Fragment N2, a caspase-3-generated RasGAP fragment, inhibits breast cancer metastatic progression

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The p120 RasGAP protein negatively regulates Ras *via* its GAP domain. RasGAP carries several other domains that modulate several signaling molecules such as Rho. RasGAP is also a caspase-3 substrate. One of the caspase-3-generated RasGAP fragments, corresponding to amino acids 158–455 and called fragment N2, was previously reported to specifically sensitize cancer cells to death induced by various anticancer agents. Here, we show that fragment N2 inhibits migration *in vitro* and that it impairs metastatic progression of breast cancer to the lung. Hence, stress-activated caspase-3 might contribute to the suppression of metastasis through the generation of fragment N2. These results indicate that the activity borne by fragment N2 has a potential therapeutic relevance to counteract the metastatic process.

Metastasis is responsible for most cancer-related deaths[1] but preventing or inhibiting metastasis formation remains a challenge. Therefore, understanding the molecular mechanisms involved in the metastatic cascade is crucial to develop therapeutical antimetastatic drugs. Metastatic progression is a complex multistep process that includes the escape of cancer cells from the primary tumor, the intravasation into the lymphatic or hematogenous systems, the extravasation into the parenchyma of new distant sites and the colonization of these sites.[2] These steps are associated with increased motility, invasiveness, cell–cell binding modulation and decreased adhesion of cells to their substratum.[2] Targeting specifically the molecular pathways that affect cell adhesion and migration represents prime anticancer strategies.

Among the most commonly deregulated signaling proteins in cancer are the Ras protein.[3] These are activated by guanine nucleotide exchange factors (GEFs) and negatively modulated by GTPase-activating proteins (GAPs).[4] There are ten different GAPs that regulate Ras[5] including DAB2IP and Rasal2 that have recently been found to be dual tumor and metastasis suppressors.[6,7] Whether other RasGAPs act as metastasis suppressors remains unknown.

p120 RasGAP (from now on referred to as RasGAP) is more than a mere negative modulator of the Ras pathway *via* its GAP domain.[8] Indeed, its N-terminal moiety contains multiple domains, including SH2 and SH3 domains, that positively modulate kinases such as Cdk1.[9] The N-terminal region of RasGAP, in contrast to its C-terminal end, can lead to Ras activation.[10] This can explain, for example, why there is a weaker basal Ras activity in cells lacking RasGAP compared to wild-type cells.[11] RasGAP can therefore negatively or positively control Ras activation in a manner that is probably cell and stimulus dependent. RasGAP is also a caspase-3 substrate. The RasGAP/caspase-3 pair forms a stress-sensing module that induces survival signals in homeostasis-perturbing conditions and apoptosis in the presence of excessive stress.[12] Stress sensing by this module relies on differential cleavage of RasGAP at low and high caspase-3 activity. In the presence of a low stress, caspase-3 cleaves RasGAP once, generating an amino-terminal fragment, called fragment N, that efficiently promotes cell survival in a Ras/PI3K/Akt-dependent manner.[10,13,14] When the stress reaches unsustainable levels, caspase-3 further cleaves fragment N into two smaller fragments, called N1 and N2, that no longer have the ability of stimulating Akt.[15] This terminates the Akt-dependent protective signals, thereby favoring cell death.

Fragment N2 favors proapoptotic signaling, in cancer cells but not in nonmalignant cells, in response to various anticancer agents.[16] Recently, fragment N2 was found to increase the adhesive capacity of cells.[17] This activity is carried by a ten-amino acid sequence within fragment N2 that corresponds to amino acids 317–326 of RasGAP.[17] These findings

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What's new?

Cancer's ability to spread all over the body is what makes it most deadly, but metastasis remains a tough problem to solve. Researchers are scrutinizing the molecular changes that allow a cancer cell to break away from a tumor and colonize another part of the body, and one key player is the Ras protein family, which in turn can be stifled by proteins called RasGAPs. In this paper, the authors investigated a caspase-3-generated fragment of one of the RasGAPs (p120 RasGAP), already known to sensitize cancer cells to anti-cancer drugs. They found that this fragment, called N2, can also halt metastasis. These results suggest that activation of caspase-3, which creates the fragment, could help kill off tumors and prevent metastasis.

prompted us to test the ability of fragment N2 to inhibit metastatic progression *in vivo*. Here, we show that fragment N2 hampers malignant cells to escape the primary tumor site. This indicates that an internal portion of RasGAP, which can be released by caspase-3 cleavage, can act as a metastasis suppressor.

Material and Methods

Cell lines, cell culture, lentiviral infection, Western blotting and wound-healing assay

All cell lines were maintained in Dulbecco's modified Eagle's medium as previously described.[17] Recombinant lentivirus production,[18] Western blotting and wound healing assays were performed as previously reported.[17]

Generation of stable 4T1 clones

4T1 cells were transfected with the pEGFP-C1 and pTK-Hyg (3:1 ratio) or with GFP-HA-hRasGAP[158–455] and pTK-Hyg (3:1 ratio) using the calcium phosphate method as previously reported.[17] The cells were selected using 200 {micro}g/ml hygromycin B until appearance of colonies. The cells were then maintained in 100 {micro}g/ml hygromycin B. GFP-positive colonies were picked, and screened by microscopy and immunoblotting. Plasmids used in this study are described in Supporting Information methods.

Antibody description

The antibodies used in this study were obtained from the following sources: anti-GFP (JL-8) (Clontech, Moutain View, CA; ref: 632381; 1:2,500), anti-RasGAP (Enzo Life Sciences, Lausen, Switzerland; ref: ALX-210–860-R100; 1:250), anti-β-actin (Chemicon International, Billerica, MA; ref: MAB1501; 1:5,000) and anti-HA (Covance, Princetown, NJ; ref: MMS-101r; 1:1,000). The secondary antibodies were IRDye800-conjugated anti-mouse IgG (Rockland, Gilbertsville, PA; ref: 610-132-121; 1:5,000) and AlexaFluor680-conjugated anti-rabbit IgG (Molecular Probes, Paisley, United Kingdom; ref: A21109; 1:5,000).

4T1 orthotopic model

We carried out orthotopic implantations as previously described[19] and under authorization license (Swiss Animal Protection Ordinance; permit number 2379). Briefly, Balb/c female mice, obtained from Charles River (Bois des Oncins, France), were injected in the right mammary fat pad with 100,000 murine mammary cancer 4T1-derived stable clones in 20% Matrigel (BD Biosciences, San Jose, CA; ref: 354248; diluted in PBS). The mice were sacrificed after 29 days and analyzed for the presence of lung metastases. The analyses involving TAT-RasGAP$_{317–326}$ injection are described in Supporting Information methods.

Primary tumor and metastasis measurement

Tumor volumes were quantified as described earlier.[20] For metastasis analyses, five equidistant sections per lung were performed and stained with hematoxylin/eosin (H/E). The number of metastatic foci was reported as the mean of the five slides per organ and normalized to the maximal effect per experiment. The metastatic index was calculated by dividing the normalized number of metastatic foci by the corresponding mouse tumor weight (normalized to the maximal effect per experiment).

Experimental metastasis assay

Experimental metastasis assays were performed as previously reported.[21] Experimental details appear in Supporting Information methods. Briefly, nude NMRI mice were injected with MDA-MB-231-Luc cells stably expressing fragment N2 and the firefly luciferase (control cells only expressing the latter) and sacrificed after 46 days. Bioluminescence of the lungs was assessed as described in Supporting Information methods.

Statistical analysis

The statistical tests were performed using the R software (version 2.11.0). The tumor growth and migration assays were analyzed by repeated measurement ANOVAs. All metastasis and tumor size data were analyzed by nonparametric Mann–Whitney *U*-tests. The Bonferroni correction was applied when more than one comparison was performed. Asterisks denote statistical differences (*p-value < 0.05; **p-value < 0.01 after Bonferroni corrections). Box plot description appears in Supporting Information methods. Except when displayed as box plots, the results were expressed as mean ± 95% confidence intervals.

Results

To evaluate the role of fragment N2 during metastatic progression, we used the well-established 4T1 murine breast cancer model.[22] 4T1 cells, when implanted orthotopically in the

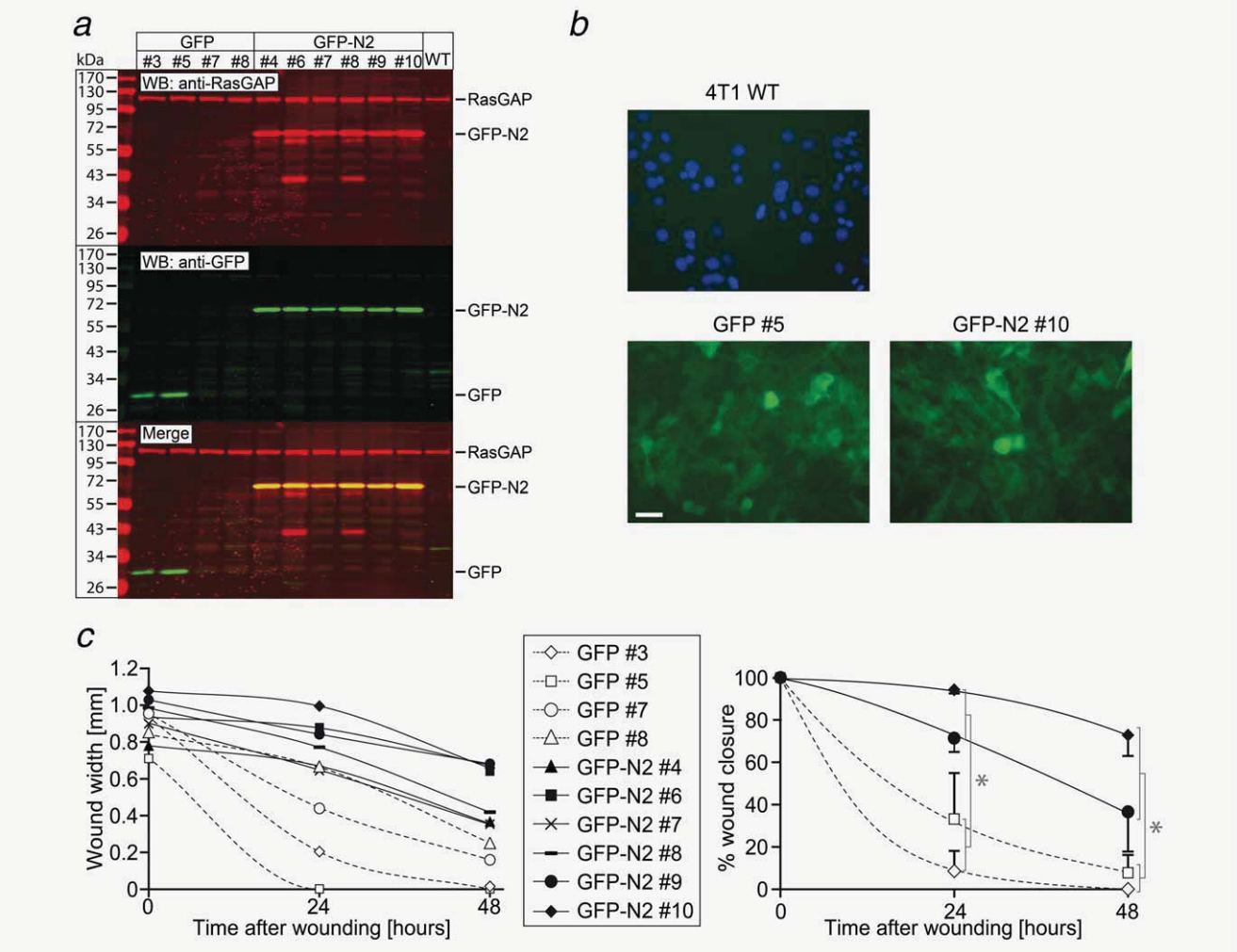

Figure 1. Stable expression of fragment N2 inhibits cell migration. (*a*) Stable 4T1 clones were screened for the expression of GFP and GFP-N2 by immunoblotting against GFP and RasGAP. (*b*) Representative images of GFP-positive 4T1 stable clones. The nucleus of untransfected 4T1 cells is also shown by Hoechst-33342 staining. Scale bar: 50 μm. (*c*) Stable 4T1 clones were subjected to wound-healing scratch assays. The left panel displays the progression of wound width over time for every clones ($n = 1$ experiment). The right panel displays the percentage of wound closure for the clones that were selected for further experiments ($n = 4$ experiments). Asterisks denote significant differences between the indicated groups after repeated measurement ANOVA. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

mammary fat pad of syngeneic Balb/c mice, efficiently metastasize to the lungs.[22] Therefore, we generated several 4T1 clones expressing fragment N2 fused to a green fluorescent protein (GFP) or GFP alone as controls. Most of these clones express elevated levels of GFP and GFP-fragment N2 (from now on referred to as GFP-N2) as revealed by immunoblotting (Fig. 1*a*) and fluorescence microscopy (Fig. 1*b*). All clones expressing fragment N2 displayed impaired motile capacity (Fig. 1*c*). Of note, even the GFP-N2 #6 and #8 clones in which GFP-fragment N2 is degraded exhibited reduced migration.

As fragment N2 efficiently inhibits migration, we evaluated its *in vivo* capacity to prevent metastasis formation to the lungs. We selected two GFP (#3 and #5) and two GFP-N2 (#9 and #10) clones to minimize the risk of generating clone-specific effects. When implanted in the mammary gland, all clones produced similarly sized primary tumors (Fig. 2*a*) and of comparable weight after 29 days (Fig. 2*b*). This indicates that fragment N2 does not affect tumor take and tumor growth. In contrast, the number of lung metastasis was significantly lower in mice bearing primary tumors that express fragment N2 (Fig. 2*c*). Consistently, the metastatic index (number of metastasis corrected for the primary tumor weight) of the N2-expressing clones was significantly lower than the metastatic index of GFP-expressing clones (Fig. 2*d*). These data indicate that expression of fragment N2 impairs metastatic progression. Fragment N2 did not affect the epithelial-to-mesenchymal transition (EMT), a process implicated in metastatic dissemination,[23] because the levels of E-cadherin or vimentin, two hallmark molecules modulated

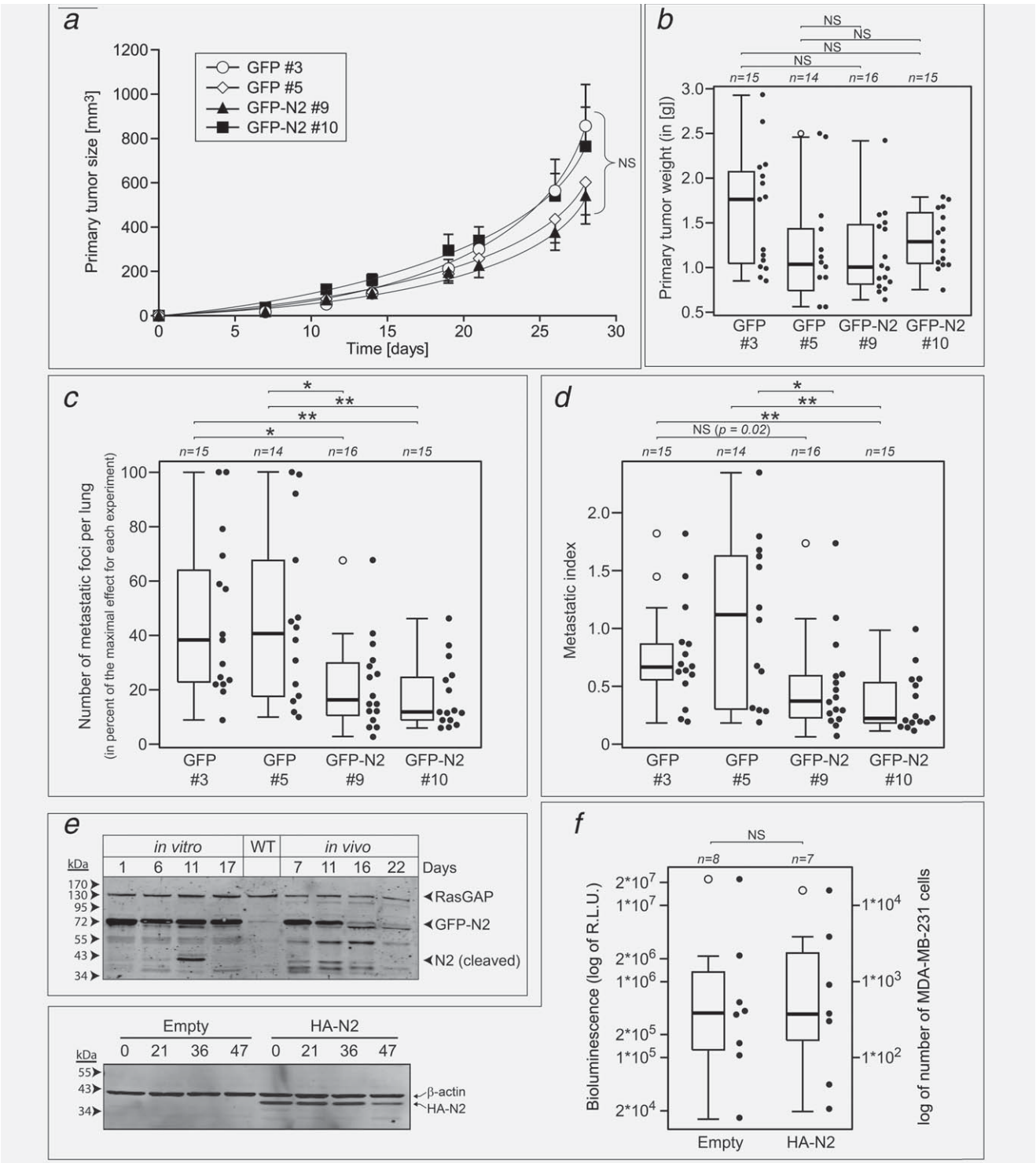

Figure 2. Fragment N2 inhibits metastatic progression. (*a*–*e*) Balb/c mice were injected with four stable 4T1 clones expressing GFP (#3 and #5) or GFP-N2 (#9 and #10). The experiments were performed thrice independently using four to six mice per condition in each experiment. The data were pooled per condition. The figure displays the tumor growth (*a*), the tumor weight after sacrifice (*b*), the normalized number of metastatic foci per lung (*c*) and the metastatic index (*d*). Of note, when pooling GFP clones together and GFP-N2 clones together, the GFP-N2 group exhibits a highly significant metastatic index decrease over the GFP group ($p = 0.00012$). (*e*) 4T1-GFP-N2 #10 cells were implanted orthotopically (*in vivo*) or maintained in culture (*in vitro*). Immunoblotting against RasGAP was performed for the samples taken at the indicated times. The ~40-kDa fragment detected at 11 days *in vitro*, and apparently in the *in vivo* condition as well, corresponds to the similarly sized fragment observed in the GFP-N2 #6 and #8 clones (see Fig. 1*a*, upper blot). Based on the size of this fragment and the fact that it is recognized by the anti-RasGAP antibody, it can be assumed that it contains the entirety of fragment N2. (*f*) An experimental metastasis assay was done by injecting MDA-MB-231-Luc cells infected with fragment N2 (or its empty vector). The bioluminescence and the number of lung-invading cells are reported in the graph. Seven to eight mice were analyzed per condition. Immunoblotting against the HA tag and β-actin is displayed on the left for cells that were maintained in culture during the time frame of the assay. In panels *b*, *c*, *d* and *f*, the results are shown as box plots on the left while raw data points are displayed on the right.

during EMT, remained unchanged in 4T1 clones overexpressing fragment N2 (data not shown).

To determine the stability of fragment N2 expression in the 4T1 clones *in vivo*, we injected five mice with 4T1 GFP-N2 #10 cells, sacrificed them at different time points and analyzed the expression of GFP-N2. This experiment revealed that expression of GFP-N2 was partially lost 16 days after tumor implantation and completely abrogated 22 days after implantation (Fig. 2*e*). In contrast, the GFP-N2 #10 clone cultured *in vitro* did not lose fragment N2 expression during this time frame (Fig. 2*e*). Loss of fragment N2 expression from tumors growing in mice may be an indication that this fragment exerts some tumor-suppressive effects. One could therefore conceive that if fragment N2 expression was not progressively disappearing during the *in vivo* metastasis development experiment, the 4T1 clones initially expressing fragment N2 would have produced much fewer metastases than what is actually observed.

Cell migration is considered to contribute to metastasis at two discrete steps in the metastatic cascade: at an early metastatic stage when cells escape from the primary tumor and again at a later stage when cells extravasate and invade the parenchyma of the newly colonized site. The 4T1 *in vivo* model does not allow differentiating whether fragment N2 inhibits escape from the primary tumor, colonization of the secondary site or both. To assess whether fragment N2 inhibits the capacity of circulating tumor cells to invade tissues, the breast cancer MDA-MB-231 cell line expressing the luciferase gene and freshly infected with lentiviruses expressing or not fragment N2 was injected in the bloodstream of nude mice. Their capacity to colonize lungs was then evaluated by measuring the luciferase activity in these organs 46 days after the injection. Figure 2*f* shows that fragment N2 did not alter the capacity of MDA-MB-231 cells to invade the lungs. There was no or minimal loss of fragment N2 in the fragment N2-infected cells during the time course of this experiment (blot of Fig. 2*f*). Altogether, these experiments indicate that fragment N2 inhibits metastatic progression by preventing cancer cells from escaping the primary site but not by inhibiting colonization of distal sites.

As fragment N2 inhibits migration, a logical follow-up was to assess whether the cell-permeable TAT-RasGAP$_{317–326}$ peptide, which bears the same anti-cancer activities as fragment N2,[17] was also able to inhibit metastasis formation. TAT-RasGAP$_{317–326}$, when injected intraperitoneally, was shown to accumulate in subcutaneously established HCT116 tumors and to improve the effects of chemotherapy on preventing the growth of these tumors.[20] Unfortunately, HCT116 tumors do not metastasize to other organs and hence could not be utilized here to investigate the antimetastatic potential of TAT-RasGAP$_{317–326}$. Therefore, we used the 4T1 model instead. However, this model is not ideal in the present setting because ^{125}I-labeled TAT-RasGAP$_{317–326}$ injected intraperitoneally failed to accumulate in primary 4T1 tumors (Supporting Information Fig. 1A). Our conclusion that the peptide did not accumulate in the tumor derives from the fact that the radioactive values in the tumor were even lower than the residual signal found in the blood (Supporting Information Fig. 1A). In contrast, it accumulated in the liver as shown previously (Supporting Information Fig. 1A).[20] Moreover, it is impossible to test if the weakly delivered TAT-RasGAP$_{317–326}$ dose has sufficient functional effects as 4T1 cells are not sensitive to genotoxins and could therefore not be tested for sensitization to apoptosis. Nevertheless, we tested whether the RasGAP peptide could affect the ability of 4T1 cells to metastasize. Starting at the time when 4T1 tumors cells were injected in the fat pad, mice were injected thrice a week with 1.6 mg/kg TAT-RasGAP$_{317–326}$. The primary tumor size and weight were not affected by the peptide treatment (Supporting Information Figs. 1B and 1C). Twenty-five days after tumor injection, the mice were sacrificed and the lungs were analyzed for the presence of metastases. TAT-RasGAP$_{317–326}$-treated mice did not display fewer metastatic foci (Supporting Information Fig. 1D). As the peptide does not apparently accumulate in 4T1 tumors, the negative nature of these experiments does not allow us to conclude whether TAT-RasGAP$_{317–326}$ inhibits or not metastasis formation.

Discussion

Cancer therapy still suffers from a lack of metastasis-specific drugs. Only a few of them are used in the clinics to treat specific cancers, whereas the majority remains in early clinical trials.[24] Here, we provide the proof of concept that the fragment N2 of RasGAP acts as a metastasis suppressor. Hence, compounds bearing fragment N2 activities have the potential to function as anti-metastatic drugs. A cell-permeable protease-resistant ten-amino acid peptide corresponding to a short region of fragment N2 is interesting in this context. This compound, called TAT-RasGAP$_{317–326}$, efficiently sensitizes cancer cells to various antitumor treatments, both *in vitro* and *in vivo*.[16,20] It also increases cell adherence, blocks cell migration and prevents invasion.[17] Therefore, this TAT-RasGAP$_{317–326}$ peptide has the potential to inhibit metastasis development *in vivo*. Unfortunately, the experiment we have performed here did not allow us to determine whether TAT-RasGAP$_{317–326}$ acts as a metastasis blocker because this compound failed to accumulate in breast-implanted 4T1 tumors. Thus, testing this hypothesis requires that compounds with fragment N2 activities and being able to accumulate at sufficient levels in primary tumors and/or target organs are developed. The prototypical issues associated with peptide therapeutics are the clearance by the liver, the weak selectivity of delivery and the peptide short half-lives. In the case of TAT-RasGAP$_{317–326}$ this last concern has been circumscribed by using D-amino acids for its synthesis but there is still room for improvement concerning the first two issues.[25,26] The development of small molecules mimicking the activity of TAT-RasGAP$_{317–326}$ is a suited alternative. We recently found that deleted in liver cancer-1 (DLC1), a RhoGAP and metastasis suppressor, was required for TAT-RasGAP$_{317–326}$

to prevent migration.[17] Work based on the interaction between fragment N2 and DLC1 could potentially lead to the development of a small molecule with TAT-RasGAP$_{317–326}$-like activities.

Fragment N2 can be produced endogenously in response to stress,[12] although probably not to the levels obtained in the clones used in our study. One could anticipate that the endogenous fragment N2 plays some physiological roles, potentially in the context of malignant transformation. In cancer development, premalignant cells experience oncogenic stress that induces caspase activation leading to apoptosis in many but not all cells.[27] There are indeed cases, such as in breast cancer, where caspase-3 activity is higher in malignant tissues than in corresponding normal ones.[28] Because of caspase activation, the surviving cells may produce fragment N2 that has then the potential to exert two tumor-suppressor functions. First, fragment N2 can render cancer cells more sensitive to stress-induced death and therefore contribute to their elimination, if the first wave of caspase activation failed to do so. Second, fragment N2 can prevent dissemination of surviving cancer cell, hence blocking metastasis formation.

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