin RNA (shRNA) reverses hyaluronan/CD44v9/cMet-associated signaling, decreases androgen receptor function, and increases apoptotic pathway responses.

These results strongly suggest that interaction of androgen receptor and CD44v9 is essential for regulating hyaluronan-mediated invasiveness in prostate cancer and provide evidence for an important paracrine role for stroma-derived HGF in regulating the effects of androgen receptor- and hyaluronan/CD44v9-dependent malignant signaling in prostate cancer.

EXPERIMENTAL PROCEDURES

Cell Culture

Human prostate normal epithelial PWR1E cells (43), DT-dependent transformed human prostate epithelial LNCaP cells, and prostate cancer-associated myofibroblast cells (from ATCC, Manassas, VA) were maintained in their respective media. DT-independent C4-2 cells were derived from LNCaP cells (44, 45) (gift from Dr. C. Voelkel-Johnson, Medical University of South Carolina) and were maintained in the medium as described (20, 43–45). PWR1E cells produce remarkably less hyaluronan, CD44 variants, AR expression, and activity (Figs. 1 and 2), whereas C4-2 and LNCaP cells have high PSA activity (Fig. 4), increased HA synthesis (data not shown), and substantial expression of CD44 variants (Fig. 4).

Preparation of Stable Transfectants

All transfections were done using Lipofectamine (Invitrogen). The HAS2 (hyaluronan synthase 2) stable transfectant of PWR1E cells (PWR1E-HAS2) and vector transfectant (PWR1E-vector) clones were prepared using pCIneo-HAS2 and pCI-neo vector and were selected in the presence of 500 μg/ml geneticin (8, 17, 18, 20, 22).

RNA Silencing

CD44 Variant shRNA Cloning in pSicoR Vectors—Double-stranded oligonucleotide cassettes for CD44v9 shRNA, CD44v5 shRNA, and CD44v10 shRNA were prepared. The linearized pSicoR vectors were ligated to the double-stranded oligonucleotide cassettes (18). The resulting pSicoR-CD44/variant shRNA transfectants constitutively silence respective CD44 variants in the cells. pSicoR-CD44v9 shRNA silences CD44v9 or any variant that contains v9, such as CD44v6–9.

Androgen Receptor shRNA Cloned in pRS Vectors—Scrambled shRNA (scrshRNA) was cloned into the pRS vector, and the androgen receptor shRNA plasmids are from Origene.

Anchorage-independent Growth

The soft agar tetrazolium assay was done as described previously (3).

Cell Lysis, Immunoprecipitations, and Western Blotting

Preparation of cell lysates, SDS-PAGE, and immunoprecipitation analyses were done as described previously (17, 22).

Lipid-rich Microdomains

Separation of lipid raft fractions was done with the lysates of C4-2 cells that had been pretreated with HGF (10 ng/ml for 12 h) or that were transfected with scrambled silencing RNA (control siRNA), with HAS2 siRNA, with pSicoR scrambled

shRNA (control shRNA), or with pSicoR-CD44v9 shRNA (v9 shRNA) prior to induction with HGF. Transfected and treated cells were extracted in 1% Triton X-100-containing buffer and adjusted to ~45% sucrose. This was then overlaid with equal volumes of 35 and 5% sucrose for step gradient centrifugation as described in our earlier work (22). Eight fractions (300 μl) were collected from the bottom up of the gradients using a minipump, and 50 μl from each fraction was immunoprecipitated with cMet antibody and was Western blotted for p-cMet, androgen receptor, HSP90, p110α/PI3K, p85/PI3K (regulatory subunit of PI3K), and CD44. This analysis revealed that phospho-cMet (p-cMet) was distributed throughout the gradient, whereas androgen receptor, HSP90, p110α/PI3K, p85/PI3K, and CD44 were enriched in the middle fractions (fractions 2–5) of the gradient (data not shown).

Because fractions 2–5 from the gradients correspond to the putative rafts as described in our previous work (22), these pooled fractions were collected to study protein/protein interaction by immunoprecipitating with antibody against cMet (Fig. 8*A*) or CD44 (Fig. 8*B*). Western blots were prepared from the immunoprecipitates with antibodies against cMet, p-cMet, and CD44 as well as for the p110α and p85 subunits of PI3K, androgen receptor, and β-actin.

The cells were also treated with 5 mM methyl-β-cyclodextrin in phosphate-buffered saline for 1 h at 37 °C, which extracts cholesterol from cell membranes and causes disassembly of lipid rafts prior to extraction and gradient centrifugation. This treatment caused all of the p-cMet, HSP90, p110α/PI3K, p85/PI3K, and CD44 to concentrate in the bottom fractions (fractions 6–8) of the gradient. Some of the p-cMet remained in the top fractions (fractions 2–5) from the gradient, whereas the rest was concentrated in the bottom fractions (fractions 6–8). The differences in amounts of p-cMet, androgen receptor, and CD44 present in pooled fractions (fractions 2–8) can be calculated from cells treated with methyl-β-cyclodextrin *versus* the amounts present in these fractions (fractions 2–8) from cells that were not treated with methyl-β-cyclodextrin. Thus, estimations of the amounts of lipid raft-associated p-cMet, androgen receptor, and CD44 present in the cMet-immunoprecipitated fractions (fractions 2–8) of step gradient centrifugation from the lysates of cells treated with or without methyl-β-cyclodextrin were obtained. These amounts are plotted in Fig. 8*C* (the data for CD44 are not shown).

RT-PCR for CD44 Variants

Primers for human CD44 variants were used as described previously (46, 47).

Androgen/Receptor-mediated Gene Expression

Gene expression was measured with a transient co-transfection assay using PSA-luciferase (Prof. Hung, University of Texas MD Anderson Center, Houston, TX) in combination with pSV-β-galactosidase (Promega).

Preparation of Conditioned Medium and Measurement of HGF, PSA, and Hyaluronan in Co-culture Medium Experiments

To prepare conditioned medium from carcinoma cells, confluent carcinoma cells were washed three times and incubated for 24 h in step down medium (SDM; Dulbecco's modified Eagle's medium supplemented with 0.25% fetal bovine serum plus 2 μg/ml heparin). The conditioned media were collected and processed for various treatments as indicated below. Prostate cancer-associated myofibroblasts (5×10^4 cells/cm^2) were seeded on 48-well culture dishes, and the cells were than maintained in SDM for 24 h. HGF, hyaluronan, and PSA secreted in the media were measured by ELISA kits (HGF (R & D Systems) and PSA (Abazyme)) and by an ELISA-like method (hyaluronan) (15, 17–20, 48).

In Vitro Invasion Assays

Conditioned Medium Co-culture—The cells (5×10^4 cells/cm^2) were seeded on 24-well Matrigel invasion chamber plates and cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. HGF was added to the medium of the lower chambers, and cells were cultured for 72 h. Invasive cells that migrated to the underside of the membrane were stained with crystal violet and counted by light microscopy.

Co-cultivation of Carcinoma Cells and Prostate Cancer-Associated Myofibroblasts—Human prostate cancer-associated myofibroblasts (5×10^4 cells/cm^2) were initially seeded on 24-well plates and cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum for 24 h. The medium was changed to fresh medium supplemented with 1% fetal bovine serum. Carcinoma cells (5×10^4 cells/cm^2) were then seeded on the inserts of invasion chamber plates and cultured for 72 h. Invasive cells that migrated to the underside of the membrane were stained with crystal violet and counted by light microscopy.

Cell Adhesion Assay

Cells (1×10^6) were seeded in 24-well plates previously coated with supernatant from the respective transfected cultures of C4-2 and LNCaP cells treated with 10 ng/ml DT or with 10 ng/ml DT and 500 μg/ml hyaluronan and incubated for various times. After washing, adherent cells were stained with crystal violet, counted, and dissolved in 10% acetic acid. Absorbance was measured at 595 nm, and results are presented as percentage of adherence of non-transfected cells as control.

Statistical Analyses

All data were analyzed with Student's *t* test using Primer of Biostatistics software, Version 5.0. Statistical significance was determined and expressed as $p < 0.05$.

RESULTS AND DISCUSSION

Increased Hyaluronan Production Transforms Normal Prostate Epithelial PWR1E Cells to Androgen Independence and Up-regulates CD44v9 Splice Variants—Previously, we showed that experimental up-regulation of hyaluronan in non-transformed epithelial cells can lead to enhanced hyaluronan/cell interactions and cell survival signaling pathway activity and thus acquisition of transformed cell properties (15, 17–20, 22). To extend these phenomena in PWR1E cells, which have very low hyaluronan synthesis compared with other prostate cancer cells (~2–5% of that of C4-2, LNCaP, and PC3 cells (data not shown)), we up-regulated hyaluronan synthesis by overexpres-

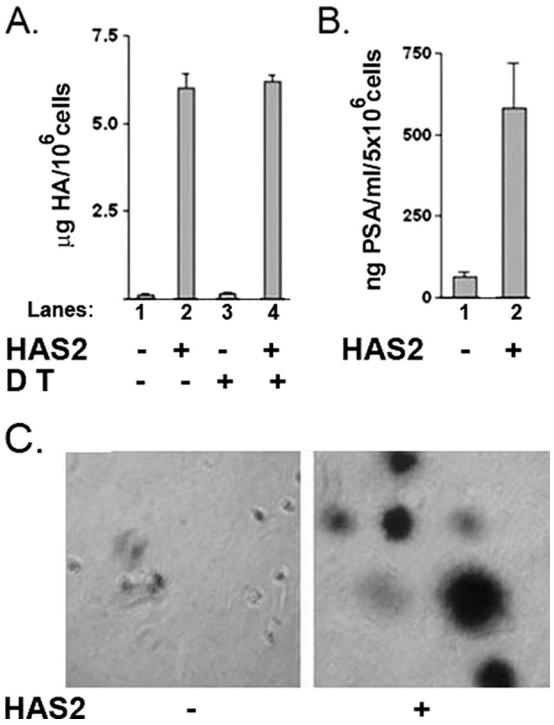

FIGURE 1. **Increased expression of HA promotes transformation of normal epithelial cells by augmenting expression and activation of the AR gene and soft agar colony formation.** *A–C*, increased HA synthesis transforms PWR1E cells to be non-responsive to DT (10 ng/ml). HA and PSA in the medium were measured by an ELISA-like method (*A*) and the ELISA method (*B*), and colony formation in soft agar was detected by tetrazolium dye (*C*). The data in these experiments are representative of three experiments and, where indicated, are the mean of three experiments ± S.D. (*error bars*).

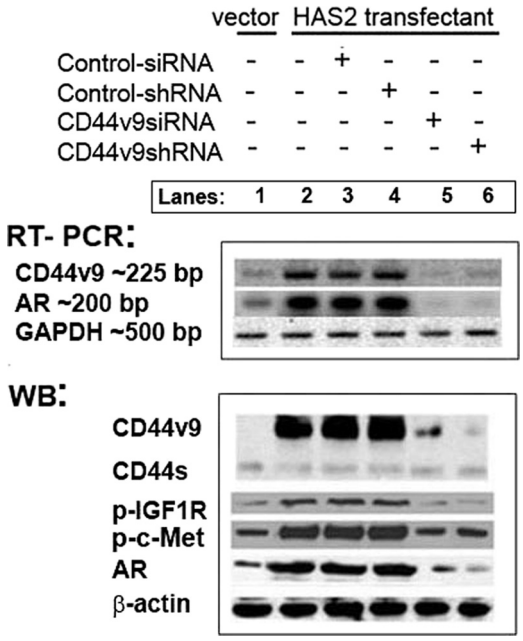

FIGURE 2. **Increased HA stimulates CD44v9 expression, and silencing CD44v9 inhibits expression of AR and activation of cMet and IGF1R.** Empty vector transfectant (*lane 1*) and untreated PWR1E-HAS2 (*lane 2*) were controls. PWR1E-HAS2 stable transfectants were transfected with control siRNA (*lane 3*), control shRNA (*lane 4*), CD44v9 siRNA (*lane 5*), or pSicoRCD44v9 shRNA (*lane 6*) plasmids (0.5 μg/5 $\times$ 10^5 cells). Expressions of proteins and message levels were determined by Western blots (*WB*) from the lysates and mRNA by RT-PCR from the total RNA of the transfectants. The data in these experiments are representative of three experiments.

sion of HAS2 and created a PWR1E-HAS2 stable transfectant cell line. To determine whether hyaluronan alone is sufficient to stimulate the androgen receptor function in PWR1E-HAS2 cells independent of the presence of DT, we first measured hyaluronan in vector and HAS2 transfectant cells in the presence and absence of DT (10 nM for 2 h at 37 °C). Hyaluronan produced and secreted in the medium by the PWR1E-HAS2 cells was 30–50-fold higher (Fig. 1*A*, *lanes 2* and *4*) than by the vector transfectant cells (*lanes 1* and *3*). Moreover, hyaluronan synthesis in the PWR1E-HAS2 cells was independent of DT treatment (*lane 2* compared with *lane 4*), as expected. Further, a large up-regulation of CD44v9 protein with little or no change in CD44s protein was observed in PWR1E-HAS2 cells compared with vector transfectant cells (Western blots in Fig. 2, *lane 1* compared with *lane 2*). Thus, the hyaluronan produced by the PWR1E-HAS2 cells will interact with the up-regulated CD44v9, which may then induce phenotypic changes by altering androgen receptor activity that favors tumor cell metastatic processes. We then showed that the increased hyaluronan production correlated with an ~8-fold increase in PSA production (Fig. 1*B*) and with increased colony formation in soft agar (Fig. 1*C*), ~300 colonies for PWR1E cells *versus* 7–10 colonies for PWR1E-vector cells. These results strongly suggest that endogenously produced, high levels of hyaluronan in the PWR1E-HAS2 cells transform them into a malignant phenotype.

CD44v9 shRNA Silences CD44v9 Expression, but Not CD44s, and Regulates Androgen Receptor Expression—In addition to CD44v9 mRNA and androgen receptor mRNA, their corresponding proteins were significantly increased in the PWR1E-HAS2 cells compared with the vector transfectants (Fig. 2, *lanes 2* compared with *lanes 1*). Further, p-IGF1R and p-cMet proteins were also significantly increased (Fig. 2, *lane 2* compared with *lane 1*) in the PWR1E-HAS2 cells. During splicing, CD44 may acquire variable exon 9 alone (CD44v9), or the same exon may be present in combination with other variant exons, such as v6 through v9 (CD44v6–v9). However, no amplicon corresponding to v6–v9 was detected by RT-PCR in the PWR1E vector-transfected cells (data not shown). We therefore determined whether down-regulation of CD44v9 in PWR1E-HAS2 cells inhibits up-regulation of androgen receptor and prevents activation of IGF1R (p-IGF1R) and cMet (p-cMet) in CD44v9 siRNA and CD44v9 shRNA transfection experiments. To address this possibility, oligonucleotides for CD44v9 shRNA were cloned in U6 promoter-based pSicoR vectors as described (49). This pSicoR-CD44v9 shRNA releases the shRNA constitutively in the transfected PWR1E-HAS2 cells. Fig. 2 shows that transfection of PWR1E-HAS2 cells with neither the control siRNA nor the control shRNA vectors inhibited protein expression of CD44v9 or CD44s (Fig. 2, *lanes 3* and *4*). In contrast, both CD44v9 siRNA and CD44v9 shRNA transfections significantly decreased mRNA and protein expression of CD44v9 and of androgen receptor (70–90%) and also decreased p-cMet and p-IGF1R, with little or no effect on protein expression of CD44s (Fig. 2, *lanes 5* and *6* compared with *lanes 3* and *4*). The antibody against CD44 (HCAM) that was used in the Western blot detected both the CD44 variant and CD44s forms. However, the variant form was abolished, leaving CD44s unchanged in the Western blot when the cells were treated with CD44v9

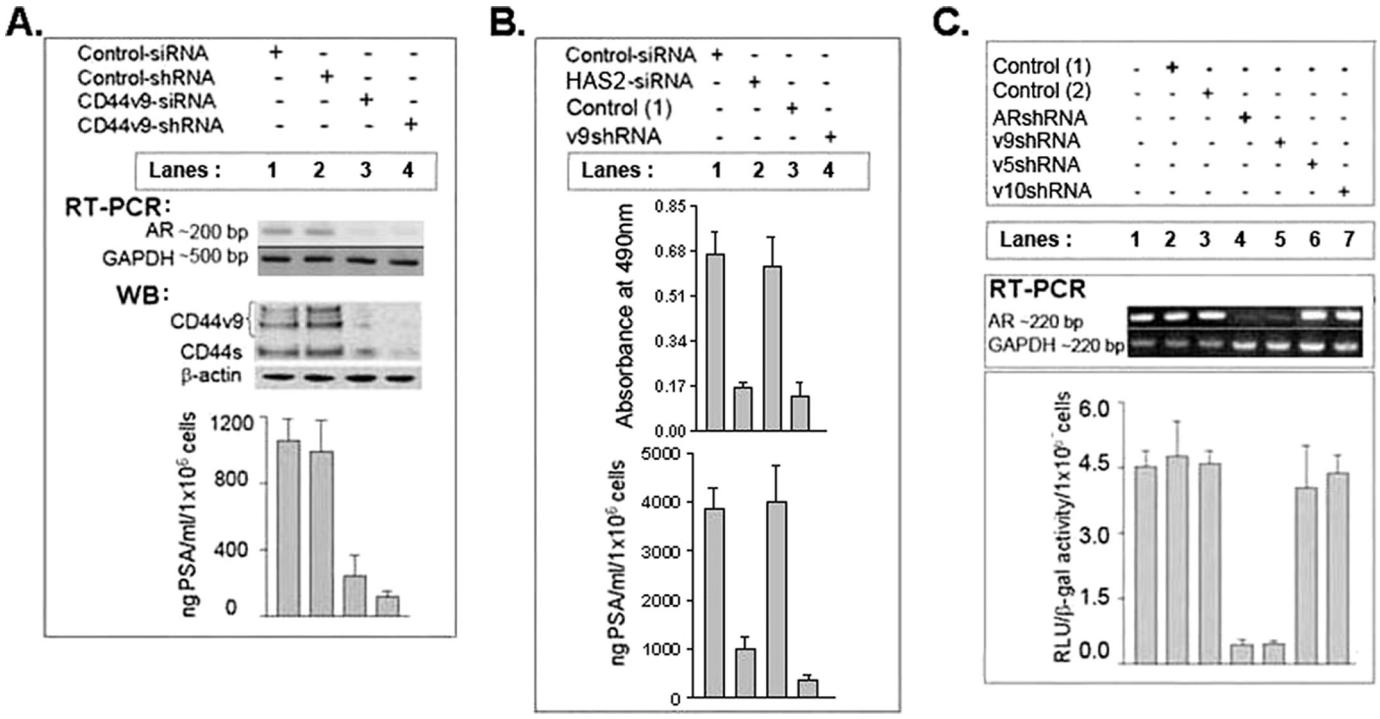

FIGURE 3. **Endogenous HA/CD44v9 interactions are required for AR gene expression, cell adhesion, and cell proliferation.** *A–C*, an endogenous HA/CD44v9 interaction regulates AR-mediated gene expression. *A*, C4-2 cells were transfected with scrambled siRNA (control siRNA; *lane 1*), pSicoR scrambled shRNA (control shRNA; *Control (1)* (*lane 2*)), CD44v9 siRNA (*lane 3*), or pSicoR-CD44v9 shRNA (*v9shRNA*; *lane 4*) plasmids. PSA secreted in the media was measured by an ELISA method. Expressions of CD44v9 at protein and message levels were analyzed by Western blot (*WB*) and by RT-PCR. The *lanes* in RT-PCR are compared with *lanes* of Western blot and *bars* of PSA secretion against each treatment. *B*, HA/CD44 interaction is required for AR gene expression and cell proliferation. C4-2 (20×10^3) cells were transfected with control siRNA (*lane 1*), HAS2 siRNA (*lane 2*), pSicoR scrambled shRNA (*Control (1)*, *lane 3*), or CD44v9 shRNA (*lane 4*). Cell proliferation was measured by an MTS assay and expressed as the mean absorbance at 490 nm/20×10^3 cells/90 min. PSA contents were measured by an ELISA. The *lanes* for cell proliferation of the *upper panel* are compared with *lanes* of PSA secretion against each treatment. *C*, CD44v9 regulates AR expression and function, whereas CD44v5 and CD44v10 do not. C4-2 cells were transfected with pSicoR scrambled shRNA (*Control (1)*, *lane 2*), pRS scrambled shRNA (*Control (2)*, *lane 3*), pRS-AR shRNA (*lane 4*), pSicoR-CD44v9 shRNA (*lane 5*), pSicoR-CD44v5 shRNA (*v5shRNA*) (*lane 6*), or pSicoR-CD44v10shRNA (*v10shRNA*) (*lane 7*). Non-transfected C4-2 cells were used as control (*lane 1*). The mRNAs for AR and glyceraldehyde-3-phosphate dehydrogenase mRNAs were measured by RT-PCR. In a separate experiment, the transcriptional activities of AR were measured, and the results are expressed as RLU/β-galactosidase activity. The *lanes* in RT-PCR are compared with *bars* of transcriptional activity of AR (RLU/β-galactosidase activity) against each of the plasmids. The data in these experiments are representative of three experiments and, where indicated, are the mean of three experiments ± S.D. (*error bars*).

shRNA, indicating that the variant form that HCAM recognized was CD44v9. The results in Figs. 1 and 2 clearly indicate that the HAS2 overexpressing PWR1E-HAS2 clone acquired many properties of the transformed phenotype, including increased colony formation in soft agar and elevated production of PSA (Fig. 1), most likely due to overexpression of androgen receptor and CD44v9 and activation of cMet and IGF1R (Fig. 2). Importantly, hyaluronan synthesis in CD44v9 shRNA-treated PWR1E-HAS2 cells was only reduced 10–15% (data not shown), indicating that CD44v9 is not directly involved in regulating the synthesis of hyaluronan; rather, the presence of CD44v9 may be required for the increased binding of hyaluronan (40, 50) to induce the signaling that maintains the high expression of androgen receptor, p-IGF1R, and p-cMet for cell survival.

Interaction of Androgen Receptor with CD44v9 Is Required for Endogenous Hyaluronan-mediated Malignant Behavior in Prostate Tumor C4-2 and LNCaP Cells—Increased expression of CD44v9 in prostate cancer cells is a crucial mediator of oncogenic signaling that mediates metastasis (33, 51, 52). Therefore, we probed the mechanistic functions of hyaluronan/CD44v9 interaction in prostate cancer cells (C4-2 and LNCaP cells).

First, we inhibited expression of CD44v9 in C4-2 cells by silencing CD44v9. Both CD44v9 siRNA and CD44v9 shRNA transfections significantly decreased mRNA expression of androgen receptor and protein expression of CD44v9 (Fig. 3*A*, *lanes 3* and *4* compared with *lanes 1* and *2*). PSA production was also significantly decreased (Fig. 3*A*, *bottom*).

Second, we silenced HAS2 enzyme in C4-2 cells, which diminished the amount of endogenous hyaluronan that could interact with CD44v9. The results showed that HAS2-siRNA inhibited PSA production and cell proliferation by ~75% (Fig. 3*B*, *lane 1* compared with *lane 2*) compared with CD44v9 shRNA, which reduced PSA production ~77% and cell proliferation ~85% (Fig. 3*B*, *lane 3* compared with *lane 4*). Overall, these data suggest that hyaluronan/CD44v9 interaction regulates androgen receptor activation/function. In a separate experiment, silencing HAS2 significantly decreased the amount of hyaluronan synthesized (~90%) (data not shown).

To determine the specificity of the shRNA construct on androgen receptor expression, we tested CD44v5 shRNA and CD44v10 shRNA. Fig. 3*C* shows that shRNA constructs for CD44v5 and CD44v10 (*lanes 6* and *7*) do not inhibit androgen receptor mRNA expression, in contrast to shRNA constructs for CD44v9 and androgen receptor (*lanes 4* and *5*), which also inhibit androgen receptor function, as indicated by the inhibition of PSA-luciferase promoter activity (*bottom*, *lanes 4* and *5*). These results strongly suggest that hyaluronan/CD44v9-asso-

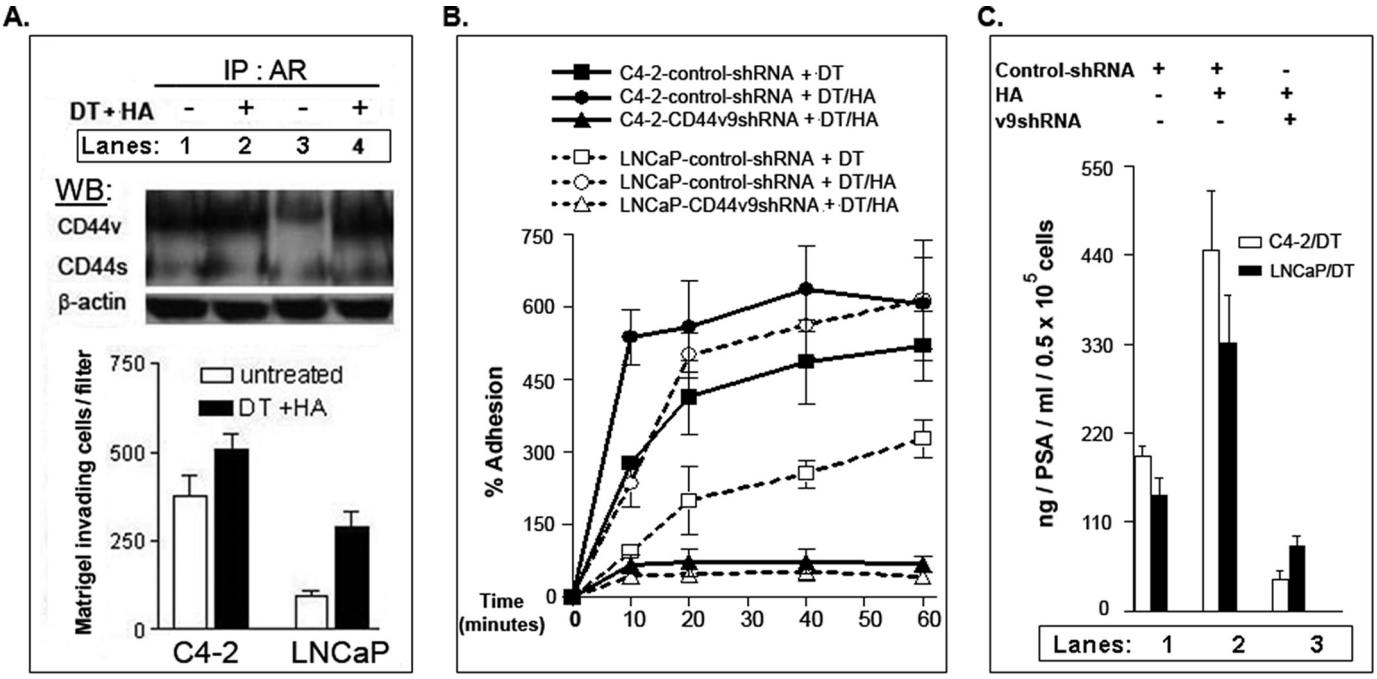

FIGURE 4. **AR regulates HA-mediated interaction of AR and CD44v9, cell invasion, and AR gene function.** *A*, *top*, results of immunoprecipitation (*IP*) with AR followed by Western blot (*WB*) analysis of CD44 protein in the lysates of C4-2 treated with or without 10 ng/ml DT, 500 μg/ml HA for 24 h. *A*, *bottom*, DT/HA-treated and -untreated C4-2 cell (5×10^4 cells/cm^2) invasion in 24-well plates containing a Matrigel chamber on PCMFs (5×10^4 cells/cm^2). The *lanes* in the Western blot are compared with those of invasion against each of the treatments. *B*, C4-2 cells (1×10^6), LNCaP cells (1×10^6), or their transfectants with pSicoR scrambled shRNA (control shRNA) or pSicoR-CD44v9 shRNA (v9shRNA) plasmids were cultured in the 24-well tissue culture wells coated with supernatant from the respective cultures treated with 10 ng/ml DT or 10 ng/ml DT plus 500 μg/ml HA. Adherent cells were analyzed. Results are presented as percentage of adherence of non-transfected cells as control. *C*, the experiments in *B* were repeated for a fixed time period of 60 min. The data in this experiment are presented as control shRNA (*lane 1*), control shRNA plus HA (*lane 2*), and CD44v9 shRNA plus HA (*lane 3*). The cells were cultured for 48 h and then incubated with serum-free medium for 24 h. PSA secreted in the media was measured by ELISA. The data in these experiments are representative of three experiments and, where indicated, are the mean of three experiments ± S.D. (*error bars*).

ciated signal activation specifically regulates androgen receptor expression and function in prostate cancer cells.

In order to understand the relationship between DT-induced androgen receptor signaling and hyaluronan/CD44v9-associated signal activation, we analyzed the expression of CD44v9 and Matrigel invasion of LNCaP cells (DT-dependent) and of C4-2 cells (DT-independent), which were pretreated with both DT (10 ng/ml) and hyaluronan (500 μg/ml) for 24 h. Fig. 4*A* shows that C4-2 cells treated with DT and hyaluronan have only a small increase of CD44v9 in immunoprecipitates of androgen receptor (Western blots in *lane 1* compared with *lane 2*) and a moderate (~1.3-fold) increase in Matrigel invasion in co-cultures with prostate cancer-associated myofibroblasts (*white bar* compared with *black bar*, *C4-2 lanes*). In contrast, DT-sensitive LNCaP cells treated with DT and hyaluronan have a large increase of CD44v9 in the androgen receptor immunoprecipitates (Western blots in *lane 3* compared with *lane 4*) and a large (~3-fold) increase in the invasion assay (*white bar* compared with *black bar*, *LNCaP lanes*). These results suggest that endogenous levels of hyaluronan are sufficient to saturate CD44v9 and resulting signaling pathways in C4-2 cells, which therefore do not respond further to the addition of exogenous hyaluronan. Further, the addition of DT alone does not change either CD44v9 mRNA or protein expression levels in C4-2 cells, whereas in LNCaP cells, DT stimulated both CD44v9 mRNA and protein expression 1.5–1.8-fold (data not shown). These results indicate that DT/hyaluronan-induced CD44v9 signaling in LNCaP cells increases responses similar to the full endogenous responses of hyaluronan-mediated CD44v9 signaling levels in C4-2 cells.

Matrix adhesion and modulation are important factors in tumor progression (53), and CD44 has been suggested to contribute to tumor progression via hyaluronan binding (23). Because increased cell survival of cancer cells is supported by the tumor matrix, we investigated whether CD44v9 contributes to adhesive matrix production via an interaction with hyaluronan in DT-dependent LNCaP cells and DT-independent C4-2 cells. Fig. 4*B* shows assays using transfected C4-2 and LNCaP cells adhering to culture plates incubated for various times after treatment with their respective conditioned medium isolated from 48-h cultures from 1) control shRNA-transfected C4-2 cells (*filled squares*) and control shRNA-transfected LNCaP cells (*open squares*) treated with 10 ng/ml DT; 2) control shRNA-transfected C4-2 cells (*filled circles*) and control shRNA-transfected LNCaP cells (*open circles*) treated with 10 ng/ml DT and 500 μg/ml hyaluronan; and 3) CD44v9 shRNA-transfected C4-2 cells (*filled triangles*) and CD44v9 shRNA-transfected LNCaP cells (*open triangles*) treated with 10 ng/ml DT and 500 μg/ml hyaluronan. The DT treated C4-2-control shRNA cells without (*filled squares*), or with hyaluronan treatments (*filled circles*) showed similar adhesion patterns with time over 60 min, with the latter reaching a somewhat higher plateau (~600% (with hyaluronan treatment) and ~500% (without hyaluronan treatment) of the control non-transfected C4-2 cells set at 100%). DT/hyaluronan-treated transfected C4-2-CD44v9 shRNA cells (*filled triangles*) showed very low

adhesion, only about 15% of the respective control shRNA-transfected cells (*filled squares*). DT-treated transfected LNCaP control shRNA cells (*open squares*) showed slower adhesion and only reached ~300% of the DT/hyaluronan treated transfected LNCaP control shRNA cells (*open circles*) by 60 min. Interestingly, DT/hyaluronan-treated transfected LNCaP control shRNA cells (*open circles*) showed adhesion equivalent to that of DT/hyaluronan-treated transfected C4-2-control shRNA cells (*filled circles*) by 60 min. CD44v9 shRNA reversed most of the adhesion effects by the concurrent hyaluronan and DT stimulations (LNCaP (*open triangles*) *versus* C4-2 (*filled triangles*)), suggesting that 1) that these elevated tumorigenic effects (*i.e.* cell adhesion (Fig. 4*B*) and cell invasion (Fig. 4*A*)) indeed resulted from the activation of the DT/hyaluronan/CD44v9 signaling in LNCaP cells and from hyaluronan/CD44v9 signaling in C4-2 cells, and 2) these increased tumorigenic responses in LNCaP cells in response to DT/hyaluronan/CD44v9 interaction are similar to the full constitutive hyaluronan/CD44v9 interaction-mediated oncogenic signaling in C4-2 cells.

Fig. 4*C* shows that both cell types have nearly the same PSA responses in 48-h cultures. The shRNA control-transfected cells cultured in DT/hyaluronan (*lane 2*) synthesized ~2-fold higher levels of PSA than cells cultured in DT alone (*lane 1*). Cells transfected with CD44v9 shRNA showed significantly decreased PSA contents (*lane 3*).

These findings in Fig. 4 suggest that 1) androgen receptor directly interacts with CD44v9 (co-immunoprecipitation experiment; Fig. 4*A*, *WB*); 2) in the absence of hyaluronan, LNCaP cells have moderate adhesion (Fig. 4*B*, *open squares versus open circles*); 3) in the absence of DT + hyaluronan, LNCaP cells have limited invasiveness compared with that of C4-2 cells (Fig. 4*A*, *bottom*), and 4) DT/hyaluronan-induced CD44v9 signaling in DT-dependent LNCaP cells correlates with constitutively activated hyaluronan/CD44v9 signaling of DT-independent C4-2 cells (Fig. 4, *A* (*black bars* compared with *white bars*), *B* (*filled circles versus unfilled circles* at 60 min), and *C* (*lane 2*, *white bars* compared with *black bars*)).

Carcinoma-derived HGF Inducers Activate Prostate Cancer-associated Myofibroblasts to Synthesize HGF, Which Stimulates Hyaluronan Production and Invasion in Tumor/Stroma Interaction—As reported previously, basic fibroblast growth factor, IGF1, HST-1/FGF-4, interleukin-1, TNF-α, platelet-derived growth factor, epithelial growth factor, TGF-α, and PGE2 can stimulate HGF production in human fibroblasts (54–59). Therefore, we tested the effect of antibodies to various growth factors/cytokines or their receptors on the production of HGF in prostate cancer-associated myofibroblasts cultured in conditioned medium from C4-2 cells. Inhibition of HGF production by a specific antibody could be indicative of the HGF-stimulatory effect of growth factors/cytokines in conditioned medium from C4-2 cells. Fig. 5*A* shows that an IGF1 antibody (Ab) completely abrogated HGF production in prostate cancer-associated myofibroblasts. TNFα Ab showed ~60% inhibition, and marginal inhibitions were observed for IL1R Ab and basic fibroblast growth factor Ab. No inhibition was found with the antibody against platelet-derived growth factor. Thus, major HGF-inducing factors in the conditioned medium of C4-2 cells were IGF1 and TNFα, suggesting that these factors derived from tumor cells can activate neighboring fibroblasts to secrete HGF, which in turn may be responsible for up-regulating CD44v9 variant expression on the tumor cells, ECM degradation, and concomitant cell invasion and tumorigenesis.

Carcinoma cells capable of inducing HGF production in stromal cells may be particularly susceptible to a more malignant progression through tumor/stroma interactions. Prostate cancer-associated myofibroblasts cultured for 24 h in 24-well plates without conditioned medium from C4-2 cells secrete less than 0.30 ng/ml HGF (data not shown). Fig. 5*B* shows that hyaluronan increased ~2 fold in prostate cancer-associated myofibroblasts cultured in conditioned medium (CM) alone (*lane 1*) and 4–5-fold in conditioned medium supplemented with HGF (*lane 3*). The increase in hyaluronan production in the presence of HGF was suppressed by the anti-HGF antibody (Fig. 5*B*, *lane 3 versus lane 4*). In addition, hyaluronan in prostate cancer-associated myofibroblasts cultured with conditioned medium from C4-2 cells without HGF stimulation when treated with anti-HGF antibody also decreased hyaluronan synthesis to the same basal level (Fig. 5*B*, *lane 2*), indicating that HGF in the conditioned medium is responsible for the increase in hyaluronan (*lane 1*).

We then tested whether synthesis of stroma-derived HGF is linked to CD44v9 in cancer cells by silencing expression of CD44v9 in cancer cells. Prostate cancer-associated myofibroblasts were cultured in the presence of conditioned medium prepared from cultures of C4-2 cells that were treated with control IgG or anti-HGF or were transfected with control shRNA or CD44v9 shRNA. Fig. 5*C* (*top*) shows that HGF synthesis by the prostate cancer-associated myofibroblasts was increased more than 10-fold in conditioned medium from control IgG and from C4-2 control shRNA cultures (*lanes 1* and *2*), whereas the conditioned media from anti-HGF and from C4-2 CD44V9 shRNA cultures (*lanes 3* and *4*) completely blocked this increase to the basal level of prostate cancer-associated myofibroblasts cultured in normal medium alone (*PCMF lane*). The results provide strong evidence that silencing CD44v9 in C4-2 cells inhibits the production of HGF in prostate cancer-associated myofibroblasts. Thus, CD44v9 shRNA reduces these malignant characteristics by antagonizing HGF/cMet signaling amplification, as seen in Fig. 2.

Parallel to the increase in hyaluronan concentration (Fig. 5*B*), the number of C4-2 cells that invaded Matrigel increased ~3–4-fold by co-culture of prostate cancer-associated myofibroblasts with C4-2 cells directly or with C4-2 cells transfected with pSicoR-control shRNA (Fig. 5*C*, *bottom*, *lanes 1* and *2*). In contrast, co-culture with C4-2 cells transfected with CD44v9 shRNA reduced Matrigel invasion to nearly control levels (Fig. 5*C*, *bottom*, *lane 4* compared with *PCMF lane*). Co-culture with C4-2 cells treated with anti-HGF also reduced Matrigel invasion significantly (Fig. 5*C*, *bottom*, *lane 3* compared with *lane 1*). These observations provide strong evidence that growth factors/cytokines in conditioned medium (primarily IGF1 and TNFα) from cancer cells induce hyaluronan and HGF synthesis in the stromal cells (prostate cancer-associated myofibroblasts). These stroma-secreted molecules (hyaluronan and HGF) in turn induce CD44 splicing (v9) in the cancer (C4-2)

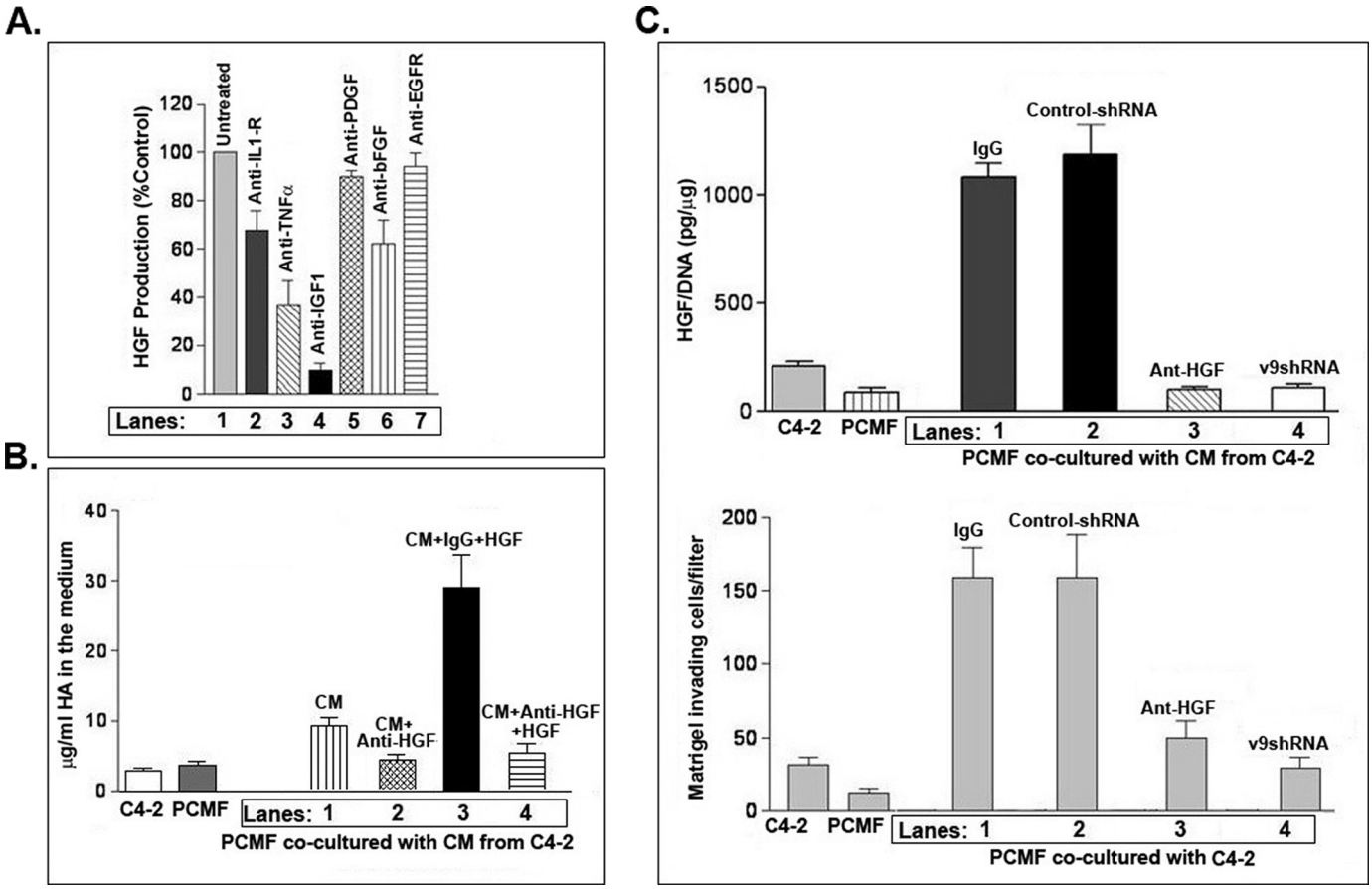

FIGURE 5. **Carcinoma cells induce HGF in PCMFs that stimulates HA production and HA/CD44v9-induced invasion.** *A*, PCMFs (5×10^4 cells/cm^2) were co-cultured in CM from the C4-2 cells (5×10^4 per/cm^2) in the absence or presence of antibodies (10 μg/ml) to IL1R (*lane 2*), TNF-α (*lane 3*), IGF1 (*lane 4*), platelet-derived growth factor (*lane 5*), basic fibroblast growth factor (*lane 6*), or epithelial growth factor receptor (*lane 7*). HGF contents in the supernatants of the co-cultures were determined by ELISA. *B*, C4-2 cells (5×10^4 cells/cm^2) were grown in the absence and presence of control IgG or of 10 μg/ml anti-HGF Ab for 24 h, followed by treatment with 10 ng/ml HGF for another 48 h. CM were isolated from these cultures. PCMFs (5×10^4 cells/cm^2) were grown in the CM (in 1:1 (v/v) CM/SDM as described under "Experimental Procedures") from untreated C4-2 cells (*lane 1*), CM from the C4-2 cells that were treated with HGF-antibody (*lane 2*), fresh SDM plus CM from IgG-treated C4-2 cells (*lane 3*), or fresh SDM plus CM from the HGF-antibody treated C4-2 cells (*lane 4*), followed by culture for 24 h. The concentrations of HA in the media of these CM co-cultures were determined by an ELISA-like method. *C*, *top*, CM from the C4-2 cells (5×10^4 cells/cm^2) pretreated with IgG (10 μg/ml) or with HGF-antibody (10 μg/ml) or transfected with control pSicoR scrshRNA or pSicoR-CD44v9 shRNA were prepared. PCMFs were grown in the CM from the C4-2 cell (in 1:1 (v/v) CM/SDM) and cultured for another 24 h: IgG treatment (*lane 1*), HGF antibody treatment (*lane 2*), control shRNA transfection (*lane 3*), and v9shRNA transfection (*lane 4*). HGF in the media was measured by an ELISA method, and the ratios of HGF/total DNA content (by measuring fluorescence of the Hoechst dye 33258) for the cells were plotted (*y* axis) for various treatments (*x* axis). *C*, *bottom*, PCMFs (in the well) were co-cultured with C4-2 cells (in the insert) treated with IgG (*lane 1*), transfected with pSicoR scrambled shRNA (*lane 2*), treated with anti-HGF antibody (*lane 3*) (10 μg/ml), or transfected with pSicoR-CD44v9 shRNA (*v9shRNA*) (*lane 4*) (5×10^4 cells/200 μl) of the invasion chamber and were incubated for 3 days. Invasive cells that migrated to the underside of the membrane were counted. The data are presented as mean $\pm$ S.D. (*error bars*) of three experiments.

cells, resulting in hyaluronan/CD44v9 interaction on the tumor cells in the tumor/stroma environment that increases invasiveness of the cancer cells.

HGF, which can be derived from a variety of tissues, is known to be elevated in the serum of men with metastatic prostate cancer (36, 60). In the prostate, stromal cells secrete HGF, which acts locally on prostate epithelial cells expressing its receptor, the tyrosine kinase c-Met. Prostate cancer can also express HGF via stimulation by interleukin-1β, platelet-derived growth factor, basic fibroblast growth factor, vascular endothelial growth factor, and epithelial growth factor derived from stromal cells (61). Thus, we examined dose responses of HGF on C4-2 cell survival and invasion. Fig. 6, *A* and *B*, shows that maximal stimulatory effects of HGF for cell proliferation and migration were 10–20 ng/ml, and these levels of HGF can be correlated with the physiological level of serum HGF (2–10 ng/ml) in prostate cancer (36, 60). These levels of HGF are dependent upon the dynamic balance of cell proliferation, differentiation, metastasis, and apoptosis through interactions between cells and their microenvironment. Our results show that HGF has a modest mitogenic effect on C4-2 cells (Fig. 6*A*), whereas HGF strongly affects cell migration (Fig. 6*B*). Fig. 6*C* shows that the optimum time for HGF-induced hyaluronan production is ~12–16 h. Thus, in subsequent experiments, we used 10 ng/ml HGF for 12 h when necessary. The results shown in Figs. 5 and 6 indicate that interaction of hyaluronan with the carcinoma cell receptor CD44v9 has significant effects on stromal fibroblasts and probably accounts for the existence of activated fibroblasts that have been observed in stromal tissues associated with tumor tissue (62).

Synergy between Hyaluronan/CD44v9 Signaling and HGF/cMet Signaling—To explore whether CD44v9 is also required for the activation of cMet/PI3K signaling in C4-2 cells, we examined the activation of cMet and AKT, a downstream target

of PI3K signaling, after treating serum-starved cells with 10 ng/ml HGF for various times. After 24 h of serum starvation, the relative expression levels of CD44 variants and p-AKT were low (Fig. 7*A*, *lane 1*). Stimulation of these cells with 10 ng/ml HGF resulted in up-regulation of CD44 variants (Fig. 7*A*, *lanes 3* and *5*) and p-AKT expression (Fig. 7*B*, *lane 3*). This stimulation was apparent after 4 h (data not shown) and peaked at 12 h (5.5 ± 1.2-fold) (Fig. 7*A*, *lane 5 versus lane 1*). An ~3-fold (3.14 ± 0.49) increase was observed after 8 h of stimulation as compared with the unstimulated state (Fig. 7*A*, *lane 3 versus lane 1*). In contrast, expression of the CD44s (standard form) showed less variation upon serum starvation and HGF stimulation. Overall, these results indicate that CD44 alternative splicing (CD44v9) is up-regulated by 10 ng/ml HGF at 12 h of treatment. The activation of cMet was consistently inhibited >75% using CD44v9 shRNA (Fig. 7*B*, p-cMet in *lane 4* compared with *lane 3*) after treatment with HGF for 12 h. The strong inhibition (>75%) of phosphorylation on cMet after treatment with CD44v9 shRNA suggests that downstream targets of cMet (63) are inhibited by CD44v9 shRNA treatment. This is indeed the case, as shown for signal transduction of p-AKT (Fig. 7*B*, *p-AKT*, *lane 3 versus lane 4*). Next we demonstrated that the PI3K inhibitor LY 294002 (10 μM) inhibited by ~80% of transcriptional activation of androgen receptor (Fig. 7*C*, *lane 2 versus lane 1*), and ~90% inhibition of C4-2 cell migration was found with 10 μM LY294002 (Fig. 7*D*, *lane 3 versus lane 1*). Likewise, cell migration was inhibited ~55% using androgen receptor shRNA (Fig. 7*D*, *lane 2*). Our studies demonstrate that the direct interaction of p-cMet and HGF-dependent cell survival activities requires the CD44v9 isoform and represents a strong relationship among androgen receptor, CD44v9, and PI3K/AKT activation (Fig. 7, *A*–*C*).

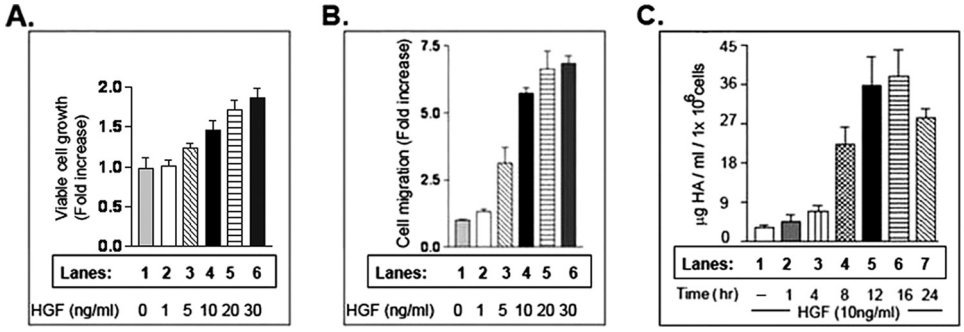

FIGURE 6. **HGF induces cell proliferation/migration and HA production.** *A*, 70% confluent C4-2 cells (2.5×10^4) were grown in serum-depleted medium (see legend to Fig. 3) in the presence of various doses of HGF for 72 h, and cell proliferation was measured by counting viable cells in a trypan blue dye exclusion assay with a hemocytometer. *B*, the wells of a 24-well Matrigel invasion chamber contained 400 μl of SDM with various doses of HGF. Migration of C4-2 cells (2×10^4 cells/200 μl of medium were added in the insert) to the lower side of the membrane were counted (12 different fields). *C*, C4-2 cells were treated with 10 ng/ml HGF for various times and were allowed to grow for another 24 h in SDM, and HA released in the medium was measured by an ELISA-like method. The data are presented as mean ± S.D. (*error bars*) of three experiments.

Previous reports by Lin *et al.* (7) have documented hyaluronan-augmented formation of an androgen receptor CD168-containing complex in LNCaP cells. Our earlier studies demonstrate that hyaluronan/CD44 variant interaction induces a signaling complex that includes HSP90 and Cdc37 (17, 22). The androgen receptor is one the clients of HSP90, which protects the client proteins from degradation. We predicted that androgen receptor will be present in the signaling complex and that inhibition of expression of CD44v9 in prostate cancer cells will lead to breakdown of the multimeric signaling com-

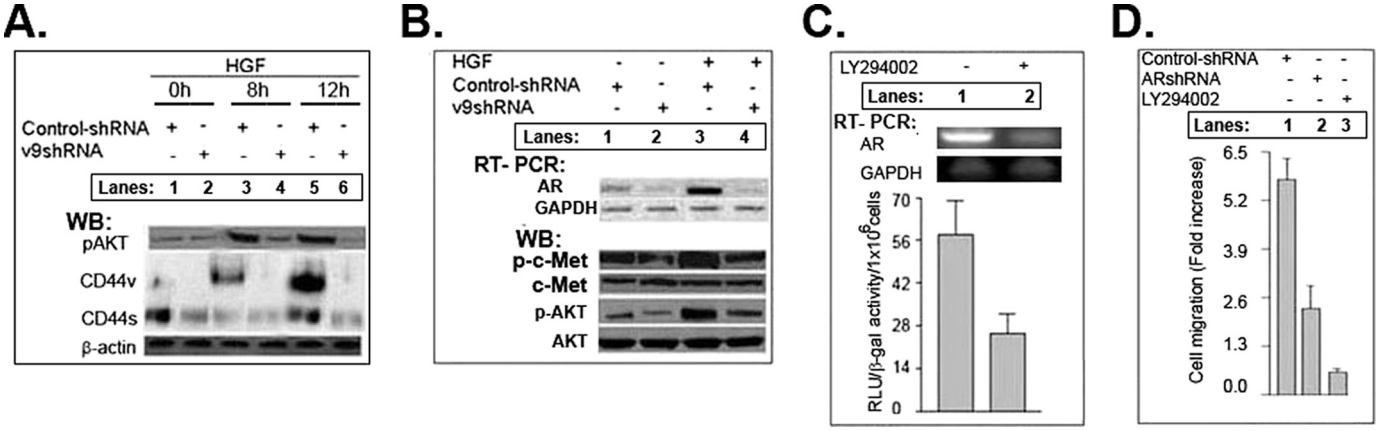

FIGURE 7. **Down-regulation of CD44v9 inhibits PI3K (PI3K)/AKT activation, AR gene expression, and cell migration in C4-2 cells.** *A*, C4-2 cells transfected with pSicoR scrshRNA (control shRNA) or pSicoR-CD44v9 shRNA (*v9shRNA*) were cultured with HGF (10 ng/ml). Cell lysates were extracted at the indicated time points and were analyzed by Western blot (*WB*) for p-AKT, AKT (data not shown), CD44, and β-actin. *B*, C4-2 cells transfected with control shRNA (*lanes 1* and *3*), or pSicoR-CD44v9 shRNA (*lanes 2* and *4*) were cultured to 70% confluence. After a 24-h incubation in SDM, cells were stimulated with HGF (10 ng/ml) for 12 h. Cells were then cultured for 72 h; cell lysates were prepared and analyzed for p-cMet, cMet, p-AKT, or AKT by Western blot; and mRNAs were determined for AR and glyceraldehyde-3-phosphate dehydrogenase by RT-PCR. Note that basal HGF secretion of C4-2 cells in this medium is <0.10 ng/ml. *C*, C4-2 cells were cultured without (*lane 1*) or with LY294002 (10 μM) (*lane 2*) for 12 h, followed by transfection with PSA-luciferase and pSV-β-galactosidase, and grown for 48 h. Cell extracts were prepared and used for luciferase and β-galactosidase assays, and results are expressed as RLU/β-galactosidase activity. *D*, C4-2 cells were treated with LY294002 (10 μM) for 12 h (*lane 3*). C4-2 cells were transfected with pRS scrshRNA (*control shRNA*; *lane 1*), or pRS-AR shRNA (*ARshRNA*; *lane 2*). The transfected cells (2×10^4 cells/200 μl of medium) were added in the transwell inserts and allowed to migrate for 16 h at 37 °C. Migrated cell numbers were compared with that of untreated control cells and expressed as -fold increase. The data in these experiments are representative of three experiments and, where indicated, are the mean of three experiments ± S.D. (*error bars*).

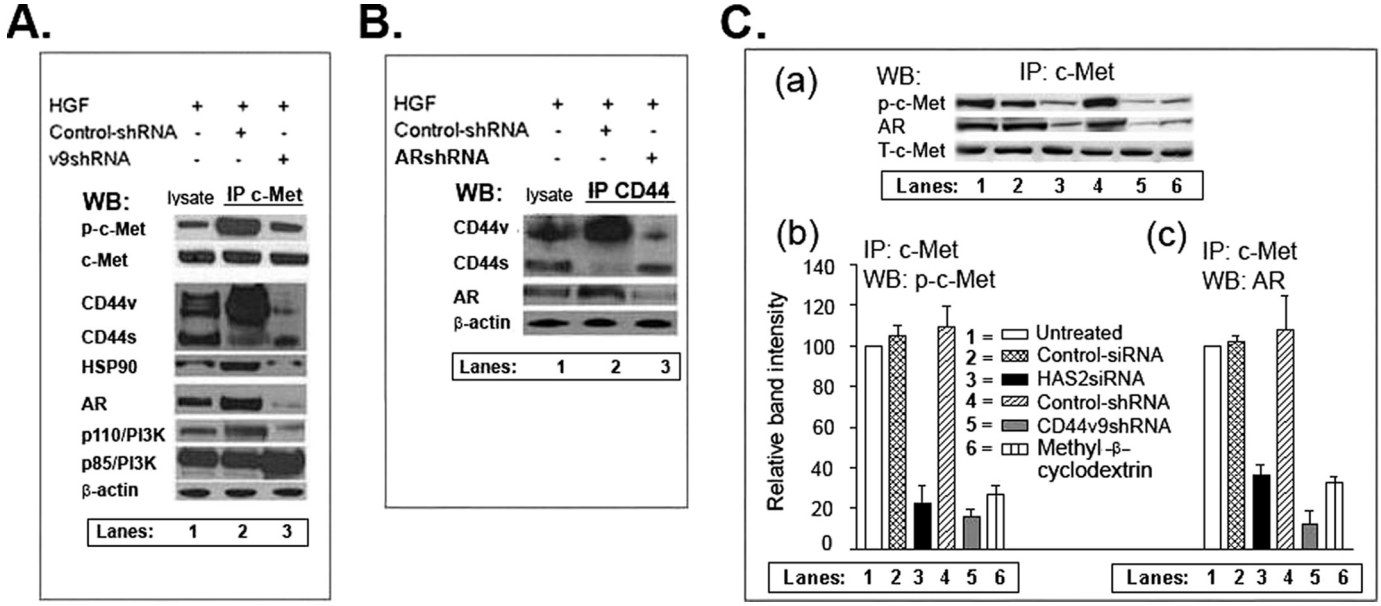

FIGURE 8. **Silencing CD44v9 inhibits assembly of p-cMet, AR, HSP90, P110α/PI3K, and CD44 into lipid raftlike structures.** *A*, lipid raft fractions 2–5 of the gradients were isolated by sucrose density centrifugation from lysates of C4-2 cells that had been pretreated with HGF (10 ng/ml for 12 h) or were transfected with pSicoR scrambled shRNA (*Control shRNA*) or pSicoR-CD44v9 shRNA (*v9shRNA*) prior to treatment with HGF. Immunoprecipitates (*IP*) were prepared with antibodies against cMet and Western blotted (*WB*) with antibodies against cMet, p-cMet, and CD44 as well as for the p110α/PI3K, p85/PI3K, AR, and β-actin. *B*, lipid raft fractions (fractions 2–5) from the same cultures were immunoprecipitated with a human CD44 (*HCAM*) antibody and then Western blotted for human CD44v9 (~140 kDa), CD44s (~90 kDa), AR (~65 kDa), and β-actin (~55-kDa loading control). *C* (*a–c*), C4-2 cells untreated (*lane 1*) or transfected with scrambled siRNA (*control-siRNA*; *lane 2*), HAS2 siRNA (*lane 3*), pSicoR scrambled shRNA (*Control shRNA*, *lane 4*), pSicoR-CD44v9 shRNA (*CD44v9 shRNA*, *lane 5*), or methyl-β-cyclodextrin (*lane 6*). After 72 h of culture, components of signaling complex were dissociated by 5 mM methyl-β-cyclodextrin at 37 °C for 1 h. Most of the components (AR, CD44, p-cMet, etc.) after dissociation with methyl-β-cyclodextrin reside in the bottom fractions 6–8, whereas some p-cMet resides in fractions 2–5. Thus, fractions 2–8 were isolated and pooled and immunoprecipitated with cMet, and these immunoprecipitates were Western blotted for p-cMet (*a* and *left bars* in *b*) and AR (*a* and *right bars* in *c*). The bands in *b* and *c* were analyzed by densitometry. The data in all of these experiments are representative of three experiments and, where indicated, presented as mean ± S.D. (*error bars*) of three experiments.

plex, loss of the androgen receptor, and consequently loss of its function. We investigated whether CD44v9 shRNA will perturb HGF-inducible CD44/cMet interaction, thus implicating endogenous hyaluronan in the assembly or stabilization of this complex. As described under "Experimental Procedures," we prepared lipid raft fractions from sucrose gradient centrifugation of lysates of C4-2 cells that had been pretreated with HGF (10 ng/ml for 12 h) or transfected with pSicoR scrshRNA or pSicoR-CD44v9 shRNA prior to induction with HGF. We then pooled fractions 3–5 from the gradients because these correspond to the putative rafts as described in our previous work (22). We immunoprecipitated these pooled fractions with antibody against cMet or CD44 and Western blotted the immunoprecipitates with antibodies against cMet, p-cMet, and CD44 as well as for the p110α (catalytic) and p85 (regulatory) subunits of PI3K, androgen receptor, and β-actin. We probed for androgen receptor because it associates with HSP90 (64), a chaperone essential for RTK activity (65), and for p110α/PI3K because it has been shown to be associated with cMet (20, 66). Fig. 8*A* shows that the cMet immunoprecipitate in the scrshRNA control (Fig. 8*A*, *lane 2*) exhibits a high content of p-cMet, CD44v9, HSP90, androgen receptor, and p110α/PI3K, all of which are greatly diminished in the cMet- immunoprecipitate from the lipid raft fraction of the lysate from CD44v9 shRNA-treated C4-2 cells (Fig. 8*A*, *lane 3* compared with *lane 2*). As expected, CD44s, total Met (cMet), and the p85/PI3K levels were not altered (Fig. 8*A*).

Because CD44v9 is necessary for cMet activation, androgen receptor probably interacts directly with CD44v9, and knockdown of androgen receptor blocks the expression of HGF-induced CD44v9 (Fig. 8*B*, *lane 3 versus lane 2*). In addition, CD44v9 could not be immunoprecipitated with cMet in cells that were not treated with HGF (data not shown). In this case, another antagonist of hyaluronan/CD44v9 interactions, HAS2 siRNA, should show a similar effect. Thus, we compared the ability of HAS2 siRNA to inhibit the endogenous interaction of hyaluronan with CD44v9 in C4-2 cells with treatments of CD44v9 shRNA and methyl-β-cyclodextrin. Cell lysate gradient fractions 2–8 were isolated from C4-2 cells that were transfected with HAS2 siRNA, CD44v9 shRNA, control siRNA, or control shRNA or that were treated with methyl-β-cyclodextrin, as described under "Experimental Procedures." Immunoprecipitate with antibody to cMet was isolated, and Western blots for p-cMet and androgen receptor were prepared (Fig. 8*C*, *a*) and quantitated by densitometry (Fig. 8*C*, *b* and *c*). HAS2 siRNA treatment inhibited both p-cMet (~80%) (*b*) and androgen receptor (~60%) (*c*) in the immunoprecipitate compared with control siRNA treatment (*lane 2 versus lane 3*). For comparison, CD44v9 shRNA treatment inhibited both p-cMet (~85%) (*b*) and androgen receptor (~90%) (*c*) in the immunoprecipitate compared with control shRNA treatment (*lane 4 versus lane 5*). The result with methyl-β-cyclodextrin treatment was nearly the same as that for HAS2 siRNA (*lane 3 versus lane 6*). Moreover, HAS2 siRNA, CD44v9 shRNA, and methyl-β-cyclodextrin have no effect on the total amount of cMet (data not shown). Therefore, HAS2 siRNA and CD44v9 shRNA treatments had very similar or more pronounced effects than that of methyl-β-cyclodextrin (Fig. 8*C*). Overall, the results in

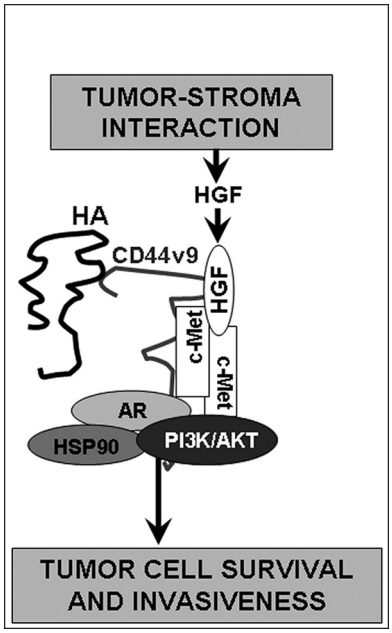

FIGURE 9. **Proposed model for the cross-talk between stromal HGF and tumor cell-derived HA/CD44v9/Met-induced signaling in prostate cancer cells.** Cancer cells and stroma-derived fibroblasts influence each others' development. The extracellular domain of CD44 variants that contain the sequence encoded for v9 and its interaction with HA are required for the HGF-dependent activation of its receptor Met and its downstream anti-apoptotic malignant signaling involving PI3K/AKT and AR. HSP90 protects AR and AKT from degradation. Fibroblast (PCMF)-derived HGF and HA synthesized in response to HGF and cancer cell-derived AR, CD44v9, and cMet are involved in the malignant behavior in DT-independent prostate cancer cells.

Fig. 8, *A–C*, provide strong evidence that signaling components p-cMet, CD44v9, P110α/PI3K, HSP90, and androgen receptor form a lipid raft-associated, multimeric complex and that this signaling complex is important for hyaluronan/CD44v9-induced downstream signaling and androgen receptor stability necessary for cell survival and invasiveness.

Model Consistent with Results (Fig. 9)—Our experimental results in this study indicate that 1) HA/CD44v9 interaction is an important co-regulator of HGF/cMet and its downstream anti-apoptotic activities, including androgen receptor expression and function, therefore regulating androgen receptor; 2) hyaluronan induced by stroma-derived HGF interacts with CD44v9 to activate cMet (p-cMet formation) to form the signaling complex in lipid raftlike structures, and this complex is necessary for hyaluronan/CD44v9-induced downstream signaling, androgen receptor stability, and cell invasiveness; 3) CD44v9 exons are necessary for androgen receptor expression and function, and knockdown of androgen receptor has a strong influence on CD44v9 expression and hence hyaluronan/CD44v9-induced functions. Thus, the interactions between carcinoma cells and stroma-derived myofibroblasts can be mediated by cross-talk between CD44v9 variants derived from carcinoma (epithelial C4-2) cells and HGF and hyaluronan derived from stromal (prostate cancer-associated myofibroblast) cells. Such mutual interaction may exist in prostate tumor tissues and may explain how tumor cells acquire more malignant phenotypes that depend on stromal tissues. Our recent work showed that delivery of CD44v6 shRNA/nanoparticles to colon tumors inhibited tumor growth by perturbing hyaluronan/CD44v6 signaling in colon cancer cells (18). This innovative therapeutic approach (18) can be made versatile to deliver CD44v9 shRNA using CD44v9 shRNA/nanoparticles to target tumors at one or more of these levels: the microenvironment (stromal factors, such as HGF), receptor-based signals (CD44v9 and Met), and signal tranducers, such as androgen receptor, PI3K/AKT, and HSP90 (model in Fig. 9).

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