

## Chromogranin A Regulates Tumor Self-Seeding and Dissemination

Eleonora Dondossola, Luca Crippa, Barbara Colombo, Elisabetta Ferrero, and Angelo Corti

### Abstract

Cancer progression involves the seeding of malignant cells in circulation and the colonization of distant organs. However, circulating neoplastic cells can also re-infiltrate the tumor of origin. This process, called "tumor-self seeding," can select more aggressive cells that may contribute to cancer progression. Here, using mouse mammary adenocarcinoma models, we observed that both tumor self-seeding and organ colonization were inhibited by chromogranin A (CgA), a protein present in variable amounts in the blood of cancer patients. Mechanism studies showed that CgA inhibited the shedding of cancer cells in circulation from primary tumors, as well as the re-infiltration of tumors and the colonization of lungs by circulating tumor cells. CgA reduced gap formation induced by tumor cell-derived factors in endothelial cells, decreased vascular leakage in tumors, and inhibited the transendothelial migration of cancer cells. Together, our findings point to a role for circulating CgA in the regulation of tumor cell trafficking from tumor-to-blood and from blood-to-tumor/normal tissues. Inhibition of the multidirectional trafficking of cancer cells in normal and neoplastic tissues may represent a novel strategy to reduce cancer progression. *Cancer Res*; 72(2); 449–59. ©2011 AACR.

### Introduction

A common feature of most life-threatening tumors is their propensity to seed tumor cells in circulation and to spawn metastasis in distant organs (1). This process requires a complex cascade of events that involve tissue invasion, intravasation, survival in circulation, arrest in target tissues, extravasation, invasion, and organ colonization (2). Recent studies have shown that circulating tumor cells, either derived from primary tumors or from metastatic colonies, can re-infiltrate their tumor of origin (or other tumor masses) in a process that is called "tumor-self seeding" (3, 4). On the basis of data obtained in animal models, it has been proposed that tumor cells acquire, after their excursion outside their site of origin, a more aggressive phenotype that may contribute to accelerate the growth of primary tumors and the breeding of metastatic progenies (3). Studies on the mechanisms that regulate the trafficking of cancer cells in normal and neoplastic tissues may be, therefore, of great experimental and clinical interest.

It has been reported that hematogenous metastasis can originate from the proliferation of endothelium-attached tumor cells (5). Tumor cells can also secrete factors that alter

the endothelial barrier function, facilitating their migration across endothelial cells (6). For example, VEGF-expressing tumor cells can promote, via VEGF, the disruption of VE-cadherin- $\beta$ -catenin complex in the lung endothelium of mice, facilitating their extravasation (7). Furthermore, the enhancement of VE-cadherin-dependent endothelial barrier function with activated protein C has been shown to limit cancer cell extravasation in normal tissues (8). Thus, whereas the presence of vessels with tight endothelial barriers may represent an impediment for the infiltration of normal tissues, the presence of leaky vessels in tumors might facilitate the shedding of cancer cells in circulation and tumor re-infiltration. On the basis of these notions, we have hypothesized that factors that regulate the endothelial barrier in tumors and in normal tissues can also regulate tumor self-seeding and dissemination.

We have previously shown that chromogranin A (CgA), an acidic glycoprotein secreted by normal and neoplastic neuroendocrine cells, neurons and granulocytes (9), can enhance the endothelial barrier function in normal tissues and protect vessels from vascular leakage (10). CgA is released in circulation in normal subjects at about 1 nmol/L levels and in several pathologic conditions, including cancer, at higher levels in the nanomolar range (11). The CgA1-78 fragment, called vasostatin-1 (VS-1), can inhibit the formation of gaps in endothelial cell monolayers and the permeability to macromolecules induced by TNF- $\alpha$ , VEGF, and thrombin (10, 12). Thus, CgA and its N-terminal fragment are efficient inhibitors of the endothelial barrier alterations caused by different inflammatory and proangiogenic stimuli, which are potentially released in the tumor microenvironment.

These notions prompted us to investigate whether circulating CgA might affect the tumor self-seeding process and the dissemination of cancer cells to normal tissues. To this aim, we

**Authors' Affiliation:** Division of Molecular Oncology and IIT Network Research Unit of Molecular Neuroscience, San Raffaele Scientific Institute, Milan, Italy

**Note:** Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

**Corresponding Author:** Angelo Corti, Division of Molecular Oncology, San Raffaele Scientific Institute, via Olgettina 58, 20132 Milan, Italy. Phone: 39-02-26434802; Fax 39-02-26434786; E-mail: corti.angelo@hsr.it

doi: 10.1158/0008-5472.CAN-11-2944

©2011 American Association for Cancer Research.

studied the ability of circulating CgA (exogenous and endogenous) to affect the self-seeding and the dissemination of murine mammary adenocarcinoma tumors. We show that pathophysiologic levels of CgA are sufficient to significantly inhibit both processes. Furthermore, we show that CgA can inhibit the seeding of tumor cells in circulation, the homing of circulating cells to tumors, and the colonization of lungs in mouse models. Finally, we provide experimental evidence to suggest that an important mechanism underlying these effects is the enhancement of the endothelial barrier function against tumor cell-derived propermeabilizing factors.

## Materials and Methods

### Cell lines, drugs, antibodies, and reagents

Mouse B16-F10 melanoma cells were from American Type Culture Collection and TSA cells were provided by Dr. Giulia Casorati. TSAThy1.1 cells were generated as previously reported. These cells were cultured as described previously (13, 14). Ser-Thr-Ala-CgA<sub>1-78</sub> (VS-1; ref. 15), human CgA (hCgA), and murine CgA (mCgA) were prepared by recombinant DNA technology and expression in *E. coli* cells as described (15–17). Anti-human CgA monoclonal antibody (mAb) 5A8 (epitope CgA53–57), B4E11 (epitope CgA68–71), A11 (epitope CgA81–90; all IgG1k), and anti-Thy1.1 mAb 19E12 were produced and characterized as described previously (14, 16, 18). Sulfate latex beads 4% w/v, 5 μm were from Invitrogen.

### Animal models

Studies on animal models were approved by the Ethical Committee of the San Raffaele Scientific Institute and carried out according to the prescribed guidelines. To study the effect of CgA on tumor self-seeding and metastatic dissemination, syngeneic immunocompetent BALB/c mice (8-week-old; Charles River Laboratories) were challenged with TSA or TSAThy1.1 cells subcutaneously ( $2 \times 10^5$  cells) and/or intravenously ( $4 \times 10^4$ ). Alternatively, C57BL/6 mice were injected, intravenously, with  $6 \times 10^4$  B16-F10 melanoma cells resuspended in 0.9% sodium chloride. Mice were treated intravenously with hCgA or VS-1 (in 0.9% sodium chloride containing 100 μg/mL of human serum albumin) or with other drugs in 0.9% sodium chloride. Drug doses and treatment schedules for each experiment are reported in figures and figure legends. The presence of disseminated TSAThy1.1 cells in the lungs of BALB/c mice (Thy 1.2) and in recipient tumors was detected by fluorescence-activated cell sorting (FACS) analysis of lungs or tumors, after excision and disaggregation, with an antibody specific for the Thy1.1 antigen (mAb 19E12). Furthermore, the number of TSA and B16 colonies in the lungs of mice was counted with the help of a stereomicroscope.

### FACS analysis, reverse transcriptase PCR, antibody detection, and *in vitro* assays

The methods for Thy1.1 cells detection [FACS analysis, reverse transcriptase PCR (RT-PCR)], antibody binding assay and other *in vitro* assays (cell proliferation, migration, invasion, transendothelial migration, and permeability) are reported in "Supplementary Methods."

## Results

### Recombinant hCgA inhibits mouse mammary adenocarcinoma self-seeding

To investigate the effect of CgA on tumor self-seeding, we used a syngeneic mouse mammary adenocarcinoma model based on the subcutaneous implantation of TSA and TSAThy1.1 cells (TSA cells genetically engineered to express the Thy1.1 allele) in controlateral flanks of immunocompetent mice that express the Thy1.2 allele. Immunohistochemical analysis of TSA tumors with an anti-Thy1.1 antibody (mAb 19E12), done 21 days after tumor implantation, revealed the presence of islets of Thy1.1<sup>+</sup> cells of different size in TSA tumors (Fig. 1A), indicating that the TSA model is suitable for studies on tumor self-seeding. Quantification of TSAThy1.1 cells that infiltrated TSA tumors in mice was obtained by FACS analysis with the anti-Thy1.1 antibody (Fig. 1B). This assay can accurately quantify TSAThy1.1 cells added either to cultured TSA cells or to cells obtained from TSA tumors (Supplementary Fig. S1A and S1B).

Administration of recombinant human CgA (hCgA, 0.3 μg/day, i.v.) to mice bearing both tumors (a dose that achieves physiologically relevant blood levels) reduced the number of Thy1.1<sup>+</sup> cells recovered from TSA tumors (Fig. 1C and D) but not the tumor volume and the TSA/TSAThy1.1 tumor weight ratio. These results suggested that the effect on tumor infiltration was not due to changes in the tumor masses and that hCgA can inhibit the seeding of TSAThy1.1 cells into TSA tumors.

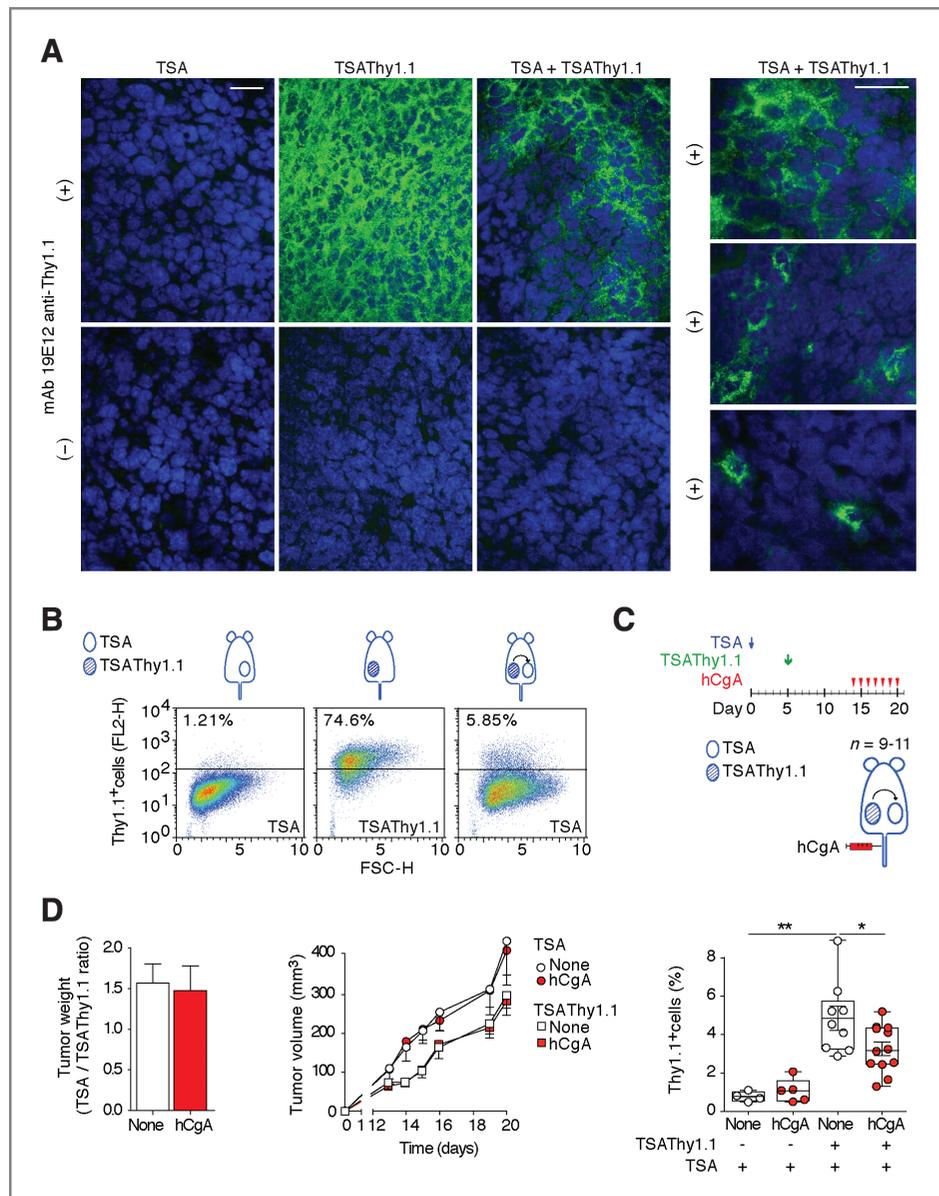
### Recombinant hCgA inhibits the seeding of tumor cells in circulation by primary tumors

To dissect the mechanism of action we studied, first, the effect of hCgA on the shedding of cancer cells in circulation by subcutaneous TSAThy1.1 tumors. To this aim, we treated tumor-bearing mice with or without hCgA and we analysed by FACS the presence of Thy1.1<sup>+</sup> cells in circulation at day 15. The results showed a reduced number of Thy1.1<sup>+</sup> cells in blood samples obtained from mice treated with hCgA, either freshly isolated or incubated for 5 days in cell culture medium containing G-418 (to select TSAThy1.1 cells genetically engineered with the pRS1Neo-Thy1.1 vector; Fig. 2A and Supplementary Fig. S2). The average number of circulating cell, expressed as the percentage of white blood cells, decreased from  $0.40 \pm 0.2\%$  to  $0.03 \pm 0.03\%$  in CgA-treated mice. We also analyzed the mRNA extracted from circulating cells at day 15 by RT-PCR with primers selective for aminoglycoside-3'-phosphotransferase (APH, a pRS1Neo-Thy1.1 sequence that is absent in the mouse genome and is present in TSAThy1.1 cells). The results showed reduced levels of APH mRNA in CgA-treated mice (Fig. 2A). These results, together, suggest that hCgA can reduce the seeding of tumor cells in circulation by primary tumors.

### Recombinant hCgA inhibits the homing of circulating tumor cells to primary tumors

To further dissect the mechanism of action of hCgA in tumor self-seeding, we then studied the effect of this protein on the homing of circulating TSAThy1.1<sup>+</sup> cells (injected into the tail

**Figure 1.** Exogenous hCgA inhibits tumor self-seeding. A and B, detection of Thy1.1<sup>+</sup> cells in tumor tissue sections obtained from mice bearing TSA or TSAThy1.1 tumors alone, or bearing both tumors, by immunohistochemistry (A) and by FACS analysis (B) with anti-Thy1.1 antibody. Immunofluorescence analysis of frozen tissue sections was done using mAb 19E12 as a primary anti-Thy1.1 antibody (3  $\mu$ g/mL) and MOM Immunodetection Kit (Vector Laboratories). Nuclei were stained with 4',6-diamidino-2-phenylindole (Molecular Probes). The right panels show magnification of different fields. Bars, 25  $\mu$ m. FACS analysis with mAb 19E12 was carried out on cells obtained after tumor excision and disaggregation. C and D, experimental design and effect of hCgA on tumor self-seeding. Mice bearing TSAThy1.1 and TSA tumors in different anatomical compartments were treated with hCgA (0.3  $\mu$ g) at the indicated time. Tumor weight and volumes (mean  $\pm$  SE) and percentage of Thy1.1<sup>+</sup> cells present in TSA tumors at day 21, as measured by FACS analysis with mAb 19E12, are shown (cumulative results of 2 independent experiments,  $n = 9-11$ ; box-plots with median, interquartile, and min/max values). \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  by  $t$  test (2-tailed).



vein of mice) to subcutaneous TSA tumors. Concomitant administration of hCgA reduced the infiltration of circulating Thy1.1<sup>+</sup> cells into TSA tumors, as measured by FACS. No effect was observed on TSA tumor growth (Fig. 2B). These results suggested that circulating hCgA can impair the infiltration of primary tumors by circulating tumor cells.

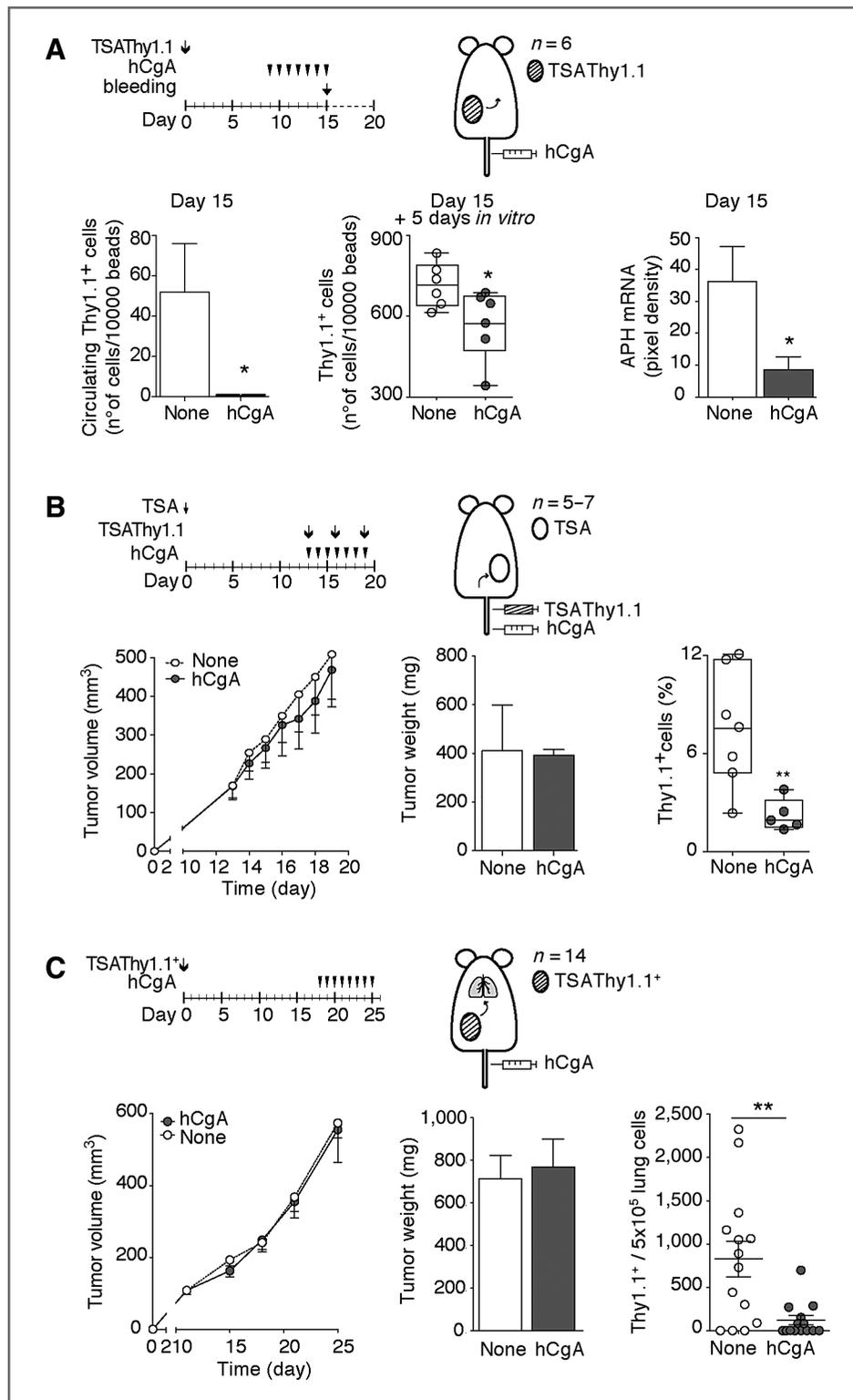
### Recombinant hCgA inhibits the dissemination of mammary carcinoma cells from primary tumors to the lungs

We then investigated whether hCgA can also inhibit the dissemination of TSAThy1.1 cells from primary tumors to the lungs of mice. TSAThy1.1 tumor-bearing mice were treated daily without or with hCgA (0.3  $\mu$ g) and the number of tumor cells that infiltrated the lungs was analyzed by FACS at day 25.

A reduced number of Thy1.1<sup>+</sup> cells was observed in the lungs of mice treated with hCgA, compared with the controls (Fig. 2C). Also in this case, the treatment did not affect the growth of primary tumors (Fig. 2C), suggesting the reduction of Thy1.1<sup>+</sup> cells present in the lungs was not caused by reduction of TSAThy1.1 cells in the primary tumors.

### Recombinant hCgA inhibits lung colonization by circulating mammary carcinoma and melanoma cells

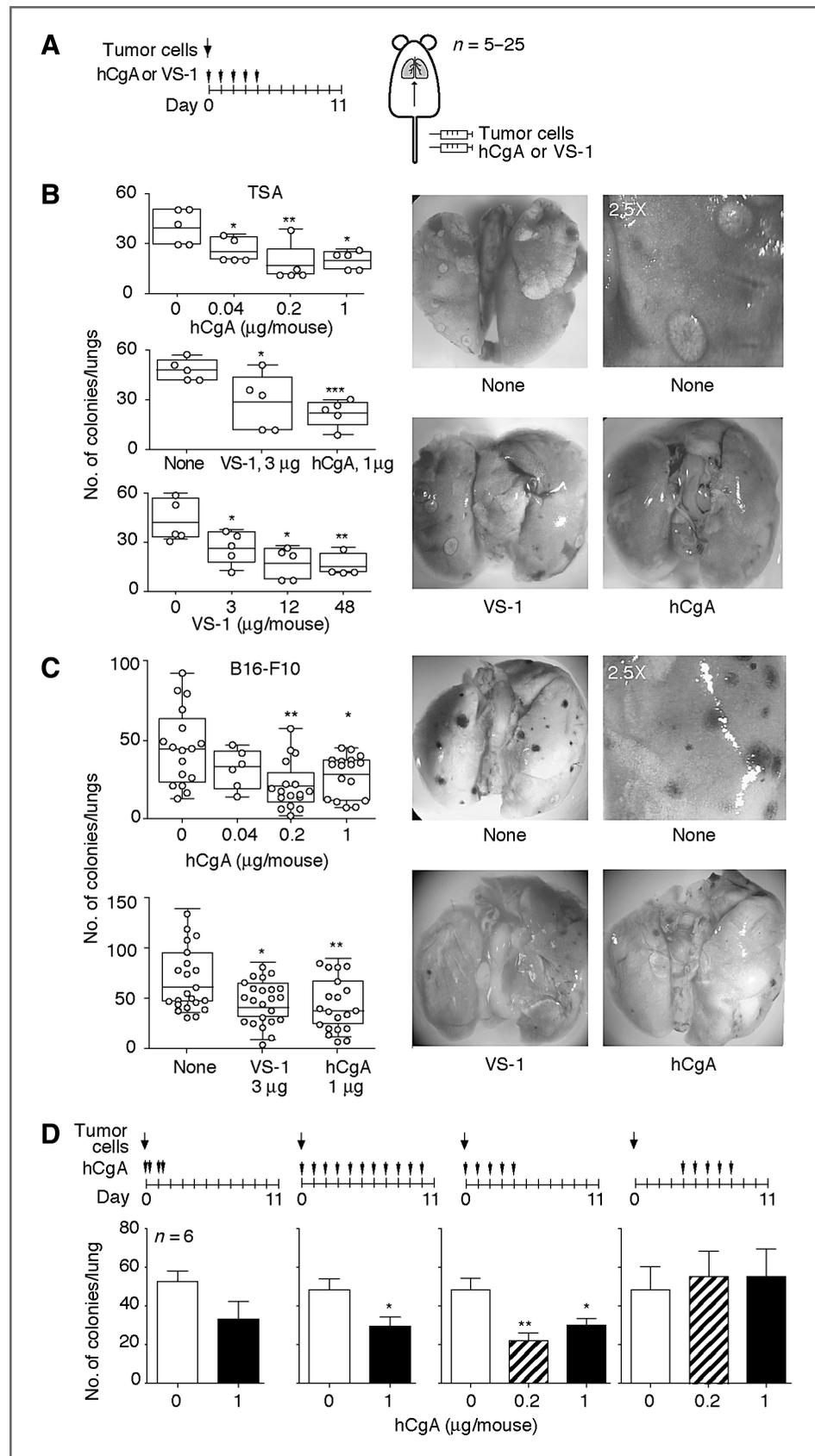
To assess whether the inhibitory effect of hCgA on tumor dissemination also involved inhibition of lung infiltration and colonization by circulating tumor cells, we studied the effect of hCgA on the formation of tumor cell colonies in the lungs of mice injected with TSA cells into the tail vein. Daily administration of hCgA (see Fig. 3A for a schematic representation of



**Figure 2.** Exogenous hCgA inhibits the bidirectional trafficking of tumor cells from subcutaneous tumors to the circulation and vice versa and the dissemination of TSAThy1.1 cells from primary tumors to the lungs. **A**, effect of hCgA on the seeding of tumor cells in circulation. Mice bearing TSAThy1.1 tumors were treated with hCgA (0.3  $\mu$ g) at the indicated time and bled at day 15. The effect of hCgA on circulating TSAThy1.1 cells was estimated by measuring (i) Thy1.1<sup>+</sup> cells present in the blood of mice by FACS (at day 15 and after 5 days in culture); the unspecific binding (checked with blood samples from normal mice) was subtracted from each sample. (ii) RT-PCR of aminoglycoside-3'-phosphotransferase (APH) mRNA (a protein encoded by the pRS1Neo-Thy1.1 vector used to generate the TSAThy1.1 cells, see Supplementary Methods). Mean  $\pm$  SE, *n* = 6. **B**, effect of hCgA on the homing of circulating tumor cells to primary tumors. TSA tumor-bearing mice were injected with TSAThy1.1 (intravenously) and treated with hCgA (0.3  $\mu$ g) as indicated. Tumor volume and weight (mean  $\pm$  SE) and percentage of Thy1.1<sup>+</sup> cells present in TSA tumors at day 20 are shown (*n* = 5-7, box plots with median, interquartile, and min/max values). **C**, TSAThy1.1 tumor-bearing mice were treated with hCgA (0.3  $\mu$ g) as indicated. Tumor volume, weight, and number of Thy1.1<sup>+</sup> cells that infiltrated the lungs are shown (mean  $\pm$  SE, cumulative results of 2 independent experiments, *n* = 14). \*, *P* < 0.05; \*\*, *P* < 0.01 by *t* test (2-tailed).

the experiment) decreased the number of TSA colonies in the lungs (Fig. 3B). To verify that this phenomenon was not a peculiarity of TSA cells, we used a similar model based on B16-F10 melanoma cells. hCgA inhibited lung colonization also in

this model (Fig. 3C). Notably, hCgA could inhibit tumor cell engraftment when mice were treated from day 0 to 1, or 0 to 4, or even 0 to 11 but not from day 4 to 9 (Fig. 3D), indicating that hCgA could affect the early steps of lung colonization, likely the



**Figure 3.** Exogenous hCgA and VS-1 reduce the number of lung colonies formed by circulating TSA mammary adenocarcinoma and B16-F10 melanoma cells. A, schematic representation of the experiment reported in (B) and (C): mice were injected with TSA or B16-F10 tumor cells (intravenously) and with CgA or VS-1 (intravenously) at the indicated time. At day 11, mice were killed and the lungs were inspected for the presence of tumor cell colonies. B and C, effect of hCgA and VS-1 on the lung colonization by TSA (B) or B16-F10 (C) cells. Cumulative results of several independent experiments ( $n = 5$  or  $6$  mice per experiment; box plots with median, interquartile, and min/max values). D, effect of various treatment schedules on lung colonization by B16-F10 cells (indicated on the top of each panel with arrows). Bars represent the mean  $\pm$  SE ( $n = 6$  mice per group). \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ , and \*\*\*,  $P < 0.001$  by  $t$  test (2-tailed).

seeding process. Administration of recombinant VS-1, the N-terminal fragment of hCgA, could also decrease the number of lung colonies in both models (Fig. 3B and C), suggesting that the N-terminal region of hCgA contains a functional site.

These results, altogether, suggested that recombinant hCgA might inhibit the dissemination of tumor cells in mice by affecting not only the tumor tissue compartment (i.e., the seeding of cancer cells into circulation and tumor-self seeding) but also by affecting the engraftment of circulating tumor cells in normal tissues.

#### **Endogenous CgA inhibits lung colonization by circulating mammary adenocarcinoma cells**

We then investigated the role of endogenous CgA on the dissemination of cancer cells in mice. To this aim, we studied the effect of the anti-hCgA mAbs 5A8, B4E11, and A11 (16, 18) on lung colonization by circulating TSA cells. Studies on antibody cross-reactivity showed that mAb 5A8, but not B4E11 and A11, could recognize mCgA (Fig. 4A). Thus, mAb B4E11 and A11, which belong to the same IgG1(k) isotype of 5A8, could represent good negative controls. Systemic administration of mAb 5A8, but not of B4E11 and A11, increased the number of lung colonies made by circulating TSA cells (Fig. 4B). These results suggested that normal levels of endogenous mCgA contribute to regulate the engraftment of circulating tumor cells in the lungs.

The effect of omeprazole, a drug known to induce the release of hCgA in circulation in patients (19, 20), was also investigated. Administration of omeprazole to mice (1 mg, i.v.) increased the circulating levels of mCgA from  $0.9 \pm 0.08$  to  $1.75 \pm 0.27$  nmol/L after 4 days (Fig. 4C). Thus, omeprazole is a valid tool for inducing a sustained increase of circulating mCgA in mice.

Repeated administration of omeprazole significantly decreased the number of lung colonies formed by TSA cells (Fig. 4D). This effect was neutralized by mAb 5A8 but not by mAb B4E11 (Fig. 4D), suggesting that it was mediated by mCgA. Thus, a moderate increase of endogenous mCgA, as obtained by pharmacologic treatment with omeprazole, can further reduce the colonization of lungs by circulating tumor cells.

#### **hCgA inhibits the transendothelial migration of mammary adenocarcinoma cells through endothelial cell monolayers**

To further investigate the mechanism of action of CgA we studied, first, the effect of hCgA on TSA cell viability, proliferation, migration, invasion, and transendothelial migration *in vitro*. hCgA did not affect the viability and the proliferation of TSA cells (Fig. 5A and B), whereas it increased the migration of these cells from the upper chamber to the lower chamber of transwell systems coated without or with Matrigel (in migration and invasion assays, respectively; Fig. 5C). No proteolytic processing of CgA occurred during the assays, as indicated by Western blot analysis of cell supernatants (Fig. 5C). Furthermore, hCgA did not alter the production of MMP-2 and MMP-9 by TSA cells, two enzymes that play important roles in tumor cell invasion (Fig. 5D). Finally, hCgA inhibited the migration of TSA cells through endothelial cell monolayers (Fig. 5E). hCgA could also inhibit the transendothelial migration of B16-F10

cells (Fig. 5E) as well as their motility in the migration and invasion assays (Supplementary Fig. S3). The reduced motility may explain the stronger inhibition observed in the transendothelial migration assay, compared with TSA cells. No proteolytic processing of CgA occurred during the assays, and no effect of hCgA on cell proliferation was observed also in the case of B16-F10 cells (Supplementary Fig. S3 A–C).

#### **Exogenous hCgA and endogenous mCgA enhance the endothelial barrier function in TSA tumors**

We have previously shown that CgA can enhance the endothelial barrier function in the liver (10). To assess whether CgA can enhance the endothelial barrier function also in tumors, which could be crucial for the regulation of tumor cell trafficking, we investigated the effect of exogenous and endogenous CgA on the penetration of the anti-Thy1.1 antibody (mAb 19E12) in TSAThy1.1 tumors. Tumor-bearing mice were pretreated with hCgA, or with the neutralizing anti-CgA mAb 5A8, and then with mAb 19E12. Tumors were excised, disaggregated, and analyzed by FACS to assess the binding of anti-Thy1.1 antibody to tumor cells. hCgA and mAb 5A8 decreased and increased, respectively, the amount of antibody molecules that reached the tumor cells, compared with controls (Fig. 6A and B). These results suggested that CgA, either exogenously administered or endogenously produced, can regulate the vascular leakage in tumors.

#### **hCgA protects the endothelial barrier against TSA cell-derived propermeabilizing factors**

To investigate whether TSA mammary adenocarcinoma can release factors capable to disrupt the endothelial barrier integrity, we carried out endothelial permeability assays with TSAThy1.1 cell supernatants (TSA-SN). TSA-SN induced VE-cadherin-dependent junction disassembly and gap formation and increased the paracellular transport of fluorescein isothiocyanate (FITC)-dextran through endothelial cell monolayers to an extent similar to that induced by TNF, a well known propermeabilizing cytokine. Notably, hCgA inhibited all these effects (Fig. 6C and D).

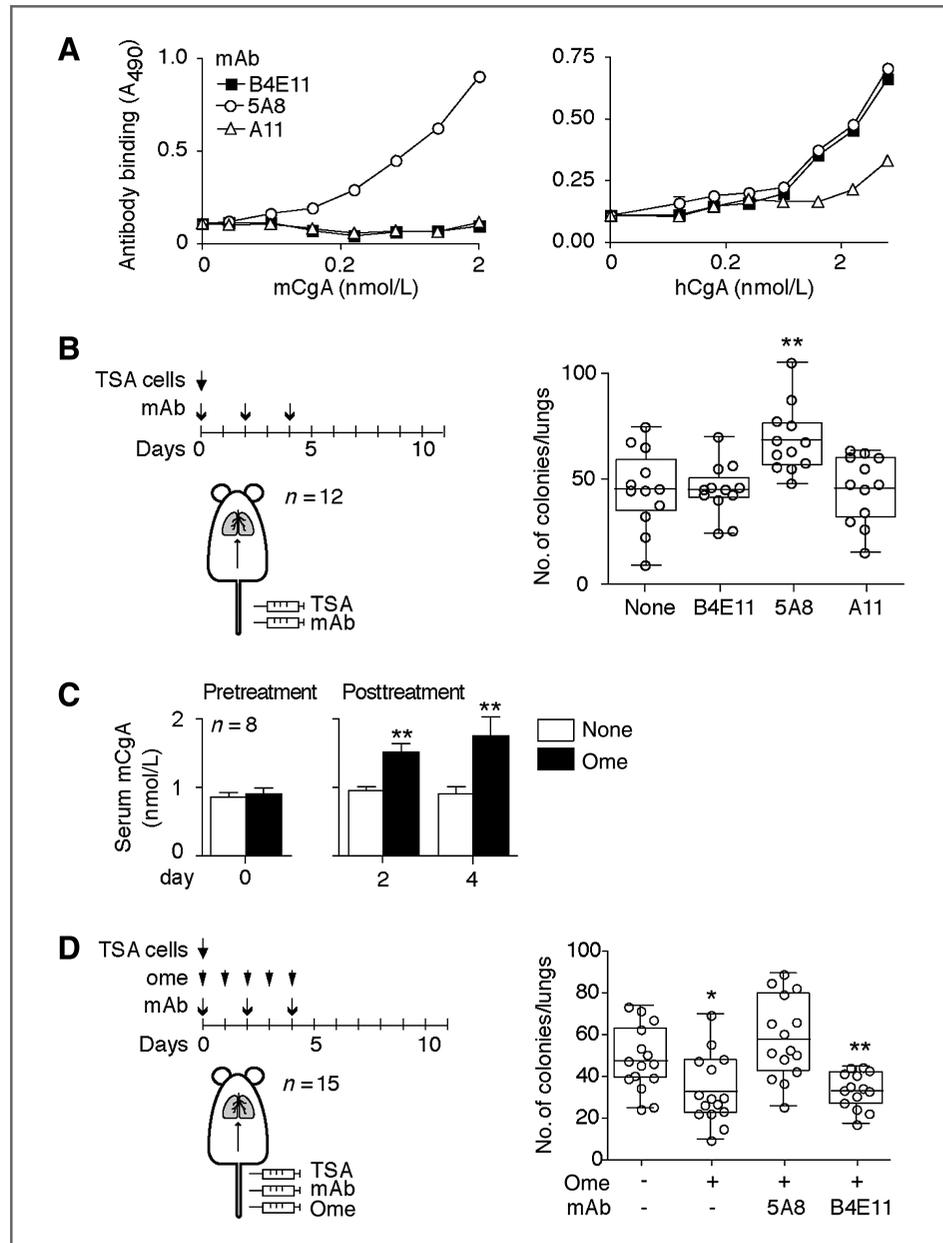
Using a multianalyte profiling approach, we then characterized the cytokines and chemokines secreted by TSAThy1.1 cells. Interestingly, various cytokines and chemokines capable to disrupt the endothelial barrier integrity were present in the cell supernatant, including TNF, VEGF, and interleukin 1 (IL-1; Supplementary Table S1). Remarkably, these cytokines were detected also in tumor tissue extracts (Supplementary Table S2).

These results suggested that an important mechanism underlying the reduced transendothelial migration of cancer cells is related to the protective effect exerted by hCgA on the endothelial barrier function.

#### **hCgA affects TNF-induced expression of proteins involved in cytoskeleton rearrangement in endothelial cells**

As changes in endothelial cell shape and cytoskeleton rearrangement have been implicated in the regulation of the endothelial barrier, we then carried out high-throughput

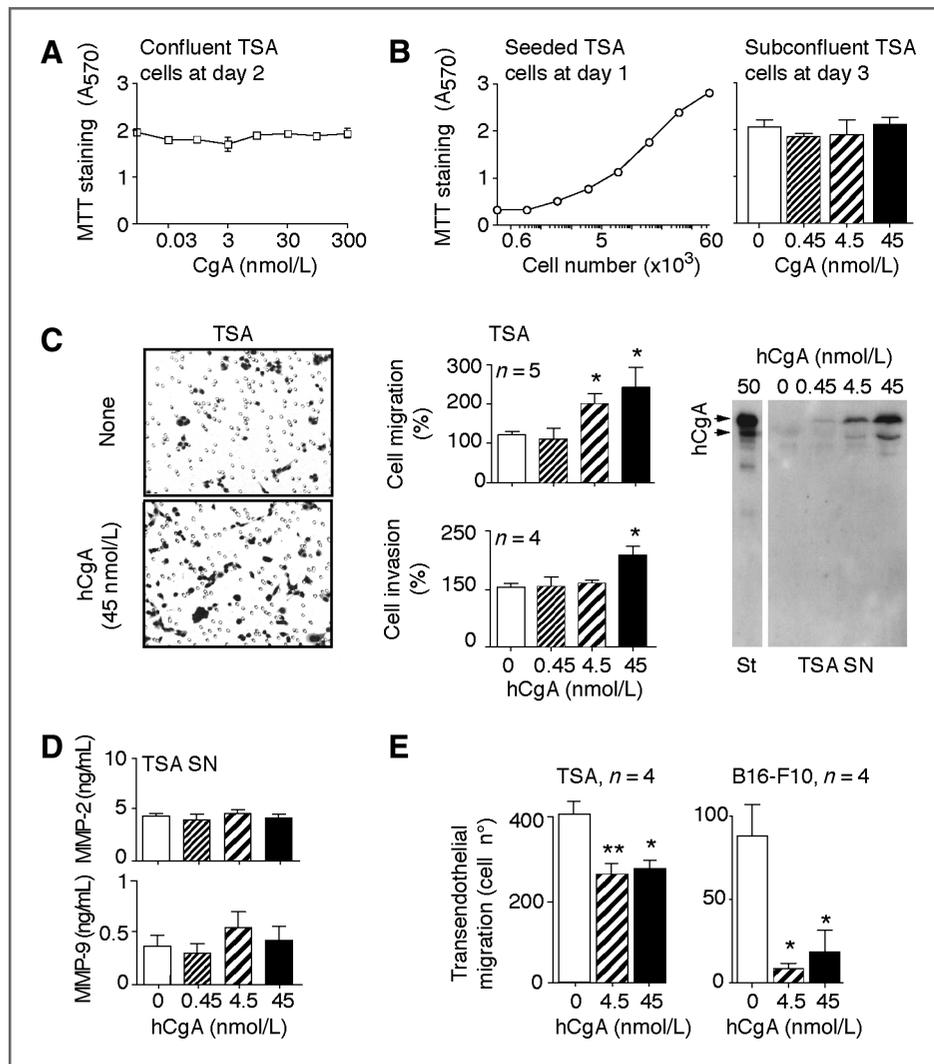
**Figure 4.** Lung colonization by TSA cells is enhanced by neutralization of endogenous mCgA with mAb 5A8 (A and B), whereas it is inhibited by induction of endogenous mCgA with omeprazole (C). A, cross-reactivity of the anti-human CgA mAbs 5A8, B4E11, and A11 with murine (left) and human (right) CgA, by ELISA. B, effect of anti-CgA mAbs on lung colonization by circulating TSA cells. Mice ( $n = 12$ ) were injected with TSA cells (intravenously) and treated with anti-CgA mAbs (10  $\mu\text{g}/\text{mouse}$ ) as indicated. The number of tumor cell colonies at day 11 is shown (cumulative results of 2 independent experiments). C, effect of daily administration of omeprazole (ome; 1 mg/mouse, intravenously) on serum mCgA (mean  $\pm$  SE,  $n = 8$ ). Mice were bled on days 0, 2, and 4 and serum was analyzed by ELISA assay. D, effect of omeprazole and anti-CgA mAbs (5A8 and B4E11) on lung colonization by TSA cells. Mice were injected with TSA cells (intravenously), omeprazole (1 mg/mouse) and mAbs (10  $\mu\text{g}/\text{mouse}$ ) as indicated. Cumulative results of 3 independent experiments ( $n = 5$  mice per experiment, total  $n = 15$ ; box plots with median, interquartile, and min/max values). \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  by  $t$  test (2-tailed).



expression profiling of endothelial cells treated with hCgA, alone or in combination with TNF. Both proteins could affect the expression of several intracellular molecules known to be involved in the regulation of the cytoskeleton (Supplementary Fig. S4; ref. 21). For example, hCgA increased the amount of phosphorylated cofilin (as also checked by Western blot analysis, Supplementary Fig. S5), whereas TNF increased phospho-ezrin, MEKK1, and several other proteins. Notably, hCgA inhibited various TNF-induced effects (Supplementary Fig. S4), suggesting that this mechanism may contribute to the inhibitory effect exerted by hCgA on endothelial barrier function.

#### hCgA inhibits the secretion of inflammatory cytokines and chemokines in the tumor microenvironment

Finally, we observed that hCgA can reduce the levels of various inflammatory cytokines and chemokines in the tumor microenvironment, such as IFN $\gamma$ , IL-1 $\alpha$ , IL-1 $\beta$ , lymphotactin, and MIP-1 $\beta$  (Supplementary Table S2). As some of these proteins may contribute to promote an inflammatory microenvironment and to increase the leakiness of vessels in tumors, the inhibition of their production in the tumor microenvironment might represent an additional mechanism for the enhancement of the endothelial barrier function by CgA.



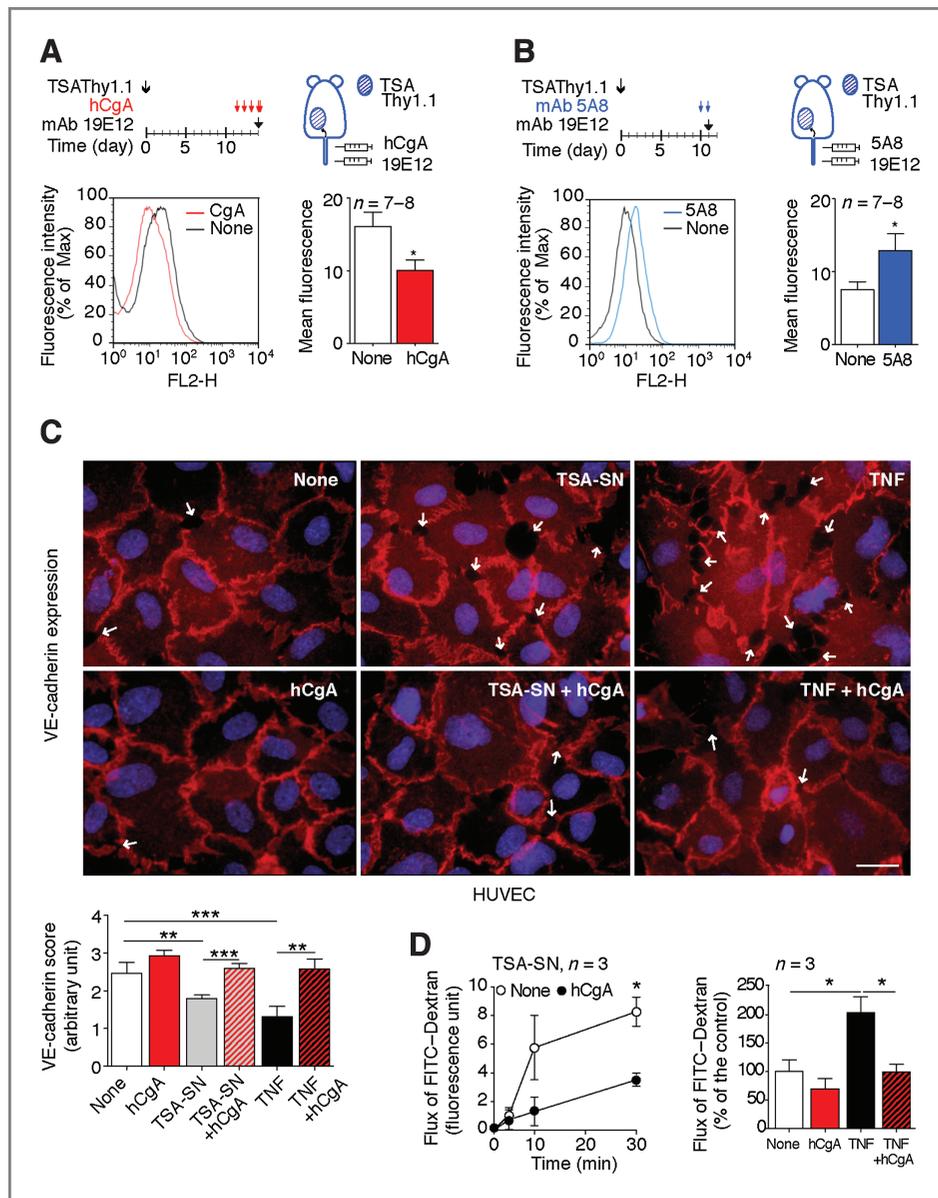
**Figure 5.** Effect of hCgA on tumor cell viability, proliferation, migration, metalloproteinase production, invasion, and transendothelial migration. **A**, effect of hCgA on TSA cell viability. **B**, effect of hCgA on TSA cell proliferation. Left: calibration curve; right: subconfluent tumor cells (5,000 cells per well) were incubated with hCgA for 76 hours and stained with MTT. Two experiments were conducted. Bars represent the mean  $\pm$  SE of 4 wells of one representative experiment. **C**, effect of hCgA on TSA cell migration and invasion. TSA cells were left to migrate through filters of transwell systems for 20 hours at 37°C in the presence of different amounts of hCgA. Cells migrated to the lower side of the filter were counted (10 random fields). Photographs of filters and quantitative data (mean  $\pm$  SE of 4 or 5 independent experiments) are shown. Western blot analysis with anti-CgA mAb B4E11 of cell supernatants recovered at the end of cell migration assay (as compared with the initially added material, St) is also shown. **D**, effect of hCgA on the production of metalloproteinase 2 (MMP-2) and MMP-9 by TSA cells. Bars represent the cumulative data (mean  $\pm$  SE) of 2 independent experiments, each conducted in triplicate. **E**, effect of hCgA on TSA and B16-F10 cell transendothelial migration (see Supplementary Methods). Each bar represent the mean  $\pm$  SE of 4 independent experiments. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  by *t* test (2-tailed).

## Discussion

The results show that circulating CgA can inhibit the dissemination of cancer cells from primary tumors to the lungs in murine models of mammary adenocarcinoma. Studies aimed at dissecting the mechanism of action have shown that CgA can inhibit (i) the shedding of cancer cells in circulation by primary tumors, (ii) the homing of circulating tumor cells to primary tumors (necessary for the self-seeding process), and (iii) the engraftment in the lungs by circulating tumor cells (another important step of the metastatic cascade). As tumor self-seeding can contribute to select more aggressive and metastatic cancer cells (3), it is possible that all these mechanisms contribute to the inhibitory effects exerted by CgA on the dissemination of cancer cells from primary TSA mammary adenocarcinoma to the lungs.

How does CgA reduce tumor self-seeding and dissemination? The doses of CgA used in our models could not affect the growth of primary tumors. Hence, the effect on metastasis formation was not related to changes in the mass

of primary tumors, pointing to other mechanisms. It has been previously shown that tumor cells can secrete factors that alter the endothelial barrier function, which may facilitate their migration across endothelial cells (6, 7). According to this view, we have observed that cultured TSA cells release soluble factors that promote (i) the disassembly of VE-cadherin from adherence junctions, (ii) the formation of intercellular gaps, and (iii) the paracellular transport of macromolecules in endothelial cell monolayers. CgA inhibited all these effects. CgA also inhibited the migration of TSA cells through endothelial cell monolayers. Multianalyte profiling of TSA cell supernatants and tumor tissue extracts showed that various cytokines, including TNF and VEGF among others, were present in TSA cell supernatants as well as in the tumor microenvironment. Remarkably, CgA could counteract the pro-metastatic activity of these cytokines. The hypothesis that CgA enhances the endothelial barrier function also *in vivo* is supported by the observation that administration of CgA to TSAThy1.1 tumor-bearing mice could inhibit the penetration of an anti-Thy1.1 antibody into tumors, whereas the



**Figure 6.** CgA inhibits vascular leakage in tumors and the endothelial barrier alteration induced by TSA cell-derived factors *in vitro*. **A** and **B**, effect of hCgA and mAb 5A8 on the penetration of the anti-Thy1.1 mAb 19E12 in subcutaneous TSAThy1.1 tumors. Tumor-bearing mice were treated with hCgA (0.3  $\mu$ g) or with mAb 5A8 (10  $\mu$ g, to neutralize mCgA) and then with mAb 19E12, as indicated. Tumors were excised 4 or 24 hours later, respectively, disaggregated and analyzed by FACS. The percentage of antibody-positive cells, as stained with Alexa Fluor 546 conjugate anti-mouse IgG, is shown (mean  $\pm$  SE,  $n = 7-8$ ). **C**, effect of CgA on VE-cadherin disassembly and gap formation (arrows) induced by TSA-SN and by TNF in confluent human umbilical vein endothelial cell (HUVEC). VE-cadherin was detected with a rabbit anti-VE-cadherin antibody and goat anti-rabbit Alexa Fluor 546 conjugate (red). Nuclei were stained with 4',6-diamidino-2-phenylindole (blue). Bar plot show the mean  $\pm$  SE of a score of VE-cadherin disassembly, obtained by evaluating the number of gaps and their size in 15 different fields (score 0, 1, 2, 3, 4:  $>20$ , 11–19, 4–10, 1–3, 0 gaps per field, respectively). Bar, 10  $\mu$ m. **D**, effect of hCgA on endothelial cell permeability induced by TSAThy1.1 cell supernatant (TSA-SN) and by TNF. The assay was carried out with transwell systems coated with HUVEC monolayers (see Supplementary Methods). The flux of FITC-dextran through the endothelial cell monolayers at various time points (left) or after 30 minutes (right) is shown. Each curve and bar represents the mean  $\pm$  SE of 3 independent experiments. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$  by *t* test (2-tailed).

neutralization of endogenous CgA with mAb 5A8 promoted the penetration of the anti-Thy1.1 antibody. These findings, altogether, suggest that CgA can reduce tumor self-seeding and dissemination by enhancing the endothelial barrier function, which may be critical for the migration of tumor cells from tumor tissue-to-blood and vice versa.

Regarding the mechanism of action, we have shown previously that the CgA fragment VS-1 can inhibit TNF-induced phosphorylation of p38-MAPK, by a pertussis toxin-sensitive mechanism, as well as VEGF-induced phosphorylation of ERK in endothelial cells (12, 22), that is, 2 signaling pathways that have been implicated in endothelial permeability (23, 24).

Furthermore, the present results of high-throughput expression profiling of endothelial cells treated with CgA, alone or in combination with TNF, suggest that CgA can affect the expression of many proteins involved in the regulation of cell cytoskeleton rearrangement, a process critical for endothelial cell shape change and vascular permeability.

The results of multianalyte profiling of the tumor microenvironment also suggest that CgA can reduce the levels of various inflammatory cytokines and chemokines (e.g., IFN $\gamma$ , IL-1 $\alpha$ , IL-1 $\beta$ , lymphotactin, and MIP-1 $\beta$ ) that may contribute to promote an inflammatory microenvironment and to increase the vascular leakage in tumors. On the basis of these observations, we think that CgA can inhibit tumor cell trafficking through endothelium by improving the endothelial barrier alterations directly, by affecting endothelial cells, and indirectly, by reducing the levels of inflammatory cytokines in tumor tissues. Notably, impairment of transendothelial cell migration and tumor cell dissemination by CgA was observed also with the B16-F10 melanoma model, suggesting that this phenomenon was not a peculiarity of TSA cells.

The biologically active doses of recombinant human CgA used in our experimental models (0.3–1  $\mu$ g, i.v.) generate circulating levels of 3 to 10 nmol/L. CgA is present in the serum of normal subjects at about 1 nmol/L levels (25). Higher circulating levels (in the nanomolar range) have been detected in patients with neuroendocrine tumors and in subpopulations of patients with breast cancer, prostate cancer, small and non-small cell lung cancer (11, 26–28), or in patients with heart failure, renal failure, hypertension, rheumatoid arthritis, giant cell arthritis, sepsis, and atrophic gastritis, or treated with proton pump inhibitors (9, 25, 26, 29, 30). Thus, the doses used in this study are biologically relevant as they are in the range of those found in patients with nonneuroendocrine tumors.

Notably, we observed that neutralization of endogenous circulating CgA with the anti-CgA mAb 5A8 increases the formation of neoplastic colonies in the lung of mice injected with TSA mammary adenocarcinoma cells, whereas the induction of circulating CgA with omeprazole decreased colony formation in a CgA-dependent manner. These results support

the hypothesis that the endogenous CgA plays a role in the regulation of tumor cell dissemination.

As discussed above, our study addresses the role of circulating CgA on metastasis formation by nonneuroendocrine tumors. The role of locally produced CgA, as it may occur in patients with neuroendocrine tumors, was not investigated in this study. Although increased circulating levels of CgA in cancer patients with advanced nonneuroendocrine tumors are associated with worse performance status, advanced stage, and worse prognosis (28), to our knowledge, no information is available on the correlation between CgA levels and circulating tumor cells or with the probability of onset of metastatic disease in patients with early-stage tumors.

In conclusion, the results of this work show that pathophysiologically relevant levels of circulating CgA (as it may occur in patients with nonneuroendocrine tumors) can regulate tumor self-seeding and dissemination of tumor cells in mice, pointing to an important role of this protein. The loss of endothelial barrier integrity caused by tumor cell-derived factors and the inhibitory effects exerted by CgA could represent important mechanisms for the positive and negative modulation of tumor self-seeding and metastatic dissemination processes. These findings suggest that drugs that directly or indirectly improve the endothelial barrier function and reduce the multidirectional trafficking of cancer cells in tumors and in normal tissues may reduce cancer progression in patients.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

#### Grant Support

This work was supported by Associazione Italiana per la Ricerca sul Cancro, and Alleanza Contro il Cancro and FIRB (Italy).

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.

Received September 3, 2011; revised November 17, 2011; accepted November 23, 2011; published OnlineFirst December 2, 2011.

#### References

- Gupta GP, Massague J. Cancer metastasis: building a framework. *Cell* 2006;127:679–95.
- Fidler IJ. The pathogenesis of cancer metastasis: the 'seed and soil' hypothesis revisited. *Nat Rev Cancer* 2003;3:453–8.
- Kim MY, Oskarsson T, Acharyya S, Nguyen DX, Zhang XH, Norton L, et al. Tumor self-seeding by circulating cancer cells. *Cell* 2009;139:1315–26.
- Norton L, Massague J. Is cancer a disease of self-seeding? *Nat Med* 2006;12:875–8.
- Al-Mehdi AB, Tozawa K, Fisher AB, Shientag L, Lee A, Muschel RJ. Intravascular origin of metastasis from the proliferation of endothelium-attached tumor cells: a new model for metastasis. *Nat Med* 2000;6:100–2.
- Khanna P, Yunkunis T, Muddana HS, Peng HH, August A, Dong C. p38 MAP kinase is necessary for melanoma-mediated regulation of VE-cadherin disassembly. *Am J Physiol Cell Physiol* 2010;298:C1140–50.
- Weis S, Cui J, Barnes L, Cheresh D. Endothelial barrier disruption by VEGF-mediated Src activity potentiates tumor cell extravasation and metastasis. *J Cell Biol* 2004;167:223–9.
- Van Sluis GL, Niers TM, Esmon CT, Tigchelaar W, Richel DJ, Buller HR, et al. Endogenous activated protein C limits cancer cell extravasation through sphingosine-1-phosphate receptor 1-mediated vascular endothelial barrier enhancement. *Blood* 2009;114:1968–73.
- Helle KB, Corti A, Metz-Boutigue MH, Tota B. The endocrine role for chromogranin A: a prohormone for peptides with regulatory properties. *Cell Mol Life Sci* 2007;64:2863–86.
- Ferrero E, Scabini S, Magni E, Foglieni C, Belloni D, Colombo B, et al. Chromogranin A protects vessels against tumor necrosis factor alpha-induced vascular leakage. *FASEB J* 2004;18:554–6.
- Corti A. Chromogranin A and the tumor microenvironment. *Cell Mol Neurobiol* 2010;30:1163–70.
- Blois A, Srebro B, Mandalà M, Corti A, Helle KB, Serck-Hanssen G. The chromogranin A peptide vasostatin-I inhibits gap formation and signal transduction mediated by inflammatory agents in cultured bovine pulmonary and coronary arterial endothelial cells. *Reg Pept* 2006;135:78–84.
- Colombo B, Curnis F, Foglieni C, Monno A, Arrighoni G, Corti A. Chromogranin A expression in neoplastic cells affects tumor

- growth and morphogenesis in mouse models. *Cancer Res* 2002;62:941–6.
14. Moro M, Pelagi M, Fulci G, Paganelli G, Dellabona P, Casorati G, et al. Tumor cell targeting with antibody-avidin complexes and biotinylated tumor necrosis factor alpha. *Cancer Res* 1997;57:1922–8.
  15. Corti A, Sanchez LP, Gasparri A, Flavio C, Longhi R, Brandazza A, et al. Production and structure characterization of recombinant chromogranin A N-terminal fragments (vasostatins): evidence of dimer-monomer equilibria. *Eur J Biochem* 1997;248:692–9.
  16. Corti A, Longhi R, Gasparri A, Chen F, Pelagi M, Siccardi AG. Antigenic regions of human chromogranin A and their topographic relationships with structural/functional domains. *Eur J Biochem* 1996;235:275–80.
  17. Gasparri A, Sidoli A, Sanchez LP, Longhi R, Siccardi AG, Marchisio PC, et al. Chromogranin A fragments modulate cell adhesion. Identification and characterization of a pro-adhesive domain. *J Biol Chem* 1997;272:20835–43.
  18. Ratti S, Curnis F, Longhi R, Colombo B, Gasparri A, Magni F, et al. Structure-activity relationships of chromogranin A in cell adhesion. Identification and characterization of an adhesion site for fibroblasts and smooth muscle cells. *J Biol Chem* 2000;275:29257–63.
  19. Sanduleanu S, Stridsberg M, Jonkers D, Hameeteman W, Biemond I, Lundqvist G, et al. Serum gastrin and chromogranin A during medium- and long-term acid suppressive therapy: a case-control study. *Aliment Pharmacol Ther* 1999;13:145–53.
  20. Giusti M, Sidoti M, Augeri C, Rabitti C, Minuto F. Effect of short-term treatment with low dosages of the proton-pump inhibitor omeprazole on serum chromogranin A levels in man. *Eur J Endocrinol* 2004;150:299–303.
  21. Pavlidis P, Noble WS. Matrix2png: a utility for visualizing matrix data. *Bioinformatics* 2003;19:295–6.
  22. Belloni D, Scabini S, Foglieni C, Veschini L, Giazzon A, Colombo B, et al. The vasostatin-I fragment of chromogranin A inhibits VEGF-induced endothelial cell proliferation and migration. *FASEB J* 2007;21:3052–62.
  23. Breslin JW, Pappas PJ, Cerveira JJ, Hobson RW 2nd, Duran WN. VEGF increases endothelial permeability by separate signaling pathways involving ERK-1/2 and nitric oxide. *Am J Physiol Heart Circ Physiol* 2003;284:H92–100.
  24. Koss M, Pfeiffer GR 2nd, Wang Y, Thomas ST, Yerukhimovich M, Gaarde WA, et al. Ezrin/radixin/moesin proteins are phosphorylated by TNF-alpha and modulate permeability increases in human pulmonary microvascular endothelial cells. *J Immunol* 2006;176:1218–27.
  25. Ceconi C, Ferrari R, Bachetti T, Opasich C, Volterrani M, Colombo B, et al. Chromogranin A in heart failure; a novel neurohumoral factor and a predictor for mortality. *Eur Heart J* 2002;23:967–74.
  26. O'Connor DT, Bernstein KN. Radioimmunoassay of chromogranin A in plasma as a measure of exocytotic sympathoadrenal activity in normal subjects and patients with pheochromocytoma. *New Engl J Med* 1984;311:764–70.
  27. Portela-Gomes GM, Grimelius L, Wilander E, Stridsberg M. Granins and granin-related peptides in neuroendocrine tumours. *Regul Pept* 2010;165:12–20.
  28. Gregorc V, Spreafico A, Floriani I, Colombo B, Ludovini V, Pistola L, et al. Prognostic value of circulating chromogranin A and soluble tumor necrosis factor receptors in advanced nonsmall cell lung cancer. *Cancer* 2007;110:845–53.
  29. Pieroni M, Corti A, Tota B, Curnis F, Angelone T, Colombo B, et al. Myocardial production of chromogranin A in human heart: a new regulatory peptide of cardiac function. *Eur Heart J* 2007;28:1117–27.
  30. Taupenot L, Harper KL, O'Connor DT. The chromogranin-secretogranin family. *New Engl J Med* 2003;348:1134–49.

# Cancer Research

The Journal of Cancer Research (1916–1930) | The American Journal of Cancer (1931–1940)

## Chromogranin A Regulates Tumor Self-Seeding and Dissemination

Eleonora Dondossola, Luca Crippa, Barbara Colombo, et al.

*Cancer Res* 2012;72:449-459. Published OnlineFirst December 2, 2011.

**Updated version** Access the most recent version of this article at:  
doi:[10.1158/0008-5472.CAN-11-2944](https://doi.org/10.1158/0008-5472.CAN-11-2944)

**Supplementary Material** Access the most recent supplemental material at:  
<http://cancerres.aacrjournals.org/content/suppl/2011/12/02/0008-5472.CAN-11-2944.DC1>

**Cited articles** This article cites 30 articles, 15 of which you can access for free at:  
<http://cancerres.aacrjournals.org/content/72/2/449.full.html#ref-list-1>

**Citing articles** This article has been cited by 2 HighWire-hosted articles. Access the articles at:  
</content/72/2/449.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](mailto: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](mailto:permissions@aacr.org).