SNAI1 Is Required for Tumor Growth and Lymph Node Metastasis of Human Breast Carcinoma MDA-MB-231 Cells

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Abstract

The transcription factor, SNAI1 (Snail), has recently been proposed as an important mediator of tumor invasion because of its role in E-cadherin down-regulation and induction of epithelial-mesenchymal transition. In human breast cancer, the expression of SNAI1 and/or the homologous SNAI2 (Slug) has been associated with E-cadherin repression, local or distant metastasis, tumor recurrence, or poor prognosis in different tumor series. However, the specific contribution of either factor to breast tumor progression is still unclear. We have analyzed the role of SNAI1 in human breast cancer by loss of function studies and provide evidence of a major role for SNAI1 in both primary tumor growth and metastasis of human breast carcinoma MDA-MB-231 cells. Specific silencing of SNAI1 by short hairpin RNA induces a decrease in mesenchymal and proinvasive markers (MMP9, ID1, SPARC) in MDA-MB-231 cells, concomitant with reduced in vitro invasive behavior. More importantly, stable SNAI1 silencing in MDA-MB-231 cells leads to a dramatic reduction of in vivo tumor incidence and growth rate. Tumors induced by MDA-MB-231-SNAI1–silenced cells show extensive necrotic regions and a significant decrease in invasive and angiogenic markers. Moreover, SNAI1 silencing increases the sensitivity of MDA-MB-231 cells to chemotherapeutics relevant in breast cancer treatments, gemcitabine and docetaxel. Remarkably, analysis of cell lines derived from lymph node metastasis indicates that SNAI1 expression is required for metastatic dissemination. [Cancer Res 2007;67(24):11721–31]

Introduction

Local tumor invasion represents the first step of the metastatic cascade of carcinomas. The invasion of carcinoma cells requires profound changes in the cell adhesion, polarity, and migratory properties of tumor cells, collectively known as epithelial-mesenchymal transition (EMT). Loss of functional E-cadherin is an essential event for EMT and is considered a hallmark of the process (1, 2). Similar to the situation occurring during development, EMT is thought to be a dynamic process during tumor progression, operating at specific stages such as local invasion and intravasation, with potential reversion by the converse mesenchymal-epithelial transition (MET) process (1–3). Huge efforts made in the last few years has led to the characterization of several E-cadherin transcriptional repressors (3). Between them, two members of the Snail family of transcription factors, SNAI1 (also known as Snail) and SNAI2 (also known as Slug; ref. 4), have been characterized as strong E-cadherin repressors and major EMT inducers (5–7) both during embryonic development and tumor progression (1, 4, 8). Accumulated evidence has revealed that Snail factors have additional functions beyond E-cadherin repression and EMT induction (reviewed in ref. 4). SNAI1 could act as a cell cycle regulator through the repression of cyclin D2 and induction of p21 expression (9), although apparently in a cell type/context-dependent manner (10). Additionally, Snail family members have been characterized as antiapoptotic molecules. Snail-expressing cells survive serum deprivation and are resistant to apoptosis induced by proapoptotic stimuli or genotoxic agents (9, 11). Snai2 expression also gives hematopoietic progenitors radioresistance to cell death (12, 13). All these findings support the fact that Snail genes could act primarily as survival factors and inducers of cell movement, apart from their role in EMT (4).

The last few years have also experienced mounting interest for the role of different EMT inducers in human tumors. Although the results of expression analyses need to be interpreted with care because of the paucity of specific reagents, SNAI1 and/or SNAI2 expression has been detected in an increasing number of carcinomas, synovial sarcomas, and melanomas (reviewed in ref. 3), in many cases, associated with E-cadherin repression, lymph node status, and/or distant metastasis. In breast carcinomas, SNAI1 expression is associated with E-cadherin repression, metastasis, and/or poor clinical outcome (14–18). Likewise, the expression of SNAI2 has been associated with poor clinical outcome in breast and ovarian tumors (18, 19) and correlated with a partially differentiated phenotype in breast carcinoma, suggesting that SNAI1 and SNAI2 might have different effects on the individual or collective invasive behavior of breast cancers (17). Significantly, SNAI1 has been recently implicated in tumor recurrence in breast cancer mouse models and, importantly, microarray data set analysis of human breast cancers correlated elevated SNAI1 expression with decreased relapse-free survival (20). Notwithstanding, a direct role for SNAI1 in tumor progression of breast cancer and its relation with EMT or other Snail-associated functions has yet to be established.

To address this issue, we have performed loss of function studies in the highly undifferentiated human breast carcinoma MDA-MB-231 cell line, using stable SNAI1 interference. We present evidence that SNAI1 knockdown significantly reduces the invasive phenotype at the same time that increases the chemosensitivity
of MDA-MB-231 cells to clinically relevant chemotherapeutics in breast cancer, gemcitabine and docetaxel. More importantly, in vivo studies indicate that SNAI1 silencing dramatically reduces tumor occurrence and the tumor growth potential of MDA-MB-231 cells. In addition, analyses of distant lymph nodes and derived cell lines support a direct role for SNAI1 in tumor dissemination. Together, these data strongly support a major role for SNAI1 in breast cancer tumor growth and metastasis.

Materials and Methods

Cell culture. MDA-MB-231 cells and their derived cell lines were grown in DMEM (Life Technologies) supplemented with 10% fetal bovine serum, 2 mmol/L of l-glutamine, and antibiotics at 37°C in a humidified 5% CO2 atmosphere.

Generation of expression vectors and stable cell lines. The generation of pcDNA3-Sna1-HA vector has been previously described (21). pcDNA3-mutS-Sna1-HA was constructed using pcDNA3-Sna1-HA as a template for site-directed mutagenesis following standard protocols. The oligonucleotides used for amplification carrying five silent point mutations were 5'-TG CAC ATG TCG CAC AGC GTG CTT GTC ACC TG-3' and 5'-AG CTG GTG CGA CGT CAT GT CAT CTT CAC GGC CAG GCT G-3'. The generation of short hairpin RNAs (shRNA), containing specific oligonucleotide sequences against EGFP or mouse/human Snai1, cloned into the pSuperior-Puro vector (Oligoengine), has been recently described (22). All transfections were performed using LipofectAMINE 2000 (Life Technologies). pSuperior vectors (shEGFP and shSNAI1) were transfected in MDA-MB-231 cells and selection performed with 1 µg/mL of puromycin for 2 to 4 weeks. pcDNA3-mutS-Sna1-HA was transfected in MDA-MB-231-shSNAI1-C2/C4 cell lines and selected with 400 µg/mL of G418 for 4 to 6 weeks. Ten clones were isolated after shRNA transfection and individually characterized, or collected as pooled clones in control transfections.

Reverse transcription-PCR and quantitative RT-PCR. Total RNA was isolated from the different cell lines and reverse transcription-PCR (RT-PCR) analyses were performed as previously described, using specific primers for human/mouse Sna1, human SNAI2, and GAPDH (6, 7). Specific primers for human SNAI2 are provided in the Supplemental Material. RT-PCR of tumors was performed as recently described (21). For quantitative RT-PCR (qRT-PCR), cDNA from cells and tumors was synthesized using the High-Capacity cDNA Archive Kit (Applied Biosystems). Specific primers for qRT-PCR analysis are indicated in the Supplemental Material. qRT-PCR reactions were performed as described (10) with internal normalization to 18s rRNA. Three replicates from each cDNA were analyzed.

The data are presented relative to mRNA levels of control shEGFP cells.

Cell extracts and Western blot analysis. Preparation of whole cell extracts and Western blot analysis with the appropriate antibodies was performed as described (6, 7, 10). The primary antibodies used are indicated in the Supplemental Material.

Gelatin zymography. Gelatin zymography was performed essentially as described (10, 22) on 24-h conditioned media of confluent cultures from the indicated cell lines. The gels were finally stained with Coomassie brilliant blue R250, and the gelatinolytic activities detected as clear bands against a blue background.

Invasion assays. Invasion assays on modified Boyden chambers (0.8 µm pore filters) coated with collagen type IV gel were performed as previously described (6, 10), starting with 1 × 105 seeded cells, and counting of cells into the lower part of the filter 24 h after seeding.

Tumorigenesis, spontaneous metastasis assays, and obtainment of lymph node-derived cell lines. Parental human MDA-MB-231 cells and derived clones from subconfluent cultures were orthotopically injected (1 × 106 in 0.05 mL serum-free growth medium) into the left fifth mammary fat pad of female BALB/c immunocompromised mice aged 8 weeks (Charles River). The growth of tumors was measured every 2 days by the determination of two orthogonal external diameters using a caliper. When tumors reached a size of 0.4 cm3, they were surgically excised and processed for histology, immunofluorescence, and RT-PCR analysis. A minimum of 10 tumors from each cell line were generated and, at least 4 different tumors derived from each cell line were analyzed. For spontaneous metastasis assay, primary tumors were excised when they reached a volume of 0.15 cm3. Afterwards, mice were kept alive for an additional 6 months and then sacrificed. The contralateral lymph nodes were then surgically removed, carefully dissected, and cultured for 3 to 4 weeks in the presence of puromycin (except for those derived from animals injected with parental cells) to select for antibiotic-resistant human MDA-MB-231 cells. Mice were housed and maintained under specific pathogen-free conditions and used in accordance with institutional guidelines approved by the Use Committee for Animal Care.

Histologic, immunohistochemical, RT-PCR, and terminal nucleotidyl transferase-mediated nick end labeling analyses of primary tumors. Histologic, immunostaining, and RT-PCR analyses of tumors were basically performed as previously described (10, 21) on paraffin and/or frozen sections or tumor fragments. Antibodies used in immunostaining are indicated in the Supplemental Material. For DNA fragmentation analysis, paraformaldehyde-fixed cryostat sections were analyzed by the terminal nucleotidyl transferase-mediated nick end labeling method using the In situ Cell Death Detection kit (Roche) according to the manufacturer's protocol.

Drug sensitivity assays. Drug sensitivity was analyzed with the minimal drug concentration inducing 80% reduction of cellular growth compared with nontreated cells (IC80). Exponentially growing cells were treated with 100 µmol/L of docetaxel (Taxotere, Aventis Pharma, S.A.; ref. 23) or 6 ± 2 µmol/L of gemcitabine (Gemzar, Lilly S.A.; ref. 24) for 48 h. Treated and control cells were then trypsinized, and after centrifugation and washing twice in PBS, stained with Annexin V-FITC/propidium iodide using the Annexin V/FITC kit (MBL International) according to the manufacturer's instructions. Stained cells were analyzed by FASCAN cytometry. Results show the mean ± SD of three independent assays.

Results

Stable SNAI1 silencing in MDA-MB-231 cells increases E-cadherin transcripts and decreases the expression of mesenchymal markers. To analyze the role of SNAI1 in the tumor growth and metastasis of breast carcinomas, we stably silenced SNAI1 in dedifferentiated human MDA-MB-231 cells as highly tumorigenic and weakly metastatic (25), expressing high levels of SNAI1 and SNAI2, and lacking E-cadherin (Fig. 1A and C; data not shown). In order to obtain stable SNAI1-silenced clones, MDA-MB-231 cells were transfected with a pSuperior-shSNAI1 vector designed to recognize human/mouse Snai1 mRNAs, but not the SNAI1L pseudogene transcript (26), and probed to efficiently silence Sna1 in mouse carcinoma cells (10). After drug selection, at least 10 clones were isolated and characterized for SNAI1 expression; between them, clones MDA-MB-231-shSNAI1-C2 and MDA-MB-231-shSNAI1-C4 (hereafter called shSNAI1-C2 and shSNAI1-C4) were selected as the most representative (Fig. 1A and B). As a control, an RNAi-resistant form of mouse Snai1 (mutS; see Supplementary Fig. S1A) was stably transfected into shSNAI1-C2 and shSNAI1-C4 clones. After drug selection, cells were pooled (shSNAI1-C2+mutS, shSNAI1-C4+mutS). As an additional control, an irrelevant shRNA against EGFP was stably transfected in MDA-MB-231 cells (shEGFP cells). Stable expression of shSNAI1 leads to almost complete inhibition of SNAI1 expression at both mRNA and protein levels in MDA-MB-231 cells without significantly affecting SNAI2 expression (Fig. 1A–C). Overexpression of the RNAi-resistant mutS-Sna1 in shSNAI1-C2+mutS and shSNAI1-C4+mutS cells led to similar or even higher levels of SNAI1 expression than those of parental MDA-MB-231 cells (Fig. 1A–C). Due to the high similarity of the mouse and human Snai1 genes (4, 8), the mutS-Sna1 allele is detected in the PCR and qRT-PCR
reactions using oligonucleotides against human SNAI1 (Fig. 1A and B, top and left, respectively), as well as by cross-reaction of SNAI1 antibodies (Fig. 1C, top), accounting for the increased SNAI1 levels in cells carrying the mutS-SnaI allele. The expression and nuclear localization of the mutS-SnaI protein in overexpressing clones was also confirmed by immunofluorescence (Supplementary Fig. S1, HA staining). Up-regulation of hsNAI2 mRNA was also observed after reexpression of the mutS-SnaI allele (Fig. 1B, middle); a fact that could not be attributed to a mispriming effect, as confirmed by product analyses (data not shown). Rather, this observation suggests the existence of cross-regulation between Snai1 factors, as supported by up-regulation of the hsNAI2 promoter by Snai1. Because SNAI1 is a bona fide EMT inducer (6), the effect of SNAI1 silencing on epithelial (E-cadherin) and mesenchymal markers (fibronectin and vimentin) was analyzed. Stable SNAI1 knockdown in MDA-MB-231 cells induced a modest increase in E-cadherin mRNA levels up to 2.5-fold (Fig. 1B, right) and, importantly, this effect was fully reverted by overexpression of the mutS-SnaI allele (Fig. 1B, compare hE-CD levels from shSNAI1-C2 and -C4 with shSNAI1-C2+mutS and -C4+mutS, respectively). Despite the increased transcripts, E-cadherin protein expression could not be detected in the SNAI1-interfered clones (data not shown). In contrast, a strong decrease in the expression and organization of the mesenchymal marker fibronectin was observed in shSNAI1-C2 and -C4 clones; an effect also suppressed by the overexpression of mutS-Sna1 (Fig. 1C; Supplementary Fig. S2). Reduced vimentin levels in shSNAI1 cells were only detected by Western blotting (Fig. 1C). Taken together, these results suggest that SNAI1 silencing induces a partial MET in MDA-MB-231 cells.

SNAI1 silencing inhibits the invasive behavior of MDA-MB-231 cells. To study the effect of SNAI1 silencing in the invasive behavior of MDA-MB-231 cells, we first analyzed the expression levels of recently described Snai1 target genes involved in invasion and metastasis such as MMP2, SPARC, or ID1 and the related ID2 factor (25, 27–29). SNAI1 silencing in MDA-MB-231 cells induces a strong decrease in SPARC and ID1 transcripts (Fig. 2A) and total protein levels (Fig. 2B), in agreement with previous reports supporting a direct relation between SPARC and SNAI1 in melanoma progression (30, 31). In contrast, ID2 mRNA levels strongly increased after SNAI1 silencing, in concordance with the reported role of ID2 in the maintenance of a differentiated, noninvasive phenotype in breast cells (29). Importantly, the effects of SNAI1 silencing on the expression of SPARC and ID genes were fully reverted after overexpression of the mutS-SnaI allele (Fig. 2A and B). On the other hand, transcript levels of another SNAI1 target, MMP2 (32, 33), do not vary after SNAI1 silencing or overexpression of mutS-Sna1 (Fig. 2A, top), probably because MMP2 levels in parental MDA-MB-231 cells are very low (Fig. 2C). In contrast, MDA-MB-231 cells exhibit high levels of MMP9 activity, another SnaI target (22). MMP9 activity was strongly reduced by SNAI1 silencing and fully reverted by reexpression of the mutS-SnaI allele (Fig. 2C). These results support the specific effect of SNAI1 silencing on SPARC, ID1, and MMP9 regulation.

To address if the molecular changes observed after SNAI1 silencing in MDA-MB-231 cells influence the invasive phenotype, in vitro invasion assays were performed. SNAI1 silencing dramatically reduced the ability of MDA-MB-231 cells to migrate into collagen IV matrices, and >50% reversion was achieved after

E. Cubillo, H. Peinado, and A. Cano, unpublished results.

Figure 1. SNAI1 silencing induces reexpression of E-cadherin and down-regulation of mesenchymal markers. Characterization of the clones obtained after stable transfection of shSNAI1 in MDA-MB-231 cells. A, RT-PCR analyses of human SNAI1 (top) and mouse Snai1 (middle) mRNA levels in parental MDA-MB-231 cells, two stable clones generated after shSNAI1 transfection (shSNAI1-C2 and shSNAI1-C4) and two stable cell lines generated after expression of a RNAi-resistant Snai1 (mutS) in shSNAI1-C2 and shSNAI1-C4 cells (shSNAI1-C2+mutS, shSNAI1-C4-mutS). MDA-MB-231 cells stably transfected with shEGFP are included as controls. GAPDH mRNA levels (bottom) are charge controls. B, qRT-PCR analysis of the mRNA levels of human SNAI1 (left), human SNAI2 (middle), and human E-cadherin (hE-CD; right) of the cell lines described above. Transcript levels in the different cell types were normalized to shEGFP mRNA levels. Columns, mean of three replicates obtained from each cDNA sample; bars, SD. C, Western blot analysis of the protein levels of the human/mouse Snai1, human SNAI2, and the mesenchymal markers fibronectin and vimentin in independent clones and control cells as in A and B. Western blot of α-tubulin is shown as a loading control.
mutS-Snai1 reexpression in the SNAI1-silenced clones (Fig. 2D), confirming that SNAI1 silencing decreases the invasive phenotype of MDA-MB-231 cells.

SNAI1 interference dramatically decreases tumorigenicity of MDA-MB-231 cells. To get further insights into the biological relevance of SNAI1 interference, we analyzed its effect on the tumorigenic properties of MDA-MB-231 cells. Two independent SNAI1-silenced clones (shSNAI1-C2/C4) and derived cells after mutS-Snai1 expression (C2+mutS/C4+mutS) were orthotopically injected into the mammary fat pad of nude mice, in parallel with

Figure 2. SNAI1 silencing decreases the invasive capabilities of MDA-MB-231 cells. A, qRT-PCR analysis of the mRNA levels of human MMP2, SPARC, ID1, and ID2 in parental MDA-MB-231, control shEGFP cells, stable shSNAI1 clones (shSNAI1-C2 and -C4), and stable cell lines obtained after reexpression of the mutS-Snai1 into shSNAI1-C2 and -C4 cells. B, Western blot analysis of total protein levels of human ID1 and SPARC in the independent cell lines, as in A. Western blot of α-tubulin is shown as a loading control. C, Zymography assay of MMP9-secreted activity performed on the conditioned medium of cells described in A. MMP2 activity is shown as control. D, analysis of the invasive phenotype of the cell lines in transwell filters coated with collagen type IV matrix. Cells were plated above the matrix and allowed to migrate for 24 h. After this time, the cells which migrated into the lower chamber were trypsinized and counted. Columns, mean of three independent assays; bars, SD. **, *P < 0.01, ANOVA analysis.
parental and control MDA-MB-231-shEGFP cells. As shown in Fig. 3A, the incidence of primary tumors induced by parental and control MDA-MB-231-shEGFP, stable SNAI1–interfered clones (C2 and C4), and stable cell lines obtained after reexpression of mutS-Sna1. The cell lines were injected into the mammary fat pad of 8-week-old female nude mice. Incidence is represented as the percentage of the induced tumors per mice at 2 mo postinjection. B, analysis of the tumor growth potential (tumor latency and growth rate) of the cell lines above after their orthotopic injection into the mammary fat pad of nude mice. Points, mean of two independent assays performed with five mice per cell line; bars, SD. *** P < 0.001, ANOVA analysis. C, RT-PCR analysis of human SNAI1 and SNAI2 and mouse Snai1 expression performed on RNA samples isolated from individual tumors generated by the cell lines indicated above. Two tumors generated from each indicated cell type are shown. GAPDH mRNA levels are included as a loading control. D, qRT-PCR analysis of the mRNA levels of human E-cadherin, SPARC, ID1, and ID2 performed on RNA samples isolated from individual tumors generated by the clones indicated above. Columns, mean of cDNAs extracted from two independent tumors for each clone; bars, SD.

Furthermore, a dramatic reduction in the growth rate of tumors induced by SNAI1-silenced cells was detected (Fig. 3B). More than 95% reduction in the volume of shSNAI1-C2 and C4 xenografts was observed at 40 days postinjection, together with a 2-fold increase in tumor latency (days to reach a tumor size of 0.1 cm³), compared
with tumors induced by parental and control cells. Importantly, both the tumor incidence and tumor growth rate were almost completely recovered after reexpression of the mutS-Snai1 allele (Fig. 3A and B, compare shSNAI1-C2 or shSNAI1-C4 with C2+mutS and C4+mutS, respectively, and with control cells).

RT-PCR analyses of the xenografts indicated that SNAI1 transcripts remained silenced in all tumors derived from shSNAI1 clones, whereas no significant changes were observed in the expression of the homologous SNAI2 gene (Fig. 3C). Indeed, SNAI2 silencing in MDA-MB-231 cells does not reduce tumor incidence or tumor growth rate (data not shown), further supporting a specific effect of SNAI1 silencing in the tumorogenic behavior of breast carcinoma MDA-MB-231 cells. Interestingly, an increase of up to 6-fold in E-cadherin mRNA levels and strong decrease in SPARC transcripts were detected in shSNAI1-C2/-C4 xenografts (Fig. 3D, top). Similar to the behavior observed in vitro, those expression changes were abrogated in the xenografts from shSNAI1-mutS-Sna1 cells (Fig. 3D, top). Expression of ID2 in tumors was similar to that observed in culture with up to 1.8-fold mRNA increase detected in tumors from shSNAI1-silenced cells (Fig. 3D). Surprisingly, the expression of ID1 in tumors was inverse to the pattern observed in culture (Fig. 3D), compare with Fig. 2A, ID1), indicating a differential mechanism of regulation for ID1 expression between ex vivo and in vivo suggestive of influence of the tumor stroma.

To get further insights into the effect of SNAI1 silencing on the tumor phenotype, histologic and immunohistochemical analyses were performed on tumors of similar size. No significant differences were detected in the histology of shSNAI1-C2 or -C4 xenografts regarding tumors induced by control or mutS-Sna1–overexpressing cells (Fig. 4A, a–f; data not shown). However, major differences were found in the number and extension of necrotic areas detected within the tumors, being much more prominent in shSNAI1-C2/C4 xenografts (Fig. 4A, a–f; data not shown). Concomitantly with the increase in necrotic areas, an increase in the number of apoptotic cells (up to 2.7-fold) was detected within the shSNAI1-C2/C4 xenografts compared with tumors from control cells (Fig. 4B, j–k; data not shown). Moreover, immunostaining against Ki67 showed that SNAI1 silencing induced a drastic reduction in the tumor proliferative potential (Fig. 4A, g–l; data not shown). Immunofluorescence analyses of invasive and angiogenic markers confirmed the data obtained by qRT-PCR: strongly increased cytoplasmic ID2 expression (Fig. 4B, g–h) and notable reduction in the number of MMP9 and CD31-positive cells in shSNAI1-xenografts (Fig. 4B, a–c and d–f, respectively). The changes in the above markers and in the apoptotic and proliferative tumor potential were fully recovered after the expression of mutS-Sna1 in the shSNAI1 cells (Fig. 4A and B, compare right panels with the rest of the panels), confirming the specific effect of SNAI1 silencing in the tumorogenic behavior of MDA-MB-231 cells.

SNAI1 expression favors distant lymph node metastasis. We then analyzed the consequences of SNAI1 knockdown in the metastatic capability of MDA-MB-231 cells. For that purpose, a spontaneous metastasis assay was performed following surgical removal of primary tumors induced by orthotopic injection of control and SNAI1-silenced MDA-MB-231 cells. Six months thereafter, the mice were sacrificed and organs (lung, liver, kidney, and spleen) analyzed for the appearance of metastatic lesions. No macrometastases were detected in either organ (data not shown) in agreement with the low spontaneous metastatic potential of parental MDA-MB-231 cells (25). However, most animals exhibited inflammation at the contralateral limb lymph node. The existence of distant lymph node metastasis was, therefore, analyzed. Cell lines were derived from the lymph nodes after careful dissection and growth in culture to select for human MDA-MB-231 cells and to avoid the growth of mouse cells. Only six cell lines out of 24 dissected lymph nodes were obtained: one of six MDA-MB-231 (named 231-20), two of six MDA-MB-231-shEGFP (named shEGFP-OI and shEGFP-2D), one of six shSNAI1-C2 (named shSNAI1-C2-2D), and two of six shSNAI1-C4–Injected mice (named shSNAI1-C4-SM and shSNAI1-C4-OI; see Supplementary Fig. 3A for the scheme). To further discard the selection of immortalized mouse cells, the presence of the human amelogenin gene, frequently used in forensic tests for human DNA and gender determination (34), was tested by PCR. All selected cell lines, except 231-20, were positive for the female form of amelogenin gene (Supplementary Fig. 3B), confirming their human origin and their derivation from distant lymph node metastasis of the primary tumors induced by the indicated MDA-MB-231-derived cells.

We then analyzed the expression of SNAI1 in the lymph node–derived cell lines. Surprisingly, SNAI1 expression was detected by qRT-PCR and Western blot in all five lymph node–derived cell lines, regardless of whether the origin of the primary tumor was from control cells or from any of the shSNAI1-silenced clones, to similar or even higher levels than those present in parental MDA-MB-231 cells (Fig. 5A and B). The lack of SNAI1 interference observed in the lymph node–derived shSNAI1-C2-2D, shSNAI1-C4-SM, and shSNAI1-C4-OI cells could not be attributed to the loss of the shSNAI1 expression cassette by DNA reorganization, as the pSuperior-shRNA vector, containing the specific 64 nucleotide control or SNAI1 hairpin, was detected in all lymph node–derived cell lines (Supplementary Fig. 3C). Up-regulation of endogenous SNAI2, at both mRNA and protein levels, was also detected in most of the analyzed lymph node–derived lines compared with parental MDA-MB-231 cells (Fig. 5A and B). To obtain additional information, the tumorogenic potential of the lymph node–derived cell lines shSNAI1-C2-2D and shSNAI1-C4-SM cells was analyzed by orthotopic injection. Tumors induced by lymph node–derived shSNAI1-C2-2D and shSNAI1-C4-SM cells grew at the same rate as those induced by parental MDA-MB-231 cells (Fig. 5C), in contrast to the reduced tumor growth potential of MDA-MB-231-shSNAI1-C2 and -shSNAI1-C4 cells (see Fig. 4B). Analysis of E-cadherin, SPARC, ID1, and ID2 transcripts in tumors induced by lymph node–derived cells revealed similar levels to those found in tumors induced by parental MDA-MB-231 cells (data not shown).

Collectively, the in vivo data suggest a strong selective pressure in favor of SNAI1- and SNAI2-expressing cells within the more aggressive/metastatic subpopulation of MDA-MB-231 breast carcinoma cells. These results are also in agreement with the previously reported role for SNAI1 in the induction of tumor recurrence in breast carcinoma (20).

**Stable silencing of SNAI1 confers sensitivity to chemother-apy.** Previous studies on SNAI1 expression and function have characterized SNAI1 as a complex factor able to induce different biological effects, including cell cycle arrest and resistance to genotoxic damage–induced apoptosis (3, 4). To address whether those biological properties were affected by SNAI1 silencing in MDA-MB-231 cells, we analyzed the proliferative properties of SNAI1-silenced cells, not detecting significant differences compared with parental cells when grown in the presence or absence of serum (Supplementary Fig. S4).
We then tested if SNAI1 silencing confers sensitivity to genotoxic damage induced by docetaxel and gemcitabine, two chemotherapeutics commonly used to treat breast cancer (23, 24). Although both drugs provoke modest levels of apoptosis in MDA-MB-231 cells, SNAI1 silencing induced a 2-fold higher apoptotic response under either chemotherapeutic treatment, an effect fully abrogated after the reexpression of mutS-Snai1 in the shSNAI1 clones (Fig. 6). Importantly, treatment of the lymph node–derived cell

Figure 4. SNAI1 silencing decreases tumor proliferation and induces apoptosis, necrosis, and a less invasive/angiogenic phenotype. A, histologic and proliferation analysis of tumors induced by control MDA-MB-231-shEGFP cells, one representative SNAI1-interfered clone (shSNAI1-C2) and its corresponding control after stable reexpression of mutS-Snai1 (shSNAI1-C2+mutS). Low magnification (a–c) and high magnification images (d–f). Insets, magnified regions (a–c). Necrotic areas (arrows). Immunostaining of the proliferation antigen Ki67 (g–i). B, immunofluorescence analysis of frozen sections of the tumors indicated above. Sections show staining for MMP9 (a–c), CD31 (d–f), and ID2 (g–i). Detection of apoptotic cells was performed by terminal nucleotidyl transferase–mediated nick end labeling assay (j–l). Note the increase in necrotic and apoptotic cells as well as the decrease in MMP9 and CD31 (white arrows), and the increase in ID2 stain in tumors induced by shSNAI1-C2 cells. Bars, 50 µm.
lines (shSNAI1-C2-2D, shSNAI1-C4-SM, and shSNAI1-C4-OI) with either drug induced a low apoptotic response, similar to that exhibited by parental and control cells (Supplementary Fig. S4), further supporting the association between SNAI1, tumorigenic/metastatic behavior, and resistance to apoptosis in MDA-MB-231 cells.

Taken together, the in vivo and in vitro data reported here indicate that SNAI1 expression confers properties to breast carcinoma cells that go further from repression of E-cadherin and induction of EMT, conferring tumor growth advantage, resistance to chemotherapeutic drugs, and invasion and metastatic capabilities.

Discussion

Breast cancer is a heterogeneous disease with diverse metastatic behavior and is still poorly understood. When breast carcinomas remain confined to breast tissue, cure rates exceed 90%. As cells spread, however, long-term survival decreases depending on the extent and the sites of colonization (35). Attempts to predict the probability for local, regional, or distant recurrence in a clinical setting have yielded several useful markers of poor prognosis (36). In this context, recent findings reveal the existence of metastasis gene signatures expressed by primary tumors (25, 37). Interestingly, many of the deregulated genes enable tumor cells to acquire a more invasive phenotype elicited by the loss of cell-cell adhesion and increased proteolysis and motility, characteristic features of EMT.

Loss of E-cadherin molecule, a caretaker of the epithelial phenotype, is at the crossroads of EMT (2). The E-cadherin repressors, SNAI1 and SNAI2, have emerged as master regulators of EMT during development and tumor progression (2, 3, 8). In breast cancer, in particular, SNAI1 expression has been detected at the

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**Figure 5.** Metastatic lymph node–derived cell lines express high levels of SNAI1 and SNAI2 and recover the tumorigenic potential. A, qRT-PCR analysis of the mRNA levels of human SNAI1 (left) and human SNAI2 (right) of parental MDA-MB-231 cells, two cell lines derived from lymph node metastasis from control MDA-MB-231-shEGFP (shEGFP-OI and shEGFP-2D), one cell line derived from lymph node metastasis from MDA-MB-231-shSNAI1-C2 (shSNAI1-C2-2D), and two cell lines derived from lymph node metastasis from MDA-MB-231-shSNAI1-C4 (shSNAI1-C4-SM and shSNAI1-C4-OI) cells. B, Western blot analysis of the protein levels of human SNAI1 and SNAI2 in parental MDA-MB-231 cells and independent lymph node–derived clones as in A. Western blot of α-tubulin is shown as loading control. C, analysis of the tumorigenic potential of the shSNAI1-C2-2D and shSNAI1-C4-SM compared with parental MDA-MB-231 cells by orthotopic injection into the mammary fat pad of nude mice. See the similar tumor latency and tumor growth rate of all analyzed cell lines.
SNAI1 Silencing and Inhibition of Breast Tumor Growth

A

Docetaxel, 100 nmol/L

% Apoptosis

MDA-MB-231

shEGFP

shSNAI1-C2

shSNAI1-C2 + mutS

shSNAI1-C4

shSNAI1-C4 + mutS

B

Gemcitabine, 6 pmol/L

% Apoptosis

MDA-MB-231

shEGFP

shSNAI1-C2

shSNAI1-C2 + mutS

shSNAI1-C4

shSNAI1-C4 + mutS

Figure 6. SNAI1 silencing induces sensitivity to chemotherapy. Analysis of the apoptosis induced after 48 h of treatment with 100 nmol/L of docetaxel (A) or 6 pmol/L of gemcitabine (B) in parental MDA-MB-231 and control shEGFP, stable SNAI1-interfered clones (shSNAI1-C2 and -C4) and stable cell lines obtained after reexpression of mutS-Sna1. Columns, mean of three independent assays; bars, SD. **, P < 0.01; ***, P < 0.001, ANOVA analysis.

invasive regions coincidently with down-regulation of E-cadherin, and has been associated with lymph node status (14–17) or tumor recurrence (20). These observations support the fact that SNAI1 plays a role in breast cancer progression mediating tumor spread and metastasis. Reasoning that SNAI1 could also act on other target genes that actively contribute to the invasive and migratory phenotype as well as to the cell survival and proliferation capabilities (reviewed in ref. 3), we sought to knock down SNAI1 expression in breast cancer cells to block tumor growth, invasion, and metastasis.

To this end, we used the human breast cancer cell line MDA-MB-231 derived from the pleural effusion of a breast cancer patient suffering from widespread metastasis years after the removal of her primary tumor (38). The designed shRNA against SNAI1 specifically interferes with mouse and human SNAI1 mRNAs without affecting the expression of the closely related SNAI2 gene or the human SNAI1 pseudogene (ref. 10; this work). Our results show that stable blockade of SNAI1 expression in MDA-MB-231 cells leads to a partial MET process associated with increased E-cadherin transcripts and, concomitantly, to down-regulation of fibronectin, SPARC, and ID1 and reduced invasiveness. SPARC and ID1 are sporadically expressed in primary human breast tumors (39–41) and have been recently associated with EMT because both genes were found up-regulated by the expression of E47 and Snail factors in epithelial cells (27, 28). Moreover, both SPARC and ID1 belong to the proposed “lung metastasis gene-expression signature” previously identified in selected single-cell–derived progenies from MDA-MB-231 cells that have metastatic tropism to the lung (25). SNAI1 was probably not associated with this gene signature by Minn and coworkers because SNAI1 is already highly expressed in the parental MDA-MB-231 cells. Indeed, a role for SNAI1 in lung metastasis could be inferred from the fact that several genes of the “lung metastasis signature” are direct or indirect targets of Snai1 such as ID1, SPARC, or MMP2 (3).

Consistent with the reversion of invasiveness after SNAI silencing is the observation that ID2 expression was up-regulated in the MDA-MB-231 transfectants. Interestingly, ID2 expression has been associated with reduced cellular invasion and better prognosis in human breast tumors (29). Furthermore, although no changes in MMP2 were observed, the activity of the closely related metalloproteinase, MMP9, another Snai1 target (22), was strongly down-regulated in these cells. Together, these results indicate that blockade of endogenous SNAI1 expression in MDA-MB-231 cells affects the expression of several genes involved in the invasive phenotype and it may explain the reduced invasive behavior of the shSNAI1–derived cells (Fig. 2D). Importantly, all the effects detected after SNAI1 silencing were completely overcome after the expression of a RNAi-resistant Snai1 allele, confirming the specific effect of SNAI1 in the invasive phenotype of MDA-MB-231 cells. Strikingly, Snai1 transcript up-regulation has not been detected in several studies of invasive carcinoma cells (42–44), in contrast to SNAI2 up-regulation. This can be explained, at least in part, to the lack of specificity of the Snai1 probes to discriminate the highly homologous hSNAI1 gene and hSNAI1L pseudogene transcripts (26). More interestingly, those observations could indicate that SNAI1 function is mainly regulated at the posttranscriptional level (16, 21) in breast carcinoma cells; a hypothesis that certainly deserves further clarification.

Our present study also provides evidence of the important role of SNAI1 in the tumorigenic behavior of MDA-MB-231 cells. Histopathologic analyses indicate that tumors induced by MDA-MB-231-shSNA1 cells have extensive necrotic areas, increased apoptosis, and reduced proliferation compared with tumors derived from MDA-MB-231 controls. These features could account for the observed decrease in tumor incidence and growth rate of MDA-MB-231-shSNA1 cells compared with parental and control cells. Importantly, the shSNAI1–mediated effects on tumorigenicity were almost completely abrogated after reexpression of the RNAi-resistant Snai1 allele on MDA-MB-231-shSNA1 cells, thus discarding the nonspecific effects of the SNAI1 shRNA. Thus, in at least the MDA-MB-231 system, our data show for the first time a direct link between SNAI1 expression and growth potential of primary human breast tumors, thus providing additional information for the proposed role of SNAI1 in breast tumor recurrence (20).

Concerning the effects of SNAI1 expression on metastatic ability, we could not evidence the presence of lung, liver, or other organ metastasis either from MDA-MB-231 controls or from SNAI1–silenced tumors (even 6 months after the removal of the primary
tumors), in agreement with the reported low metastatic potential of parental MDA-MB-231 cells in spontaneous metastasis assays (25). However, a strong inflammatory response and the presence of metastatic cells was observed in the contralateral lymph nodes of most injected animals. Surprisingly, analysis of derived cell lines from dissected lymph nodes (5 out of 24) showed that all of them, including those derived from SNAI1-silenced clones, express SNAI1 at similar or even higher levels than those found in parental MDA-MB-231 cells (Fig. 5). The fact that the shRNAi expression cassette is present in all the lymph node–derived cell lines, including shSNAI1-derived cells, suggests that epigenetic modifications (45, 46) and/or additional unknown mechanisms, are working to avoid shSNAI1 expression and/or effective SNAI1 interference. Whatever the mechanism, taken together, these results suggest that a strong pressure operates into the tumor population to favor the selection of SNAI1-expressing cells enabling them to develop lymph node metastasis. Of note, these results are in agreement with previous studies showing a positive correlation between SNAI1 mRNA expression and lymph node status (14, 15). Moreover, the levels of SNAI2 were also found to be increased in the majority of the lymph node–derived cells, suggesting that high expression of SNAI2 might also be important for lymph node metastases of breast tumors, in addition to the proposed SNAI2 contribution to local invasion and/or tumor effusions (17, 19). The coordinated expression of SNAI1 and SNAI2 factors found in metastatic lymph nodes reinforces the interplay or synergy between both E-cadherin repressors in the metastatic progression of breast tumors. The fact that SNAI1 silencing is sufficient to abrogate the local invasion and tumor growth potential of MDA-MB-231 cells suggests that other E-cadherin repressors/EMT inducers, such as Twist1 or Zeb factors, might preferentially act downstream of SNAI1/SNAI2 at invasion or subsequent steps of breast tumor progression (3). This model also fits with the reported involvement of Twist1 in intravasation and angiogenesis of breast carcinoma cells (47, 48), and of Zeb2/SIP1, indeed a Snail target (49), in breast carcinoma effusions (19).

It is also tempting to speculate whether the prosurvival properties of the Snail family members are necessary for distant lymph node metastases. In this context, the observation that SNAI1 silencing leads to a significant increase in the chemosensitivity of MDA-MB-231 cells to at least two of the chemotherapeutic agents more frequently used in clinics, reinforces the SNAI1 prosurvival action.

In summary, the studies presented here highlight the contribution of SNAI1 expression to the local invasion and growth of primary breast tumors, but importantly, to distant lymph node metastasis and chemoresistance as well. These findings provide additional support to SNAI1 as a potential therapeutic target for human breast cancer.

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References

Correction: SNAI1 Silencing and Inhibition of Breast Tumor Growth

In the article on SNAI1 silencing and inhibition of breast tumor growth in the December 15, 2007 issue of Cancer Research (1), the final sentence of the first paragraph on page 11730 should read as follows: "This model also fits with the reported involvement of Twist1 in intravasation and angiogenesis of breast carcinoma cells (47, 48) and of Zeb2/SIP1 in breast carcinoma effusions (19), and with the fact that Zeb1 has been identified as a Snail1 target (49)."


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