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Maspin influences response to doxorubicin by changing the tumor microenvironment organization

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Altered degradation and deposition of extracellular matrix are hallmarks of tumor progression and response to therapy. From a microarray supervised analysis on a dataset of chemotherapy-treated breast carcinoma patients, maspin, a member of the serpin protease inhibitor family, has been the foremost variable identified in non-responsive versus responsive tumors. Accordingly, in a series of 52 human breast carcinomas, we detected high maspin expression in tumors that progressed under doxorubicin (DXR)-based chemotherapy. Our analysis of the role of maspin in response to chemotherapy in human MCF7 and MDAMB231 breast and SKOV3 ovarian carcinoma cells transfected to overexpress maspin and injected into mice showed that maspin overexpression led to DXR resistance through the maspin-induced collagen-enriched microenvironment and that an anti-maspin neutralizing monoclonal antibody reversed the collagen-dependent DXR resistance. Impaired diffusion and decreased DXR activity were also found in tumors derived from Matrigel-embedded cells, where abundant collagen fibers characterize the tumor matrix. Conversely, liposome-based DXR reached maspin-overexpressing tumor cells despite the abundant extracellular matrix and was more efficient in reducing tumor growth. Our results identify maspin-induced accumulation of collagen fibers as a cause of disease progression under DXR chemotherapy for breast cancer. Use of a more hydrophilic DXR formulation or of a maspin inhibitor in combination with chemotherapy holds the promise of more consistent responses to maspin-overexpressing tumors and dense-matrix tumors in general.

Resistance of tumors to chemotherapeutic drugs remains a major clinical challenge for cancer treatment. Although such resistance has been attributed mainly to mechanisms by which tumor cells avoid apoptosis, evidences indicate that the tumor microenvironment also plays a major role.[1] Remodeling of the extracellular matrix is a normal biological process, and its dynamic interaction with tumor cells is crucial for cellular homeostasis and tissue remodeling. Altered degradation and deposition of the extracellular matrix are hallmarks of tumor progression and response to therapy. Maspin (serpinB5), a member of the serpin family of serine protease inhibitors, was originally identified as a gene down-modulated in invasive breast cancer and proposed as a class II tumor suppressor.[2] Although currently classified as a non-inhibitory serpin since there are no known target molecules,[3] maspin is likely to act as a serpin-like inhibitor of serine protease-like targets[4] based on reports of high maspin expression in normal human mammary epithelial cells but decreased expression in breast tumor cells and absence in metastatic cells.[5] Maspin localizes primarily in the cytoplasm but can also localize in the nucleus, in secretory vesicles, extracellularly and at the cell surface, although the regulation of maspin trafficking is currently unknown.[6,7] Despite extensive studies of the role of intracellular and membrane-associated maspin in myriad biological functions,[8] the function of secreted maspin is still unclear. To date, no data on the predictive value of maspin expression in breast cancer have been reported.

Here, we provide evidence that overexpression of maspin in breast carcinomas, leading to collagen fiber accumulation, underlies unresponsiveness to doxorubicin (DXR) by blocking the drug's diffusion.

Key words: maspin, extracellular matrix, response to doxorubicin, drug delivery
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Material and Methods

Patients

Breast carcinoma core biopsies were obtained from 52 patients before treatment with anthracycline-based chemotherapy at Fondazione IRCCS Istituto Nazionale dei Tumori (INT) of Milan

What's new?

Alterations in the extracellular matrix can cause resistance of tumors to chemotherapeutic drugs. Here, the authors identify the serpin-like serine protease inhibitor maspin as a gene upregulated in its expression in breast tumors that progress despite doxorubicin treatment. In mice xenografted with maspin-overexpressing tumor cells, enhanced collagen fiber accumulation and decreased diffusion of the chemotherapeutic drug was observed. The study indicates that more hydrophilic doxorubicin formulations or the combination with a maspin inhibitor could be useful in the treatment of drug-resistant, dense matrix breast tumors.

and for whom response to neoadjuvant chemotherapy was available. Supporting Information Table 1 lists the patients' characteristics. All procedures were in accordance with the Helsinki Declaration. Biospecimens used for research consisted of leftover material of samples collected during standard surgical and medical approaches at Fondazione IRCCS-INT. Samples were donated by patients to the Institutional BioBank for research purposes, and aliquots were allocated to this study after approval by the Institutional Review Board and a specific request to the Independent Ethical Committee of Fondazione IRCCS-INT.

Cell culture, plasmids and infection

Human ovarian carcinoma SKOV3 cells and breast carcinoma MCF7 and MDAMB231 cells, able to grow in athymic mice, were purchased from ATCC (Rockville, MD). The cell lines exhibited distinct morphologic features and were authenticated by the Fragment Analysis Facility at INT, using the Stem Elite ID System-Promega. Profiles were matched to their original profiles in the cell line database at ATCC. Cells were maintained in RPMI 1640 medium (Sigma Chemical Co., St. Louis, MO) supplemented with 10% (v/v) fetal calf serum (FCS) (Sigma) and L-glutamine in a humidified chamber (95% air, 5% CO_2) at 37°C.

The plasmid pLENTI6/maspin, encoding human maspin and used to stably infect parental cells, was produced by amplifying full-length human maspin cDNA from the pCMVXL4-maspin expression vector (Origene Technologies, Inc., Rockville, MD) and subcloning the fragment in the SpeI and XhoI sites of pLENTI6 (Invitrogen, Carlsbad, CA). Lentiviral particle formation and cancer cell infection were performed according to the manufacturer's recommendations. Individual colonies were randomly picked, expanded, tested for maspin production by qRT-PCR and Western blot and maintained in the presence of blasticidin (Invitrogen).

Proliferation and DXR response assays

Relative cellular growth in DXR-treated or untreated cells was measured using the sulforhodamine B assay. Optical density was evaluated with an ELISA microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA). Results for proliferation were normalized to those of control samples, and IC_{50} (μM DXR) was determined from the dose-response curves.

Protein extraction, Western blotting and antibodies

Protein fraction was extracted from cells grown *in vitro* or xenotransplanted in athymic mice and analyzed by Western blotting as described.[9] Extracellular maspin was detected as described.[7] Antibodies used were: anti-vinculin (Sigma), anti-maspin (BD Bioscience, San Jose, CA), anti-P-AKT (Ser 473), anti-total AKT, anti-phospho-p44/42-MAPK and anti-p44/42-MAPK (Cell Signaling Technology, Beverly, MA), anti-actin (Sigma) and anti-mouse collagen I (Abcam, Cambridge, UK).

Mouse monoclonal antibody (MAb) against human maspin MPI1 was produced and selected as described.[10] Briefly, Balb/c mice were immunized three times with recombinant GST-maspin protein (NeoMarkers, Fremont, CA), and splenocytes were fused with NS0 mouse myeloma cells and seeded in 96-well plates. Hybridoma supernatants were recovered for selection by ELISA in plates precoated with recombinant maspin or GST (NeoMarkers), and Ig isotypes were determined using IsoStrip (Roche, Mannheim, Germany).

In vivo growth

Experimental protocols were approved by the Ethics Committee for Animal Experimentation of Fondazione IRCCS-INT. About 6- to 8-week-old athymic mice were purchased from Charles River (Calco, Italy). Care and use of the animals was in accordance with institutional guidelines. Mice (4/group) were injected subcutaneously or into the mammary fat pad in both flanks with 1×10^6 SKOV3 or 5×10^6 MDAMB231 cells in growth medium diluted 1:1 with Matrigel (BD Bioscience). To generate an extracellular matrix-rich model, 10^6 SKOV3 cells embedded in 100 μL of Matrigel were injected in athymic mice. DXR or DOXIL was administered in SCID and nude mice (4 and 6 mg/kg, respectively) intravenously once per week for 3 weeks starting when tumors reached a median volume of 100 mm^3. One week before the beginning of DXR treatment, 4 mg/kg MPI1 or unrelated antibody was administered intraperitoneally twice weekly for 3 weeks. Tumors were calibrated twice weekly and tumor volume was calculated as $0.5 \times d_1^2 \times d_2$, where d_1 and d_2 are the smaller and largest diameters, respectively.

Confocal microscopy

To assess drug sequestered in tumor stromal fibers, tumors with a median volume of 200 mm^3 grown in athymic mice were removed at different time points after DXR or DOXIL injection, fixed in 4% paraformaldehyde and OCT, frozen in liquid nitrogen, sectioned (5-μm) and processed for immunofluorescence (IF) labeling. DXR and DOXIL content were

analyzed in a time course of 5 min to 24 hr after injections. Because DXR and DOXIL peaks content in tumors derived from mock tumor cells were observed at 15 min and 24 hr, respectively (not shown), drug content was compared in maspin-overexpressing versus matched mock cells at these times. Slides were saturated with 3% BSA and incubated with primary antibodies: rabbit pAb anti-human HER2-C18 clone (Santa Cruz Biotechnology, CA), rabbit pAb anti-human HER1 (Cell Signaling Technology), or rat MAb anti-mouse CD31 (BD Bioscience) and incubated with specific Alexafluor-conjugated (Invitrogen) secondary antibody.

To determine maspin localization within cells grown *in vitro* and *in vivo* and in tumor tissues, cells, frozen xenografts and FFPE human specimens after fixation and permeabilization steps were stained with mouse MAb anti-human maspin (BD Bioscience) rabbit MAb anti-mouse collagen I (Abcam) or rabbit pAb anti-human collagen I (Millipore, MA), respectively, and incubated with specific Alexafluor-conjugated (Invitrogen) secondary antibody. Nuclei were visualized by DRAQ5. Coverslips were mounted on glass slides using Prolong (Calbiochem, San Diego, CA) and examined with a differential interference contrast equipped (DIC) confocal microscope (Microradiance 2000, BioRad) equipped with Argon (488 nm), Green HeNe (543 nm) and Red diode (633 nm) lasers. Images were obtained using a 60× oil immersion lens (512 × 512 or 1,024 × 1,024 pixels) and analyzed using Image-Pro Plus v. 7.0.1 (MediaCybenetics) software.

Immunohistochemical (IHC) analyses

IHC staining of human core biopsies and tumors grown in mice was carried out on formalin-fixed, paraffin-embedded (FFPE) sections. Ki67 (Clone MIB-1, Dako, Glostrup, Denmark) and collagen IV (Acris antibodies GmbH, Germany) staining were analyzed as described.[11,12] Maspin was analyzed using mouse anti-human maspin MAb (BD Bioscience), and collagen content, using COLI and COLIII antibodies (Novus Biological, Littleton, CO). Blood vessel content was analyzed by CD31 staining using anti-mouse CD31 (BD Bioscience) antibody. Antigen retrieval was carried out by heating slides for 6 min at 95°C in 5 mM citrate buffer, pH 6.0. Immunoreactions were enhanced by streptavidin–biotin–peroxidase, followed by counterstaining with Carazzi hematoxylin. H&E-stained FFPE sections were used to assess the average density of stroma. Tumors were considered maspin-positive if they showed at least 20% immunoreactive cells.

RNA extraction and quantitative real-time PCR

Total RNA was extracted from cell lines and xenografts with Trizol® (Invitrogen) and from FFPE tissue with the High Pure FFPE RNA extraction kit (Roche, Basel, Switzerland) according to the manufacturer's instructions. cDNAs were reverse-transcribed from 1 μg of total RNA in a 20-μL volume with SuperScript II (Invitrogen) using oligo-dT primers for cell lines and random-hexamer primers for FFPE breast tissues. qRT-PCR was performed using Applied Biosystem Taqman assays (Maspin: Hs.00985285_m1, COL1A2: Mm00483888_m1) on the ABI Prism 7900HT sequence detection system (Applied Biosystems). Data were normalized to GAPDH in experiments on cell lines and to the combined expression levels of genes MPRL19 and PMM1, selected by GeNorm software[13], in experiments on FFPE breast tissues. Gene expression levels were calculated by the comparative Ct method using untreated samples mock cells as the reference in experiments on cell lines and using Universal Human Reference RNA (Stratagene, La Jolla, CA) in experiments on FFPE breast tissues.

Statistical analysis

Public gene expression data of Chang *et al.*[14] was used for analysis with no additional data manipulation. Genes differentially expressed in responsive versus non-responsive patients were identified in *t*-test in significance analysis of microarray (SAM)[15] using an ECM gene list[16] and BRB Array tools developed by Richard Simon and Amy Peng Lam.[17] Absence of association between two categorical variables was tested by chi-square test. Two-sided $p < 0.05$ was considered statistically significant. Differences between groups using a two-tailed unpaired Student's *t*-test were considered significant at $p < 0.05$.

Results

Association between maspin expression and chemotherapy response in breast carcinomas

Supervised analysis of published genomic data on breast cancer patients neoadjuvantly treated with docetaxel,[14] using "disease progression under treatment" as a supervised variable, identified maspin as a significantly differentially expressed variable (13.4-fold higher in progressive vs. non-progressive tumors under chemotherapy [FDR = 0.03, $p < 0.001$]). qRT-PCR analysis of core biopsies from 52 patients treated in our Institute with anthracycline-based neoadjuvant therapy revealed significantly higher maspin levels in patients with progressive disease as compared with responders ($p = 0.0016$) (Fig. 1*a*); IHC analysis of these 52 breast tumor core biopsies revealed maspin positivity in 87% (14/16) of non-responsive versus 31% (11/36) of responsive tumors ($p = 0.0002$) (Fig. 1*b*). Notably, collagen content was elevated in 72% (18/25) of maspin-positive biopsies versus 33% (9/27) of negative tumors ($p = 0.0067$) (Fig. 1*b*). No significant differences were found in tumor cell proliferation, as evaluated by Ki67 staining (77% maspin-positive vs. 61% maspin-negative tumors showed nuclear positivity in more than 10% of tumor cells), and in tumor vascularity, as evaluated by CD31 staining (52% maspin-positive vs. 67% maspin-negative tumors showed high vessels content), suggesting that maspin may exert its inhibitory effect on drug efficacy by influencing collagen deposition/degradation in the tumor extracellular matrix rather than by inhibiting angiogenesis or tumor cell division reported to be associated with sensitivity to chemotherapeutic drugs.[18]

Stable expression of maspin in breast and ovarian carcinoma cells

To begin to investigate the role of maspin in drug response, MDAMB231 and MCF7 breast carcinoma and SKOV3 ovarian carcinoma cells were infected with maspin-expressing or control lentiviral particles, and stable clones (MCF7_M1, _M2; 231_M1, _M2; SKOV3_M1, _M2) expressing various levels of maspin were subcloned (Figs. 2*a* and 2*b*). Maspin localized primarily in the cytoplasm of overexpressing cells, as assessed by confocal microscopy (Fig. 2*c*), and in the extracellular space, as assessed by Western blot of the matrix deposited by MCF7_M1, 231_M1, SKOV3_M1 cells after complete removal of the cells (Fig. 2*d*). Consistent with previous reports,[19,20] SKOV3_M1 cells showed higher adhesion on a fibronectin matrix and less migration as compared with mock cells (Supporting Information Fig. S1).

In vitro proliferation assays revealed no significant differences between maspin-overexpressing and matched mock cells (Supporting Information Fig. S2), and the growth-inhibitory effect of DXR in both maspin-expressing and matched mock cells was essentially the same (IC_{50} [μM]: MDAMB231 mock: 0.5 ± 0.25, M1: 0.5 ± 0.09, M2: 0.42 ± 0.08; MCF7 mock: 0.53 ± 0.33, M1: 0.48 ± 0.29, M2: 0.59 ± 0.07; SKOV3 mock: 0.57 ± 0.18, M1: 0.45 ± 0.18, M2: 0.41 ± 0.08) (Supporting Information Fig. S2), indicating that maspin overexpression does not impact *in vitro* response of cells to DXR.

Effects of maspin expression on tumor growth, microenvironment organization and response to DXR treatment

Consistent with *in vitro* studies, maspin expression had no impact on growth of MDAMB231 and SKOV3 cells injected into the mammary fat pad in SCID mice and subcutaneously in nude mice, respectively (Supporting Information Fig. S3), with the same percentage of Ki67-positive nuclei found in tumors from mice xenografted with maspin-expressing and mock cells. By contrast, DXR-mediated growth inhibition was higher in mock tumors as compared with maspin-expressing tumors (Fig. 3*a*), and, unlike tumor sections from mock cell-injected mice, tissue sections from maspin-expressing xenografts

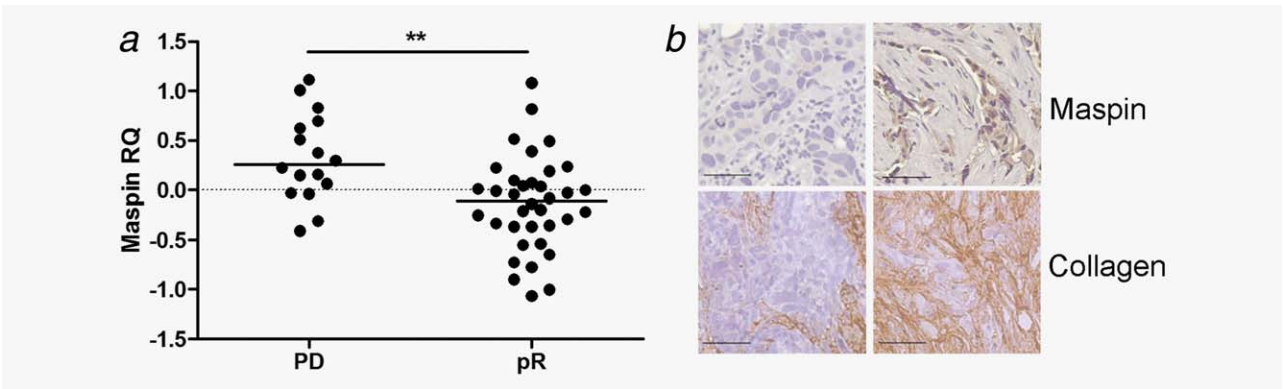

Figure 1. Correlation of maspin expression in breast core biopsies with response to chemotherapy. (*a*) qRT-PCR of maspin mRNA assessed in FFPE core biopsies from patients treated with neoadjuvant chemotherapy. Relative maspin mRNA expression was normalized to PMM1 and MRPL19 as reference genes. Each *dot* corresponds to a tumor. PD: progressive and stable disease; pR: partial response. $^{**}p < 0.01$ by unpaired *t*-test. (*b*) Representative IHC staining of maspin and collagen expression (Collagen I-III) in maspin-negative (**left**) or -positive (**right**) tumors. Scale bar, 60 μm.

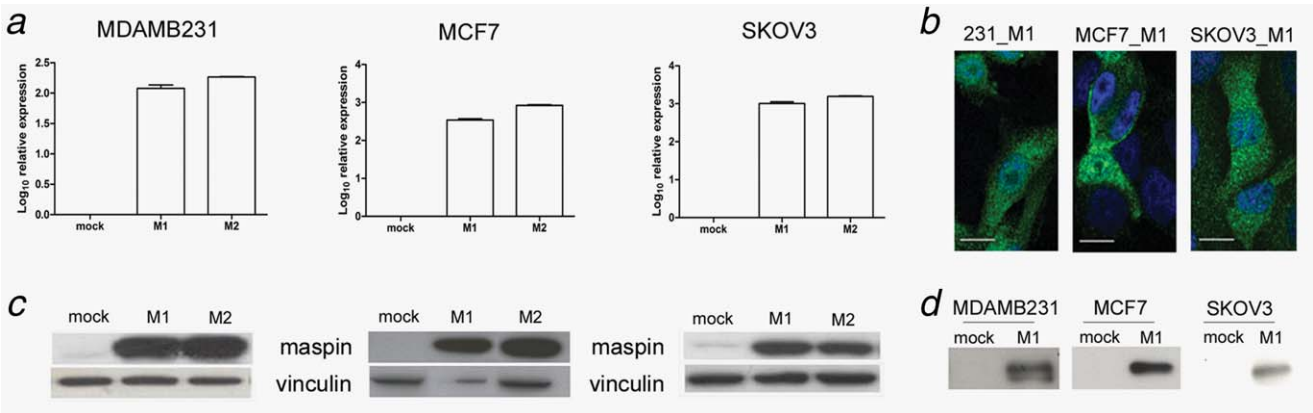

Figure 2. Characterization of stable maspin transfectants. (*a*) qRT-PCR and (*b*) Western blot of maspin in stable transfectants and mock MDAMB231 (**left**), MCF7 (**middle**) and SKOV3 (**right**) cells. Data are mean ± SD ($n = 3$). (*c*) Confocal microscopy analysis of maspin in M1 clones (*green*: maspin, *blue*: DRAQ5). Scale bars, 10 μm. (*d*) Western analysis of maspin in the matrix deposited by M1 clones.

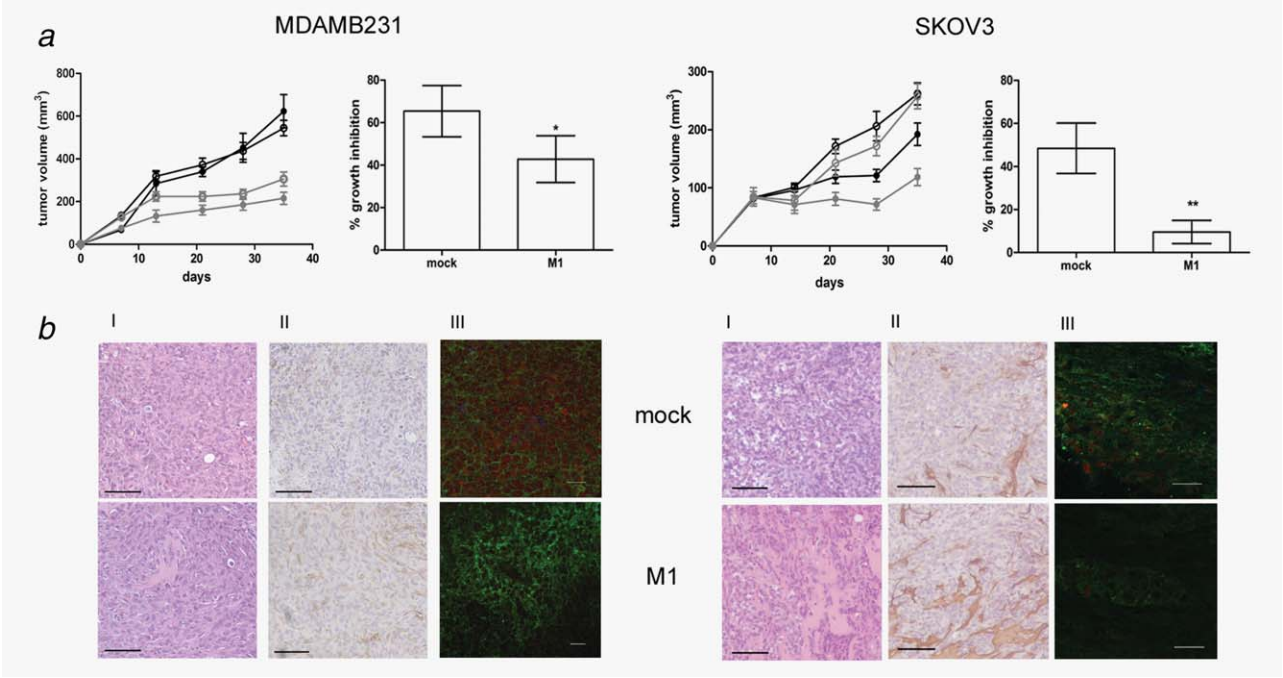

Figure 3. Effect of maspin expression on DXR response. (*a*) Tumor volume and percentage tumor growth inhibition by DXR at day-35 after injection of 231_M1 (**left**) and SKOV3_M1 (**right**) and mock cells. (●) Untreated mock cells, (○) untreated M1 cells, (●) DXR-treated mock cells, (○) DXR-treated M1 cells. Data are mean ± SD ($n = 8$). *$p < 0.05$, **$p < 0.01$ by unpaired *t*-test. (*b*) IHC staining of xenografts from mice. Representative results are shown for H&E staining (I) and collagen IV immunostaining (II) in mock- and maspin-positive (M1) xenografts from injection of 231 (**left**) or SKOV3 (**right**) cells. Scale bars, 100 μm. (III) DXR (*red*) localization evaluated by confocal microscopy (MDAMB231, *green*: HER1, *blue*: CD31; SKOV3, *green*: HER2, *blue*: CD31). Scale bars, 20 μm.

displayed a matrix organization characterized by collagen fiber accumulation (Fig. 3*b*, panels I and II). Western blot analysis of the protein fraction extracted from cells grown *in vivo* also revealed collagen accumulation in M1 tumors using anti-mouse, but not anti-human collagen antibodies (Fig. 4*a*), indicating that the increase in this extracellular matrix protein did not derive from enhanced collagen production by maspin-expressing tumor cells. In addition, no differences in COL1A2 expression between M1 and mock tumors were detected in qPCR analysis using primers specific for the murine sequence (Fig. 4*b*), suggesting that the observed collagen accumulation in maspin-expressing tumors likely derived from inhibition of its degradation rather than its enhanced production. Because recombinant maspin has been shown to reduce collagen degradation *in vitro*,[7] and because M1 cells released maspin in the extracellular space *in vitro,* we evaluated the localization of maspin both in M1 xenografts and maspin-positive human breast carcinomas by confocal microscopy analysis of tumor tissue sections. As shown in Figure 4*c*, maspin labeling was found in the extracellular space and co-localized with collagen I in both types of samples, suggesting that maspin interaction likely inhibits collagen susceptibility to proteolytic enzymes.

Based on several studies suggesting that the adhesion of tumor cells to the extracellular matrix might play a role in resistance to therapy-derived apoptotic stimuli,[21–24] the interaction of maspin-expressing cells with increased collagen fibers could result in direct activation of survival signaling to affect DXR efficacy.[25] However, Western blot analysis revealed no significant differences in pAKT/AKT and pMAPK/MAPK ratios in high collagen-enriched M1 clone as compared with mock tumors (Supporting Information Fig. S4). Thus, the decreased susceptibility of maspin-expressing xenografts to DXR does not appear to be induced by signaling events resulting from direct cell adhesion with the tumor microenvironment. To test whether the different matrix organization of maspin-expressing xenografts affected DXR diffusion, we examined the spatial distribution of DXR *ex vivo* in mice bearing tumors overexpressing maspin or not. Confocal microscopy analysis of cryostatic sections from 231_M1 and SKOV3_M1 tumors showed a dramatically decreased number of cells with DXR-positive nuclei as compared with mock tumors (Fig. 3*b*, panel III) at 15 min from drug injection. Few and comparable DXR-positive nuclei were detected in 231_M1 and 231_mock mouse xenografts at 30 min from DXR administration (data not shown). Mean vessel densities, quantified as the average number of IHC $CD31^+$ vessels, showed no differences in tumor vascularization between mock and M1 xenografts. Thus, the impairment of DXR diffusion in maspin-expressing tumors compared with mock tumors derives from collagen high content that impedes drug diffusion.

Based on the hypothesis that a block in maspin activity might increase drug diffusion and responsiveness, we derived a mouse anti-maspin monoclonal antibody (MPI1), which

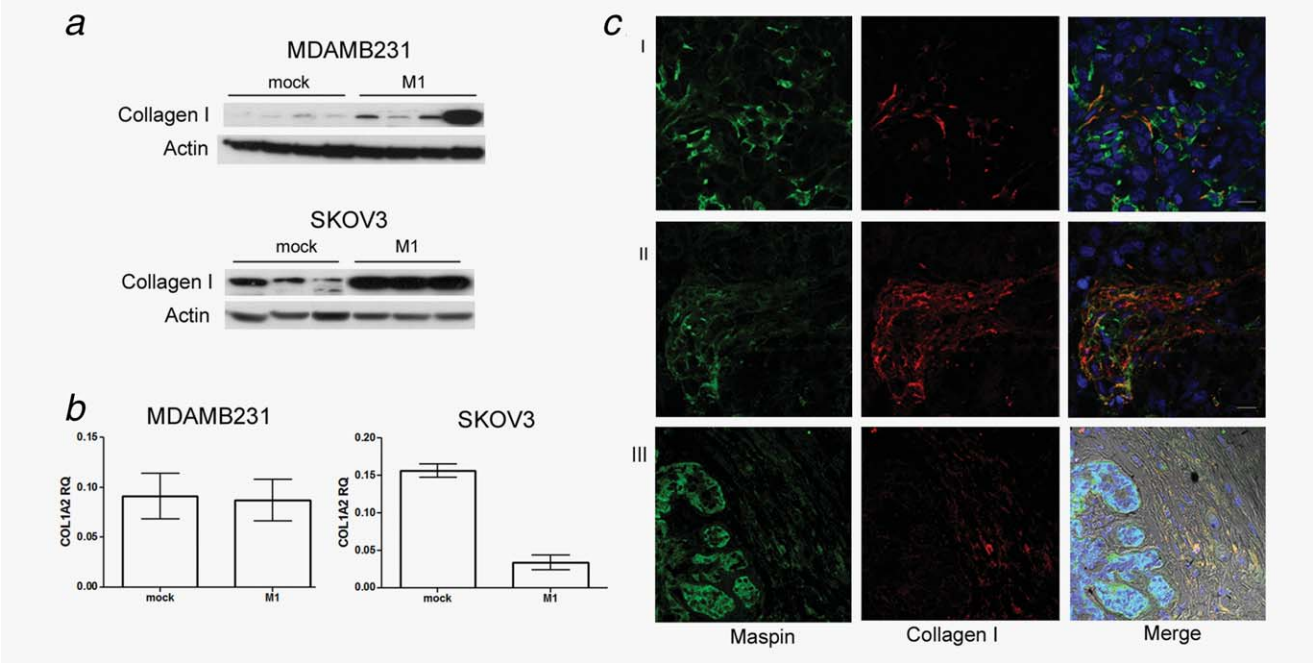

Figure 4. Effect of maspin expression on microenvironment organization. (*a*) Western blot and (*b*) qRT-PCR of mouse collagen I in tumors derived from 231_M1 (**left**) and SKOV3_M1 (**right**) and mock cells. Data are mean ± SD ($n = 4$). (*c*) Representative confocal microscopy of maspin and collagen I in I: 231_M1, II: SKOV3_M1 xenografts and III: human specimen (*green*: maspin; *red*: collagen I; *blue*: DRAQ5; *gray*: DIC). Scale bars, 10 μm.

recognized maspin in ELISA assay, blocked adhesion of SKOV3_M1 cells on a fibronectin matrix and increased 231_M1 cell migration *in vitro* (Supporting Information Fig. S5). Mice injected with 231_M1 cells and treated with MAb MPI1 IgG1 antibody plus DXR showed tumor growth inhibition of approximately 72% versus 49% by DXR alone; treatment with an unrelated antibody did not affect tumor growth (Figs. 5*a* and 5*b*). Thus, blocking maspin activity significantly increased DXR sensitivity. Analysis of the tumor microenvironment organization revealed a decreased collagen content in MPI1-treated tumors versus untreated tumors (Fig. 5*c*), suggesting that blocking maspin led to a change in the extracellular matrix framework and a consequent increase of DXR activity.

Effect of collagen accumulation on tumor growth and on response to DXR

To test whether maspin-induced collagen accumulation *per se* accounts for low DXR diffusion and resistance, we examined the effect of DXR in nude mice xenotransplanted with SKOV3 cells mixed or not with Matrigel, which contributed to the collagen-enriched microenvironment of xenografts (Supporting Information Fig. S6). DXR-mediated reduction of tumor growth was significantly greater in tumors grown in the absence of Matrigel compared with Matrigel-mixed tumors (Supporting Information Fig. S6). Moreover, confocal microscopy analysis revealed lower uptake of DXR in Matrigel-mixed tumors than in tumors grown without Matrigel (Supporting Information Fig. S6), supporting the notion that decreased drug delivery and efficacy is associated with collagen accumulation.

Antitumor effect and diffusion of liposome-based DXR (DOXIL)

To test whether a more hydrophilic DXR formulation might bypass the collagen-enriched impairment of drug delivery and efficacy, we investigated the diffusion and efficacy of liposome-based DXR (DOXIL). DXR localized in tumor cell nuclei of both maspin-expressing and non-expressing xenografts (Fig. 6*a*), and DXR localization was similar in tumors derived by injection of cells mixed or not with Matrigel treated with DOXIL (Supporting Information Fig. S6). Thus, this drug formulation appears to diffuse more readily than free DXR in a collagen fiber-enriched microenvironment. Indeed, DOXIL treatment significantly reduced growth of both SKOV3-M1 and 231-M1 xenografts in the same percentage of tumors derived from mock cells (Fig. 6*b*).

Discussion

Identification of tumor cell molecular features associated with response to specific drugs has become a major goal in oncobiology in an effort to better select patients likely to benefit from a particular treatment. Our present study provides strong evidence that a microenvironment impairment in drug diffusion due to maspin-dependent increased collagen deposition leads to DXR chemoresistance. We find that breast

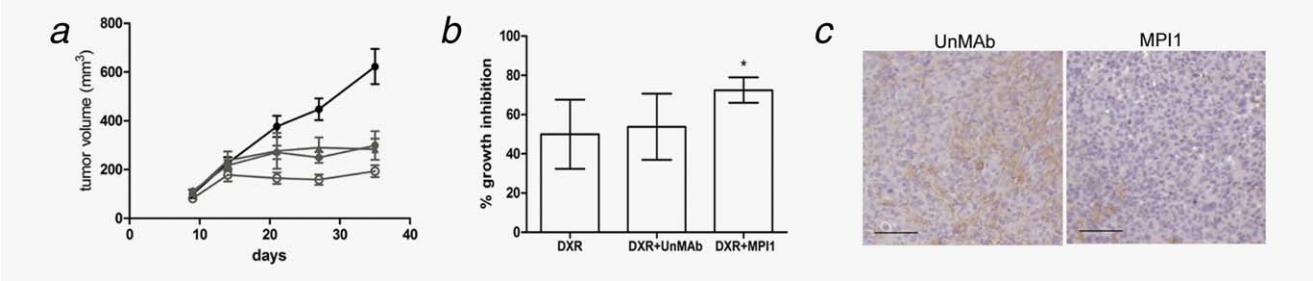

Figure 5. Inhibition of maspin by monoclonal antibody and response to DXR. (*a*) Tumor volume of 231_M1 xenografts not treated (●) or treated with DXR alone (●) or in combination with MAb MPI1 (○) or unrelated antibody (▲). Data are mean ± SD ($n = 6$). (*b*) Percent tumor growth inhibition of 231_M1 xenografts by DXR alone or in combination with MAb MPI1 or unrelated antibody (UnMAb) at day-35 after injection. *$p < 0.05$ by unpaired *t*-test versus DXR-treated group. (*c*) Representative IHC staining for collagen I in MPI1- or UnMAb-treated 231_M1 xenografts. Scale bars, 100 μm.

carcinomas overexpressing maspin are more likely to be resistant to DXR-based chemotherapy, as demonstrated both in human primary breast carcinomas with high maspin expression levels and in mice xenotransplanted with tumor cells genetically manipulated to overexpress maspin independently of their intrinsic features. Despite the reported oncosuppressor effect of maspin,[2] its overexpression in breast carcinomas did not lead to inhibition of proliferation in either human samples or in maspin-overexpressing cells compared with low-expressing tumors and mock cells, respectively. In fact, the number of Ki67-positive cells did not differ between maspin-overexpressing and non-overexpressing human primary breast carcinomas, nor did maspin overexpression in tumor cells affect their growth rate *in vitro* or *in vivo*. Thus, despite the relevance of proliferation in driving the response to chemotherapy, the resistance of maspin-overexpressing cells is likely not related to decreased proliferation. The absent effect of maspin in blocking tumor cell proliferation may rest in the predominantly cytoplasmic and extracellular localization of this protease inhibitor in breast carcinomas. In fact, maspin subcellular localization is critical in its functions, and only its nuclear localization appears to be indicative of a tumor-suppressive role.[26–29] By contrast, recombinant maspin has been described to interact with collagen I and III,[30] reducing *in vitro* collagen degradation[7] and increasing fibrosis when transfected in prostate carcinoma cells.[4] Since in our models, collagen accumulation did not reflect increased collagen production by tumor or stromal cells, and maspin co-localized with extracellular collagen, we speculate that maspin binding to collagen present in the interstitial matrix inhibits susceptibility of collagen to proteolytic cleavage, thereby enabling its accumulation within the tumor cells. Although we cannot exclude the possibility that DXR resistance of maspin-expressing tumor cells also derives from greater adhesion to the extracellular matrix compared with mock tumor cells, as found in our *in vitro* analyses and as shown to confer chemotherapy resistance through downstream anti-apoptotic signals,[22,25,31] AKT and MAPK phosphorylation in SKOV3 and MDAMB231 tumors expressing maspin or not were similar, indicating that in our models,

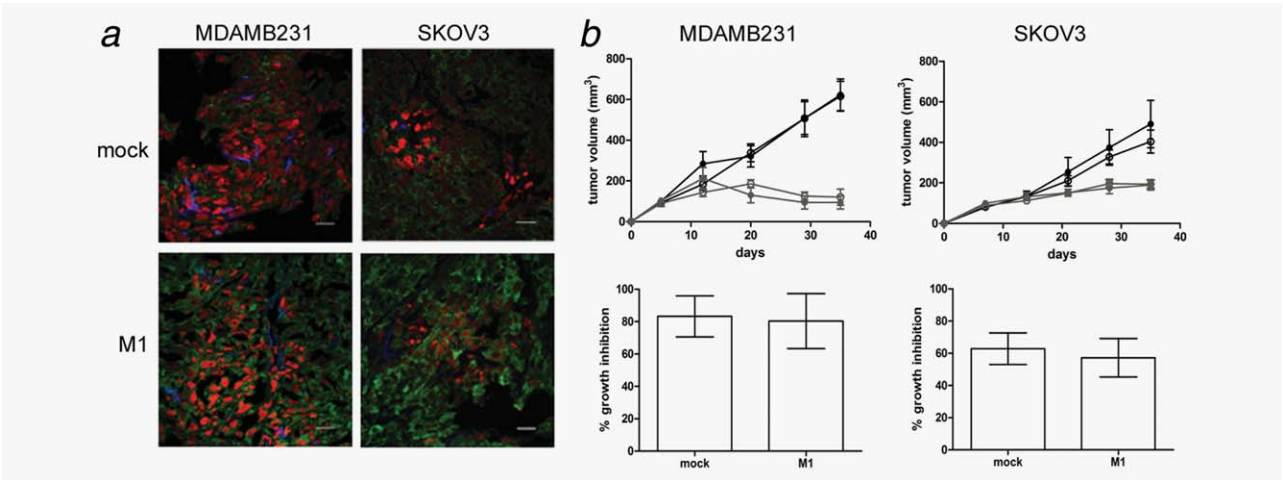

Figure 6. Effect of maspin expression on response to DXR-liposome treatment. (*a*) DXR-liposome diffusion evaluated by confocal microscopy in tumors of mice injected with 231_M1, SKOV3_M1 or mock cells (*red*: DXR, *green*: HER1 or HER2, *blue*: CD31). Scale bars, 20 μm. (*b*) Tumor volume and percent tumor growth inhibition by DXR-liposomes at day-35 after injection of 231_M1 (**left**) and SKOV3_M1 (**right**) and mock cells. (●) Untreated mock cells, (○) untreated M1 cells, (●) DOXIL-treated mock cells, (○) DOXIL-treated M1 cells. Data are mean ± SD ($n = 8$).

increased survival pathway activation was at least not the main mechanism through which maspin-overexpressing tumor cells resist chemotherapy. Thus, DXR resistance of maspin-overexpressing tumors appears to reflect a peculiar collagen accumulation found in both human specimens and in our models.

It has been reported that the tumor microenvironment can induce drug resistance by preventing drug penetration into the tumor.[32] Moreover, collagen I released by fibroblasts decreases drug uptake by cancer cells, thus mediating resistance to chemotherapy,[33] and drug penetration can be enhanced by decreasing the fibroblast pool and consequently the extracellular matrix produced.[34,35] In this context, we found that although DXR diffusion occurred in maspin-overexpressing xenografts, as assessed based on spatial distribution, it was significantly impaired in such tumors compared with those that do not express maspin. Moreover, no differences in the *in vitro* DXR growth inhibitory effect in maspin-expressing and mock cells were found, while DXR diffusion, as well as sensitivity to this anthracycline, was significantly decreased in collagen-enriched xenografts derived from injection of tumor cells embedded in basement membrane matrix as compared with non-mixed cells. Despite the reported maspin-driven antiangiogenic *milieu*,[36,37] we observed no significant hypovascular phenotype in our maspin-expressing models or in maspin-positive human specimens, indicating that maspin-associated drug resistance does not mainly depend on antiangiogenic activity of this protease inhibitor, but rather on maspin-induced accumulation of collagen fibers. However, since high pressure deriving from high collagen content can decrease vessel function,[38] it cannot be excluded that this mechanism also contributes in inducing drug resistance.

The enhanced susceptibility to DXR of maspin-overexpressing xenografts in mice treated concomitantly with an anti-maspin MAb further supports the role of maspin in drug efficacy through remodeling of the tumor microenvironment, as demonstrated by decreased fiber accumulation in tumors of mice treated with this antibody. These findings suggest the promise of administration of reagents that inhibit a collagen remodeling inhibitor such as maspin, in combination with a standard chemotherapeutic agent, such as DXR, as a novel therapeutic strategy to consistently achieve objective responses in breast carcinomas progressing under traditional therapy. The power of combining stroma-targeting molecules with standard chemotherapeutic agents has been demonstrated in pancreatic ductal adenocarcinomas, in which the systemic administration of an agent able to ablate hyaluronan, the primary determinant of the extracellular matrix barrier, can consistently increase tumor responses in mice.[39,40] Nevertheless, due to the role of maspin in inhibiting cell motility,[2,20] such an approach could increase the metastatic potential of tumor cells. The therapeutic potential of maspin inhibition in addition to chemotherapy treatments awaits further analyses to determine whether a block in this serine protease inhibitor improves sensitivity of tumor cells to drug treatments without increasing the number of metastases.

Impairment of DXR diffusion in maspin-positive tumors likely depends on biochemical properties of their collagen-enriched microenvironment, a notion consistent with the greater diffusion of a more hydrophilic formulation of DXR (polyethylene glycol-coated liposomes) in the microenvironment of maspin-overexpressing tumors than free DXR and the resulting significantly reduced growth of both maspin-positive and -negative xenografts. Thus, tumor stromal characteristics should be considered in choosing therapeutic drug formulations.

Although our analyses in preclinical models investigated the effect of maspin expression only on DXR efficacy, the correlation between maspin expression levels and disease progression under docetaxel treatment found in *in silico* analysis of a dataset relevant to neoadjuvantly treated breast cancer patients strongly suggests that the impaired drug diffusion consequent to maspin-induced collagen deposition is likely not restricted to anthracycline, but can also extend to drugs such as taxanes, frequently used in breast cancer treatment. Moreover, results obtained with SKOV3 cells suggest that maspin activity on collagen remodeling also applies to ovarian carcinomas, where maspin expression has been associated with poor survival despite chemotherapy.[29]

Our findings provide evidence that accumulation of collagen fibers within the tumor microenvironment due to the maspin overexpression accounts for disease progression under chemotherapy for breast carcinomas, making the microenvironment fertile ground for development of novel treatment strategies that augment existing therapy. Use of a more hydrophilic DXR formulation or of a maspin inhibitor in combination with chemotherapy may represent a promising strategy to consistently achieve responses of maspin-overexpressing tumors and dense-matrix tumors in general.

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