CaSm (LSm-1) Overexpression in Lung Cancer and Mesothelioma Is Required for Transformed Phenotypes

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CaSm (cancer-associated Sm-like) was originally identified based on elevated expression in pancreatic cancer and in several cancer-derived cell lines. It encodes a 133-amino acid protein that contains two Sm motifs found in the common snRNP proteins and the LSm (like-Sm) family of proteins. Lung tumors and mesotheliomas express high levels of CaSm mRNA and protein compared with adjacent nontumor and normal lung tissue, measured by immunohistochemistry, qRT-PCR, and Western blot analyses. In addition, several human lung cancer- and mesothelioma-derived cell lines have elevated CaSm expression. Two cell lines, transfected with and expressing antisense CaSm RNA, demonstrate altered transformed phenotypes, reducing their ability to form colonies in soft agar and tumors in SCID mice. Furthermore, RNAi-mediated reduction of CaSm RNA and protein is associated with inhibition of cellular growth. These data support the model that elevated CaSm expression in epithelial tissue contributes to the transformed state. Cell lines expressing exogenous CaSm also exhibit transformed characteristics, including increased anchorage-independent colony formation and tumor growth. Thus, the results of loss of function and gain of function studies presented both indicate that CaSm functions as an oncogene in the promotion of cellular transformation and cancer progression.

Keywords: CaSm; Sm protein; transformation; lung cancer; mesothelioma

The estimated incidence of lung cancer in the United States for 2007 is 213,380 cases with 160,390 deaths, accounting for almost 30% of cancer deaths, of which 87% are associated with smoking tobacco (1). Lung cancer is associated with asbestos exposure as well as tobacco use, with combined exposure leading to a synergistic increase in risk. In recent years a variety of genetic alterations associated with both non-small cell lung cancer (NSCLC), including adenocarcinoma and squamous cell carcinoma, and small cell lung cancer (SCLC) have been identified. These include inactivation of Rb and p53 tumor suppressor pathways, as well as loss of p16 INK4A and VHL (2). The activated ras oncogene is found in many NSCLC adenocarcinoma samples, but not frequently observed in SCLC, while the myc oncogene is amplified in many SCLC. The epidermal growth factor receptor (EGFR) is often mutated in lung cancer, and specific tyrosine kinase inhibitors have been used to therapeutically target EGFR signaling, but a minority of tumors appear to be sensitive (2). Despite these insights into the molecular

CLINICAL RELEVANCE

We demonstrate that CaSm is necessary for transformation by loss-of-function and gain-of-function experiments. Also, these studies suggest that alterations in the mRNA degradation pathway may be a mechanism for the genetic disruptions seen in cancer.

biology of lung cancer, patients with NSCLC continue to have a very poor survival, 51% at 1 year for men and 60% for women; 5-year survival is only 15% and 19% for men and women, respectively (3). Attempts to improve survival with early detection strategies such as computed tomography screening in high-risk patients have not clearly improved survival, with one study showing a significant increase in survival with early diagnosis (4) and the other study showing no increase in survival (5). Such disappointing results emphasize the need to identify new critical pathways in the pathophysiology of lung cancer, both to target treatment and to identify susceptible persons.

Malignant mesothelioma (MM) is also associated with occupational exposure to asbestos, but is not associated with exposure to tobacco smoke. There are three main histologic types including: epithelial, sarcomatous, and mixed/biphasic, with the epithelial type having the best prognosis. Although it is uncommon, approximately 3,000 people a year are diagnosed with the disease in the United States and many more remain at risk for the development of disease due to exposures to asbestos that occurred before 1979, when occupational protections were instituted (6). Because of the number who remain alive that were exposed, and due to the long latency between exposure and disease presentation, the number of cases of mesothelioma is expected to rise over the next several years. Very little is understood about the genetic alterations and asbestos-induced injuries leading to this malignancy. However, it is known that asbestos is particularly toxic to mesothelial cells, the progenitor cell of mesothelioma (7), and can induce cytokine release, induction of apoptosis, and DNA damage. Some of the genetic events that have been associated with the development of malignant mesothelioma are loss or inactivation of two tumor suppressor genes in the Rb/p53 pathways, p16 INK4A and p14 (8). The growth factors EGF, TGF- β , and PDGF are often overexpressed in malignant mesothelioma, leading to autocrine activation of their receptors (9). Other cancer-associated genes, such as telomerase and the anti-apoptosis genes Bcl-x_L and survivin, are expressed in most malignant mesothelioma cells (10, 11). Despite these insights, the prognosis for malignant mesothelioma is even worse than that for lung cancer, with a median survival of less than 1 year from the time of diagnosis (6). This poor survival is due to the fact that no early detection method is available and newer therapies have only added an additional one or two months of life in most cases. These dismal statistics and the expected increase in cases make it even more imperative that additional genes important in the malignant transformation of mesothelial cells are identified.

(Received in original form June 6, 2007 and in final form January 9, 2008)

This work was supported in part by grants from the National Institutes of Health (RO1 ES011323, to P.M.W. and A.M.B.), the U.S. Department of Defense (N00014-96-1-1298, to D.K.W.), and the National Science Foundation (1SC EPSCoR 3100-Z136, to D.K.W.).

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Am J Respir Cell Mol Biol Vol 38, pp 671–678, 2008

Originally Published in Press as DOI: 10.1165/rcmb.2007-0205OC on January 24, 2008

Internet address: www.atsjournals.org

CaSm (Cancer-associated Sm-like) was originally identified on the basis of elevated expression in pancreatic cancer and in several cancer-derived cell lines, and was found to be required for the maintenance of the transformed phenotype of pancreatic cancer cell lines (12). Elevated CaSm expression is not limited to pancreatic cancer; increased levels of CaSm mRNA are also observed in prostate cancer (13).

There are eight LSm proteins, numbered 1 through 8, that form two functionally different seven-membered ring structures (14–16). CaSm (hLSm1) is a subunit of LSm 1–7, which functions in message degradation. This complex is localized in cytoplasmic P bodies (17, 18), where it binds to mRNA and enhances activity of the decapping enzymes DCP1 and DCP2 (19). After decapping, the 5' to 3' exonuclease Xrn1p is recruited to the complex and degrades the mRNA (15, 19). LSm 2–8 functions in mRNA splicing and is found in the nucleus (20).

In the current study we demonstrate that CaSm overexpression observed in NSCLC and malignant mesothelioma contributes to malignant transformation. Investigating the function of this gene in two thoracic malignancies that have very different biological behaviors may help define the specific pathways affected by CaSm expression and lead to identification of therapeutic and diagnostic tools for management of these highly lethal cancers.

MATERIALS AND METHODS

Immunohistochemistry of Tumor Samples

Human lung cancer and mesothelioma samples were obtained from the Hollings Cancer Center Tumor Bank, Medical University of South Carolina. A total of 13 patients were scored for CaSm expression; all specimens were formalin-fixed and paraffin embedded. In addition, tissue microarray (TMA) slides were obtained from the NCI Tissue Array Research Program (TARP) and also stained for CaSm expression. Deparaffinized tissue sections were rehydrated, endogenous peroxidase activity was blocked using 3% H₂O₂ in methanol, and antibody-binding sites were blocked with 1.5% normal horse serum. For antigen retrieval, slides were microwaved in citrate buffer antigen retrieval solution (Vector Laboratories, Burlingame, CA) three times for 5 minutes. Affinity-purified rabbit anti-CaSm antibody was used at 4 µg/ml and slides were incubated overnight at 4°C. The rabbit anti-CaSm antibody was generated using a synthetic peptide corresponding to the twenty C-terminal amino acids of CaSm covalently coupled to bovine serum albumin (Rockland, Gilbertsville, PA). Vector Rabbit Impress kit (Vector Laboratories) was used as secondary antibody. Sections were stained using DAB and counterstained with hematoxylin. Adjacent sections of all blocks and a TARP slide were also stained using hematoxylin and eosin. All sections were examined and scored independently by a pathologist (M.F.). Presence of staining was compared with that of normal lung tissue present in the same section. No staining was observed with negative control samples (absence of primary antibody or incubation with rabbit IgG). TMA slides were examined and scored independently by a pathologist (R. Harley, Medical University of South Carolina, Charleston, SC).

Cell Culture and Tumor Samples

The mesothelioma cell lines, MesoSA1 and Met5A, were cultured in LHC-MM medium (Biosource; Camarillo, CA), all other cell lines were cultured in RPMI 1640 (CellGro, Herndon, VA). H2052, Met5A, MSTO, A549, and other LC cell lines were obtained from ATCC (Rockville, MD). The LRK1A cell line was obtained from Steven M. Albelda, M.D., University of Pennsylvania. Frozen tumor and non-tumor samples were obtained from the Hollings Cancer Center Tumor Bank as approved by the MUSC institutional review board, and all samples were devoid of identification. Normal bronchial epithelial cells were purchased from Clonetics (Walkersville, MD) and cultured in bronchial epithelial growth medium with appropriate growth factors.

RT-PCR and Real-Time qPCR

Total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA) and further purified for qPCR using RNeasy kits and DNase Set from

Qiagen (Valencia, CA). cDNA was prepared using Superscript III (Invitrogen) with 1 to 5 µg total RNA as template. PCR was performed in 25-µl reaction volume with TaqGold Polymerase (Applied Biosystems, Foster City, CA). Fragments were visualized on 2% agarose gels by ethidium bromide staining. Real-time PCR was performed with 1 µl of a 1:10 dilution of cDNA using Platinum SYBR-Green qPCR Supermix UDG (Invitrogen) on a Roche Lightcycler (Roche, Indianapolis, IN). The cycling conditions for all genes were: pre-incubation at 50°C for 2 minutes, 95°C for 2 minutes, followed by 30 to 50 cycles of denaturation at 94°C for 10 seconds, annealing at 55°C for 10 seconds, and extension for 30 seconds at 72°C, with a single data acquisition at the end of each extension. All ramping was done at 20°C per second. The relative expression of each gene was quantified on the basis of cycle threshold value using the LinReg PCR program. To compare relative amounts of RNA between samples, data were normalized to S26. Primers used are as follows; CaSm forward (qPCR) GACTGG AAAAGGAGAGTGACA (nt396–417); CaSm forward (PCR) ATGA ACTATATGCCTGGCACC (nt165–186); CaSm reverse (qPCR and PCR) GGGCAAAAGATTAGTACTCATC (nt555–575); S26 forward (qPCR) GCGAGCGTCTTCGATGC; S26 reverse CTCAGCTCCTTA CATGGG; actin forward GCTCGTCGTCGACAACGGCTC; actin reverse CAAACATGATCTGGGTCATCTTCTC. The actin primers were part of the Superscript RT-PCR kit purchased from Invitrogen.

Western Blot Analysis

Frozen tissue samples were pulverized in LN₂ and suspended in RIPA buffer (150 mM NaCl, 50 mM Tris, pH 7.4, 1% TritonX 100, 0.1% SDS, 1% Na Deoxycholate, and 8% protease inhibitors [Sigma Chemical Co., St Louis, MO]) and further dispersed with a dounce homogenizer. Extracts were centrifuged to remove insoluble material and supernatants collected for Western blots. Cell cultures were lysed in RIPA buffer and treated as above. Proteins were separated on a 12.5% acrylamide gel and transferred to a nitrocellulose membrane (Schleicher and Schuell, Keene, NH). After blocking, the membrane was incubated overnight in 2 µg/ml affinity-purified anti-CaSm IgY and for 1 hour in horseradish peroxidase (HRP)-goat anti-IgY (Aves Labs, Portland, OR). After washing, the membrane was incubated in HRP-labeled secondary antibody. The membrane was developed in West Pico Chemiluminescent substrate (Pierce, Rockford, IL) for 5 minutes and exposed to Kodak MR film. As a loading control, the membrane was incubated for 1 hour with mouse monoclonal β-Actin (Sigma) followed by goat anti mouse IgG (Caltag, Burlingame, CA) and developed as above.

The chicken anti-CaSm antibody was made using full-length bacterial expressed protein injected into laying hens (Aves Laboratory, Portland, OR). IgY was isolated from eggs and affinity-purified with full-length CaSm protein covalently linked to agarose (Pierce). The specificity of this antibody was demonstrated by the absence of immunoreactive proteins in Western blots of extracts prepared from cell lines that do not express CaSm mRNA.

Western blots of tumor and adjacent nontumor tissue were quantified using Kodak Molecular Imaging software. Densitometry values were determined for CaSm and actin bands and the ratio of the values for each sample calculated. Values reported are the means of three separate Western blots. To compare the expression of CaSm in the different cell lines, the protein bands were quantified using Kodak Molecular imaging system and standardized to the GAPDH expression. The values are the mean of three independent Western blots with at least two different protein extracts for each cell line. The value for the normal bronchial epithelial cells is the mean of three independent extracts.

Transfections

A549 and Meso SA1 cells were transfected with the pSG5neo plasmid containing the cloned antisense CaSm using Fugene (Roche). Stable clones were selected with 500 µg/ml G418, and those expressing antisense RNA were expanded and characterized. LRK1A cells were transfected with pSG5neo plasmid with the full-length CaSm sequence or pCDNA3 plasmid containing a FLAG-tagged CaSm.

RNA Interference

Sequences for siRNA were identified within the CaSm sequence using BLAST search technology to find 21mers with no sequence identity to

Figure 1. Immunohistochemical demonstration of CaSm protein overexpression in lung cancer and mesothelioma. Paraffin-fixed sections stained with rabbit anti-CaSm antibody using Vectastain anti-rabbit Impress reagent with DAB and hematoxylin counterstain showing high levels of cytoplasmic expression of CaSm in tumor tissue. (A) Adenocarcinoma: *a*, *d*, and *e*, mucinous adenocarcinoma; *b* and *c*, nonmucinous adenocarcinoma (bar in *a* is 100 μ m). (B) *a*–*c*, squamous cell carcinoma. (C) Bronchial alveolar carcinoma. (D) Mesothelioma: *a*, biphasic mesothelioma, sarcomatoid portion; *b*, biphasic mesothelioma, epithelial portion; *c*, epithelial mesothelioma; *d*, epithelial mesothelioma (bar is 50 μ m). (E) Normal lung (bar is 50 μ m). Magnification is the same in A–D, *a*–*c*.

other genes. Two sequences were selected; CaSm 1 corresponds to nucleotides 104 to 125 and CaSm 2 to nucleotides 328 to 347 of the cDNA sequence. Double-stranded 2'-ACE RNA oligomers were synthesized and purified by Dharmacon Research (Lafayette, CO). In addition, a control oligonucleotide pool with no known sequence homology to the human genome was purchased from Dharmacon. Transfection was carried out using Oligofectamine (Invitrogen) in 24-well tissue culture dishes with cells at 20 to 30% confluence according to manufacturer's instructions using 4 pmoles dsRNA and 10 μ l Oligofectamine transfection reagent.

Anchorage-Dependent and -Independent Growth Analyses

Anchorage-dependent growth studies were performed using MTT (methylthiazolyl-diphenol-tetrazolium; Sigma) assays. Parental and transfected cells were plated at 1,000 cells per well in a 96-well tissue culture plate. At set times, 10 μ l of 5 mg/ml MTT (Sigma) was added per well and incubated for 4 hours at 37°C; 100 μ l of 0.01 M HCl, 10% SDS was added and the plate incubated overnight to dissolve the tetrazolium salt. The plate was read in a microplate reader at 560 nm and 650 nm, with the difference recorded.

Anchorage-independent growth was measured by soft agar colony formation. Cells were suspended at 5×10^3 cells/ml in 0.3% agarose and layered over 0.6% agarose. Cells were provided fresh media by soft agar overlay weekly. Colonies greater than 100 μ m in diameter were counted after 3 weeks of incubation. Values shown are the mean and standard deviation of three independent experiments.

Animal Studies

Animal handling and procedures were approved by the Medical University of South Carolina Animal Studies Committee. Cells were harvested using trypsin and washed once with medium, once with PBS, and re-suspended at 1×10^7 cells/ml. One hundred microliters was injected subcutaneously into the flank of SCID-Beige mice (Taconic, Germantown, NY) and monitored weekly. In addition, cells were re-suspended at 2×10^7 cell/ml in 0.5 ml PBS and mixed with 0.5 ml Matrigel (BD Biosciences, Bedford, MA) and each mouse injected with 100 μ l. Tumors were measured by length and width and volume, calculated using the formula $V = L \times W^2 \times \pi/6$. When any animal showed signs of distress or its tumor reached a size of 2.0 cm³ it was killed.

Statistics

Student's *t* test with a Bonferroni adjustment was used to determine significance of the anti-sense anchorage-independent colony formation assay. The Kruskal-Wallis test was used to determine significance among the different CaSm-transfected cell lines in anchorage-independent colony formation. Kaplan/Meyer equations were used to calculate survival curves and log rank test done to determine significance.

RESULTS

CaSm Is Expressed at Elevated Levels in Lung Cancer and Mesothelioma

To determine the expression levels of CaSm in lung and mesothelial tissues, formalin-fixed and paraffin-embedded tumors from the MUSC Tumor Bank were stained by immunohistochemistry. Adenocarcinoma, squamous cell carcinoma, and bronchial alveolar carcinoma showed intense cytoplasmic staining for CaSm compared with adjacent nontumor lung tissue (Figures 1A, 1B, and 1C). The four mesothelioma samples examined also stained intensely for CaSm expression (Figure 1D). Normal lung showed low levels of cytoplasmic CaSm expression in macrophages and plasma cells as well as very low levels in fibroblasts. Bronchial epithelium demonstrated superficial low intensity staining along the edges of the bronchi. Type 1 alveolar cells had no staining, while type 2 cells had some very weak staining (Figure 1E). Evaluation of an additional 25 lung tumor samples (11 adenocarcinoma, 10 squamous cell carcinoma, 2 bronchial alveolar carcinoma, and 2 with unknown diagnoses) and 3 normal lungs on tissue microarray (TMA) slides from the NCI (TARP) demonstrated 4+ staining on all tumor samples with minimal staining in the normal samples (data not shown).

CaSm expression levels were also measured by quantitative real-time PCR and Western blot analysis using a different set of archived samples of lung cancer and mesothelioma and adjacent nontumor lung tissue. Two out of three mesotheliomas showed elevated CaSm mRNA expression compared with normal lung

tissue and a pleural effusion from a patient without cancer, while the other sample had lower expression (Figure 2A). All four lung cancer samples demonstrated higher CaSm expression than adjacent nontumor tissue (Figure 2B). Protein was also extracted from three of the lung cancer samples and analyzed by Western blot. Consistent with RNA data, elevated CaSm protein expression was also found in each of the tumor samples compared with the nontumor tissue (densitometry values being two to three times higher in the tumor samples) (Figure 2C). Lung cancer and mesothelioma cell lines were also analyzed for CaSm protein expression by Western blot. Compared with normal human bronchial epithelial cells (NHBE), expression was high in three NSCLC cell lines (A549, H23, and H358) and one SCLC cell line (H69), as well as five of six cell lines derived from malignant mesotheliomas (M25, M82, Meso SA1, H2052, and MSTO), and in Met5A, a mesothelial cell line immortalized by transfection with SV40 large T antigen (Figure 2E). One cell line derived from a patient with epithelial mesothelioma (LRK1A) had low levels of CaSm expression, equivalent to NHBE cells.

Development of a Sarcomatous Mesothelioma Cell Line

In order to study the characteristics of mesothelioma, we developed a cell line from the pleural fluid from a patient with sarcomatous mesothelioma. We specifically chose to study sarcomatous mesothelioma cells because this type of mesothelioma is the most resistant to all types of therapy and associated with the worst outcome (6). Cells were separated from contaminating erythrocytes and cultured in LHC-MM cell culture medium. After several days of culture individual colonies were isolated, and one colony, designated MesoSA1, was expanded. It was confirmed to be mesothelial by positive immunohistochemical staining for vimentin, keratin, and Wilm's tumor antigen (data not shown). High levels of CaSm expression in MesoSA1 were confirmed by Western blot analysis (Figure 2D). The transformed phenotype of MesoSA1 was demonstrated by *in vitro* and *in vivo* tumorigenesis assays. Anchorage-independent growth was shown by the ability of the cell line to grow in soft agar (Figure 3A). *In vivo* tumorigenicity was indicated by the ability to form tumors by subcutaneous injection of cells (1×10^7 cells in 100 μ l PBS) into the flanks of SCID/Bg mice. All mice injected developed tumors within 4 weeks (Figure 3B).

Cell Lines Expressing Antisense CaSm RNA Have Reduced Anchorage-Independent Growth and Tumorigenic Potential

To determine whether CaSm up-regulation is required for malignant phenotypes in mesothelial and lung cells, we used antisense to reduce expression of CaSm in the mesothelioma cell line MesoSA1 and the lung cancer cell line A549. Cells were transfected with a pSGneoSK plasmid vector expressing antisense CaSm. After selection, individual clones were screened by Northern blot analysis using single-stranded probes to detect antisense and sense CaSm mRNA (data not shown). Clones that indicated the presence of antisense RNA and knockdown of endogenous mRNA were expanded and assayed for *in vitro* anchorage-independent growth and *in vivo* tumor formation. The two MesoSA1 antisense clones, S1C2 and S2A2, gave rise to significantly fewer colonies compared with the parental cell line (Figure 3A). The parental mesothelioma cell line MesoSA1 formed 175 ± 52 colonies while antisense clones S1C2 and S2A2 formed 26 ± 13 and 10 ± 4 colonies, respectively. In addition, S1A5, an isolated MesoSA1 transfected clone that did not express the antisense mRNA developed colonies (177 ± 77) to a level similar to that observed for the MesoSA1 parental cell line. A549 parental lung cancer cells formed 441 ± 77 colonies, and a nonexpressing clone, A1C2, formed 628 ± 100 colonies, while two antisense-expressing clones (A1B6 and A2B6) had

Figure 2. CaSm mRNA and protein are expressed at higher level in mesothelioma and lung tumor tissue compared with adjacent nontumor tissue and in lung cancer- and mesothelioma-derived cell lines. (A) Quantitative real-time PCR using RNA extracted from a mesothelioma tumor (meso 1) or two pleural effusions (meso 2 and 3) showing elevated CaSm expression in two of the three cancers compared to a pleural effusion (PE) from a subject without cancer ("normal" lung). (B) Quantitative real-time PCR of RNA from five matched tumor (dark grey bars) and adjacent nontumor tissue (light grey bars) from non-small cell lung cancer (NSCLC). The diagnosis of the samples are: 1, squamous; 2, adenocarcinoma; 3, squamous; 4, adenocarcinoma; and 5, large cell carcinoma. The nontumor tissue was taken during surgery from an adjacent normal-appearing area. (C) Western blot of CaSm protein expression in three of the five NSCLC cases above. Tumor tissue shows elevated CaSm expression compared with nontumor tissue. Numbers below bands are mean quantitative values from densitometry from three individual Western blots. β -Actin is shown as a loading control. (D) Western blot of mesothelioma and lung cancer cell lines. A549, H23, and H358 are NSCLC-derived cell lines; H69 is an SCLC-derived cell line; Met5A is an immortalized mesothelial cell line; LRK1A, M25, M82, MesoSA1, H2052, and MSTO are mesothelioma-derived cell lines; and NHBE is normal human bronchial epithelium. GAPDH is shown as loading control. To compare the expression of CaSm in the different cell lines, the protein bands were quantified using Kodak Molecular imaging system and standardized to the GAPDH expression. The value for the normal bronchial epithelial cells is the mean of three independent extracts. Numbers below bands are mean quantitative values from densitometry from three individual Western blots using different cell extracts.

13 and 5 colonies, respectively (Figure 3A). These experiments demonstrate that reducing CaSm expression inhibits anchorage-independent growth of these cancer cell lines, a hallmark of the transformed phenotype.

Figure 3. CaSm expression is correlated with cellular transformation. (A) Anchorage independent growth of antisense CaSm-transfected clones. Parental cells and antisense-expressing clones were grown in soft agar for 3 weeks. The number of colonies greater than 100 μ m was determined. Values are mean of three independent experiments. Number of colonies for each transfected clone was compared with the parental cells using a two-sample *t* test. The *P* value for each antisense clone compared with parental cells was < 0.001 . For the MesoSA1 cells and clones a Bonferroni adjustment was done, indicating a significant *P* value of < 0.017 . (B and C) Tumor formation *in vivo* of antisense CaSm-transfected clones. Kaplan-Meier curves for (B) MesoSA1 and (C) A549 cells and MesoSA1 and A549 cells stably transfected with CaSm antisense. Cells were injected into SCID-Bg mice at Day 0, and tumor growth was monitored weekly. Five animals per group were used for the MesoSA1 cells and antisense clones, and 15 animals per group were used for the A549 cells and antisense clones. The data were plotted in Kaplan-Meier curves and analyzed using the log rank test and found to be significantly different (for MesoSA1 *P* value = 0.0018) (for A549 log-rank *P* value is < 0.0001). Densitometry values for CaSm protein expression in the extracted tumors are shown in C below labels. Values are the means from three Western blots on two tumors from A549 parental cells and three from each antisense clone. A2B6 protein expression is significantly lower than the parental A549 expression ($P < 0.02$). No tumors grew with the MesoSA1 clones.

The effect of reduced CaSm expression on growth of MesoSA1 and A549 *in vivo* was evaluated by subcutaneous injection into immunodeficient SCID-beige mice. Animals were monitored for 2 to 7 months and tumor size was measured using digital

calipers. Tumors were palpable with the Meso SA1 parental cell line less than 20 days after injection, whereas the two antisense clones did not develop palpable tumors during the 2 months the animals were monitored (Figure 3B). The data were plotted using Kaplan-Meier analysis, and the log-rank *P* value was 0.0018. Similarly, mice injected with the A549 parental cells developed tumors much faster than those injected with the antisense CaSm clones. All of the mice injected with A549 were killed due to tumor burden by 3 months, whereas mice injected with the antisense clones survived for up to 4 (clone A1B6) and 7 months (clone A2B6). Kaplan-Meier analysis gave a log-rank *P* value of < 0.0001 for both A1B6 and A2B6 compared with parental A549 cells. A few of the tumors from the A549 clones were removed, and the protein was extracted and analyzed for CaSm expression by Western blot and densitometry. The tumors from one of the antisense clones showed a lower CaSm expression than the tumors from parental A549. Parental A549 had a densitometry value of 0.97 (mean of two), three tumors derived from A1B6 had a mean of 1.13 and three tumors derived from A2B6 had a mean of 0.54 ($P < 0.02$ compared with A549) (Figure 3C). Interestingly, A2B6 was also the slower growing of the two tested antisense CaSm clones. Thus, reducing CaSm expression results in at least a 3-fold inhibition in the ability of mesothelioma and lung cancer-derived cell lines to grow as tumors *in vivo*.

Inhibition of CaSm Expression Using siRNA Results in Reduced Cell Growth

We have demonstrated that multiple stable antisense clones fail to grow independent of anchorage. To examine the phenotypes of pools of cells rather than selected individual clones, we performed complementary loss-of-function studies using siRNA (21, 22). Two small interfering RNAs (siRNAs) were designed for use in gene silencing experiments. MesoSA1 cells were transfected with the two identified CaSm siRNAs every 3 days (Days 0, 3, 6), and growth of these cells was monitored using MTT. Protein expression of CaSm was significantly reduced 8 days after transfection (2 days after final transfection [Figure 4B]). siRNA-mediated down-regulation of CaSm resulted in more than 90% reduction in mRNA (data not shown) and 80% protein expression. Growth of cells transfected with either of the CaSm-specific siRNAs was significantly slower than the parental cell line (Figure 4A) or cells transfected with a control oligonucleotide pool. Although the control oligonucleotide pool also had an effect on cell growth, this effect became less apparent with time compared with the siRNA oligomers. Therefore, reduction of CaSm protein expression inhibits MesoSA1 cell growth.

Ectopic CaSm Expression Increases Growth *In Vitro* and *In Vivo*

Mesothelioma and lung cancer cell lines that express high levels of CaSm exhibit nonadherent growth and form tumors when injected into mice. One mesothelioma cell line, LRK1A, does not overexpress CaSm relative to NHBE cells and does not grow well independent of anchorage *in vitro* or as tumors in mice. To determine if increased expression of CaSm could elicit these transformed phenotypes in this cell line, LRK1A cells were transfected with a plasmid containing the full-length open reading frame of the CaSm gene controlled by the CMV promoter. Individual clones were selected with G418 and expanded for characterization. Clones were analyzed by RT-PCR and several were found to express CaSm mRNA, at levels above those found in the parental cell line (Figure 5A). CaSm protein was also examined by Western blot analysis of protein lysates prepared from these clones (Figure 5B), and a corresponding increase in CaSm protein level was found.

Figure 4. CaSm siRNA reduces cell growth. (A) MesoSA1 cells were transfected with two different CaSm siRNA oligomers and a pool of RNA oligomers (20nt) without homology to any known human mRNA sequence (NS pool) or maintained in medium (Control) and cell number was assessed using MTT for 8 days. Transfections were carried out on Days 0, 3, and 6 as indicated by the arrows. (B) MesoSA1 cells transfected with two different CaSm siRNA oligomers as above were harvested on Day 8 after transfection and analyzed by Western blot, which shows reduced CaSm protein levels compared with control and a nonspecific pool of RNA oligomers.

Two independent pools that express CaSm protein were analyzed for colony formation in soft agar, and both were found to form significantly more colonies than the parental cell line (Figure 5C).

Both the selected clones and pools of transfected cells were next tested for their ability to form subcutaneous tumors in immunodeficient mice. Two experiments were performed. For the first experiment, 1×10^6 cells were injected in PBS; for the second experiment, the same numbers of cells were mixed with Matrigel before injection into the flanks of SCID/Bg mice. The number of tumors formed per mouse injected with the designated clone or pool of transfected cells were monitored over time. Each of the clones formed palpable tumors ($>50 \text{ mm}^3$) faster than the LRK1A parental cells (Table 1). All mice injected with the pool of transfected cells developed large tumors ($>200 \text{ mm}^3$) by 2 weeks, while mice injected with the parental cells developed fewer palpable tumors over time (Table 1). The ability of cells to grow independent of anchorage and form tumors in immunocompromised mice is a hallmark of malignant cells. Thus, increasing CaSm expression promotes malignant progression.

DISCUSSION

Our current studies demonstrate that CaSm is required to maintain the transformed phenotype of mesothelioma and lung cancer cells and can function as an oncogene in the promotion of malignant phenotypes. Up-regulation of CaSm protein is seen in malignant cells from all lung cancer and mesothelioma samples analyzed by immunohistochemistry. By qPCR and

Figure 5. Increased CaSm expression leads to a more transformed phenotype in a non-CaSm-expressing mesothelioma cell line. (A) The mesothelioma cell line LRK1A was transfected with a full-length CaSm expression plasmid using Fugene and clones selected. Total RNA was isolated using Trizol, cDNA was prepared, and CaSm PCR performed as described in MATERIALS AND METHODS. Actin PCR was performed as loading and cDNA quality control. (B) Western blot of LRK1A clones showing CaSm protein expression. (C) Nonadherent growth was assayed using soft agar colony formation assay. Cells were suspended in 0.3% agarose, and colonies greater than $100 \mu\text{m}$ were counted after 4 weeks of incubation. $P < 0.025$ for Pool 3 and $P < 0.05$ for Pool 6.

Western blot analyses, 100% of the lung tumors and 67% of the mesotheliomas show increased CaSm expression compared with nontumor or normal tissue. The remaining mesothelioma sample shows lower expression compared with normal lung tissue. Thus, we have shown overexpression of CaSm in 16 adenocarcinomas, 13 squamous cell carcinomas, 3 bronchial alveolar

TABLE 1. EXPRESSION OF CaSm IN LRK1A CELLS LEADS TO INCREASED TUMOR GROWTH IN IMMUNOCOMPROMISED MICE

	EXPERIMENT 1			
	1 wk	2 wk	4 wk	7 wk
LRK1A	0/3	0/3	0/3	1/3
P	0/2	0/2	1/2	2/2*
RR	0/3	1/3	1/3	3/3*
JJ	1/3	2/3	2/3	2/3
QQ	0/3	1/3	2/3	3/3*
TT	0/3	0/3	2/3	3/3*
	EXPERIMENT 2			4 wk
	2 wk	3 wk	4 wk	
LRK1A	0/5	1/5	3/5	
RR	0/4	1/4	4/4*	
QQ	1/5	2/5	3/5	
TT	1/5	3/5	3/5	
POOL 3	5/5*	5/5*	5/5*	

SCID-beige mice were injected with 10^6 CaSm-transfected LRK1A cells, and tumor growth was monitored. Experiment 1 used cells suspended in PBS; experiment 2 used cells suspended in Matrigel. Numbers are palpable tumors (volume $> 50 \text{ mm}^3$) per mouse injected. Tumors were measured twice weekly.

* Clones or pool that had 100% tumor growth at each time point.

carcinomas, and 5 out of 6 mesotheliomas compared with 4 normal lung samples. The difference in CaSm expression between tumor and adjacent tissue in the immunohistochemistry is greater than that seen with Western blot because RNA and protein were extracted from frozen tissue that was not characterized for the amount of tumor in each, and these are likely to contain differing amounts of tumor tissue relative to nontumor epithelium and stromal cells. In addition to the overexpression observed in the primary tissues, several lung cancer- and mesothelioma-derived cell lines had high levels of CaSm expression. Thus, in addition to pancreatic (12, 23, 24) and prostate cancer (13), lung cancer and malignant mesothelioma exhibit elevated levels of CaSm expression.

Inhibition of CaSm expression by stable antisense expression reduces the ability of lung cancer and mesothelioma cells to grow independent of anchorage and form tumors in immunocompromised mice. CaSm protein expression is significantly reduced in tumors derived from one of the A549 antisense clones that had the slower *in vivo* growth characteristics, but not in another clone that also showed growth reduction albeit to a lesser extent. Because loss of CaSm expression inhibits cell proliferation and the selection process favors colonies that grow well, it is expected that with continuous culture knockdown would be lost due to growth advantage of those cells that lose antisense expression.

Although we show growth inhibition with the two independent siRNA oligomers, the nonspecific siRNA also shows some inhibition of growth. This control siRNA has toxicity in many cell lines we have tested. Toxic and off target effects are often seen with such control oligomers (25). We expect that this is contributing to the reduction in cell growth seen with the nonspecific siRNA. However, the CaSm-specific siRNA reduces the CaSm protein expression, while the nonspecific control does not. Since we only see significant knockdown of protein with CaSm-specific siRNAs but not with the nonspecific control, a significant antiproliferative effect is caused by loss of CaSm expression. This was demonstrated using two different CaSm target sequences, also supporting the conclusion that the effect observed is mediated by reduced CaSm protein expression.

In addition, ectopic expression of CaSm in mesothelioma cells enhances soft agar colony formation and tumor formation. All of these characteristics are indicative of the transformed phenotype supporting the model that CaSm is an oncogene.

Previous work demonstrated that CaSm resulted in transformation of NIH 3T3 cells (13). Similarly, Yan and coworkers have shown that ectopic expression of CaSm in a mouse pancreatic cancer cell line that does not normally express CaSm leads to increased cell proliferation, anchorage-independent growth, and *in vivo* tumor formation (26). These studies used mouse cell lines, whereas the studies presented here demonstrate that CaSm can transform human mesothelioma cells. Mouse cell lines can be transformed by transfection with combinations of two oncogenes, such as E1A + Ras or myc + Ras; in contrast, human cells are not transformed by these combinations (27). One experimental protocol used to transform human cells requires inactivation of the p53 and Rb tumor suppressor genes and the protein phosphatase 2A along with expression of telomerase and an oncogenic allele of Ras (28, 29). These data support the model that human cells are more difficult to transform. The LRK1A cells are derived from a human mesothelioma, but do not demonstrate typical characteristics of transformation such as anchorage-independent growth and tumor formation. Overexpression of CaSm in these cells increases their transformed phenotype as they are now able to form tumors in mice. Thus, CaSm can function as a cancer-promoting oncogene, in human cells as well as in mouse cells.

Inhibition of CaSm expression by infection with an adenovirus expressing antisense CaSm was correlated with decreased anchorage-dependent proliferation associated with a G2/M block. CaSm levels were correlated with reduced expression of cyclin B, cdk1, and nucleophosmin B23 and increased expression of NEK2 (13, 23, 24). Cyclin B is active during the transition from G2 to M phase of the cell cycle. Reduction of this protein would inhibit passage of cells through mitosis and slow cell division leading to the observed reduced proliferation and G2/M block. The mechanism whereby CaSm affects the expression of these cell cycle regulatory proteins is unknown. Genetic deletion of the yeast homolog of CaSm (spb8/Lsm1) resulted in increased stability of short-lived mRNA (30). CaSm is a subunit of the complex that targets mRNA for degradation (17); thus, changes in message stability may contribute to these effects. The increase in CaSm expression seen in cancer cells could also affect complex formation and hence mRNA stability, leading directly or indirectly to changes in expression of many of the genes involved in transformation. Supporting this notion, an increased half-life of p21 mRNA was found after reduction of CaSm (hLSm1) expression in human prostate cells (13). Up-regulation of CaSm in cancer could also shift the equilibrium between the two Lsm complexes, which would also effect RNA splicing.

Increased expression of LSm1 in immortalized breast cells induced growth factor-independent proliferation and anchorage-independent growth (31). Thus, in addition to affecting anchorage-independent growth and tumor formation, LSm1 expression reduces growth factor dependence, another property of cancer cells. Interestingly, CaSm is located on chromosome 8p11-12, a region frequently amplified in breast cancer (30).

In conclusion, we have shown that CaSm is up-regulated in lung cancer and malignant mesothelioma. Inhibition of CaSm expression by either antisense or RNAi leads to reduced anchorage-dependent and -independent growth *in vitro* and tumor formation *in vivo*. Significantly, CaSm expression increased transformed phenotypes in mesothelioma cells. Thus, CaSm expression contributes to cellular transformation and tumor formation. Future studies directed toward understanding CaSm-mediated alterations of cellular RNA processing pathways will provide mechanistic insight into how CaSm overexpression leads to changes in the transcriptome and/or spliceosome of cancer cells.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

Acknowledgments: The authors acknowledge the technical assistance of Edward Jones, Marie DiFrancesco, Melissa Savia, and Margaret Romano (Hollings Cancer Center Tumor Bank), and Dr. Russell Harley, M.D., for scoring the tissue arrays. The authors also thank Dr. Victoria Findlay for critical evaluation of the manuscript.

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