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Hyaluronan suppresses prostate tumor cell proliferation through diminished expression of N-cadherin and aberrant growth factor receptor signaling

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Abstract

Hyaluronan (HA) production has been functionally implicated in prostate tumorigenesis and metastasis. We previously used prostate tumor cells overexpressing the HA synthesizing enzyme HAS3 or the clinically relevant hyaluronidase Hyal1 to show that excess HA production suppresses tumor growth, while HA turnover accelerates spontaneous metastasis from the prostate. Here, we examined pathways responsible for effects of HAS3 and Hyal1 on tumor cell phenotype. Detailed characterization of cell cycle progression revealed that expression of Hyal1 accelerated cell cycle re-entry following synchronization, whereas HAS3 alone delayed entry. Hyal1 expressing cells exhibited a significant reduction in their ability to sustain ERK phosphorylation upon stimulation by growth factors, and in their expression of the cyclin dependent kinase inhibitor p21. In contrast, HAS3 expressing cells showed prolonged ERK phosphorylation and increased expression of both p21 and p27, in asynchronous and synchronized cultures. Changes in cell cycle regulatory proteins were accompanied by HA-induced suppression of N-cadherin, while E-cadherin expression and β -catenin expression and distribution remained unchanged. Our results are consistent with a model in which excess HA synthesis suppresses cell proliferation by promoting homotypic E-cadherin mediated cell-cell adhesion, consequently signaling to elevate cell cycle inhibitor expression and suppress G1 to S phase transition.

Keywords

Hyaluronan; prostate cancer; cadherin; integrin; cell adhesion; cell cycle

INTRODUCTION

Hyaluronan (HA) has been implicated in normal cellular growth control, as well as the altered cell proliferation in many tumor types (reviewed in [1]). A strong correlation between the accumulation of HA in pathologically graded clinical specimens and the

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diagnosis or prognosis of cancer has been reported for carcinoma of the breast [2], colon [3], bladder [4] and prostate [5]. Manipulation of the HA synthesizing enzymes (HAS) most highly expressed in each tumor type has additionally demonstrated that the involvement of HA is more than correlative, and that its effects on tumor progression are at least partially dependent on the quantity of HA produced by the tumor cell [6–8]. Notably, the combined immunochemical quantification of HA and its primary turnover enzyme hyaluronidase 1 (Hyal1) has been shown to predict biochemical recurrence of prostate cancer [9,10]. Thus, it is important to characterize the respective roles of the HA synthases and hyaluronidases in component processes of tumor progression.

HA is a polymer of alternating glucuronic acid and N-acetylglucosamine. Its synthesis is catalyzed by plasma membrane embedded HA synthase enzymes HAS1, HAS2 and/or HAS3 [11]. Newly synthesized polymers emerge from the cell surface as they extend in length. In animal models, it has independently been shown that HA overproduction by HA synthase enzymes [12], and elevated hyaluronidase activity conferred by Hyal1 overexpression [13], will each promote tumor growth in a dose responsive manner, but will also suppress tumor growth when present at levels beyond an empirically determined threshold value. We previously found that prostate tumor cells overexpressing either HAS2 or HAS3 exhibited accumulation of HA on the cell surface in a pericellular matrix, which was dispersed by coexpression with the HA turnover enzyme Hyal1 [6]. Excess HA production by either HAS2 or HAS3 led to significant decreases in proliferation of cultured cells, and in orthotopic tumor growth in mice [6]. Growth impairment was reversed and tumor growth was accelerated dramatically when cells co-overexpressed both the HA synthesis and HA turnover enzymes. Cell cycle analysis further revealed that increased HA turnover accelerated, while HA synthesis diminished, the rates of cell cycle re-entry after synchronization.

Progression through the cell cycle is dependent on external signals in the microenvironment until the restriction point [14], beyond which the cell is committed to proceed through the cycle irrespective of external cues. Growth factors and extracellular matrix (ECM) proteins are among the signals crucial for tight regulation of cell cycle progression through specific cascades [15]. The Ras/extracellular-signal-regulated kinase (ERK) mitogen activated protein (MAP) kinase pathway is critical for transmission of extracellular signals to the nucleus [16,17]. Nuclear ERK phosphorylates transcription factors that activate an array of immediate early genes, such as cyclin D1, which are responsible for cell cycle control. In response to growth factors, passage through the restriction point is determined by induction of cyclin D1, followed by activation of cyclin dependent kinases (CDK). CDK inhibitors inactivate the CDKs, thereby stalling progression through the cell cycle. Two CDK inhibitors, p21^{Cip} (p21) and p27^{Kip} (p27), specifically limit progression through G1 and S phase [18–21].

Cell-ECM interactions synergize with growth factors to support adhesion-dependent proliferation and normal cell cycle progression in signaling cascades originating from ECM ligation of integrin receptors [22]. Contact inhibition can override ECM or growth factor signals as a result of increased cell-cell adhesion and alternative signaling mediated through activity of the classical cadherins E-cadherin and N-cadherin [23]. We previously found that $\alpha 2\beta 1$ and $\alpha 4\beta 1$ integrins were downregulated in HAS3 overexpressing cells, resulting in diminished adhesion to ECM proteins collagen and fibronectin [24]. In this study, we have tested the hypothesis that the predominant mechanism of growth modulation by Hyal1 and HA is altered signaling at the cell cycle checkpoint transition from resting (G1) to DNA synthesis (S) phase, specifically activated downstream of surface receptors involved in cell-ECM and cell-cell adhesion. We investigated the effect of cell cycle arrest and release on ERK phosphorylation in prostate tumor cells expressing HAS3 or Hyal1, and the subsequent

status of cell cycle regulatory proteins. Use of signaling pathways impinging on ERK activation by growth factors was examined relative to those engaged through integrin-ECM ligation or cadherin-mediated cell-cell interactions.

MATERIALS AND METHODS

Cell Culture, Materials, and Reagents

22Rv1 human prostate adenocarcinoma cells were purchased from ATCC and maintained in RPMI 1640 medium containing 10% fetal bovine serum. Selection, characterization, and maintenance of 22Rv1 Tet-ON, GFP (vector control), Hyal1 and HAS3 stable transfectants in standard medium containing 1.5 mg/ml G418 was carried out as described previously [24,25]. For inducible expression, 22Rv1 Tet-ON cells were transiently transfected with pTRE-Tight tetracycline-inducible expression vectors into which the coding sequences for GFP, Hyal1 or HAS3 had been inserted [24,25]. Transgenes were induced 8h after transfection by addition of 2 µg/ml doxycycline to the media and maintained for another 48h prior to analysis. Transgene induction was verified in each condition tested by western blot, quantification of HA production, or hyaluronidase activity assay, as appropriate. Antibodies against ERK1/2, phosphorylated ERK1/2, FAK, phosphorylated FAK, Cyclin A, Cyclin D1, p21 and p27 were from Cell Signaling Technologies (Beverly, MA). Anti N-cadherin 13A9 and anti β-catenin 15B8 were from Santa Cruz Biotechnologies and anti E-cadherin HECD-1 was from Zymed. The Rac1 Activation Assay Kit was obtained from Millipore (Temecula, CA). Anti-tubulin antibody, protease inhibitor cocktail, wortmannin, U0126, and nocodazole were purchased from Sigma. Phosphatase inhibitors were from Pierce. EGF and bFGF were obtained from R&D Systems (Minneapolis, MN).

Cell synchronization and cell cycle analysis

Cells were synchronized at G2/M phase by treatment with the mitotic inhibitor nocodazole (0.2 mM in serum free media) for 24 h. The cells were then washed and released with serum free media containing a growth factor cocktail of EGF (20 ng/ml) and bFGF(10 ng/ml). At time 0 h following growth factor addition and at specific time points thereafter as indicated in the figures, cells were harvested and prepared for cell cycle analysis or for western analysis of cell cycle proteins. Cell cycle analysis was conducted on single cell suspensions of paraformaldehyde-fixed, propidium iodide treated cells by flow cytometry using Modfit software as previously described [6]. Asynchronous cells were harvested from 6-well plates seeded in serum containing media. In each condition tested, hyaluronidase activity and HA production were assayed to confirm that these levels varied negligibly within control transfectants, and at the levels expected for the respective Hyal1 or HAS3 transfectants.

Western analysis

Protein content was adjusted to equivalent levels in all cell lysates following quantification by Bradford assay. Ten µg of total protein were separated by SDS-PAGE and transferred to PVDF membrane. Blocked membranes were probed for ERK1/2 or phosphorylated ERK, FAK or phosphorylated FAK, total Rac1 (all at 1:1000 in PBS/), cyclin A1 (1:2000), cyclin D1 (1:2000), p21 (1:2000), p27 (1:1000), then stripped and reprobed for the tubulin loading control (1:500,000). Cadherins and catenins were probed at 1:1000 dilution. Secondary IgG conjugated to horseradish peroxidase was used at 1:2500 dilution and blots were developed using enhanced chemiluminescence. The bands obtained from immunoblots were quantified using the Totallab TL100 V2006b software at the Proteomics Core facility of UNL. Individual protein bands were normalized to the tubulin control in all experiments. Each experiment was repeated at least three times. Statistical significance was assessed by one-way ANOVA.

Growth factor stimulation of prostate tumor cell lines

For transient stimulation of cell cycle kinases, stable transfectants were seeded at 4×10^5 cells per well of a 6-well plate in serum containing media. Subconfluent cells were serum starved for 24 h, then treated with EGF (20 ng/ml) and bFGF (10 ng/ml). After 20 min, cells were washed twice in 1x PBS and harvested in Radioimmunoprecipitation assay buffer (RIPA: 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40, and 0.25% sodium deoxycholate) containing 2 mM PMSF, 2 mM sodium orthovanadate, 2 mM sodium fluoride, 1x phosphatase inhibitor cocktail, and 1x protease inhibitor cocktail. The harvested lysates were flash frozen in liquid nitrogen and stored at -80°C until further use. For sustained ERK1/2 activation, cells were serum starved for 24 h and treated with growth factors for 0, 3, 12 and 24 h prior to harvest in RIPA lysis buffer as above.

Kinase inhibitor treatment

Subconfluent cultures of stable transfectants were treated with 10 μM U0126 (MEK inhibitor) or 100 nM Wortmannin (PI3K inhibitor) for two hours. The cells were washed with 1x PBS and harvested in RIPA lysis buffer with protease and phosphatase inhibitors. Equivalent amounts of protein (10 μg) were separated by SDS-PAGE and analyzed by western blot for phosphorylated and total ERK1/2.

Rac and FAK activation assays

Microwell plates were coated for 1 h at 37°C with 0.1 mg/ml of either BSA or fibronectin (FN) then blocked for 1 h with PBS containing 0.1 mg/ml BSA. Single cell suspensions of 22Rv1 GFP, Hyal1 and HAS3 transfectants, grown in replete medium and serum starved for 4 h, were allowed to adhere for 30 min, which is the minimum time for a significant percentage of these cells (>30%) to become adherent. Both adherent and non-adherent cells were then pelleted by 5 min centrifugation of the microwell plates at $1500 \times g$. Media were carefully aspirated from the plates. Total cell lysates were prepared from the pellets and analyzed by western blot for total Rac expression, phosphorylated and total FAK, and tubulin. Rac1 activation status in the newly adherent cells was assayed by a pulldown with recombinant PAK, which specifically recognizes the GTP bound form of Rac1, using the Rac1 Activation Assay Kit. Control experiments included pulldowns from 22Rv1 cell lysates that had been preloaded with either GDP (negative control) or GTP γ S (positive control), according to the manufacturer's instructions.

Cadherin expression analysis

Total mRNA was extracted from tumor cells in serum containing media following three washes in 1x PBS, using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. RNA was reverse transcribed with the Superscript III Single Strand Synthesis kit and amplified by PCR using the following primer sets: E-cadherin forward TCCCATCAGCTGCCAGAAA; E-cadherin reverse TGACTCCTGTGTTCTGTGA; N-cadherin forward CACTGCTCAGGACCCAGAT; N-cadherin reverse TAAGCCGAGTGATGGTCC. E-cadherin, N-cadherin and β -catenin expression in whole cell lysates was compared by western blot using monoclonal antibodies specific for the respective proteins. To examine subcellular distribution of β -catenin, cytosolic and nuclear fractions were separated by hypotonic lysis and differential extraction using standard methods. Fractionated proteins were examined by western analysis, with lamin B and tubulin detection as controls for nuclear and cytosolic specificity, respectively.

For immunofluorescence analysis, cells were grown on glass coverslips, fixed and permeabilized in Histochoice MB tissue fixative (Electron Microscopy Sciences, Hatfield, PA.). Cells were blocked with 10% goat serum in phosphate-buffered saline for thirty

minutes and exposed to primary antibodies for one hour, followed by species specific secondary antibodies conjugated to Cy3 (Jackson ImmunoResearch, West Grove, PA). Fluorescence was detected with an axiovert 200M microscope using 40x oil PLAN-Neofluar (1.3 na) objective and Slidebook 5.0 image acquisition software (Intelligent Imaging Innovations, Denver, CO).

RESULTS

Altered HA metabolism impacts cell cycle progression

In previous studies, we found that excess HA production by prostate tumor cells selected for stable overexpression of HAS3 suppressed growth in vivo and in culture [6,24]. In contrast, stable overexpression of Hyal1 enhanced the intrinsic proliferation rate of these cells in culture. To determine the underlying causes of these phenomena, we first examined proliferation in more detail using cell cycle analysis. Control (vector), Hyal1 or HAS3 22Rv1 prostate tumor cell transfectants were synchronized at the G2/M phase of the cell cycle with the mitotic arrest agent, nocodazole, released with addition of serum containing media, and evaluated by flow cytometry. Rate of cell cycle re-entry was compared by examining the percentages of each transfectant population in the DNA synthesis (S) phase after serum release. Significantly fewer HAS3 transfectants were in S phase at both 6 and 12 hours (Fig 1A), and these cells were found instead to persist throughout this period in G0/G1 (not shown). Hyal1 transfectants exhibited an opposite perturbation in cell cycle progression kinetics. By 12 h, a significantly higher percentage of cells had entered S phase, and the duration of G0/G1 was reduced by 6 h relative to control cells.

A schematic illustration of the phases of the cell cycle and the relationship among key signaling proteins that mediate progression through each phase is shown in Figure 1B. We next examined the levels of these specific cyclins and cyclin-dependent kinase (CDK) inhibitors in serum replete, unsynchronized populations of transfectants, to determine which of them might be involved in the altered cell cycle kinetics we observed. We specifically examined Cyclins A1 and D1, because they have been shown to be essential for the progress through transitions on either side of S phase, in which we observed the greatest discrepancies in our cell cycle analysis. We also focused on p21 and p27 CDK inhibitors, which have been shown to suppress progress through these transitions. The overproduction of HA in the slow-growing HAS3 transfectants was correlated in western analysis with suppression of basal cell cycle mediator Cyclin A1 (~65%, Fig 2A, 2B) and modest, but not statistically significant, decrease in Cyclin D1 (~12%), concurrent with upregulation of CDK inhibitors p21 (2.3-fold) and p27 (2-fold). In contrast, the rapidly cycling Hyal1 transfectants exhibited elevation of Cyclins A1 (~2-fold) and D1 (~110%), with suppression of p21 (~82%). We previously found, using transiently transfected tetracycline-inducible 22Rv1 cells, that induction of HAS3 significantly reduced cell proliferation, which was unaffected by induction of Hyal1 or a GFP control [24]. Examination of cell cycle mediators revealed no differences in cyclin D1 expression among these transfectants (Fig 2C), but significant increases in p21 and p27 upon induction of HAS3 (Fig 2D, 2E).

ERK activation by short term growth factor treatment is comparable among HAS3 and Hyal1 transfectants

The ERK pathway regulates cellular proliferation, differentiation, migration and invasion in tumor cells. The ERK cascade is a central element in the transmission of signals from the cell surface to the nucleus. In particular, ERK phosphorylation is an essential event mediating the transition from G1 to S phase. Therefore, we evaluated the effect of growth factors on ERK activation in cells overexpressing Hyal1 and HAS3 (Fig 3). GFP control, Hyal1 and HAS3 transfectants were serum starved for 24 hours, then treated with mitogenic

factors EGF and bFGF for 20 min and analyzed by western blot for levels of transiently phosphorylated ERK (pERK) relative to total ERK (tERK). As would be expected in quiescent serum-deprived cells, the pERK level was negligible in all three lines when untreated. The pERK/tERK ratio increased dramatically upon growth factor addition, but the level of ERK activation was not significantly different among the three transfectants.

Hyal1 overexpression reduces sustained ERK activation without altering cyclin D1

The ERK pathway elicits different biological outcomes depending on the duration, magnitude and temporal activation of ERK [17,26]. Sustained ERK activation is critical for inducing immediate early genes during the G1 phase. One of these, cyclin D1, is needed for cell cycle reentry and progression into S phase, and is not induced by transient activation of ERK [27–30]. Therefore, we evaluated the effect of growth factors on ERK activation and cyclin D1 expression in unsynchronized Hyal1 and HAS transfectants, and compared with a time course of growth factor stimulation following 24h serum deprivation (Fig 4). Treated cells received EGF and bFGF for 3h and 12h, which correspond to mid G1 and late G1, respectively. Serum starvation (SFM) slightly increased pERK levels relative to cells in serum containing media (asynchronous, AS). Growth factor treatment of serum starved cells resulted in significant increases in pERK level in all three cell lines after 3h of stimulation (GF 3h, Fig 4A, 4B). Total ERK levels were uniform in all conditions tested. This increase in pERK was sustained up to 12h (GF 12h) in control and HAS3 cells, and remained elevated above the control in HAS3 transfectants even at 16h and 24h (not shown). However, ERK phosphorylation in Hyal1 expressing cells was diminished relative to the controls after 3h of growth factor exposure and was statistically reduced at 12h (Fig 4B), 16h and 24h (not shown). These results are consistent with altered signaling through ERK phosphorylation between the HA-overproducing HAS3 versus the HA-processing Hyal1 transfectant lines as a component of the mechanism leading to their altered cell cycle progression.

Levels of cyclin D1 were not altered significantly by serum starvation or growth factor stimulation in any of the cell lines (Fig 4A, 4C). This is in contrast to previous reports by several laboratories in which cyclin D1 levels were found to be reduced in serum starved normal fibroblasts and epithelial cells, and were elevated by growth factor induced, sustained ERK activation [31–33]. However, in our prior evaluation of these cells, we were unable to synchronize cultures by serum starvation [6]. Since the level of Cyclin D1 is relatively high in these cells at all phases of the cell cycle, the loss of normal cyclic control specifically of Cyclin D1 and its consequent overexpression during cell cycle transition times may be one explanation for the failure of these cells to synchronize in G0/G1 phase in the absence of serum factors.

Hyal1 overexpression reduces growth factor mediated ERK activation in synchronized cells

We have shown previously that treatment with nocodazole, a microtubule inhibitor, arrested 80–85 % of the 22Rv1 stable transfectant populations in G2/M phase. Upon release with serum addition, they progressed through the cell cycle. Therefore, we next examined ERK activation and Cyclin D1 expression in 22Rv1 stable transfectant populations that had been arrested with nocodazole and released from arrest by addition of growth factors. Nocodazole treatment resulted in a significant elevation of pERK in all three transfectant lines (Fig 5A, 5B, relative to Fig 4B). This is consistent with previous studies in which microtubule destabilizing agents were shown to activate kinases such as p38, ERK and JNK [34–36]. When cells were released from arrest by growth factor addition, ERK activation was sustained up to 12h in control and HAS3 transfectants, as observed in response to growth factor treatment of serum starved cells (Fig 5A, 5B). In contrast, pERK levels in Hyal1

expressing cells were diminished by 3h in the presence of growth factors, and had dropped to the baseline level of expression by 12h, as would be expected for cells that had already exited the cell cycle. Thus, although there was no significant difference among transfectants in their ability to transiently activate ERK, sustained activation of ERK by growth factors was significantly reduced by Hyal1 expression, and enhanced by HAS3-mediated HA overproduction.

Expression of cyclin dependent kinase inhibitors in Hyal1 and HAS3 transfectants inversely reflects their growth kinetics

Following nocodazole-induced G2/M arrest, Cyclin D1 expression levels were observed to be equivalent among transfectant cell populations. By 12h post-administration of growth factors, Cyclin D1 levels had decreased in all cell lines. However, relative to the control and Hyal1 lines, Cyclin D1 levels remained modestly but significantly higher in the HAS3 transfectants, consistent with a larger portion of the cells still in the mitotic phase of the cell cycle (Fig 5A). We next examined additional cell cycle regulatory components that could underlie the altered growth of the cells, specifically p27 and p21. Nocodazole arrest did not normalize expression of p27 and p21 as it did for Cyclin D1 and ERK activation (Fig 5A, 5C, 5D). Expression of p27 and p21 remained $\approx 55\%$ and $\approx 75\%$ lower, respectively, in the rapidly proliferating Hyal1 transfectants relative to control or HAS3 cells. However, growth factor treatment did not significantly alter levels of these proteins in either the Hyal1 or the control transfectants. In contrast, the slowly proliferating HAS3 transfectants exhibited growth factor induced increases in p27 and p21 expression at all time points, with values at 12h increased ≈ 2 -fold. This result suggests that the HA-dependent effects on cell proliferation and cell cycle progression occur to a large extent through altered levels of the CDK inhibitors.

ERK activation in prostate tumor cells is abrogated by the MEK inhibitor U0126

The 22Rv1 tumor cell line, either transfected or untransfected, expresses relatively high basal pERK levels that were not modulated by serum starvation in our experiments. Since ERK activation can occur downstream of several surface receptors, not exclusively through growth factor receptors, we investigated the upstream pathway responsible for ERK activation. Cells were treated with U0126, a specific inhibitor for both MEK1 and MEK2, and wortmannin, which targets the phosphoinositide-3-kinase (PI3K) pathway (Fig 6). We observed total depletion of pERK in the presence of U0126 in control (GFP), Hyal1, and HAS3 transfectants. In contrast, the PI3K inhibitor wortmannin failed to elicit any inhibition in these cells. This suggests that the constitutive ERK activation in these prostate tumor cells is due to the activation of the Ras/Raf/MEK pathway and does not involve the PI3K pathway. The uniform inhibition of pERK levels in all the transfectants also suggests that there is no alternative use of ERK activation pathways due to constitutive expression of HA metabolic enzymes. Moreover, this observation supports our interpretation of altered cell cycling primarily as a result of differential response to growth factor activation when HA metabolism is perturbed.

Effects of perturbed HA metabolism on cell cycle progression are independent of integrin-mediated Rac or FAK activation

We previously observed that overexpression of Hyal1 versus HAS3 had opposite effects on $\beta 1$ integrin expression and function. Specifically, Hyal1 transfectants expressed slightly more $\beta 1$ integrin and adhered more rapidly to fibronectin (FN) coated plates, while HAS3 transfectants lost $\approx 50\%$ of the control $\beta 1$ integrin expression and $\approx 90\%$ of their potential to adhere to FN. Since integrin ligation of ECM proteins like FN is a critical step in normal epithelial adhesion and cell cycle progression, we examined the activation state of Rac and FAK, two signaling components in the integrin pathway. Quantification of activated (GTP

bound) Rac indicated no significant differences among transfectant lysates from cells allowed to adhere for 30 min (Fig 7) or up to 4h (not shown). Similarly, levels of pFAK were not different between BSA (non-adherent) and FN-adherent cells. However, we did observe that although total FAK levels were identical among transfectants, FAK phosphorylation was significantly lower in cells expressing Hyal1, relative to control (GFP) or HAS3-expressing cells. In general, these results suggest that integrin-mediated adhesion and its consequent effects on intracellular signaling are not the key modulators of altered cell cycling in response to overexpression of Hyal1 or HAS3.

Expression of N-cadherin is significantly diminished in HA overproducing cells, but β -catenin levels and nuclear translocation are not affected

Finally, we examined the expression and function of the cell-cell contact proteins E-cadherin, N-cadherin, and their cytoplasmic binding partner β -catenin. While all of our stable 22Rv1 lines had equal E-cadherin expression, HAS transfectants had significantly lower N-cadherin both at the message and protein levels (Fig 8A, 8B). N-cadherin protein was also reduced by $\approx 50\%$ in 22Rv1 inducible HAS3 transient transfectants upon 48-hour induction of HAS3 (Fig 8C), but was unaffected by induction of GFP or Hyal1. E-cadherin protein levels, as observed in the stable constitutively expressing Hyal1 and HAS3 transfectants, were also not different upon transgene induction. This finding suggests that elevated production of HA results in nearly complete suppression of N-cadherin, since transfection efficiency of the cells is only $\approx 50\%$. We performed western analysis on cytosolic and nuclear extracts to determine if β -catenin nuclear translocation, an event downstream of cadherin signaling [37], was affected by N-cadherin suppression. The level of β -catenin in cytosol of control (GFP) transfectants was approximately 3-fold higher than in the nucleus. A similar ratio was present in Hyal1 transfected cells, and the ratio increased modestly (to $\approx 1:1$) in HAS3 transfectants. We further examined subcellular distribution of these critical adherens junction components by immunofluorescence microscopy to determine whether the cadherins were present at sites of cell-cell contact. As expected for functional E- and N-cadherins, as well as the associated β -catenin, these proteins were localized to the plasma membrane and abundant in cell-cell contacts of control and Hyal1 transfectants (Fig 8D). Consistent with the western analysis, N-cadherin expression was significantly reduced in HAS3 expressing cells, while expression and distribution of E-cadherin and β -catenin were unchanged. The residual N-cadherin was also dispersed from cellular boundaries, suggesting it was no longer functional in maintaining cellular junctions. Though cadherin-mediated adhesion mechanisms and their signaling pathways controlling cell cycle progression are relatively well known, this is the first example of a connection between endogenous HA synthesis via HAS3 and reduced N-cadherin expression in the context of decelerated cell proliferation.

DISCUSSION

Prostate tumor growth and metastasis are significantly impacted by the excessive production and turnover of HA. Our studies presented here have identified a potential mechanism for the opposing effects of Hyal1 and HAS3 overexpression on prostate tumor cell proliferation, cell cycle progression kinetics and tumorigenesis. Interestingly, entry into the DNA synthesis phase of the cell cycle is principally regulated through CDK inhibitors and not via cyclin D1 in prostate tumor cells. We further showed that HA overproduction leads to downregulation of N-cadherin expression, thereby magnifying the signal for sustained ERK phosphorylation transduced by homotypic interactions of E-cadherin independently of β -catenin nuclear translocation. The consequences of a transition to primarily E-cadherin driven signaling are an elevation in p21 and p27, and elongation of the cell cycle despite the

presence of growth factors. This effect is independent of integrin-mediated adhesion and stimulation of either Rac GTP binding or PI3K phosphorylation.

Sustained ERK activation mediates direct phosphorylation and stabilization of transcription factors, thereby prolonging their expression and activity (reviewed in [26]). When ERK is only transiently activated, transcription factors are relatively unstable and rapidly degraded. We saw no difference in transient ERK activation by growth factors within Hyal1 and HAS3 transfectants (Fig 3), which is consistent with results of studies in smooth muscle cells using exogenously added polymeric HA [38]. However, the effects we have shown here resulted from endogenous provision of HA. Increased endogenous production of HA by HAS2 overexpression in an intestinal epithelial cell line was reported to constitutively activate multiple cell surface growth factor receptors, including EGFR and ERBB2 [39]. In a similar context here, we observed a significant reduction in sustained ERK activation in Hyal1 expressing cells and prolonged ERK activation in HAS3 relative to mock transfectants (Fig 4,5). Although the increase was not statistically significant, we saw a comparable trend in ERK activation in transiently inducible HAS3 transfectants ($p < 0.1$, not shown). The outcome of sustained ERK activation in the presence of EGFR stimulation is usually upregulation of cyclin D1. Exogenous HA polymer has also been shown to reduce S-phase entry in smooth muscle cells and fibroblasts primarily by decreasing cyclin D1 levels [40]. However, we did not observe significant differences in cyclin D1 protein among Hyal1 and HAS3 tumor cell transfectants, despite the increase in sustained ERK activation in the latter. The most obvious explanation is that prostate tumor cells already overexpress cyclin D1 and are insensitive to further stimulation [41]. Although androgen has been shown to modulate cell cycle progression via cyclin D1 in the parent CWR22 xenograft [42], 22Rv1 cells have lost androgen dependence and may be unresponsive to this alternative mode of cyclin D1 regulation.

We observed that intrinsic pERK levels are elevated in 22Rv1 prostate tumor cells relative to HeLa, HEK293 and other types of cells. ERK is activated primarily by the Ras/Raf/MEK pathway by growth factors and integrins, but can also be phosphorylated downstream of PI3K, indirectly by PKC and directly by the ArfGAP, Centaurin- α 1 [43,44]. Inhibition of MEK eliminated ERK phosphorylation while a PI3K inhibitor had no effect (Fig 6), indicating that the predominant pathway for ERK activation in our system was via Ras/Raf/MEK. The 22Rv1 prostate tumor cells overexpress ERBB2 (HER2) and EGFR receptors [45], which would be expected to promote hyperactivation of Raf/Ras/MEK. This is a possible explanation for both elevated basal ERK expression and resultant elevation of cyclin D1 in 22Rv1 parental and transfected cells.

CDK inhibitors p21 and p27 were reduced in rapidly proliferating Hyal1 transfectants, while these levels were increased in the slower growing HAS3 cells. This finding is consistent with the well established role of p21 and p27 as negative regulators of the cell cycle [46,47]. However, there is increasing evidence suggesting p21 can also act as a positive modulator of cell proliferation and tumorigenesis [48–50]. The inhibitory potential of p21 is dependent on cellular context, phosphorylation state and protein-protein interaction. In the case of Hyal1 expressing cells, synchronized p21 protein levels were only $\approx 20\%$ of the level in control transfectants and were still only $\approx 70\%$ of the control level after growth factor stimulation. The low expression and modest rise in p21 may actually allow it to act in promoting G1/S transition in Hyal1 transfectants. Small amounts of p21 bound to the cyclin/CDK complexes activate the complex for cell cycle progression, whereas higher amounts inhibit the complex [47]. Therefore, the opposite result occurs upon induction of p21 in HAS3 transfectants, which already have high levels: that is, the increased p21 level inhibits growth. A further explanation for the opposing effects of p21 and p27 upregulation on Hyal1 versus HAS3 transfectants is that these proteins bind to proliferating cell nuclear antigen (PCNA), a DNA

polymerase δ processivity factor, thereby blocking DNA synthesis [51]. The reduced level of p21 in Hyal1 expressing cells may then enhance DNA synthesis and S-phase entry, while the increased p21 observed in HAS3 cells would conversely reduce DNA synthesis. Finally, it has also been previously reported that binding of HA polymers to CD44 antagonized S-phase entry in smooth muscle cells, and that this occurred through stabilization of p27 via inhibition of Skp2, a component of the p27 degradation machinery [40,52]. Our data showing p27 remained elevated in HAS3 up to 12h after mitogen stimulation are consistent with this regulatory scheme.

Mechanisms underlying effects of endogenous HA overproduction on cell cycle progression have not been fully examined. A role for the HA receptor, CD44, in mediating responses of fibroblasts and smooth muscle cells to growth factor stimulated proliferation has been well documented [53–55]. Signaling through CD44 was shown to involve interactions with ezrin and merlin, which directs the stability of focal adhesion complexes during cellular growth and motility. We examined total expression and cell surface localization of CD44, ezrin, and merlin, but found levels of all three to be equivalent among our transfectants in both contexts (Bharadwaj and Simpson, unpublished). This suggests differential CD44-mediated signaling is not the primary limit of S-phase entry in HA overproducing cells. In further support of this is our previous finding that EGF receptor phosphorylation in basal and EGF-treated states is also similar among transfectants [56]. Differential ligand-independent EGF receptor activation has been reported for polymeric versus oligomeric HA, as a result of CD44 ligation, so EGF receptor activation would be expected to differ in the HAS3 and Hyal1 transfectants if CD44 ligation was a significant event in their cell cycle control.

Adhesion to ECM proteins such as fibronectin, type I and type IV collagen, is usually a critical precursor to cell cycle progression. Since β 1 integrin-mediated adhesion is impaired in HAS3 transfectants [24], we expected to observe significant differences in engagement of signaling pathways downstream of integrins, particularly reflected in Rac GTPase, PI3K, and/or FAK phosphorylation. However, none of these responses were affected upon ECM exposure in either HAS3 or Hyal1 transfected cells (Fig 7), nor did a PI3K inhibitor block ERK phosphorylation. Thus, integrin signaling also does not appear to be the major cell surface determinant of HA-suppressed or Hyal1-enhanced cell proliferation. Further support for this interpretation derives from our use of transiently inducible HAS3 overexpression to control HA production. Inducible HAS3 diminished growth rate of 22Rv1 transfectants similarly to stable constitutive expression of HAS3, but did not affect integrin-mediated ECM adhesion or steady state cell surface integrin expression [24].

Cadherins are another class of cell surface receptors with a strong impact on cell proliferation (reviewed in [57]). We examined expression of cadherins in 22Rv1 prostate tumor cells and found that both E-cadherin and N-cadherin were present at high levels (Fig 8). Unlike what we observed for integrin expression, the induction of HA synthesis by both stable constitutive and transiently inducible HAS3 resulted in nearly complete ablation of N-cadherin expression, without affecting levels of E-cadherin. Importantly, an increase in p21 protein also occurred upon induction of HAS3, which is consistent with results of constitutive HAS3 expression. E-cadherin is a well-accepted tumor suppressor protein [58,59]. Binding of its extracellular domain to cadherins on neighboring cells is an essential component of intercellular adhesion and adherens junction formation. Ectopic expression of E-cadherin in invasive tumor cells that lack its expression has been shown to reduce proliferation, motility and invasion, while expression of N-cadherin in relatively benign cells increases their malignancy [58,60]. The phenomenon of cadherin switching has been reported in many types of dedifferentiated carcinoma [57], including prostate [61,62], and involves a change in expression from E- to N-cadherin that corresponds to malignant progression. This is consistent with our prior characterization of HA overproducing cells as

poorly tumorigenic and non-metastatic, whereas coexpression of HAS3 with Hyal1 reduced HA accumulation and induced rapid tumorigenesis and metastasis.

On a mechanistic level, proliferation was previously reported to be significantly diminished in breast and colon tumor cell lines that expressed E-cadherin in the absence of N-cadherin [63]. Moreover, when E-cadherin homotypic adhesion was engaged in subconfluent cells, S-phase entry was delayed, by a mechanism that was dependent on cadherin-associated β -catenin but did not involve increased β -catenin nuclear translocation [63]. E-cadherin homotypic cell-cell adhesion has also been found to prevent ligand activation of EGF receptor, thereby suppressing its proliferative activity [64]. The loss of N-cadherin upon HA overproduction in 22Rv1 prostate tumor cells would be expected to enhance homotypic E-cadherin ligation and magnify its consequences for delayed S-phase entry and diminished proliferation, consistent with our observations. Therefore, N-cadherin suppression is a probable mechanism by which excess HA decreases the aggressive phenotype of 22Rv1 prostate tumor cells, particularly in contrast to the Hyal1-overexpressing cells in which N-cadherin is not affected.

In summary, we report here the first demonstration of an impact of HA on cadherin expression and function in tumor cell proliferation, and an examination of the signaling pathways that mediate the altered growth and cell cycle kinetics. The mechanism for cell cycle acceleration by Hyal1 overexpression appears to be a suppression of p21 below the critical level for appropriate restriction of cells to the resting phase. We propose that excess HA produced by HAS3 has the two-fold effect of increasing E-cadherin homotypic interaction and suppressing S-phase entry, as well as elevating unliganded EGF receptor, sustained ERK activation and elevation of p21 and p27 protein. Understanding the relative impacts of these signaling modes will inform our future studies of prostate stromal and epithelial HA contributions during tumor progression in vivo. An exciting recent paper further highlights the importance of understanding mechanisms of N-cadherin suppression, since blocking its function has the potential to prevent metastasis and resistance to androgen deprivation therapy, which are the most deadly aspects of prostate cancer [65].

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Abbreviations

HA	hyaluronan
HAS	hyaluronan synthase
CDK	cyclin dependent kinase
ECM	extracellular matrix
FN	fibronectin
FAK	focal adhesion kinase
GFP	green fluorescent protein
EGF	epidermal growth factor
bFGF	basic fibroblast growth factor

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Figure 1. Re-entry to S phase following synchronization is significantly accelerated by Hyal1 expression and delayed by HA overproduction

(A) 22Rv1 cells stably expressing GFP (vector control), Hyal1 or HAS3 were synchronized in serum free medium with 0.2 mM nocodazole for 24h, then released by addition of serum (0h). At the indicated times, cells were trypsinized, fixed, stained with propidium iodide and analyzed by flow cytometry. Mean percentage of cells in S phase is plotted $\pm$ SEM. * $p < 0.05$

(B) A model for the interplay of cell cycle regulators in proliferation illustrates the prominent roles of ERK activation, cyclin D1 stabilization and cyclin dependent kinase inhibitors p21 and p27 in maintaining appropriate timing of S phase entry and exit.

Figure 2. Expression of Cyclins and cyclin dependent kinase inhibitors is altered in unsynchronized prostate tumor cells producing excess HA

Equal amounts of protein from serum replete asynchronous cultures of 22Rv1 stable transfectants constitutively expressing GFP (control), Hyal1, or HAS3 were analyzed by western blot for cyclin D1, cyclin A1, p21, p27, and tubulin (A). Expression of each cell cycle regulator was quantified by densitometric analysis of the blots normalized to the respective tubulin band. The blot is representative of three independent experiments, for which the mean $\pm$ SEM is plotted (B). Equal protein from vehicle or dox-treated (2 μ g/ml) 22Rv1 Tet-ON cells transfected with pTRE-Tight GFP (control), Hyal1, or HAS3 constructs was immunoblotted and densitometrically quantified for cyclin D1 (C), p21 (D) or p27 (E). Mean $\pm$ SEM is plotted for three experiments. In panels B–E, * $p < 0.05$.

Figure 3. Transient activation of ERK by EGF and bFGF is unaffected by altered HA metabolism

22Rv1 cells stably expressing GFP (control), Hyal1, or HAS3 were serum starved for 24 hours, then treated with growth factor cocktail (GF, 20 ng/ml of EGF and 10 ng/ml of bFGF) for 20 min. Vehicle (–) and GF treated (+) cells were harvested and lysed in RIPA buffer with protease and phosphatase inhibitors. Cell lysates were analyzed by western blot for phosphorylated and total ERK1/2, with tubulin as a loading control. The blot is representative of three independent experiments.

Figure 4. Hyal1 overexpression reduces sustained ERK activation without altering cyclin D1
 Cell populations in serum containing (asynchronous, AS) or serum free (SFM) media for 24h were treated with growth factors for 3h (GF 3h), and 12h (GF 12h). (A) At each time, cells were harvested, lysed in RIPA buffer with protease and phosphatase inhibitors, and analyzed by western blot probed with the indicated antibodies. Representative data from a single experiment are shown, along with densitometric quantification of p-ERK2 to t-ERK2 ratio (B) and cyclin D1 to tubulin ratio (C). Mean $\pm$ SEM of three independent experiments is plotted; * $p < 0.05$.

Figure 5. Expression of cyclin dependent kinase inhibitors and growth factor mediated ERK activation inversely reflect S phase entry kinetics in response to HA

GFP, Hyal1 and HAS3 transfectants were synchronized with nocodazole for 24h (Noc Arr), then released by the application of GF containing media. (A) The cells were harvested at 3h, 12h and 24h post release and assessed by western blot probed for total and phosphorylated ERK1/2, cyclin D1, p21 and p27 in comparison to synchronized cells. Representative data from one experiment are shown. (B) ERK phosphorylation was quantified by densitometry and plotted as a ratio to total ERK, normalized to tubulin. Similar quantification analysis is shown for p27 (C) and p21 (D). The mean $\pm$ SEM of three independent experiments is plotted for arrested cells and cells that have been arrested and released for 12h; * $p < 0.05$ compared to GFP in each condition.

Figure 6. ERK activation in prostate tumor cells is dependent on MEK

Subconfluent cultures of 22Rv1 stable transfectants were treated with vehicle (DMSO), 10 μ M U0126 (MEK inhibitor) and 100 nM Wortmannin (PI3K inhibitor) for 2 hours. The cells were harvested and equivalent protein was assessed by western blot for pERK, tERK and Tubulin.

Figure 7. Differential effects of Hyal1 and HA on ERK phosphorylation and cell cycle progression are not coincident with Rac or FAK activation upon integrin ligation

Single cell suspensions of 22Rv1 GFP, Hyal1 and HAS3 transfectants were allowed to adhere to microwell plates coated with either BSA or fibronectin (FN). After 30 min, plates were centrifuged for 5 min to pellet all cells. Cell lysates were prepared and analyzed by western blot for total Rac expression, phosphorylated and total FAK, and tubulin. Activated Rac (GTP bound form) was specifically immunoprecipitated for comparison among transfectants.

Figure 8. 22Rv1 prostate tumor cells express both E-cadherin and N-cadherin but only N-cadherin expression is suppressed by HAS3 and HA synthesis

(A) Total RNA was isolated from stable 22Rv1 transfectants, reverse transcribed and individual transcripts for E-cadherin, N-cadherin and GAPDH (housekeeping control) quantified by PCR as indicated. On the right, whole cell lysates were analyzed for protein levels of E- and N-cadherin by western blot. (B) Lysates were separated into cytosolic (C) and nuclear (N) fractions and subjected to western analysis using antibodies against β-catenin, lamin B (nuclear marker) or tubulin (cytosol). (C) 22Rv1 Tet-ON cells were transiently transfected with plasmids inducible for GFP, Hyal1, or HAS3 and incubated in the presence (+) or absence (–) of 2 μg/ml doxycycline for 48h. Whole cell lysates were immunoblotted for E- and N-cadherin. (D) Subcellular localization of adherens junction components in 22Rv1 transfected cells. 22Rv1 stable transfectants expressing GFP, Hyal1 or HAS3 were grown on glass coverslips overnight, then fixed and immunostained using primary antibodies specific for E-cadherin, N-cadherin, and β-catenin, as indicated, followed by secondary detection with Cy3 conjugate.