

Suppression of Tumor Development and Metastasis Formation in Mice Lacking the *S100A4(mts1)* Gene

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Abstract

The *S100A4(mts1)* protein stimulates metastatic spread of tumor cells. An elevated expression of *S100A4* is associated with poor prognosis in many human cancers. Dynamics of tumor development were studied in *S100A4*-deficient mice using grafts of CSML100, highly metastatic mouse mammary carcinoma cells. A significant delay in tumor uptake and decreased tumor incidences were observed in *S100A4(-/-)* mice compared with the wild-type controls. Moreover, tumors developed in *S100A4(-/-)* mice never metastasize. Immunohistochemical analyses of these tumors revealed reduced vascularity and abnormal distribution of host-derived stroma cells. Coinjection of CSML100 cells with immortalized *S100A4(+/+)* fibroblasts partially restored the dynamics of tumor development and the ability to form metastasis. These fibroblasts were characterized by an enhanced motility and invasiveness in comparison with *S100A4(-/-)* fibroblasts, as well as by the ability to release *S100A4* into the tumor environment. Taken together, our results point to a determinative role of host-derived stroma cells expressing *S100A4* in tumor progression and metastasis. (Cancer Res 2005; 65(9): 3772-80)

Introduction

Metastases, growth of the tumor cells at secondary sites, are determined by a complex cascade of events that involves both cancer cells and multiple factors produced by the host organism (1). Modulation of expression of numerous genes and their products is involved in this process.

Implication of *S100A4* in metastatic tumor progression has been shown by the introduction of the *S100A4* gene into nonmetastatic cell lines or the suppression of its activity in metastatic ones (2). Overexpression of the *S100A4* gene in transgenic mice also showed its metastasis-promoting activity (3, 4). Recently it has been shown that tumors of double transgenic mice bearing polyoma virus middle-T antigen (PyV-mT mice) and *S100A4*-null allele exhibited reduced level of metastasis (5). Furthermore, numerous clinical studies showed a correlation between *S100A4* expression and a poor prognostic outcome in a variety of human cancers, confirming the importance of *S100A4* in cancer progression (6–12).

S100A4 belongs to the *S100* family of small Ca^{2+} -binding proteins. Members of this family have been implicated in cytoskeletal-membrane interactions, calcium signal transduction, and cellular growth and differentiation (13). The most unusual characteristic of *S100* proteins, including *S100A4*, is their ability to act both intra-

and extracellularly. Interaction with a heavy chain of nonmuscle myosin suggests its participation in the stimulation of cell motility (14, 15). Overexpression of *S100A4* indeed leads to an increase of invasiveness and motility of the cancer cells (16, 17). Interaction with liprin β -1, another *S100A4* target, suggests its participation in cell adhesion (18). *S100A4* interacts also with the tumor suppressor p53 and regulates its trans-activating function (19). Functional significance of this interaction was confirmed by analysis of *S100A4*-null mice showing enhanced levels of spontaneous tumor development and decreased apoptosis in response to genotoxic stress (20). Tumors developed in these mice were nonmetastatic. Recently, the methionine aminopeptidase was described as a new target for *S100A4* (21). An association between *S100A4* expression, matrix metalloproteinase (MMP) activity, and metastatic potential of human tumor cells has also been reported (22, 23). These observations raised the possibility that *S100A4* participates in metastasis by interfering with cell motility, invasion, adhesion, and apoptosis. As an extracellular protein, *S100A4* stimulates neurite outgrowth and survival as well as migration of astrocytic tumor cells (24–26). It also acts as an angiogenic factor by stimulating the motility and invasiveness of endothelial cells via activation of MMP production (27, 28). This characteristic of *S100A4* in turn might contribute to its metastasis-promoting function.

S100A4 is markedly up-regulated in activated fibroblasts both in normal conditions (e.g., wound healing) and in tumor stroma (3, 29). Cocultivation of fibroblasts and tumor cells stimulates the release of *S100A4* from fibroblasts. Moreover, application of recombinant *S100A4* was able to confer a metastatic phenotype on tumor cells (30). These findings imply that *S100A4*, when released into the extracellular compartment, contributes to tumor progression by creating a favorable microenvironment for tumor invasion and neovascularization.

To directly assess the role of *S100A4* at the stroma site in tumorigenesis, we have chosen a model system where highly metastatic mammary carcinoma cells were transplanted into mice lacking the *S100A4* gene. Using this model, we showed that both tumor development and metastasis formation were retarded in these mice. The retardation of tumor development could be surmounted by addition of *S100A4* positive fibroblasts to the tumor transplants. The results obtained validate the role of stroma-derived *S100A4* in tumor progression.

Materials and Methods

Mice

Eight- to ten-week-old *S100A4(-/-)* and wild-type mice of an A/Sn genetic background (six to seven back-crosses into this strain) were used for this study. All animals were maintained according to the guidelines of the Federation of European Laboratory Animal Science Associations for the care and use of laboratory animals. Tumor cells (5×10^5) or tumor cells mixed with fibroblasts at 1:1 ratio (5×10^5) were injected s.c. to the mice.

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Each experimental set was reproduced three to five times. For all the tumor graft experiments tumor volume did not exceed 900 mm³. Mice developing tumors were monitored twice a week to determine the dynamics of tumor development. Kaplan-Meier survival curves for the experiments analyzing tumor growth were generated using Prism software. Wilcoxon log-rank test was used for the comparison of survival curves.

Cell Culture

The CSML100 and CSML0 mouse mammary tumor cell lines (31) and mouse embryonic fibroblasts (MEFs) were grown in DMEM (Gibco BRL, Taastrup, Denmark) supplemented with 10% FCS. MEF cell lines were obtained by spontaneous immortalization of primary embryonic fibroblasts derived from 14.5-day-old embryos of S100A4(+/+) and S100A4(-/-) mice according to the 3T3 protocol (32). Briefly, cells were maintained on a defined 3-day passage schedule by plating 3×10^5 cells (1×10^4 cells cm⁻²). The cells were passaged every 3 or 4 days in T25 flasks, counted, and replated at the appropriate density. MEF cells were considered immortal after approximately 25 passages, when the cells gained the ability to grow indefinitely and at a low density.

Characterization of the Mouse Embryonic Fibroblast Cell Lines

Proliferation assay. To determine the rate of cell proliferation, MEF cells were plated in triplicate. At various time points cells were harvested and the amount of the cells was determined by direct counting in Nucleocounter (Chemometec, Allerød, Denmark). The doubling time was calculated as a function of the increase of the amount of cells each 24 hours. Each experiment was repeated twice. The average \pm SD was taken as a measure for all the experiments.

In vitro wound healing assay. The monolayer of MEF cells in the OptiMem media (Gibco, BRL) supplemented with 1% FCS was wounded with a plastic loop. Wound healing was monitored and photographed in a marked area. The extent of wound healing was determined by measuring the width of a wound in 20 random sites inside the marked area using ImageQuant software. The relative migration was calculated as δ of average width at different time periods. The confidence was calculated using Student's *t* test.

Three-dimensional Matrigel invasion assay. Detailed protocol for invasion assay in three-dimensional Matrigel was described elsewhere (28). Briefly, 6×10^4 cells were incubated in a hanging drop of medium to form a clump, which then was placed on a layer of Matrigel containing DMEM and 10% FCS. The clump was covered with a drop of Matrigel and incubated at 37°C for polymerization. Extent of the outgrowth was followed in inverted microscope using 10 \times objective for 120 hours.

Histologic Analyses and Immunostaining

Animals sacrificed at the termination of the experiments were subjected to a macroscopic examination directed to reveal macroscopic metastases. Tissues were fixed in 10% formalin and embedded in paraffin. H&E-stained tissue sections were analyzed by light microscopy. Tumor tissue sections were stained with anti-S100A4 rabbit polyclonal antibodies (3), anti-F4/80 (Accurate Chemicals and Scientific Corp., Westbury, NY), anti- α -smooth muscle actin (Sigma, Broendby, Denmark), anti-Ki67, anti-CD31, and anti-CD45 (BD Biosciences, PharMingen, San Jose, CA) according to the protocol of the manufacturer. In the consecutive step, secondary anti-rabbit or anti-mouse horseradish peroxidase-conjugated antibodies (DAKO A/S, Glostrup, Denmark) were used followed by treatment with enhanced 3,3'-diaminobenzidine (Pierce, Rockford, IL) for the visualization of the positive staining. The blood vessel density was quantified by determining the amount of CD31-positive capillaries in 10 random fields from four sections of different parts of the tumor (magnification, $\times 400$). Seven tumors for each experimental group were analyzed. Data are presented as median \pm SD. The confidence was calculated using Student's *t* test.

MEF cell lines were grown on glass slides, fixed in 4% paraformaldehyde for 20 minutes, permeabilized in 1% Triton X-100 for 3 minutes, and stained with rabbit anti-S100A4, anti- α -smooth muscle actin (Sigma), anti-pan cytokeratin (Sigma), and anti-vimentin (NeoMarkers, Fremont, CA) antibodies. FITC-conjugated anti-mouse or anti-rabbit (Vector Laboratories, Burlingame, CA) secondary antibodies were used for visualization of the positive staining.

Sandwich ELISA Assay

Sandwich ELISA was used to determine the amount of S100A4 in the mouse tumor interstitial fluid. Preparation of tumor interstitial fluid was done as described (33). Fifty milligrams of tumor tissue were incubated for 2 to 4 hours at 37°C in 1,000 μ L of OptiMEM. Total amount of protein was determined and equal amounts were used for the assay. Sandwich ELISA assay was done as described (27). The sensitivity of reaction was less than 20 ng/mL. Tumor interstitial fluids from four different tumors for each experimental group were used for analysis. Data are presented as a median \pm SD. The confidence was calculated using Student's *t* test.

Results

Suppression of tumor growth in S100A4-deficient mice. We investigated the role of S100A4 in tumor growth and dissemination by use of S100A4-deficient mice in which CSML100 mammary adenocarcinoma cells have been implanted. CSML100 cells express S100A4 protein and, on s.c. injection, form rapidly growing tumors that are highly metastatic to the lungs (31). S100A4(+/+), S100A4(+/-), and S100A4(-/-) mice of matching genetic background were grafted with equal amounts of CSML100 cells. All the wild-type mice rapidly grew tumors and formed metastasis in the lungs. Tumor development in S100A4(+/-) mice was not significantly different from the wild-type animals, although a delay in tumor development was observed. Twenty-five days after injection, most mice in these experimental groups were sacrificed because of the tumor size (Table 1 and Fig. 1A). In contrast, tumor development was severely impaired in S100A4(-/-) mice. Approximately 60% of animals did not develop tumors. Mice that eventually developed tumors were sacrificed at approximately day 50 after injection, showing a considerable delay in tumor development. Tumor-bearing S100A4(-/-) mice did not develop lung metastases (Table 1). Tumor-free animals were monitored for 150 days until completion of the experiment. Kaplan-Meier survival curves derived from this experiment showed statistically significant differences in survival rates of S100A4(+/-) and (-/-) mice grafted with CSML100 tumor cells (Fig. 1A). Analysis of the dynamics of tumor growth of individual mice showed that the delay in tumor development was not caused by a slower growth rate but rather by late emergence of the tumor. If in the wild-type mice tumors were first detected on average at day 15 after injection, in S100A4(-/-) mice they first appeared at day 32 (Fig. 1B). The growth rates of tumors developed in S100A4(+/-) and (-/-) animals were comparable. In some cases, in S100A4(-/-) animals the growth rate was slower, but when the average growth rate was calculated the differences observed were not statistically significant (Fig. 1C). Determination of the amount of proliferating cells in tumor sections with the proliferation-specific Ki67 antibodies showed a similarity in the proliferation rate of tumor cells (Fig. 1D). Statistical evaluation revealed that 65.5% of cells seemed to proliferate in tumors developed in the wild-type versus 63.7% in S100A4-deficient mice. Despite the similarity in tumor growth rates, both macroscopic and histologic analyses failed to detect any metastasis in the lungs of S100A4(-/-) tumor-bearing mice. Immunohistochemical staining of the lung sections with anti-S100A4 antibodies failed to detect dormant CSML100 cells in the lungs (data not shown). S100A4(+/-) and S100A4(+/-) tumor-bearing mice, in contrast, developed multiple lung metastases in 100% of analyzed animals (Table 1).

In similar experiments the growth of nonmetastatic mammary carcinoma cells (CSML0) was compared in S100A4-null and

Table 1. Tumor growth in S100A4(+/+), S100A4(+/-), and S100A4(-/-) mice

Injected cells	Genotype of the mice	Number of the mice	Number of tumor-free mice	Tumor-free animals (%)	Lung metastases (%)
CSML100	+/+	40	1	2.5	100
CSML100	-/-	26	15	57.6	0
CSML100	+/-	18	0	0	100
CSML0	+/+	14	0	0	0
CSML0	-/-	15	4	26	0
MEF(+/+)	+/+	10	10	100	0
MEF(+/+)	-/-	10	10	100	0
MEF(-/-)	+/+	10	10	100	0
MEF(-/-)	-/-	10	10	100	0
CSML100 + MEF(+/+)	-/-	27	9	33	12
CSML100 + MEF(-/-)	-/-	24	12	50	0

wild-type mice. Although the differences in tumor growth were not as substantial as using the highly metastatic CSML100 cells, both the tumor uptake and the amount of growing tumors in S100A4-deficient mice were decreased (Table 1).

The obtained data allowed us to propose that expression of S100A4 in tumor stroma cells that are derived from the host animal is determinative both for tumor development and metastasis formation.

Altered distribution of stroma cells in tumors developed in S100A4(-/-) mice. Obtained data indicate that tumor stroma in

S100A4(-/-) mice might be impaired in fully supporting tumor development and metastasis. To understand the reasons for the deterioration of tumor development in S100A4-null mice, we did immunohistochemical analysis of the tumor sections. We assessed the role of S100A4 in the recruitment of cellular components of the tumor stroma, in particular macrophages, lymphocytes, endothelial, and fibroblast-like cells. Macrophages, as judged by the F4/80 antigen, were abundant and evenly distributed in the tumor stroma of the wild-type mice. In contrast, tumors of S100A4-deficient mice contained abundant

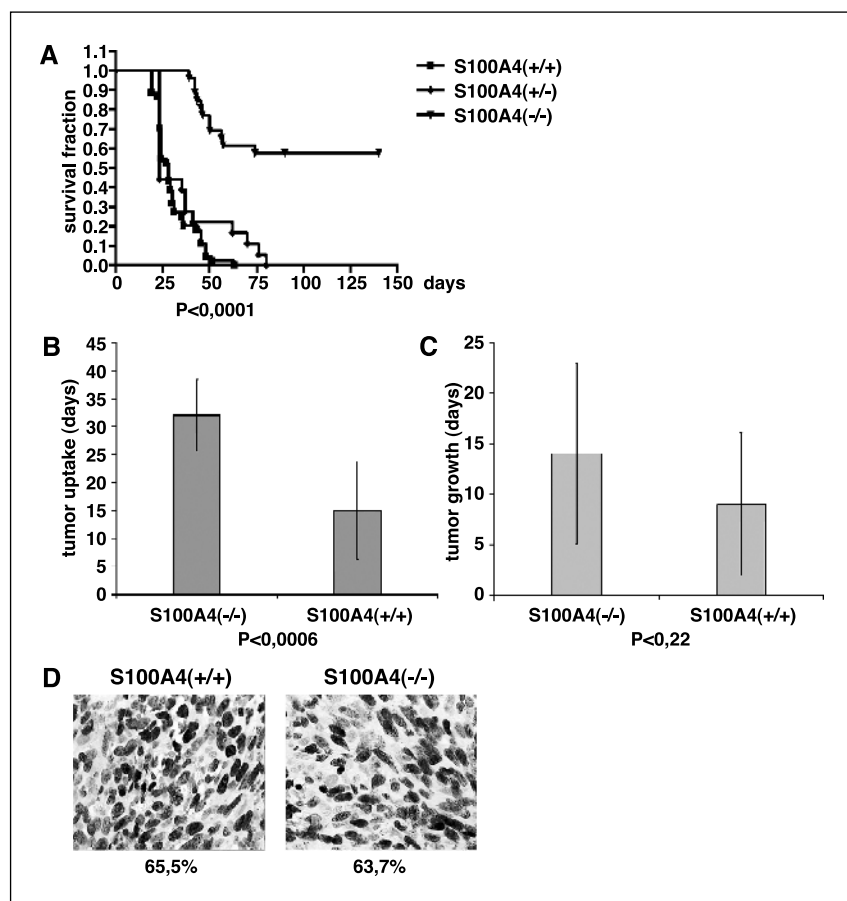


Figure 1. Dynamics of tumor development in S100A4(+/+), S100A4(+/-), and S100A4(-/-) mice grafted with highly metastatic CSML100 cells. *A*, Kaplan-Meier survival curves. *B*, average time of tumor uptake in S100A4(+/+) and (-/-) mice injected with CSML100 cells. *C*, average time of tumor growth from first detection to 900 mm³. *D*, proliferation rate of tumor cells as determined by staining of tumor sections with anti-Ki67 antibodies. Magnification, ×400.

macrophages only at the edges but not in the central part of the tumor (Fig. 2A and B). A similar distribution was observed when tumor sections were stained for lymphocyte marker (CD45) and α -smooth muscle actin (Fig. 2C-F). The α -smooth muscle actin antibodies recognize both myofibroblasts, which reflect a substantial part of tumor-associated fibroblasts (34) and smooth muscle cells that participate in the formation of blood vessels. Previously, we have shown that the S100A4 protein acts as an

angiogenic factor (27). To examine whether impairment in tumor development observed in S100A4-deficient mice is associated with alterations in angiogenesis, tumor sections were stained with antibodies against CD31, an endothelial cell-specific marker (Fig. 2G and H). Sections from tumors developed in the wild-type animals showed an even distribution of blood vessels, whereas vessels in tumors from S100A4(-/-) mice were basically concentrated on the edge of the tumor. Moreover, S100A4 deprivation was associated with ~50% reduction of vessel density (Fig. 2I).

Altogether immunohistochemical examination of the tumors revealed a substantial dissimilarity in the development of the tumor stroma in S100A4-deficient mice. We can propose that observed repression of tumor growth in S100A4(-/-) mice might be caused by the differences in the development of host-derived tumor stroma, which is deficient in S100A4 expression.

Generation and characterization of S100A4(+/+) and S100A4(-/-) embryonic fibroblast cell lines. It has been shown previously that tumor-associated mouse fibroblasts express high levels of S100A4 (3). To test the impact of S100A4 expressed in stroma on tumor development, we designed a model where S100A4 stroma deficiency would be complemented by S100A4-positive fibroblasts.

For this, we generated immortalized fibroblast cell lines (MEF) from 14.5-day-old embryos of S100A4(+/+) and S100A4(-/-) genotype. Two wild-types and two knockout MEF cell lines, named 2MEF(+/+), 4MEF(+/+), 3MEF(-/-), and 5MEF(-/-), were selected for further studies. Immunocytochemical staining of these cell lines revealed that they were negative for cytokeratin and positive for vimentin as well as α -smooth muscle actin (Fig. 3A-F). From this expression profile we conclude that on immortalization these cell lines acquired a myofibroblast-like phenotype.

Next we compared the proliferation rates of S100A4(+/+) and (-/-) MEFs. Doubling time of these cells was determined by direct cell counting of exponentially growing cultures. The doubling time of S100A4(+/+) and (-/-) MEF cell lines was comparable except for the 4MEF(+/+) cells, which divide at a slower rate than the other cell lines tested (Fig. 3G). We have determined the proliferation rates of 10 different (-/-) and (+/+) MEF cell lines and revealed that S100A4(-/-) MEFs have a tendency to proliferate faster than S100A4(+/+) MEFs (data not shown).

Cultivation of MEF cells at a low density revealed substantial differences in the morphology of the colonies formed. Wild-type MEFs developed colonies with scattered morphology, whereas S100A4-deficient MEFs formed colonies with epithelioid morphology (Fig. 4A and B). This could be explained by differences in cell adhesion and motility of different MEF types. To obtain evidence that the absence of S100A4 influences the cell motility *in vitro*, we did wound healing assays. The results of these experiments showed 2-fold difference in the relative motility of S100A4(+/+) compared with S100A4(-/-) MEFs (Fig. 4C and D).

Further, we compared the ability of S100A4(+/+) and S100A4(-/-) fibroblasts to invade the extracellular matrix in *in vitro* three-dimensional Matrigel assay. Preformed aggregates of MEFs were sealed in a thick layer of Matrigel. The ability of cells to invade the matrix was monitored for 96 to 120 hours. S100A4(-/-) MEFs, in contrast to S100A4(+/+) MEFs, were noninvasive in the three-dimensional Matrigel assay (Fig. 4E).

Reintroduction of S100A4 in the (-/-) MEF cells by infection with S100A4-expressing retrovirus results in restoration of the motility and partially of the invasive abilities of the MEF cells (data not shown).

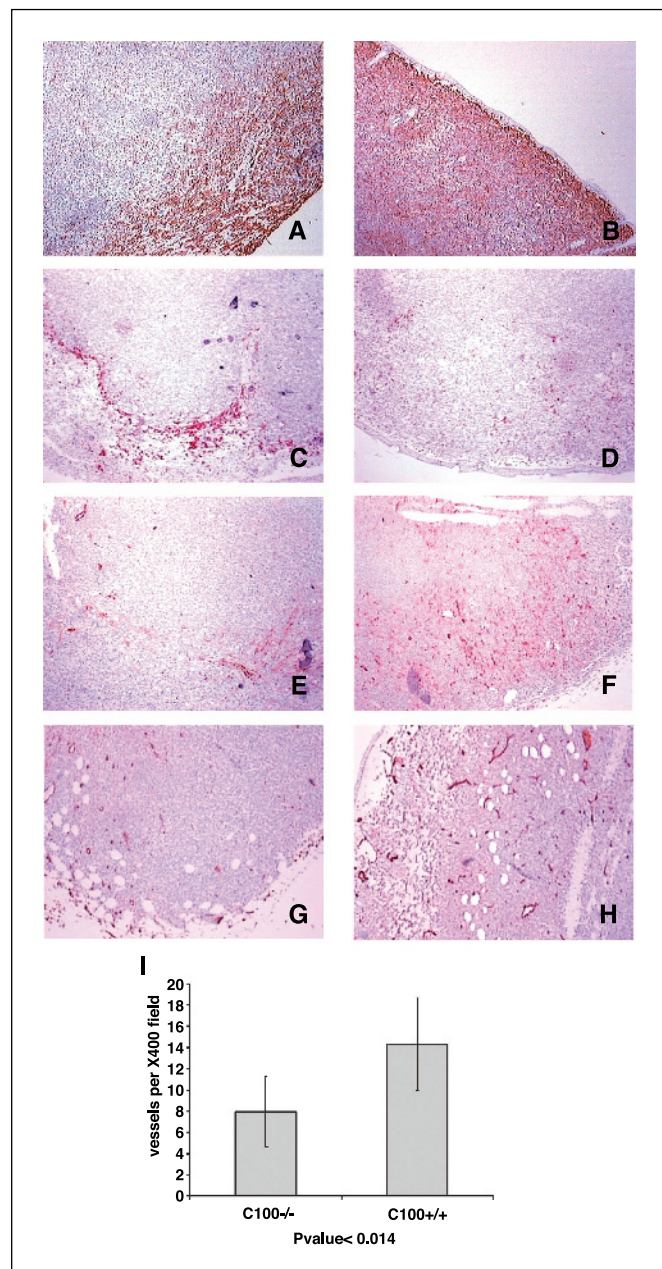


Figure 2. Immunohistochemical analysis and vessel density of CSML100 tumors developed in S100A4(+/+) [B, D, F, and H] and S100A4(-/-) [A, C, E, and G] mice. A and B, staining of the tumor sections with anti-macrophage-specific F4/80 antibodies. C and D, staining of the tumor sections with anti-lymphocyte-specific CD45 antibodies. E and F, staining of the tumor sections with anti- α -smooth muscle actin antibodies. G and H, staining of the tumor sections with anti-endothelial-cell-specific CD31 antibodies. Magnification, $\times 40$. I, average tumor vessel density as determined by quantifying the amount of capillaries immunostained with the anti-CD31 antibodies.

Collectively the data obtained showed that S100A4-deficient MEF cell lines dramatically differ in ability to move and invade the extracellular matrix. These features could be attributed to the absence of S100A4 expression.

Effect of S100A4(+/+) and S100A4(-/-) fibroblasts on CSML100 tumorigenicity in S100A4-deficient mice. To test the hypothesis that CSML100 cells supplemented with the fibroblasts expressing S100A4 could overcome retarded tumor development in S100A4(-/-) animals, we did experiments where CSML100 cells were injected together with S100A4(+/+) or (-/-) MEFs into S100A4-deficient mice. Both S100A4(-/-) and (+/+) MEFs were not tumorigenic by themselves because injection into both wild-type and S100A4 knockout mice did not show any tumor uptake (Table 1). The dynamics of the tumor development in both of the experimental groups was improved. If only 40% of S100A4-null mice grafted with CSML100 cells developed tumors (Fig. 1A), the addition of MEF cells substantially improved the tumor incidence. The percentage of animals eventually developing CSML100 tumors supplemented with S100A4(-/-) MEFs was 50% ($n = 24$), whereas the effect of S100A4(+/+) MEFs was more pronounced, reaching 67% ($n = 27$) in CSML100 + S100A4(+/+) MEF-injected mice. In two cases, tumors supplemented with S100A4(-/-) MEFs started to develop but never reached the 900 mm³ size and finally regressed. Three mice injected with CSML100 + S100A4(-/-) MEFs were sacrificed at day 11 after injection because of the size of the tumor; on autopsy it seemed to contain a thin layer of tumor cells lining a space filled with liquid (a cyst). None of these animals were excluded from the analysis, making differ-

ences in the stimulatory effect of S100A4(+/+) and S100A4(-/-) MEFs less pronounced.

Kaplan-Meier curves showed a shift in the survival of the animals injected with CSML100 cells mixed with S100A4(+/+) and (-/-) MEFs compared with the animals grafted with CSML100 cells alone (Fig. 5A and B). After ~25 days the survival of the animals grafted with CSML100 cells + S100A4(+/+) MEFs constantly decreased, and by the end of the experiment showed significant statistical difference with the animals grafted with CSML100 cells alone ($P < 0.017$). The dissimilarity of survival curves of the mice injected with CSML100 cells alone and CSML100 + S100A4(-/-) MEFs was not statistically significant ($P < 0.2$). The most striking observation was that S100A4(+/+) MEFs were able to partially restore the ability of CSML100 tumor to metastasize to the lungs, whereas CSML100 tumors supplemented with the (-/-) MEFs never metastasized (Table 1).

We therefore conclude that supplementing the CSML100 cells with expressing S100A4 tumor stroma components did at least partially restore the ability of CSML100 cells to develop aggressive metastatic tumors in S100A4(-/-) mice.

Previously, we have shown *in vitro* that tumor cells strongly stimulate the release of S100A4 protein from the fibroblasts (30). One can propose that stimulation of CSML100 tumor growth and metastasis by S100A4(+/+) MEFs could also be determined by the release of the S100A4 protein into the tumor microenvironment. We therefore compared the level of the S100A4 protein released by the tumor biopsies obtained from the mice injected with CSML100 cells alone and supplemented with S100A4(+/+) and (-/-) MEFs. Fragments of the tumor tissue isolated from the

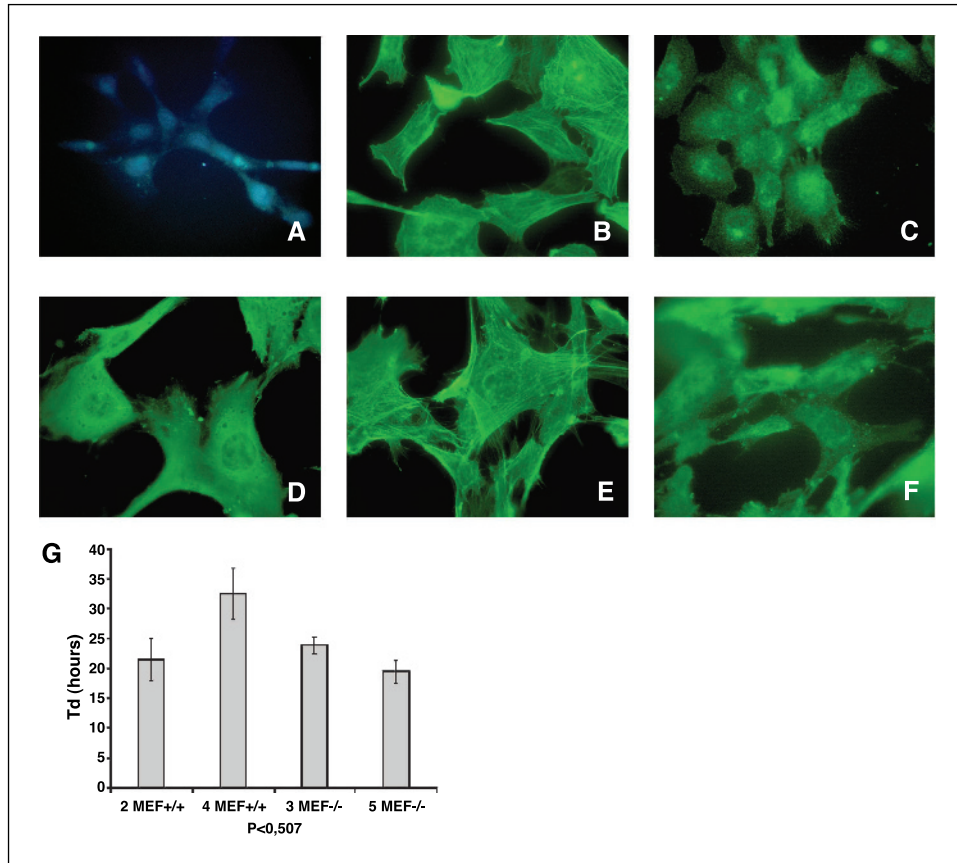
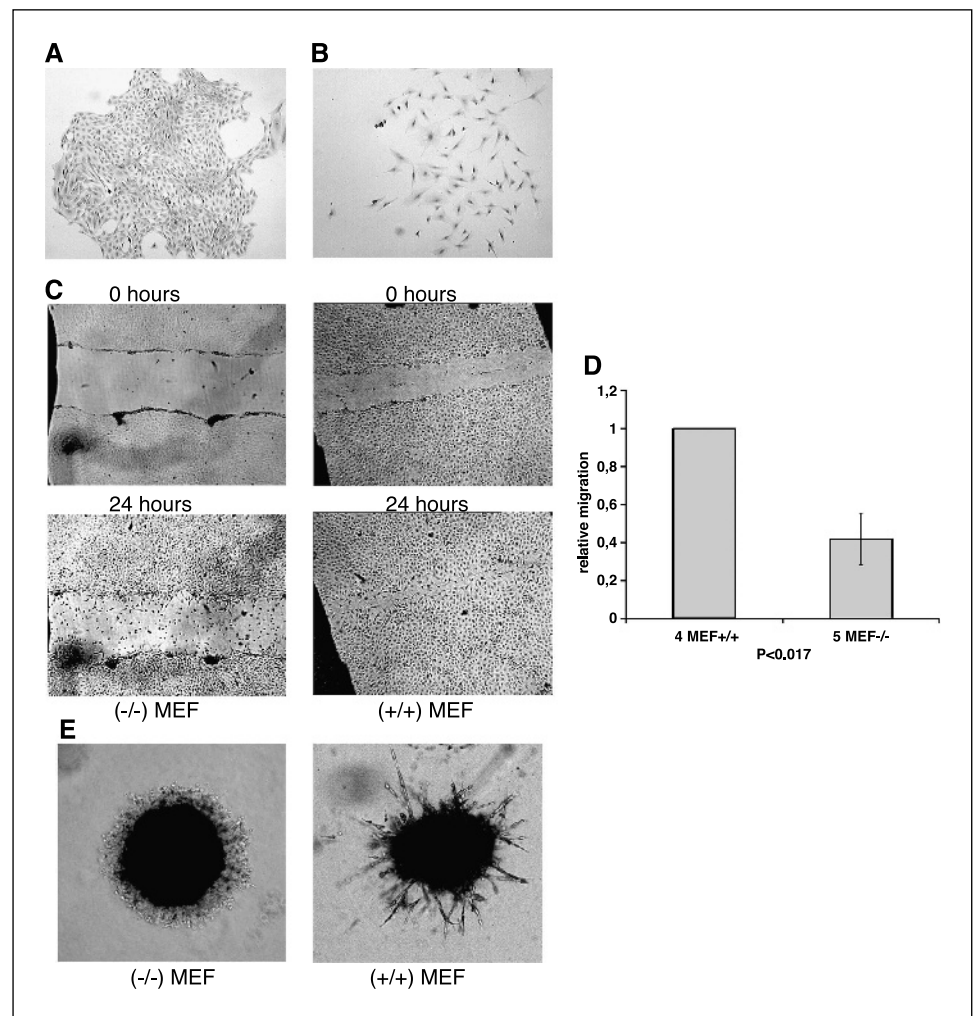


Figure 3. Characterization of S100A4(+/+) [A-C] and S100A4(-/-) [D-F] immortalized MEF cell lines. A and D, immunostaining with anti-S100A4 antibodies. S100A4(-/-) cells are negative for S100A4 staining, whereas S100A4(+/+) cells express high levels of S100A4. B and E, immunostaining with anti- α -smooth muscle actin antibodies. C and F, immunostaining with anti-vimentin-specific antibodies. Both knockout and wild-type fibroblasts are positively stained with these markers. Magnification, $\times 400$. G, doubling time of S100A4(+/+) and S100A4(-/-) immortalized fibroblast cell lines.

Figure 4. Motility and invasive properties of S100A4(+/+) and S100A4(-/-) fibroblasts. *A* and *B*, colonies formed by fibroblasts at a low density seeding: *A*, S100A4(-/-); *B*, S100A4(+/+) fibroblasts. Note scattered growth of S100A4(+/+) fibroblasts. *C* and *D*, *in vitro* wound healing by S100A4(+/+) and S100A4(-/-) fibroblasts. Relative migration of S100A4(+/+) and S100A4(-/-) fibroblasts (*D*). *E*, invasion of S100A4(+/+) and (-/-) fibroblasts in three-dimensional Matrigel. Note invasive growth of the wild-type fibroblasts.



tumor-bearing animals were incubated in serum-free medium permitting release of the proteins both from the tumor and the stroma cells (33). The amount of the released S100A4 protein (tumor interstitial fluid) was determined by sandwich ELISA. Figure 5C shows that CSML100 tumors supplied with S100A4(+/+) MEFs indeed release twice more S100A4 protein than the CSML100 tumors supplied with S100A4(-/-) MEFs ($P < 0.001$). This raises the possibility that *in vivo* the increased concentration of extracellular S100A4 in the tumor could determine its aggressive behavior.

Discussion

A substantial body of evidence associates the *S100A4* gene expression with the progression of malignant tumors. Despite the unequivocal casual relation between expression of S100A4 and the acceleration of the tumors progression, the actual mechanism of S100A4 tumor-promoting function remains unclear (2).

As a typical member of the S100 protein family, S100A4 exhibits dual extra- and intracellular functions. The intracellular S100A4 protein could interfere with vital cellular functions such as cell motility, invasion, cell division, and survival (14, 19). Modulation of these functions of the cell might play important role in stimulation of the tumor cell progression towards a metastatic phenotype. Extracellularly, S100A4 also exerts functions that might substantially

contribute to tumor progression. The extracellular S100A4 protein seems to be a cell-specific modulator of cell motility, survival, and apoptosis; it also acts as an angiogenic factor (25–27, 35). Furthermore, extracellular S100A4 stimulates production of matrix-degrading proteases (MMP) from both endothelial and tumor cells (28, 30). It has also been shown that the presence of extracellular S100A4 modulated the metastatic behavior of tumor cells. Collectively all these data strongly support the idea that extracellular S100A4 protein is determinative for its metastasis-promoting function.

The experimental mouse model studied here presents *in vivo* evidence that the expression and release of S100A4 from the tumor stroma is crucial both for stimulation of tumor development and metastasis formation. When the highly metastatic mouse mammary carcinoma cell line expressing the S100A4 protein (CSML100) was introduced into S100A4-deficient mice, we observed severe impairment in tumor incidence and tumor uptake. Moreover, we did not detect any metastasis in the lungs of S100A4(-/-) animals that grew tumors. In S100A4(+/-) we observed a tendency to retarded tumor growth; tumors that developed in the heterozygous animals were metastatic but formed smaller and less abundant lesions.¹ Heterozygous animals

¹ Our unpublished observations.

produced reduced amounts of S100A4 that support tumor development and stimulate metastasis formation only to a certain extent. This raises the possibility that expression of S100A4 restricted to tumor cells is not sufficient for stimulation of tumor growth and metastasis and points to an important role of S100A4-expressing host stroma cells as the main source of metastasis-stimulating active S100A4 protein.

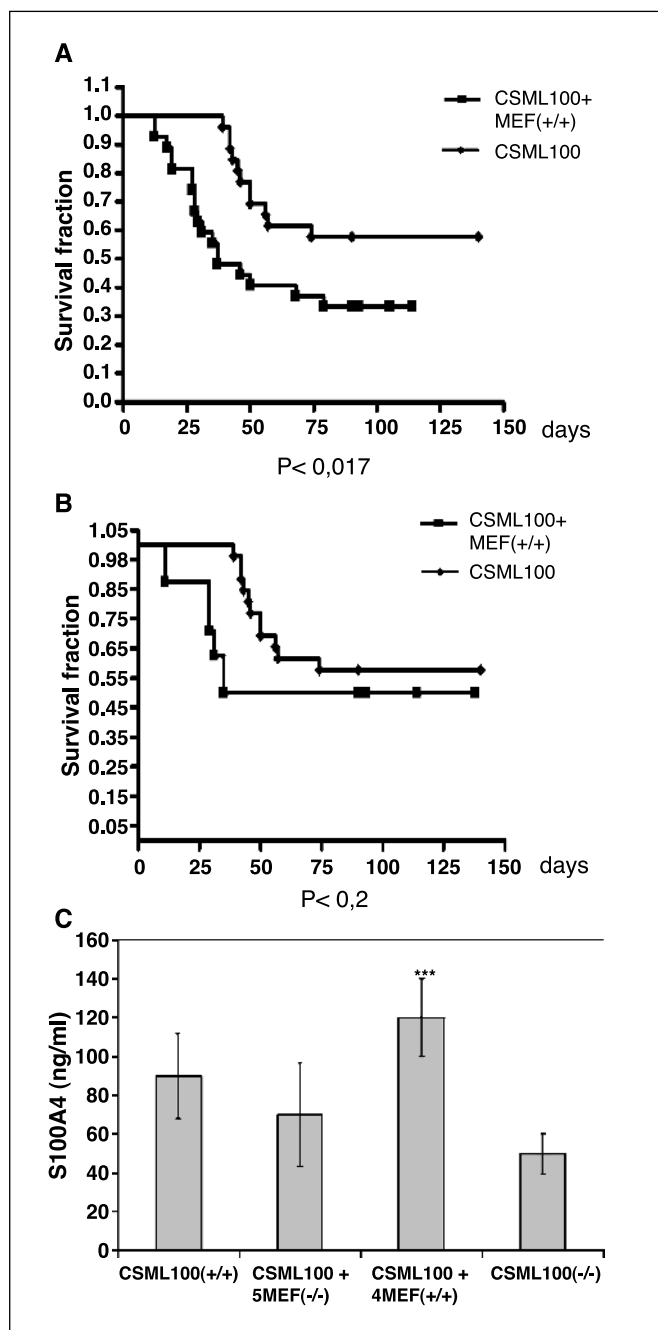


Figure 5. Tumor growth of CSML100 cells coinjected together with S100A4(+/+) and S100A4(-/-) fibroblasts in S100A4(-/-) mice. **A**, Kaplan-Meier survival curves comparing survival of CSML100 + S100A4(+/+) fibroblast tumor-bearing mice with the CSML100. **B**, Kaplan-Meier survival curves comparing survival of CSML100 + S100A4(-/-) fibroblast tumor-bearing mice with the CSML100. **C**, amount of the S100A4 protein in the tumor interstitial fluid obtained from tumor biopsies.

Retardation of tumor development was also observed when nonmetastatic CSML0 tumor cells were grafted to S100A4(-/-) mice. In general, CSML0 cells are characterized by very rapid tumor uptake and development. This indicates that these cells are less dependent on stroma for development and explains the less efficient inhibition of tumor development by S100A4-deficient stroma produced in S100A4-null mice.

Development of stroma in CSML100 tumors eventually grown in S100A4-deficient mice was impaired. We observed a decreased vessel density and an accumulation of macrophages, lymphocytes, and α -smooth muscle actin-positive cells (smooth muscle cells and myofibroblasts) in the growing edge of the tumor. It was shown previously that extracellular S100A4 stimulates motility and invasion of endothelial cells acting as an angiogenic factor. Insufficient concentration of the S100A4 protein in the tumor environment consequently leads to a decrease in the amount of newly formed blood vessels in the growing tumor.

Tumor growth is determined by an interplay between tumor cells that grow and eventually invade surrounding tissues and host-derived stroma that is recruited by tumor cells (34, 36). Atypically developed stroma of tumors grown in S100A4-deficient mice could reflect the inability of stroma cells deprived of S100A4 to move and invade the tumor mass. Both modulation of motility and invasiveness of S100A4-negative stroma cells and the insufficient amount or absence of extracellular S100A4 in tumor microenvironment might play a sufficient role in this process. S100A4 deficiency in the tumor environment could lead to changes in activation of transcription factors with subsequent modulation of tumor cell motility, invasion, and remodeling of extracellular matrix (26, 30).

It is well established that the induction of S100A4 expression leads to increased motility of tumor cells (16, 17, 37). Here we show that fibroblasts derived from S100A4-deficient mouse embryos exhibit decreased motility. Moreover, S100A4(-/-) MEFs failed to invade three-dimensional Matrigel that might be accounted as a model of stroma cells deprived of tumor extracellular matrix.

S100A4 is regarded as a fibroblast-specific marker for the cells undergoing epithelial-mesenchymal transition (38). It has been shown that tumor-associated fibroblasts in contrast to the normal fibroblasts express high levels of S100A4 (3). Furthermore, S100A4 expression is increased in activated fibroblasts and myofibroblasts of the regenerating cornea (29). One can propose that fibroblasts derived from S100A4(-/-) mice could not be efficiently recruited by a tumor because of their decreased invasive abilities and motility. This is confirmed by immunohistochemical staining of tumors obtained from S100A4-deficient mice with antibodies against α -smooth muscle actin. These antibodies recognize smooth muscle cells and myofibroblasts that represent the predominant fraction of tumor-associated fibroblasts (39). The same could be speculated for other host-derived tumor stroma cells, such as lymphocytes and macrophage, which increase expression of S100A4 on activation (40). Both tumor infiltrating leukocytes and macrophages represent a major component of tumor stroma that, by production of an array of growth factors, proteases, and angiogenic mediators, promote tumor growth, angiogenesis, and metastasis (41, 42).

It has been shown that fibroblasts after stimulation by tumor cells release S100A4. Increased concentration of S100A4 in the tumor microenvironment could contribute to tumor progression. S100A4 stimulates production of matrix-degrading proteases and modulates the cytoskeleton and focal adhesions of tumor cells. It also stimulates motility and invasion of endothelial cells by

stimulating the production of matrix degrading enzymes, thereby acting as an angiogenic factor (28, 30). These data indicate that local concentration of S100A4 in the tumor microenvironment could be determinative both for stimulation of angiogenesis and for invasion of tumor cells.

The fibroblasts represent a major cell type of the stroma compartment. Tumor-associated fibroblasts are basically represented by myofibroblasts and actively produce growth factors and other modulatory proteins that promote both tumor cell growth and invasion (34, 43). In experimental models fibroblasts are capable of stimulating epithelial tumor cell growth and progression (44, 45).

In our experimental model, we were able to restore the ability of CSML100 cells to grow tumors in S100A4-deficient mice by supplying them with fibroblasts, one of the predominant fractions of stroma cells. MEFs gain features of myofibroblasts on immortalization (34); moreover, they express high levels of S100A4 and release S100A4 on cocultivation with tumor cells (30). Therefore, we decided to generate immortalized MEFs that will mimic features of activated tumor-associated fibroblasts and supply CSML100 tumor cells with these fibroblasts to overcome the retardation of CSML100 tumor development observed in S100A4 mice. Both S100A4(+/+) and (-/-) fibroblasts were capable of stimulating CSML100 tumor development in S100A4-deficient mice. This was an expected result in light of recent studies demonstrating that tumor-associated fibroblasts play a substantial role in cancer initiation and progression (43). However, coinjection of S100A4(+/-) MEFs conferred to the CSML100 cells much more aggressive features compared with S100A4-deficient MEFs. Coinjection of CSML100 cells and S100A4(-/-) MEF led to a 10% increase in the tumor incidence, whereas coinjection of S100A4(+/-) MEFs with CSML100 cells left only 33% of animals tumor free. More striking, the metastatic ability of the CSML100 cell was partially restored.

The effect of S100A4(+/-) fibroblasts might be associated with their enhanced motility and ability to invade the extracellular matrix. On the other hand, S100A4(+/-) MEFs could release the S100A4 protein on cocultivation with tumor cells (30). We also detected enhanced amounts of S100A4 in the tumor interstitial

fluid containing proteins released from tumor biopsies obtained after coinjection of CSML100 and S100A4(+/-) MEFs to S100A4-null mice. This opens the possibility that both the differences in invasive abilities of host-derived stroma cells and the local concentration of the S100A4 protein in tumor microenvironment could contribute to stimulation of tumor growth.

We have shown here that the fibroblasts that express S100A4 stimulate tumor growth and restore the ability of a tumor to form metastasis in distant organs of S100A4-null mice. This is correlated with the ability of S100A4(+/-) fibroblasts to move and invade extracellular matrix and to release the S100A4 protein into the tumor microenvironment. Our results strongly indicate that expression and release of S100A4 by host-derived stroma cells could play a pivotal role in stimulation of tumor progression.

In recent years, more and more attention is paid to the role of tumor stroma in stimulation of tumor development and determination of its metastatic ability (36). It has been shown that molecules produced by tumor stroma, such as proteases or cytokines, dramatically influence tumor progression. Stromelysin-1 promotes in a paracrine manner homing of malignant epithelial cells (46). Tumor development is retarded in mice lacking the components of the plasminogen activator system and MMP-9 (47-51). Thrombospondin-1 suppresses tumor growth by modulating extracellular matrix remodeling and angiogenesis (52).

The S100A4 protein now emerges as a new important player in this process. Blocking of S100A4 activity in the tumor microenvironment will open new possibilities for the development of new anticancer therapies.

Acknowledgments

Received 12/20/2004; revised 2/17/2005; accepted 2/24/2005.

Grant support: Danish Cancer Society, Danish Research Council and EU "BRECOSM," and Dansk Kraefftforskning's Fond (B. Grum-Schwensen).

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We thank Birgitte Kaas, Ingrid Fossar-Larsen, and Inge Skibshøj for careful technical assistance.

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