Antisense-Mediated Suppression of Hyaluronan Synthase 2 Inhibits the Tumorigenesis and Progression of Breast Cancer

Lishanthi Udabage,1 Gary R. Brownlee,1 Mark Waltham,2 Tony Blick,2 Emma C. Walker,2 Paraskevi Heldin,3 Susan K. Nilsson,1 Erik W. Thompson,1 and Tracey J. Brown1

1Laboratory for Hyaluronan Research, Department of Biochemistry and Molecular Biology, Monash University, Clayton, Australia; 2St. Vincent’s Institute of Medical Research, Melbourne, Australia; 3Department of Medical Biochemistry and Microbiology, Ludwig Institute for Cancer Research, Uppsala, Sweden; and 4Stem Cell Laboratory, Peter MacCallum Cancer Institute, St Andrew’s Place, East Melbourne, Australia

Abstract

The progression of several cancers is correlated with the increased synthesis of the glycosaminoglycan, hyaluronan. Hyaluronan is synthesized at the plasma membrane by various isoforms of hyaluronan synthases (HAS). The importance of HAS2 expression in highly invasive breast cancer was characterized by the antisense inhibition of HAS2 (ASHAS2). The effect of HAS2 inhibition on cell proliferation, migration, hyaluronan metabolism, and receptor status was characterized in vitro, whereas the effect on tumorigenicity and metastasis was established in vivo. HAS2 inhibition resulted in a 24-hour lag in proliferation that was concomitant to transient arrest of 79% of the cell population in G0-G1. Inhibition of HAS2 did not alter the expression of the other HAS isoforms, whereas hyaluronidase (HYAL2) and the hyaluronan receptor, CD44, were significantly down-regulated. ASHAS2 cells accumulated greater amounts of high molecular weight hyaluronan (>10,000 kDa) in the culture medium, whereas mock and parental cells liberated less hyaluronan of three distinct molecular weights (100, 400, and 3,000 kDa). The inhibition of HAS2 in the highly invasive MDA-MB-231 breast cancer cell line inhibited the initiation and progression of primary and secondary tumor formation following s.c. and intracardiac inoculation into nude mice, whereas controls readily established both primary and secondary tumors. The lack of primary and secondary tumor formation was manifested by increased survival times where ASHAS2 animals survived 172% longer than the control animals. Collectively, these unique results strongly implicate the central role of HAS2 in the initiation and progression of breast cancer, potentially highlighting the codependency between HAS2, CD44, and HYAL2 expression. (Cancer Res 2005; 65(14): 6139-50)

Introduction

The interaction between a tumor and its environment is often characterized by the morphologic changes observed in the stroma of human breast cancer. One such morphologic change is the concentration of the glycosaminoglycan hyaluronan where its overproduction at the tumor invasion front has been implicated in breast cancer progression (1) and invasion (2). Hyaluronan seems to influence tumor cell behavior and cancer progression by modulating the hydration and osmotic balance in the tumor environment (3). Acting as a biomechanical molecule, hyaluronan contributes to the assembly, structural integrity, and physiologic properties of the tumor extracellular matrix. Moreover, by interaction with its specific receptors, hyaluronan is capable of intracellular signal transduction that can promote the malignant phenotype (4). The enrichment of hyaluronan in tumors can result from increased production by tumor cells (5) or from the interaction between malignant cells and stromal fibroblasts (6).

Hyaluronan is synthesized by a multi-isofrom family of transmembrane glycosyltransferases termed the hyaluronan synthases (HAS; ref. 7), whereas hyaluronan is depolymerized by a combination of enzymatic and nonenzymatic mechanisms (8). Hyaluronan polymerization occurs on the inner face of the plasma membrane where it is extruded onto the extracellular surface of the cell (9). Three eukaryotic HAS isoforms have been identified, termed HAS1, HAS2, and HAS3. The catalytic rate for each HAS isoform is different (7). HAS1 is the least active and drives the synthesis of high molecular weight hyaluronan (2,000 kDa), suggesting low constitutive levels of hyaluronan synthesis. HAS2 is the more catalytically active isoform and is also associated with synthesis of high molecular weight hyaluronan (2,000 kDa). HAS2 is implicated in developmental processes involving tissue expansion and growth, processes that are essential to the formation and maturation of the tumor environment. HAS3, the most active isoform, drives the synthesis of short hyaluronan chains (100 to 1,000 kDa), where its expression seems to be activated to produce large amounts of low molecular weight hyaluronan, which is thought to contribute to the pericellular matrix or may interact with cell surface hyaluronan receptors, triggering signaling cascades and profound changes in cell behavior.

The role of hyaluronan synthesis in tumor initiation and progression is now being elucidated by the genetic manipulation of the various HAS isoforms, where up-regulation of hyaluronan synthesis has been indicated to contribute to various aspects of cellular growth (10), tumor progression (4), and matrix assembly (11). The importance of HAS1 expression in tumorigenesis was established when HAS1 activity was chemically inhibited in a highly metastatic, HAS1-expressing carcinoma (12). When compared with the parental cell line, the inhibition of HAS1 did not alter the growth of primary tumors but it reduced pulmonary metastasis by nearly 40%. Similarly, overexpression of HAS2 in a human fibrosarcoma cell line resulted in 3-fold increase in net hyaluronan synthesis, and promoted anchorage-independent growth and tumor formation when compared with parental cells and mock transfectants (13). To test whether heightened expression of individual HAS isoforms could contribute to the
process of malignant transformation, nontransformed rat cells were transfected with HAS1-3 (10). In this cell line, overexpression of each HAS isoform promoted the formation of a pericellular coat where HAS2 produced a larger pericellular matrix than that of HAS1 or HAS3; however, the overexpression of the HAS isoforms did not promote anchorage-independent growth or increase the rate of formation of s.c. tumors. Overexpression of HAS3 is correlated with both the initiation of prostate tumor growth (14) and of the progression and metastasis of colorectal cancer (15).

Based on these recent findings that indicate the potential role of hyaluronan synthesis in cancer, preliminary studies have selectively inhibited the expression of the several HAS isoforms. In the first report of its nature, Simpson et al. (16) prepared stable antisense HAS2 and/or HAS3 transfectants in an aggressive prostate tumor cell line. Individual antisense inhibition of either HAS2 or HAS3 or concomitant expression of HAS2/3 reduced the end point tumor volume by 70% to 75% when compared with their control counterparts. Exogenous addition of 1 mg/ml of high molecular weight hyaluronan in the antisense experiments enhanced the tumor growth that was comparable with the controls. The aforementioned studies, therefore, highlight the importance of HAS isoform expression in relation to cancer, where hyaluronan synthesis can promote anchorage-independent growth and increase tumor invasiveness, properties that are hallmarks of the malignant phenotype. Moreover, these collective observations reinforce the intricate and complex relationship between the three HAS isoforms, where they seem to exhibit different functional biological characteristics in relation to normal cellular growth and in disease states such as cancer.

We have previously characterized the HAS gene expression in the highly invasive MDA-MB-231 breast cancer cell line and revealed HAS2 to be the predominantly expressed isoform. Based on its highly aggressive phenotype, we selected this cell line as an ideal candidate to study the effect of HAS2 antisense inhibition on the malignant phenotype in vitro and tumorigenicity in vivo. Moreover, this study has also considered the downstream effect of antisense inhibition of HAS2 on the gene expression of the other HAS isoforms, on hyaluronidase expression and the hyaluronan receptor, CD44. To date, this is the first study to encompass these parameters but also complements other independent research that highlights the importance of HAS2 in tumor growth and migration.

**Materials and Methods**

**Cell culture.** Aneuploid human breast adenocarcinoma cell line MDA-MB-231 (American Tissue Culture Collection, Rockville, MD) was selected based on the expression of HAS2. Cells were propagated in monolayer culture in Leibovitz L-15 medium (Sigma, St. Louis, MO), supplemented with 10% FCS, 100 units/ml penicillin, and 100 mg/ml streptomycin.

**Construction of antisense expression vector.** The cDNA open reading frame for human HAS2 was generated by designing gene-specific primers from the published sequence of Brinck and Heldin (Genbank accession no. U54804; ref. 22) and consisted of the following primers: sense, 5'-AGACTTGTGCTTGTAGTATGTATCTTCCAT-3' and antisense, 5'-GACATGGTGGATTGATGATGATCTTCCAT-3'. Total RNA harvested from exponentially dividing human dermal fibroblasts was used as the template for reverse transcription-PCR (RT-PCR), generating a 1.7 kb cDNA fragment of HAS2, which was cloned directly into pGEM-T vector (Promega, Madison, WI). The cDNA for HAS2 was subsequently subcloned into the pc-Neo expression vector (Promega) and isolated clones containing the insert in the antisense orientation (ASHAS2 construct) were identified by restriction endonuclease mapping and automated sequencing.

**Transfection and validation of MDA-MB-231 human breast cancer cells with ASHAS2 and mock constructs.** The ASHAS2-pc-Neo construct and mock control (pc-Neo vector without insert) were transfected into human MDA-MB-231 breast cancer cells using LipofectAMINE plus reagent (Life Technologies, Melbourne, Victoria, Australia) according to the manufacturer's instructions. For at least 1 month, before commencing studies, transfected cells were selected in the presence of 500 μg/ml G418 antibiotic (Promega). Stable cell lines were established by harvesting and pooling of antibiotic-resistant colonies. Confirmation of the stable incorporation of the antisense HAS2 construct into the genome was done using PCR on purified genomic DNA. In brief, a gene-specific primer for pc-Neo, 5'-GCCACAGTGGTAAAG-3', was used in combination with two specific HAS2 primers of the following sequence: GSP2 sense 5'-GCTGTGTAATTGACGTGTCGCT-3' and GSP4 sense, 5'-GCGGAGTAAACTCAGACG-3'. When used in the following combination, pCI-neo/GSP2 and pCI-neo/GSP4, expected size products of 1,443 and 2,223 bp were amplified, respectively. The products of PCR were identified by restriction endonuclease mapping and automated sequencing.

**Quantification of messenger RNA for HAS1, HAS2, and HAS3.** Real-time PCR using gene-specific primers and an internal oligonucleotide probe was used to quantitative the relative mRNA levels of HAS1, HAS2, and HAS3 in parental, mock-, and ASHAS2-transfected cells (Table 1). For CD44, an internal oligonucleotide probe was not required due to the use of a SYBR green detection system, and therefore only gene-specific primers (Table 1) were added to the PCR mix. In brief, total RNA was purified from exponentially growing cells using TRI reagent (Sigma). The total RNA was used to generate single-stranded cDNA by incubating 2 μg RNA with 0.5 μg/μl random primers and superscript reverse transcriptase.

---

**Table 1. Primer sequences used for the amplification of the different isoforms of the HAS, CD44S, and HYAL genes**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Hybridization probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>HAS1</td>
<td>5'-CCTGCTACGACGGTGTTCTCTCTA-3'</td>
<td>5'-GCCGGTCA-TCCCCCAAAG-3'</td>
<td>5'-AACCTTCTGACAGTTTCTTGAAGGC-3'</td>
</tr>
<tr>
<td>HAS2</td>
<td>5'-CAGTCTCAGCTGAGGAGGCT-3'</td>
<td>5'-TGGTGGAGAAAGTCTTGGGCT-3'</td>
<td>5'-CCATGTGTAATTGACGTGTCG-3'</td>
</tr>
<tr>
<td>HAS3</td>
<td>5'-GTGGCAGAGTCACTGCTGACACT-3'</td>
<td>5'-GTCGAGGTTCAAAGTTGGTGAAG-3'</td>
<td>5'-TCAATCATAAAGAGGAGTTAAGTTG-3'</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5'-AAGGTTAGAGGGCTGAGGTTAC-3'</td>
<td>5'-GATGTTAATAACGACCCCTGGT-3'</td>
<td>5'-TTTGGTGTTATGAGCTCC-3'</td>
</tr>
<tr>
<td>HYAL1</td>
<td>5'-GCACAGGGAAGCTCACAGATGTTATGTC-3'</td>
<td>5'-CCACTGCTACGTCAGTGAAGAG-3'</td>
<td>5'-AACTTGTTATAGGTTAGAG-3'</td>
</tr>
<tr>
<td>CD44S</td>
<td>5'-CGAGGTCATGATGTTAGCATG-3'</td>
<td>5'-AGCAATAATGTTCTACAT-3'</td>
<td></td>
</tr>
<tr>
<td>HYAL2</td>
<td>5'-GATGTTTGTACAGCTGAGGAGGCGCC-3'</td>
<td>5'-CGTACTGCTGAGGTTATGATGTCAGG-3'</td>
<td></td>
</tr>
<tr>
<td>HYAL3</td>
<td>5'-GCAGTATCGGAGGATCAGCTGCG-3'</td>
<td>5'-GCTGTTGACAGTGCGCCATCGTC-3'</td>
<td></td>
</tr>
</tbody>
</table>

---

Cancer Res 2005; 65: (14). July 15, 2005 6140 www.aacrjournals.org

Downloaded from cancerres.aacrjournals.org on April 21, 2017. © 2005 American Association for Cancer Research.
Characterization of hyaluronidase gene expression. To determine the hyaluronidase gene expression for HYAL1, HYAL2, and HYAL3, RT-PCR was done on total RNA extracted from cells in both the exponential and growth-arrested phases. The gene-specific primer sets were designed from sequences retrieved from Genbank (Table 1). Amplified sequences were visualized by agarose gel electrophoresis containing ethidium bromide and their identity confirmed by automated DNA sequencing. To quantitate the sequences retrieved from Genbank (Table 1). Amplified sequences were blocked by immersion in 0.3% H2O2 in methanol for 20 minutes. Following PBS containing 1% human serum/1% FCS where detection antibodies were attached for 24 hours. Cells were fixed in Histochoice (Sigma) for 5 to 10 minutes, counterstained with hematoxylin, dehydrated, and mounted.

Cell proliferation assay. Exponentially growing, parental, mock, and HAS2-transfected cells were plated into 24-well plates (2.5 cm2/well) at cell densities ranging from 5 × 103 to 9 × 103 cells/well. The effect of HAS2 inhibition on cell proliferation was studied for 24, 48, 72, 96, 120, and 144 hours. After the defined growth period, cells were detached using 0.25% w/v trypsin and cell number determined using a Coulter counter (Beckman, Coulter, Australia).

Immunohistochemical identification of hyaluronan synthase, hyaluronidases, and CD44. The comparative effect of HAS2 inhibition on the expression of hyaluronan synthase and hyaluronidase was performed for on parental, mock-, and HAS2-transfected MDA-MB-231 cells. Eight-well chamber slides were plated at a density of 2 × 105 cells/well and cells were attached for 24 hours. Cells were fixed in Histochoice (Sigma) for 15 minutes then washed 3 × 5 minutes in PBS. Heterophile proteins were blocked by incubation with 10% FCS for 10 minutes, followed by a PBS rinse. The antisera or antibodies were against CD44H (DAKO, Copenhagen, Denmark), HYAL1, HYAL2 (kindly donated by R. Stern, University of California, San Francisco, CA), and HAS2 (kindly donated by P. Heldin, Ludwig Institute for Cancer Research, Uppsala, Sweden) were diluted in PBS containing 1% human serum/1% FCS where detection antibodies were applied for 60 minutes at 25°C. Endogenous peroxidase activity was blocked by immersion in 0.3% H2O2 in methanol for 20 minutes. Following an additional PBS wash, swine anti-rabbit or rat anti-mouse peroxidase-conjugated secondary antisera (DAKO) was applied for 60 minutes at room temperature, followed by 3 × 5 minutes washes in PBS. Epitope was visualized with Sigma Fast 3,3′,5,5′-tetramethylbenzidine (Sigma) after application for 5 to 10 minutes at room temperature. Slides were washed in tap water for 10 minutes, counterstained with hematoxylin, dehydrated, and mounted.

Cell cycle analysis by flow cytometry. The transfected and control cells were seeded at 2 × 105 cells/25 cm2 and grown with 2 mmol/L thymidine until 50% confluent. After reaching 50% confluence, cells were grown in thymidine-free culture medium. Cells were harvested by trypsinization at 0, 4, 8, 12, 16, 20, 24, 28, 32, and 36 hours followed by fixation in 95% ethanol for 2 hours at 4°C. Cells were pretreated with RNase (100 μg/mL; Sigma) and 50 μg/mL of propidium iodide (Sigma) for 30 minutes at 37°C before determining the stage of cell cycle using a FACSCalibur analytic instrument (Becton Dickinson, San Jose, CA).

Cell migration assay. Invasion assays were done using modified Boyden chambers with polycarbonate Nucleopore membranes (Corning, Corning, NY). Precoated filters (6.5 mm in diameter, 12 μm pore size, Matrigel 100 μg/cm2) were rehydrated with 100 μL of Leibovitz L-15 media supplemented with 0.1% (w/v) bovine serum albumin (BSA; Sigma). Exponentially growing cells were harvested with trypsin/EDTA (Sigma). Before addition to the top chamber of the Boyden apparatus, 3 × 105 cells per 1 mL chamber were washed twice with serum-free growth medium containing 0.1% w/v BSA. Normal growth media containing 10% v/v FCS was used as the chemottractant. After incubation for 6 hours at 37°C, noninvaded cells on the upper surface of the filter were wiped with a cotton swab, and migrated cells on the lower surface of the filter were fixed and stained with Diff-Quick kit. Invasiveness was determined by counting cells in five microscopic fields per well, and the extent of invasion was expressed as an average number of cells per microscopic field. Each experiment was done in triplicate on 2 separate days where data is represented as percentage of migrating cells compared with the parental cell line.

Quantitation of liberated hyaluronan. Triplicate cultures of parental, mock-transfected, and ASHAS2 human breast cancer MDA-MB-231 cells were seeded at 2.5 × 105 cells/cm2 and incubated for 24, 48, 72, 96, 120, and 144 hours. At the conclusion of the incubation period, cells were harvested by trypsinization and counted using a Coulter counter. Media was used for quantitation of liberated hyaluronan. The liberated hyaluronan was quantitated using an enzyme-linked hyaluronan-binding protein assay (Corgenix, Inc., Westminster, CO). The assay was done according to the manufacturer’s instructions. In brief, duplicate 100 μL of samples and the hyaluronan standards (0, 50, 100, 200, 500, and 800 ng/mL) were aliquoted into a 96-well plate coated with hyaluronan-binding protein, and incubated at room temperature for 60 minutes. Samples were washed four times with PBS. One hundred microliters of hyaluronan-binding protein conjugated to horseradish peroxidase was added to each well at room temperature for 30 minutes. After additional PBS washes, the reaction was visualized with 100 μL of 3,3′,5,5′-tetramethylbenzidine after a 30-minute room temperature incubation. The reaction was stopped with 100 μL of 0.36 N sulfuric acid and read at 450 nm (650 nm reference) in a Bio-Rad 350 microplate reader. Growth media that had not been exposed to cells was used to determine the endogenous hyaluronan levels. Endogenous hyaluronan levels were subtracted from all hyaluronan estimation results.

Characterization of hyaluronan molecular weight using size exclusion chromatography. Cells were seeded at 7.5 × 105 cells/75 cm2 culture flask and were grown for 24 hours in growth media containing 250 μCi [3-3H]glucosamine hydrochloride (Perkin-Elmer). At the conclusion of the 24-hour incubation period, the media was removed and exhaustively dialyzed (Mr exclusion of 6 kDa) against 10 mmol/L Tris-HCl/0.15 mol/L sodium chloride/0.02% sodium azide (pH 7.4) at 4°C. The dialyze and dialysis fluid were chromatographically analyzed for the identification of [3H]hyaluronan and its degradation products. [3H]hyaluronan of >5 kDa was subjected to size exclusion chromatography in a Sephacryl S-1000 gel eluted in 0.15 mol/L NaCl/phosphate (pH 7.25), which contained 19 mmol/L NaH2PO4, 38 mmol/L Na2HPO4, and 94 mmol/L NaCl at 13.6 mL/h. The dialysis fluid (molecules <5 kDa) was subjected to size exclusion chromatography in a Superose 12 gel eluted in the above-mentioned buffer at an elution rate of 20 mL/h. Molecular weight estimations were calculated using calibration data for hyaluronan in Sephacryl S-1000 and Superose 12, where data was generated from varying molecular weight of hyaluronan ranging from 800 Da to 10,000 kDa (CPN, Prague, Czech Republic).
Pharmacia, Uppsala, Sweden). To ensure that the 2-[3H]glucosamine hydrochloride was used as a sole precursor for hyaluronan production, the nondialyzable (molecules >5 kDa) dpm was subjected to digestion by 10 TRU of Streptomyces hyaluronidase (pH 6; Calbiochem, Darmstadt, Germany) at 37°C for 24 hours. Digested material was subjected to chromatography in both Sepharoch S-1000 and Superose 12 where profiles were compared with equivalent undigested samples.

Generation of mammary fat-stunted tumors. Animal studies were conducted with full ethical approval from the relevant institutional ethics committee, and in accordance with the Australian National Health and Medical Research Councils guidelines for the care and use of laboratory animals. Five-week-old CBA nude mice (Walter and Eliza Hall, Melbourne, Victoria, Australia) were randomly divided into three groups (n = 11/group) for the generation of parental, mock, and ASHAS2 tumors. Cells were harvested in the logarithmic growth phase by scraping, resuspended to a final density of 2 × 10^6 cells in 1-L medium supplemented with 0.1% glucose ± 5 mg/mL Matrigel, and by following immediate injection into the mammary fat pad. Tumor growth was recorded twice weekly by measuring three perpendicular diameters (d1, d2, d3). Tumor volume was then calculated using the formula: (1/6)π (d1 x d2 x d3). On day 84 after initiation of the tumors, mice were humanely killed. The liver, kidneys, brain, and lungs were harvested in the logarithmic growth phase by scraping, resuspended to a final density of 2 × 10^6 cells/mL, and stored at -80°C for the generation of parental, mock, and ASHAS2 tumors. DNA was purified using phenol-chloroform methodology followed by ethanol precipitation and rehydration in a DNA lysis buffer [100 mmol/L NaCl, 20 mmol/L Tris-HCl (pH 8.0), 20 mmol/L EDTA (pH 8.0), 0.4% (v/v) SDS]. DNA was purified using phenol-chloroform methodology followed by ethanol precipitation and rehydration in a DNA lysis buffer [100 mmol/L NaCl, 20 mmol/L Tris-HCl (pH 8.0), 20 mmol/L EDTA (pH 8.0), 0.4% (v/v) SDS]. DNA was purified using phenol-chloroform methodology followed by ethanol precipitation and rehydration in a DNA lysis buffer [100 mmol/L NaCl, 20 mmol/L Tris-HCl (pH 8.0), 20 mmol/L EDTA (pH 8.0), 0.4% (v/v) SDS].

For pathologic assessment, half of the primary tumor was fixed in 4% formaldehyde and embedded in paraffin; 5 μm sections were examined after H&E staining.

Intracardiac inoculation of breast cancer cells. Before intracardiac tumor inoculation, mice were anesthetized with an i.p. mixture of ketamine (50 mg/kg) and xylazine (5 mg/kg). The MDA-MB-231 cells were prepared as previously described and resuspended to 1 × 10^6 cells/mL. The cell suspension was drawn into a 1 mL syringe fitted with a 25-gauge needle and 0.1 mL injected into the left ventricle. Mice were placed on a heated pad for recovery before returning to the cages. Periodically, radiographic analysis for bone osteolysis was done. For this, mice were anesthetized (as previously described) and X-rayed in a prone position against the X-Omat film (Eastman Kodak, Co., Rochester, NY) and exposed with X-rays of 35 kV for 30 seconds using a Cabinet X-ray system-Faxitron System, Hewlett-Packard, Co. (model MX20 with a 20 μm focal source; Faxitron X-ray Corp., Wheeling, IL). Again, animal health and survival rate was observed in accordance with the aforementioned ethical committees until their euthanasia due to one of the following medical reasons: severe weight loss (exceeding 20% of original body mass), hyperventilation, paralysis, or bone fracture. Collected organs—liver, kidneys, brain, and lungs—were removed and stored at −20°C until Alu PCR analysis was done. To determine the median survival time, a survival curve was plotted using Prism stats program (Kaplan-Meier survival) with the days elapsed following intracardiac inoculations. P value was calculated for the comparison of the survival curves.

Alu PCR quantification of metastasis. Quantitative Alu PCR was used to detect metastasis of MDA-MB-231 from the primary tumor to secondary organs. In brief, DNA was extracted by grinding samples under liquid nitrogen and resuspending in a DNA lysis buffer [100 mmol/L NaCl, 20 mmol/L Tris-HCl (pH 8.0), 20 mmol/L EDTA (pH 8.0), 0.4% (v/v) SDS]. DNA was purified using phenol-chloroform methodology followed by ethanol precipitation and reconstitution in TE buffer. The purified DNA was adjusted to a final concentration of 10 ng/mL in TE buffer (pH 7.2), aliquoted, and stored at −20°C until analysis. To remove exogenous human DNA contamination, the reaction mix, before addition of primers, was purified using phenol-chloroform methodology followed by ethanol precipitation and reconstitution in TE buffer. The purified DNA was adjusted to a final concentration of 10 ng/mL in TE buffer (pH 7.2), aliquoted, and stored at −20°C until analysis. To remove exogenous human DNA contamination, the reaction mix, before addition of primers, was treated with 17 units/mL nuclease S7 (Roche Diagnostics, Melbourne, Germany) at 37°C for 24 hours. Digested material was subjected to chromatography in both Sepharoch S-1000 and Superose 12 where profiles were compared with equivalent undigested samples.

Antisense inhibition of HAS2. Concomitant to these experiments,HAS2 and HAS3 mRNA levels were also quantitated using real-time PCR with HYAL1, HYAL2, and HYAL3 expression characterized by standard RT-PCR. To allow comparison of real-time HAS expression between transfected and parental cells, the level of each mRNA quantitated was normalized with respect to their internal GAPDH controls. When comparing the endogenous level of HAS2 mRNA expression in parental cells and mock transfectants, there were no observed differences between these cell lines. In contrast, mRNA expression in ASHAS2 stable transfected cells was decreased by 50% when compared with parental and mock transfectants, respectively (P = 0.008; Fig. 1A). Moderate HAS3 expression was also detected and was comparable in parental, mock-, and antisense-transfected cells, where HAS1 could not be detected in any of the treatment groups (data not shown). As assessed by quantitative real-time RT-PCR, an interesting observation in the ASHAS2 transfectants was the down-regulation of mRNA for CD44, which was approximately reduced by 60% when compared with the parental controls (Fig. 1B). Mock transfectants also displayed a reduction in mRNA expression for CD44 but was only ~20% to that observed in the parental control. Antisense inhibition of HAS2 significantly altered the expression of HYAL1, HYAL2, and HYAL3 (Fig. 1C). HYAL3 could not be detected in either parental or mock transfectants, but the inhibition of HAS2 resulted in the expression of HYAL3 (Fig. 1C). Inhibition of HAS2 expression, however, resulted in the down-regulation of gene expression for HYAL2 mRNA to the point where it was not detectable even after 35 cycles of PCR. This observation is reinforced by the lack of immunoreactivity of ASHAS2 MDA-MB-231 stable transfecants with the HYAL2 antibody. Both parental and mock transfectants tested positive where staining localized to the plasma membrane and also appeared as cytoplasmic vesicles (data not shown).

Inhibition of HAS2 down-regulates cell surface expression of HAS2 and CD44. Immunohistochemical detection of HAS2 with isofrom-specific monoclonal antibody showed that stable transfection

---

Cancer Res 2005; 65: (14). July 15, 2005  6142 www.aacrjournals.org

Downloaded from cancerres.aacrjournals.org on April 21, 2017. © 2005 American Association for Cancer Research.
with ASHAS2 resulted in the effective blocking of translation of the HAS2 protein (Fig. 2A). Both parental and mock-transfected cells exhibited a high degree of HAS2 expression as indicated by positive epitope staining (Fig. 2B). The reduced expression of mRNA for the hyaluronan receptor, CD44, was also sufficient to block translation of the CD44 protein (Fig. 2C). In both controls, staining for CD44 was most evident in the plasma membrane with areas of intense focal membrane staining (Fig. 2D).

**Antisense inhibition of HAS2 alters hyaluronan metabolism and modulates the molecular weight of hyaluronan produced by MDA-MB-231 breast cancer cells.** Due to the altered HAS, HYAL, and CD44 expression in ASHAS2 MDA-MB-231 transfectants, the amount of liberated hyaluronan was quantitated. ASHAS2 MDA-MB-231 transfectants liberated significantly greater quantities of hyaluronan when compared with either the parental cell line or mock transfectants (Fig. 3A). Over the duration of the experiment, ASHAS2 cultures synthesized an average of 6.94 pg hyaluronan per cell per day with one noticeable exception at 24 hours after plating—where synthesis was increased to ~15.4 pg hyaluronan per cell per day, in contrast to parental and mock transfectants that synthesized ~2 and 1.6 pg hyaluronan per cell per day, respectively. Media removed from the ASHAS2 MDA-MB-231 transfectants was highly viscous when compared with control cell lines. Digestion of the media with *Streptomyces hyaluronidase* showed that >98% of [3H]glucosamine had been incorporated into [3H]hyaluronan, with the remaining 2% of [3H] dpm was associated with Pronase digestible macromolecules of ~50 kDa. All figures represent data where this reactivity has been removed. The molecular weight of hyaluronan synthesized by parental, mock-, and antisense-transfected cells was determined by Sephacryl S-1000 size exclusion chromatography. The parental cell line synthesized three distinct molecular weight of hyaluronan, which were estimated to be 100, 400, and 3,000 kDa potentially reflecting the synthetic products of the prevalently expressed HAS isoforms, HAS2 and HAS3, respectively (Fig. 3B). In contrast, antisense HAS2 transfectants synthesized hyaluronan corresponding to a molecular weight of ~10,000 kDa where a minor fraction (5.2%) corresponding to a molecular weight 100 kDa was also detected.

**Antisense inhibition of HAS2 did not affect cell-associated hyaluronan.** The exclusion of fixed erythrocytes was used to indirectly visualize the hyaluronan pericellular matrix. When compared with parental and mock transfectants, the inhibition of HAS2 did not result in any gross difference in the thickness of the hyaluronan pericellular matrix and subsequent cell-associated hyaluronan (data not shown).

**HAS2 inhibition decreases breast cancer cell proliferation and arrests the cell cycle in G0-G1.** Comparison of the effect of antisense inhibition of HAS2 on cell proliferation and the progression of cell cycle during periods of active cell growth showed that in both parental and mock-transfected cells, a doubling of cell number occurred every 24 hours where plateau growth phase was reached at 72 hours (Fig. 4A). In ASHAS2 stable transfectants, the lack of a functional HAS2 altered cell proliferation by exhibiting a lag period of ~24 hours, reaching growth arrest by 96 to 120 hours (Fig. 4A). General cellular morphology was comparable between all experimental groups. Concomitant to these observations, flow cytometric analysis was also done on parental, mock, and ASHAS2 transfectants to determine relative DNA content at defined time points after plating at subconfluent densities. The percentage of the ASHAS2-transfected cells in the cell cycle phases G0-G1, S, and G2-M 20 hours after plating were 79%, 4%, and 5% as seen in Figs. 4B to D, respectively. In contrast, the corresponding figures in the parental cell line for the cell cycle phases, G0-G1, S, and G2-M, were ~10%, 84%, and 13%, respectively (Fig. 4B-D). Mock transfectants were comparable with the parental cell line. In contrast, antisense inhibition of HAS2 caused a

**Figure 1.** Quantitation of mRNA expression of HAS, the hyaluronidase family, and CD44 in parental MDA-MB-231 and antisense transfectants. Total RNA was extracted from exponentially dividing cultures of parental MDA-MB-231, mock transfectants, and stable clones expressing ASHAS2 mRNA. The level of mRNA for HAS2 (A) and CD44 (B) was quantitated by real-time RT-PCR. Conventional RT-PCR was used to detect the presence of the hyaluronidase genes, HYAL1, HYAL2, and HYAL3 (C). PCR products were resolved by agarose gel electrophoresis containing ethidium bromide. Band volume in stained gels was then subjected to densitometric analysis as described in Materials and Methods to allow comparison of levels for each HYAL gene expression between parental, mock-, and ASHAS2-transfected cells. Closed columns, HYAL1; open columns, HYAL2; diagonal hatched column, HYAL3. Percentage variance between triplicate determinations <2%.
transient delay (~24 hours) of entry into S phase (Fig. 4C). These results are consistent with the observation in the 24-hour lag period in growth rate observed in the proliferation assay.

Suppression of HAS2 reduces the migration of human breast cancer. Migration of the breast cancer cells was ameliorated by the inhibition of HAS2 as indicated by the inability of the ASHAS2 transfectants to migrate across a Matrigel membrane (Fig. 5). Comparison of cellular migratory rates showed that both the parental and mock transfectants displayed typical invasive phenotypes with 100% of the cell populations permeating the parental and mock transfectants. In contrast, only 7% of the HAS2 stable transfectants maintained the ability to invade the Matrigel membrane. It should be noted that this was not a result of induction of apoptosis as shown in separate experiments where cells that had not traversed the basement membrane were determined to be still viable (data not shown).

HAS2 inhibition totally inhibits the initiation and progression of primary breast cancer in vivo. Mice intradermally inoculated with parental or mock-transfected MDA-MB-231 readily established primary tumors that were comparable in growth over the duration of the 12-week experiment (Fig. 6A). In contrast, however, mice inoculated with ASHAS2 transfectants did not establish primary tumors (Fig. 6A). To ensure that the lack of tumor growth was not a result of a poor cell viability of the cell inoculum, in one set of experiments, Matrigel was also included in the inoculation medium. Again, no primary tumor could be detected over the duration of the 12-week experiment. When quantitating the spread of the primary cancer, the highly sensitive Alu PCR assay showed that metastasis in animals inoculated with parental and mock-transfected cells was most prevalent in brain and lung, but was also detected in kidneys and liver transfectants. Mice injected with MDA-MB-231 ASHAS2 did not exhibit metastasis to any organs (Fig. 6B).

Modulation of HAS2 inhibited the formation of secondary tumors and increased animal survival. When quantitating the metastasis of the breast cancer after intracardiac inoculation, animals inoculated with parental and mock-transfected cells showed prevalent spread of the cancer to the brain, liver, kidneys, lung, and bone, whereas mice injected with MDA-MB-231 ASHAS2 did not exhibit metastasis to any organs (Fig. 7A). Bone lesions were observed in several mice from the control groups, whereas ASHAS2-inoculated mice did not present with any bone lesions (data not shown). Mice inoculated with parental or mock-transfected MDA-MB-231 cells showed a significantly shorter survival period, 72 and 77 days, when compared with the ASHAS2 animals that had a mean survival time of 124 days (P = 0.0001; Fig. 7B). Due to ethical regulations being adhered to at St. Vincent’s Institute of Medical Research, ASHAS2 animals were euthanized at a point where weight loss had exceeded 20% of original starting mass.

Discussion

In this study, we characterized the consequence of antisense inhibition of the HAS2 protein on the in vitro cellular proliferation, invasive potential, and in vivo tumorigenicity and malignancy of a well-characterized breast cancer cell line. Moreover, this study has also considered the impact of HAS2 antisense inhibition on the gene expression of the other HAS isoforms, hyaluronidase genes, and the expression of functional HAS2, HYAL2, and CD44 proteins. To date, this is the first study to encompass these parameters and highlight that perturbation of a functional HAS2 protein, by antisense methodology, alters the otherwise orchestrated expression of HAS2, CD44, and HYAL2 at both the mRNA and the protein level. As a consequence of the suppression of HAS2, the MDA-MB-231 breast cancer cell line displayed decreased cellular proliferation with a transient arrest in cell cycle, ablation of migratory phenotype, alterations in hyaluronan catabolism, and inhibition of primary and secondary tumor formation.

Several studies have modulated the expression of the HAS isoforms and characterized the final effect on tumorigenesis. The overexpression of HAS1 in a mouse mammary carcinoma cell line...
increased lung metastasis (12), whereas a similar manipulation of melanoma cells increased cell migration and the formation of a pericellular matrix (17). Yet, the increased expression of HAS3 in TSU prostate carcinoma promoted increased tumorigenicity but had no effect on lung metastasis following i.v. injection of these transfectants (14). In the same paper, the authors eluded that similar observations occurred when MDA-MB-231 cells were transfected with antisense to HAS3, which resulted in attenuation of hyaluronan synthesis and inhibition of high-density cell growth. Antisense inhibition of HAS3 in the metastatic colon cancer cell line SW620 significantly decreased anchorage-independent growth concomitant to the ablation of the hyaluronan pericellular matrix (15). Our findings of decreased invasiveness and total inhibition of both primary and secondary tumor formation are unique, as the inhibition of HAS2 in a human prostate cancer cell line did not inhibit tumorigenesis, but did retard tumor growth and progression (16).

Consistent with other studies, the antisense inhibition of HAS2 did not result in total down-regulation of the mRNA. When using antisense suppression of HAS2 in keratinocytes, the lack of rat antibodies to the specific HAS isoforms prompted Rilla et al. (18) to gauge the degree of the antisense construct incorporation and subsequent functionality by direct measurement of the synthesized hyaluronan and RT-PCR detection of endogenous HAS2 mRNA. This study noted that in one stable clone, a newly synthesized hyaluronan was reduced by 50%, yet the mRNA for HAS2 was comparable with control cell lines (18). Likewise, when a human prostate adenocarcinoma cell line was transfected with antisense constructs containing HAS2 or HAS3 (19), inhibition of hyaluronan synthesis into the culture medium was observed to approximately half of that compared with mock transfectants. The same transfectants were still surrounded by a hyaluronan glycocalyx but was reduced in thickness. Transfectants harboring HAS2/3 antisense synthesized comparable amount of hyaluronan to the HAS3 antisense transfectants but did not retain a pericellular coat of hyaluronan (19). All transfectants retained endogenous expression of the HAS genes. The group later characterized the effects of this impaired hyaluronan synthesis in tumor progression in immunocompromised mice and showed individual inhibition of HAS2 or HAS3 or both could reduce end point tumor volume by as much as 75% (16).

Although the mRNA was only reduced by half, the most compelling evidence that indicates the functionality of antisense inhibition was the complete lack of detectable HAS2 protein in the ASHAS2 transfectants. The lack of protein translation in the presence of mRNA could potentially be due to the mechanistic action by which antisense exerts its effect over gene transcription/translation in a given cell type. For example, the RNase H–mediated activity, where the resultant RNA-DNA duplexes formed are hydrolyzed by action of endogenous RNase H activity, results in degradation of the target RNA and eventual inhibition of gene expression (20). Interestingly, in studies of tissues that are terminally differentiated and not undergoing proliferation, such as the central nervous system, RNase H activity is almost undetectable (20). In such cases, antisense targeting of select genes can result in only a partial decrease or even an increase in the level of the target mRNA (20), an event where antisense may function by hybrid-arrested translation resulting in the reduction of the selected protein, as observed in this study. Antisense inhibition of HAS2 in MDA-MB-231 did not alter the expression level of HAS1 or HAS3, not an unexpected finding as the HAS genes are located on separate chromosomes (21).

Our observation that the suppression of HAS2 resulted in an increase in liberated hyaluronan and also a decrease in CD44 expression is not unprecedented. Concurrent increases in hyaluronan synthesis and decreased CD44 expression is observed in Chinese hamster ovary cells transfected with the different HAS isoforms (22) and a HAS2-transfected melanoma cell line (17). The contrary to this has also been observed where a 4-fold increase in epidermal hyaluronan correlated with up-regulation of HAS2, HAS3, and CD44 following epidermal growth factor stimulation in a newborn keratinocyte cell line (23). HAS2 expression in a

**Antisense Inhibition of HAS2 Inhibits Breast Cancer**

![Figure 3. Quantification of hyaluronan synthesis and molecular weight characterization of the hyaluronan (HA) synthesized by parental and MDA-MB-231 stable transfectants harboring ASHAS2.](image)

- **A**. Cells were seeded at 2.5 × 10⁵ cells in 25 cm² culture flasks and incubated at 37°C for 24, 48, 72, 96, 120, and 144 hours. At each time point, cells were trypsinized and quantitated using an automated Coulter counter. Hyaluronan concentration in the harvested culture medium was determined using a hyaluronic acid binding protein assay as described in Materials and Methods. Hyaluronan synthesis by parental and mock-transfected MDA-MB-231 was expressed as picograms of hyaluronan synthesized per cell (pg/cell). Points, average of triplicate determinations at each time point; bars, SD. A. – o, parental MDA-MB-231; o– o, mock transfectants; o– o, ASHAS2 transfectants. B. Cells were seeded at 7.5 × 10⁵ cells in 75 cm² culture flasks and grown for 24 hours in complete medium supplemented with 250 μCi of [6-3H]glucosamine hydrochloride. To determine the molecular weight of [3H]hyaluronan in the medium, samples were subjected to size exclusion chromatography on a Sephacryl S-1000 SF eluted in 0.15 mol/L NaCl/phosphate (pH 7.25) at 13.6 mL/h. Differences in molecular weight synthesized by parental MDA-MB-231 and their transfected counterparts harboring antisense mRNA to HAS2 are shown. Eluted fractions were proven to be hyaluronan using an automated Coulter counter. Hyaluronan concentration in the harvested culture medium was determined using a hyaluronic acid binding protein assay as described in Materials and Methods. Hyaluronan synthesis by parental and mock-transfected MDA-MB-231 was expressed as picograms of hyaluronan synthesized per cell (pg/cell). Points, average of triplicate determinations at each time point; bars, SD. A. – o, parental MDA-MB-231; o– o, mock transfectants; o– o, ASHAS2 transfectants.
nonhyaluronan-producing mesothelioma cell line induced elevated hyaluronan synthesis and a concurrent 2-fold increase in CD44 expression and cell motility (24).

As a consequence of antisense HAS2 inhibition in the MDA-MB-231 cell line, we observed a concomitant down-regulation of CD44 and HYAL2. This was confirmed using real-time RT-PCR and monoclonal specific antibodies. These findings highlight the potential codependency of these three proteins and their possible role in the highly invasive phenotype of the MBA-MB 231 breast cancer cell line, where it was indicated that the down-regulation of CD44 and HYAL2 totally negated the invasive phenotype. In the context of CD44, ablation of hyaluronan interactions with this receptor has been formed to inhibit the proliferation (25) and migration of tumor cells (26).

The down-regulation of HYAL2 resulted in the expression of HYAL1 and HYAL3, suggesting the implementation of a back-up cellular mechanism. The expression of HYAL3 in the ASHAS2 transfectants is an unusual finding, as to date this enzyme is supposed to be only associated with the testis and bone marrow (27). Unlike the HAS genes, which are located on separate chromosomes (21), the three-hyaluronidase genes, HYAL1, HYAL2, and HYAL3, map to the same chromosomal region, 3p21.3 (28). Recognized absence of a functional HYAL2 protein in the MDA-MB-231 cells expressing ASHAS2 mRNA may have resulted in the increased expression of HYAL1 and recruitment of HYAL3 not normally expressed in this cell line. Despite its characterized acidic optimum required for activity, HYAL2 is also anchored to the plasma membrane via a glycosylphosphatidylinositol association (29). It has been earlier recognized that CD44 participates in the uptake and degradation of hyaluronan (30), a mechanism more recently elaborated upon by Knudson et al. (31) who postulated that the initial action of extracellular hyaluronidases would cleave high molecular weight hyaluronan to a size more susceptible for uptake. A novel model of CD44/ hyaluronan-mediated uptake now includes ASHAS2 mRNA may have resulted in the increased expression of HYAL1 and recruitment of HYAL3 not normally expressed in this cell line. Despite its characterized acidic optimum required for activity, HYAL2 is also anchored to the plasma membrane via a glycosylphosphatidylinositol association (29). It has been earlier recognized that CD44 participates in the uptake and degradation of hyaluronan (30), a mechanism more recently elaborated upon by Knudson et al. (31) who postulated that the initial action of extracellular hyaluronidases would cleave high molecular weight hyaluronan to a size more susceptible for uptake. A novel model of CD44/ hyaluronan-mediated uptake now includes HYAL2, where CD44 binds hyaluronan at the cell surface and HYAL2 performs the initial processing of hyaluronan to 20 kDa intermediate-sized fragments (32), which are then subsequently endocytosed (33). Interestingly, concomitant expression of HAS2 and HYAL2 are implicated in hyaluronan turnover during the early phases of lung injury (34). We have previously observed that the patterns of mRNA expression, as assessed by Northern blot analysis, for HYAL2 mirror that of HAS2 in exponentially dividing dermal fibroblasts,5 thereby highlighting a codependency between these two genes in the catabolism of hyaluronan. Preliminary data indicate that HAS and hyaluronidase colocalize (27) to a minor-organelle called a hyaluronosome, which is a membrane-bound structure containing HAS, hyaluronidases, and various hyaluron receptors, such as CD44 and RHAMM, an organelle that is thought to regulate the deposition of hyaluronan into the extracellular matrix. A minor-organelle of this nature could explain the coregulation and/or metabolic feedback mechanisms that seem to be present in breast cancer cells.

Examining the effect of HAS2 inhibition on the production of hyaluronan showed marked differences between the controls and transfectants. When considering the rapid proliferation of the mock transfectants and parental cell line, only a small quantity of high molecular weight hyaluronan (3,000 kDa) was detected in the cell media with the majority of liberated hyaluronan being detected in the molecular weight range of 100 to 400 kDa, potentially indicating a rapid turnover by the concerted effort of CD44, HYAL1, and HYAL2, or a poor synthetic output by the HAS2 and HAS3 enzymes. In contrast, antisense inhibition of HAS2 resulted in a significant increase in the quantity and molecular weight of liberated hyaluronan where ~95% of the hyaluronan was >10,000 kDa. The molecular weight of liberated hyaluronan produced by the ASHAS2 cells is consistent with the reported molecular weight of HAS2-generated hyaluronan (7). Five percent of the hyaluronan produced by MDA-MD 231 ASHAS2 clones was estimated to be ~100 kDa, most likely representing the synthetic product of HAS3 (7). It is interesting to note, however, that HAS3, when overexpressed in intact cells, can synthesize hyaluronan within the same molecular weight range to that observed with HAS1 or HAS2 (22). One cannot eliminate the possibility of the existence of a cellular recognition mechanism detecting a redundant HAS2 synthetic pathway resulting in the transition of HAS3 to synthesize high molecular weight hyaluronan. The significantly higher levels of hyaluronan in the ASHAS2 MDA-MB-231 media is, therefore, most likely due to a reduced turnover and not an increase in synthesis. Indeed, inhibition of hyaluronan degradation with dextran sulfate in MDA-MD-231 causes a 2- to 3-fold increase in liberated hyaluronan in the culture medium and is of a higher molecular weight when compared with cultures grown in the absence of dextran sulfate (35). The down-regulation of both HYAL2 and CD44 would dramatically impair the ability of the cells to internalize and metabolize the liberated hyaluronan via the CD44/HYAL2 proposed pathway. Therefore, with HAS3 still being expressed in the ASHAS2 transfectants, this could potentially be the source of the high molecular weight hyaluronan observed in the culture medium from the ASHAS2 transfectants. This statement is further substantiated where we have characterized HAS, HYAL, and CD44 expression in our bank of breast cancer cell lines. The cell line displaying the least invasive potential was MDA-MB-453, which only expressed the isoform HAS3; has minimal HYAL1, HYAL2, and HYAL3 expression; and did not express CD44.6 Characterization of the molecular weight of hyaluronan liberated into the culture medium from this cell line clearly defined a modal molecular weight of 10,000 kDa, which was 100% susceptible to Streptomyces hyaluronidase digestion. In addition, where hyaluronan synthesis has been characterized during postdifferentiation of adipocytes, sole expression of HAS3 coincided with the appearance of high molecular weight hyaluronan.7 It is also been shown that the standard form of CD44 and Hyal-2 are associated in a complex in MDA-MB-231 cells (36). In the same paper, the authors describe the use of small interfering RNA technology to reduce the expression of CD44 and Hyal-2 and characterization of the molecular weight of hyaluronan produced. Transient disruption of either CD44 or Hyal-2 expression resulted in the appearance a larger hyaluronan polymer (~500,000Da) in the culture medium that was not normally detected in the untreated cells. These observations are in accord with our results in that the down-regulation of CD44/HYAL2 pathway in MDA-MB-231 has altered the catabolism of hyaluronan in this cell line.

---

5 Unpublished observation.


Cellular proliferation was significantly affected by the suppression of HAS2 where a 24-hour lag period was observed in ASHAS2 transfectants when compared with controls. It is well documented that elevated concentrations of high molecular weight hyaluronan can inhibit proliferation (37) mediated by CD44/hyaluronan interactions (30). As the ASHAS2 transfectants did not express CD44, the lack of CD44/hyaluronan interaction could very well act as a temporary inhibitor of breast cancer cell proliferation. Investigation of the effect of HAS2 inhibition on cell cycle showed that when control cells were actively engaged in DNA synthesis (S phase), the majority of the antisense HAS2 population were transiently stalled in G	extsubscript{0}-G	extsubscript{1}, followed by a lower percentage entering S phase apparently 24 hours later. Although not as dramatic as our observations, these results are in accord with the finding of Rilla et al. (18) who described a similar phenomenon in epidermal keratinocytes transfected with antisense HAS2, where a delay of entry into S-phase was also observed. Overexpression of the individual HAS cDNAs in a rat cell line resulted in a higher proportion of the population in G	extsubscript{2}-M and S phases when compared with G	extsubscript{0}-G	extsubscript{1} (10). Hyaluronan synthesis and its receptors (RHAMM and CD44) have been shown to play an important role in mitosis (38, 39) and that alterations in hyaluronan metabolism, such as those observed in ASHAS2 MDA-MB-231 transfectants, could ultimately affect cellular division. The delay of entry into S-phase could be a result of the 3-fold increase in cellular hyaluronan production where the cells recognize this aberration as a signal to a cell-cycle checkpoint at G	extsubscript{0}-G	extsubscript{1}, a common phenomenon enabling cells to overcome cellular damage and initiate repair (40). As well as the aforementioned factors that illicit cell cycle arrest, lack of a functional membrane-associated hyaluronidase (HYAL2) could also compound events that cause a transiently delay upon entry into S-phase. This statement can be related to studies that observed an increase in the percentage of a human HSC3 oral squamous cell carcinoma cell line in S-phase following stable transfection with HYAL1 (41).

The absence of tumor initiation in ASHAS2 transfectants could potentially be explained by the production of large quantities of high molecular weight hyaluronan. It has been well shown that high molecular weight hyaluronan inhibits angiogenesis (42), a process that is essential for tumor progression. When a tumor reaches a critical volume, it is necessary to establish neovascularization, which enables nutrient and oxygen supply to the rapidly expanding cellular mass. In the case of the s.c. inoculated ASHAS2 tumor cells, the down-regulation of CD44 and HYAL2 may have inhibited the degradation of the antiangiogenic high molecular weight hyaluronan into small angiogenic fragments (<10 kDa); therefore, the tumor mass would very rapidly become nutrient deprived and hypoxic, which would have inhibited the formation of a palpable tumor. Indeed, where hyaluronan synthesis and matrix formation was increased by the overexpression of HAS2 in the v-Ha-ras–transformed rat fibroblast cell line, inhibition of both i.p. and s.c. tumor formation was observed (43).

A critical component in the process of metastasis is the ability for cancer cells to invade basement membranes. Accordingly, we chose the basement membrane in vitro invasion assay to examine the effect of antisense inhibition of HAS2 on invasive potential of breast cancer cells. The invasive characteristics of MDA-MB-231, as

![Figure 4](https://www.aacrjournals.org)
previously shown by Thompson et al. (44), were confirmed in this
study where both the parental and mock transfectants displayed
highly invasive properties. In 93% of ASHAS2-transfected popula-
tions, the invasive phenotype was lost, a finding that is most likely a
direct result of the loss of CD44 expression, a receptor that is
essential for cancer cell motility (45). For instance, in models such
as mammary carcinoma, where soluble forms of CD44 are
overexpressed and therefore compete with endogenous hyaluronan
for resident receptors, has resulted in inhibition of tumor
progression and metastasis (39). Likewise, perturbation of endog-
enous hyaluronan/receptor interactions by incubation with hyalur-
onan oligosaccharides can inhibit in vivo
tumor growth in cancer
cell lines, such as mammary, melanoma, and lung carcinomas (46).
It has been more recently shown that hyaluronan oligosaccharides
cause this phenomenon by attenuating the phosphoinositide 3-
kinase/Akt cell survival pathways that was shown to significantly
arrest the progression of tumor growth in vivo (47). Thus,
interactions of the hyaluronan-pericellular matrix with CD44
and/or other hyaluronan receptors and the induction of intracel-
lar cascades are important for the progress of tumor growth and
metastasis. Indeed, binding of hyaluronan to MDA-MB-231 via the
CD44/HYAL2 pathway is shown to recruit Rho kinase and NHE1
phosphorylation as well as activate cathepsin B (36). An interesting
mutual regulation between CD44 and the proteolytic form,
gelatinase B (MMP-9), which mediates tumor invasiveness, is
described (48). Overexpression of soluble CD44 inhibited tumor
invasiveness by disrupting the formation of the CD44/MMP-9. The
association of CD44 and MMP-9 is dependent of the hyaluronan-
induced clustering of plasma membrane CD44 receptors. Moreover,
yaluronan has been shown to stimulate the secretion of MMP-2 in
a time- and dose-dependent manner in human lung carcinoma via
the CD44 pathway (49). Inhibition of HAS2 in MDA-MB-231 cells
seem to have produced a cellular cascade where CD44 and HYAL2
protein are no longer expressed, causing potential disruption to the
proteolytic arsenal that a cancer cell uses to drive primary tumor
formation and stimulate the formation of new blood vessels. In this
context, no primary growth was observed in nude mice inoculated
into the mammary fat pad with MDA-MB-231, whereas the parental
and mock-transfected cells developed primary tumors
that were comparable in size throughout the study and at
experimental end point. Based on this observation alone, it is not
surprising that distant organs, such as the brain, kidney, liver, lungs,
and bone, did not display any metastatic involvement in mice
inoculated with MDA-MB-231 ASHAS2, whereas the parental and
mock-transfected cells displayed high metastatic behavior. These
results correlate well with the in vitro
observations using the
Boyden chamber methodology.

This study has identified that the HAS isoform, HAS2, is critical
in the processes of tumorigenicity in the highly metastatic breast
cancer cell line MDA-MB-231, and that antisense inhibition of this

Figure 5. Inhibition of in vitro invasiveness of MDA-MB-231 expressing
antisense mRNA to HAS2. The invasive potential of parental MDA-MB-231,
mock (vector only) and antisense HAS2-transfected cells were examined using
the Boyden chamber chemoinvasion assay as described in Materials and
Methods. The cells that had traversed the Matrigel and spread on the lower
surface of the filter were expressed as a percentage of the cell count determined
for the parental MDA-MB-231 cell line. Columns, average of triplicate
experiments done on two separate days; bars, SD. Percentage variance
between triplicate determinations <2%.

Figure 6. The effect of HAS2 antisense inhibition on the tumorigenicity and metastasis in MDA-MB-231. A, parental, mock, and ASHAS2 transfectants were
inoculated into the mammary fat pad of nude mice. Primary tumor growth was followed over a 12-week period with tumor progression recorded twice weekly. The
results graphed represent the average tumor volume (mm³) ± SE, where n = 9 to 13. B, Alu PCR was used to determine the extent of soft organ metastasis from brain,
kidney, liver, and lung as described in Materials and Methods. Results are expressed as the percentage of human tumor DNA in mouse soft organs (n = 8 per group).
envelope attenuates cellular proliferation, profoundly affects in vitro invasiveness, and inhibits the formation of primary and secondary tumors in vivo. Furthermore, this is the first study to show that a codependency exists between this isoform with two other hyaluronan-binding proteins equally important in the progression of cancer, namely CD44 and HYAL2.

Figure 7. The effect of HAS2 antisense inhibition on the metastasis and in animal survival. A, Alu PCR was used to determine the extent of soft organ metastasis after intracardiac inoculation of nude mice from brain, kidney, liver, lung, and bone as described in Materials and Methods. Results are expressed as the percentage of tumor DNA in nude soft organs (n = 9 per group). No metastasis to these organs could be detected where animals had been inoculated with MDA-MB-231 ASHAS2-transfected cells. B, survival rate of the parental, mock, and ASHAS2 transfectant mice were plotted using Prism stats program (Kaplan-Meier survival) with the days elapsed following intracardiac inoculations. There were no differences in the animal survival rate (P = 0.0840) between the parental and mock-transfected mice. Survival curve for ASHAS2 was significantly different (P < 0.0001) from the both control groups. MDA-MB-231 ASHAS2 transfectants (solid); parental cell line (short dash); mock transfectants (long dash).

Acknowledgments
Received 5/9/2004; revised 3/1/2005; accepted 5/6/2005.

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

We thank the Thomaï Breed Cancer Research Fund, which helped support the in vivo studies.

References
Antisense-Mediated Suppression of Hyaluronan Synthase 2 Inhibits the Tumorigenesis and Progression of Breast Cancer

Lishanthi Udabage, Gary R. Brownlee, Mark Waltham, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/65/14/6139

Cited articles
This article cites 46 articles, 19 of which you can access for free at:
http://cancerres.aacrjournals.org/content/65/14/6139.full.html#ref-list-1

Citing articles
This article has been cited by 19 HighWire-hosted articles. Access the articles at:
/content/65/14/6139.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.