Intratumoral heterogeneity of ADAM23 promotes tumor growth and metastasis through LGI4 and nitric oxide signals

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Intratumoral heterogeneity (ITH) represents an obstacle for cancer diagnosis and treatment, but little is known about its functional role in cancer progression. The A Desintegrin And Metalloproteinase 23 (ADAM23) gene is epigenetically silenced in different types of tumors, and silencing is often associated with advanced disease and metastasis. Here, we show that invasive breast tumors exhibit significant ADAM23-ITH and that this heterogeneity is critical for tumor growth and metastasis. We demonstrate that while loss of ADAM23 expression enhances invasion, it causes a severe proliferative deficiency and is not itself sufficient to trigger metastasis. Rather, we observed that, in ADAM23-heterotypic environments, ADAM23-negative cells promote tumor growth and metastasis by enhancing the proliferation and invasion of adjacent A23-positive cells through the production of LGI4 (Leucine-rich Glioma Inactivated 4) and nitric oxide (NO). Ablation of LGI4 and NO in A23-negative cells significantly attenuates A23-positive cell proliferation and invasion. Our work denotes a driving role of ADAM23-ITH during disease progression, shifting the malignant phenotype from the cellular to the tissue level. Our findings also provide insights for therapeutic intervention, enforcing the need to ascertain ITH to improve cancer diagnosis and therapy.

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INTRODUCTION
Cancer is a dynamic heterogeneous disease. The coexistence of tumor cells with different phenotypic traits within a primary tumor has been known since the late 1970s. This phenotypic diversity, called intratumoral heterogeneity (ITH), appears to be the end result of branched evolutionary tumor growth, fostered by increased genetic and epigenetic instability and selective pressures from the tumor environment.1-8 Recent studies, mostly using deep-sequencing technologies, have elucidated the genetic basis of ITH in different types of tumors and associated metastatic sites.9-19 Collectively, these studies have demonstrated that ITH is a ubiquitous characteristic of neoplasms, arising during disease progression, and that primary tumors are ecosystems of evolving clones with different spatial and temporal distributions.20-22

Although ITH has been an underlying concept in cancer biology for decades,1-8,23 it is not yet fully considered by clinicians and pathologists and represents a major obstacle for cancer diagnosis and treatment. This is due mainly to technical limitations in assessing ITH in the clinical setting and to our rudimentary knowledge of the functional role of ITH for cancer progression, drug resistance and metastasis.

The ADAM23 gene (also known as MDC3), encoding a non-catalytically active member of A Disintegrin And Metalloproteinase family,24 is frequently silenced in gliomas,25 breast,26-27 pancreatic,26 gastric,27 head and neck,28 colorectal29 and lung30 tumors. We have previously demonstrated that ADAM23 expression is silenced by DNA promoter hypermethylation in the advanced stages of breast and head and neck tumors.26,27 In breast cancer, we showed that ADAM23 hypermethylation is an independent prognostic factor associated with a ninefold higher risk of developing distant metastases and lower survival rates.27 In addition, we demonstrated that knockdown of ADAM23 expression in the MDA-MB-435 cell line enhanced tumor cell migration, adhesion and arrest in the lungs of immunodeficient mice.27 Here, we show for the first time that loss of ADAM23 expression, previously associated with the development of distant metastases and poor prognosis, causes a proliferative and colonization deficiency and, counterintuitively, is not sufficient to trigger metastatic dissemination. Rather, the rise of ADAM23-negative (A23neg) cells within an ADAM23-positive tumor generates ADAM23-ITH and actively enhances proliferation and promotes invasion of adjacent ADAM23-positive (A23pos) tumor cells through Leucine-rich Glioma Inactivated 4 (LGI4) secretion and nitric oxide (NO) production, respectively. Ablation of LGI4 and NO production uncouples this cellular crosstalk and attenuates tumor cell proliferation and invasion. Our findings highlight a driving role of ADAM23-ITH in cancer progression by promoting tumor growth and metastasis. Our results also provide important insights for therapeutic intervention and enforce the need to address tumor subclonal architecture to improve the diagnosis and treatment of cancer.
RESULTS
ADAM23 silencing and ITH in invasive breast carcinomas

To explore the role of ADAM23 silencing during breast cancer progression, we first analyzed if there were differences in ADAM23 mRNA expression levels between ductal breast carcinomas in situ (DCIS) and invasive ductal breast carcinomas (IDCs). Compared with normal breast, ADAM23 levels were reduced in most IDC (10/19), but not in DCIS (1/7) (Figure 1a, $P < 0.05$). We also analyzed the ADAM23 mRNA expression levels in paired in situ and invasive components of four IDCs to determine if ADAM23 silencing is homogeneous within IDC. Tumor cells from both components were laser microdissected (Figure 1b) and the heterogeneous ADAM23 expression was observed in two out of the four tumor samples analyzed (IDC2 and IDC4), most pronounced in tumor IDC4, extracted from a patient with extensive lymph node metastasis (Figure 1c).

ADAM23 protein expression was also analyzed in 12 independent IDCs. As illustrated in Figure 1d, ADAM23 protein staining was more prominently detected in breast ducts with preserved normal architecture and a gradual decrease in staining intensity was observed between the in situ and invasive components of different IDCs (Figure 1d). It is worth noting that ADAM23-ITH was observed in topographically distinct invasive areas within undifferentiated IDCs, with some invasive areas being composed by a mosaic cluster of A23(pos) and A23(neg) tumor cells (Figure 1e, in detail, and Supplementary Figure 1).

These results indicate that ADAM23 expression is downregulated during breast cancer progression and that ADAM23-ITH is a common characteristic of invasive carcinomas.

ADAM23-ITH promotes tumor cell proliferation and tumor growth

To address the functional relevance of ADAM23-ITH during cancer progression, we first examined the role of ADAM23-ITH in tumor cell proliferation using three-dimensional cultures, which more effectively mimic tumor behavior, as well as by subcutaneous tumorigenesis assays. For these experiments, we used the MDA-MB-435 cell line, which we have previously used to address the functional impact of ADAM23 silencing in tumor cell adhesion and migration. In vitro, MDA-MB-435 ADAM23-positive cells (435-A23(pos)) or their ADAM23 short hairpin RNA (shRNA) derivatives (435-A23(neg)) (Supplementary Figures 2a and b) and a mixture of 1:1 ratio of both (435-A23(neg)) were cultured as multicellular spheroids (MS), and proliferation rates were estimated by bromodeoxyuridine (BrdU) labeling. A lower percentage of BrdU-labeled cells was observed in 435-A23(neg)-MS (21%) compared with 435-A23(pos)-MS (32%) and 435-A23(neg)-MS (32%, $P < 0.01$) (Figure 2a). In agreement with the proliferative deficiency of 435-A23(neg) cells, using anchorage-independent clonogenic assays, we observed that 435-A23(neg) cells formed an equivalent number of colonies to 435-A23(pos) cells, although these colonies were on average threefold smaller, confirming that both cells have a similar growth potential.

Figure 1. ADAM23 expression levels in ductal breast carcinomas. (a) RT–qPCR analysis of ADAM23 mRNA expression in normal breast (NB) ($n=4$), DCIS ($n=7$) and IDCs ($n=19$). (b) Representative example of a laser capture microdissection of the paired in situ (top panels) and invasive (bottom panels) components of an IDC. (c) RT–qPCR analysis of ADAM23 mRNA expression in paired in situ and invasive components of four IDCs (IDC1–4). Expression levels relative to normal breast tissue and normalized to the hypoxanthine guanine phosphoribosyltransferase (HPRT) endogenous control gene are shown in (a and c). (d and e) Immunohistochemistry staining of ADAM23 protein was carried out using the polyclonal antibody anti-ADAM23 (HPA012130). (d) ADAM23 protein expression in mammary normal ducts, in situ and invasive tumor components of an IDC (IDC15 from patient no. 15). Bar represents 100 μm. (e) ADAM23-ITH is observed between topographically distinct invasive areas of an undifferentiated IDC. In detail, mosaic clusters of A23(pos) (green arrow) and A23(neg) (red arrow) tumor cells coexisting in close proximity within an invasive component. Bar represents 50 μm.
MS, despite having initially half the amount of 435-A23pos cells had similar percentages of BrdU-labeled cells as 435-A23pos/A23neg and the other groups (Figure 2e, f). Kinetics and 435-A23pos or 435-A23neg cells (Figure 2d) and, exhibited reduced tumor latency than animals injected with either (Figure 2a). Flow cytometry analysis of tumors from mice injected 435-A23pos (14/15, 93%, showed impaired tumorigenesis (5/15, 33%) when compared with retained in these tumors 10 weeks after inoculation, despite their proliferation deficiency (data not shown). Similar results were observed by immunohistochemical analysis of ADAM23 expression in 435-A23het tumors (Supplementary Figure 4). These results indicate that the coexistence of A23pos and A23neg cells in primary tumors enhance proliferation and tumor growth.

To explore this possibility, we produced 435-A23het,MS composed of 435-A23pos cells prelabeled with a red fluorescent dye (435-A23pos/DiIC18) and different ratios of unlabeled 435-A23neg cells, so that we could track the proliferation rate of both cell subpopulations in heterotypic MS. In 435-A23het,MS, the percentage of BrdU-labeled 435-A23pos/DiIC18 cells increased from 28 to 45% along with the increase in the number of 435-A23neg cells (Figure 2f, P < 0.001), indicating that, in heterotypic environments, 435-A23neg cells enhance the proliferation rate of 435-A23pos cells. In contrast, rates of BrdU labeling in 435-A23neg cells progressively decreased from 20 to 10%, with the gradual increase in 435-A23pos/DiIC18 cell number (Figure 2g, P < 0.05).

To demonstrate that ADAM23-dependent proliferative signals are not restricted to MDA-MB-435 cancer cells, we screened a panel of tumor cell lines for ADAM23 mRNA expression (Supplementary Figure 5) and performed similar experiments with SK-Mel-37 melanoma cells (SK-A23pos) and their ADAM23-shRNA derivatives (SK-A23neg) (Supplementary Figures 2c and d). In vitro, SK-A23neg,MS showed a smaller percentage of BrdU-labeled cells than SK-A23pos,MS (Supplementary Figure 6a). In vivo, subcutaneous tumorigenicity of SK-A23neg cells (5/7) was not impaired compared with SK-A23pos cells (5/7), although significant differences in growth kinetics and tumor size were observed (Supplementary Figure 6b, P < 0.01). Notably, SK-A23neg mixtures were more tumorigenic (8/8) and showed reduced tumor latency.
compared with both homotypic groups (Supplementary Figure 6c, \( P < 0.05 \)).

Altogether, our findings demonstrate that ADAM23 expression is important for tumor growth at the primary site and that ADAM23 silencing is associated with an accentuated deficiency in cell proliferation, but not with reduced clonogenic cell survival. Moreover, we demonstrated that in ADAM23-heterotypic environments, the proliferation of the A23\(^{\text{pos}}\) subpopulation is enhanced by A23\(^{\text{neg}}\) cells, compensating for the intrinsic proliferative deficiency of the latter subpopulation. Most importantly, our results suggest that ADAM23-ITH can promote tumor growth.

LGI4 mediates the proliferative crosstalk in ADAM23-heterotypic environments

LGI4 (also known as LGIL3), a member of the LGI family, is a putative secreted protein\(^ {34} \) involved in proliferation in the nervous system\(^ {35} \) and a known ADAM11, ADAM22 and ADAM23 ligand\(^ {35} \)–\(^ {37} \). We found that LGI4 mRNA expression was eightfold higher in 435-A23\(^{\text{neg}}\) than in 435-A23\(^{\text{pos}}\) cells (Figure 3a, \( P < 0.01 \)). To determine whether LGI4 produced by 435-A23\(^{\text{neg}}\) cells is responsible for the enhanced cellular proliferation of 435-A23\(^{\text{pos}}\) cells in 435-A23\(^{\text{het}}\)-MS, we examined BrdU labeling in 435-A23\(^{\text{neg}}\)/MS composed of 435-A23\(^{\text{neg}}\) cells with or without ablation of LGI4 (435-A23\(^{\text{neg}}\)/shLGI4 or 435-A23\(^{\text{neg}}\)/shCtrl, respectively) (Figure 3b). Consistent with a proliferative function of LGI4, the percentage of BrdU labeling in 435-A23\(^{\text{neg}}\)/shLGI4-MS (20%) was significantly lower than in 435-A23\(^{\text{neg}}\)/shCtrl-MS cells (28%) and 435-A23\(^{\text{pos}}\)-MS (34%) (Figure 3c, \( P < 0.001 \)). Moreover, the 435-A23\(^{\text{pos}}\)/DiIC\(_{18}\) subpopulation showed an increased percentage of BrdU labeling, from 32 to 40%, when cocultured with 435-A23\(^{\text{neg}}\)/shLGI4 cells (\( P < 0.001 \)), but not when cocultured with 435-A23\(^{\text{neg}}\)/shCtrl cells (31%, Figure 3d). As expected, LGI4 knockdown in 435-A23\(^{\text{neg}}\) cells significantly impaired by 43% the previously observed enhanced growth of A23\(^{\text{neg}}\) subcutaneous tumors (Figure 3e). In agreement with an ADAM23-mediated proliferative crosstalk, LGI4-dependent tumor growth was exclusively determined by the coexistence of A23\(^{\text{neg}}\) and A23\(^{\text{pos}}\) cells, as subcutaneous inoculums of 435-A23\(^{\text{neg}}\)/shLGI4 or 435-A23\(^{\text{neg}}\)/shCtrl alone were essentially non-tumorigenic, because of the absence of ADAM23 expression.

To our knowledge, this is the first demonstration of the relevance of LGI4/ADAM23 interactions for tumor cell proliferation and tumorigenesis. Moreover, our results show that LGI4 is the key factor mediating the proliferative crosstalk between A23\(^{\text{neg}}\) and A23\(^{\text{pos}}\) cells in ADAM23-heterotypic environments.

Figure 3. LGI4 mediates the proliferative crosstalk in ADAM23-heterotypic environments. (a) RT-qPCR analysis of LGI4 expression in 435-A23\(^{\text{pos}}\) and 435-A23\(^{\text{neg}}\) cells. Expression levels relative to MDA-MB-435 wild-type cells and normalized to hydroxymethylbilane synthase (HMBS) endogenous control gene are shown \((n = 4)\). **\( P < 0.01 \) using Student’s t-test. (b) 435-A23\(^{\text{neg}}\) cells were stably transduced with two different LGI4 shRNAs sequences (shLGI4 nos. 1 or 2) or with control shRNA (shCtrl) and LGI4 mRNA expression levels relative to cells transfected with shCtrl were determined as described above \((n = 3)\). ***\( P < 0.001 \) using one-way analysis of variance (ANOVA). (c) 435-A23\(^{\text{pos}}\) and 435-A23\(^{\text{neg}}\) cells transfected with shLGI4 (A23\(^{\text{neg}}\)/shLGI4) or shCtrl (A23\(^{\text{neg}}\)/shCtrl) were allowed to aggregate as MS and proliferation was evaluated as described above \((n = 10\ 000 \text{ cells per MS, 6 MS per group})\). ***\( P < 0.001 \) using one-way ANOVA. (d) Proliferation of 435-A23\(^{\text{pos}}\)/DiIC\(_{18}\) cells in 435-A23\(^{\text{neg}}\)/MS composed of 90% of A23\(^{\text{neg}}\)/shCtrl or A23\(^{\text{neg}}\)/shLGI4 cells was determined as described above \((n = 10\ 000 \text{ cells per MS, 4 MS per group})\). NS, nonsignificant, ***\( P < 0.001 \) using one-way ANOVA. (e) Subcutaneous A23\(^{\text{neg}}\) tumor growth constituted of \(8 \times 10^5\) 435-A23\(^{\text{neg}}\) cells stably transduced with two LGI4 shRNAs sequences (grouped as shLGI4) or with shCtrl and \(2 \times 10^5\) 435-A23\(^{\text{pos}}\) cells. A23-homotypic inoculums \((8 \times 10^5\ 435-A23^{\text{neg}} \text{ or } 2 \times 10^5\ 435-A23^{\text{pos}} \text{ cells})\) were included as controls. **\( P < 0.01 \) using one-way ANOVA. Error bars indicate s.e.m. in all experiments.
ADAM23-ITH promotes tumor cell invasion and metastasis

We next examined the role of ADAM23-ITH in tumor cell invasion and metastasis using MS embedded in collagen matrices, as well as spontaneous metastasis assays.

Ten days after embedment in collagen matrices, 435-A23\(^{\text{neg}}\)-MS were visibly more invasive than 435-A23\(^{\text{pos}}\)-MS (Figure 4a). The average speed of invasion in 435-A23\(^{\text{neg}}\)-MS was up to four times higher than in 435-A23\(^{\text{pos}}\)-MS (4.7 and 1.6 μm/h, respectively, \(P < 0.01\), Figure 4b). It is worth noting that an invasive phenotype was accomplished in the noninvasive SK-Mel-37 cell line after ADAM23 ablation. The average invasion speed was 4.5 times higher (\(P < 0.01\)) for SK-A23\(^{\text{neg}}\)-MS than for SK-A23\(^{\text{pos}}\)-MS (Supplementary Figure 7). Accordingly, ADAM23 overexpression in CMS5a cells significantly inhibited invasion in collagen matrices (Supplementary Figure 8).

We also carried out spontaneous metastasis assays by inoculating 435-A23\(^{\text{pos}}\), 435-A23\(^{\text{neg}}\) and a 1:1 mixture of both (435-A23\(^{\text{het}}\)) into the mammary fat pad of immunodeficient mice. As expected, the percentage of animals that developed tumors was lower in mice inoculated with 435-A23\(^{\text{neg}}\) cells (9/12, 75%) than with 435-A23\(^{\text{pos}}\) cells (14/14, 100%, \(P < 0.05\), Figure 4c), but no significant differences in tumor growth, final sizes or tumor latency were observed between the groups when inoculated with the Matrigel into the mammary fat pad (Figures 4c and d). Unexpectedly, as ADAM23 silencing has been previously associated with metastasis development and poor prognosis,\(^{27}\) we observed no significant differences in the number of animals that developed metastases between the 435-A23\(^{\text{neg}}\) (2/12, 16%) and 435-A23\(^{\text{pos}}\) (5/14, 36%, \(P > 0.05\)) groups (Table 1). As observed for the subcutaneous tumorigenesis assays, ADAM23 silencing remained stable throughout the spontaneous metastasis assay and, interestingly, metastatic lesions from animals bearing 435-A23\(^{\text{het}}\) tumors displayed either a positive or negative pattern of ADAM23 mRNA expression, probably associated with the...
different ADAM23 expression status of their founder’s tumor cells (Supplementary Figure 9).

It is worth noting that the metastatic potential of 435-A23het tumors was threefold higher than that of tumors composed exclusively of 435-A23neg cells (11/23, 48%; P ≤ 0.05) (Table 1) and, in vitro, 435-A23het-MS had a similar invasive behavior to 435-A23neg/het-MS, despite having initially half the amount of 435-A23neg cells (Figure 4b, P < 0.001). In addition, we observed a positive correlation between the percentage of A23neg cells within the MS and the average invasion speed (Figure 4e, r = 0.67), indicating that ADAM23-ITH can enhance tumor invasion and metastasis.

To explore this possibility, we produced A23het-MS, composed of GFP-labeled 435-A23pos cells (435-A23pos/GFP) and RFP-labeled 435-A23neg cells (435-A23neg/RFP), so that we could track the distance traveled daily over 12 days by both cell subpopulations in the heterotypic spheroids. We observed an increase in 60% in the invasion speed of 435-A23pos/GFP cells in A23het-MS compared with that in A23pos/MS (2.5 and 1.5 μm/h, respectively; Figure 4f, P < 0.01). No difference in the invasion speed of 435-A23pos/GFP cells within A23neg/MS was observed (data not shown). Consistent with a cooperative invasive pattern, we frequently noticed interlaced subpopulations of 435-A23pos/GFP and 435-A23neg/RFP cells within single-cell bundles from A23het-MS (Figure 4g).

Next, we performed haptotactic migration assays to determine whether the increased invasive behavior of A23pos cells is strictly dependent on the generation of a less resistant migratory pathway by A23neg cells in collagen matrices, or if A23neg cells also actively influence the migratory behavior of A23pos cells through paracrine signals. We observed that the number of migrating cells was higher in 435-A23het (250 cells per mm2) than in 435-A23pos cultures (22 cells per mm2, P < 0.001), but no significant differences were observed with 435-A23neg (205 cells per mm2, P > 0.05; Figure 4h). Consistent that A23neg cells ablated completely the invasive behavior of 435-A23pos cells in the presence of 435-A23neg/RFP cells, increasing NO levels by 56% (Figures 5a and b, P < 0.01). Similarly, SK-A23pos cells produced threefold more NO than SK-A23neg cells (Supplementary Figure 11, Figure 5d). Whereas the 30% reduction in endogenous NO production (Figure 5e, P < 0.01) did not alter the intrinsic invasive behavior of 435-A23pos cells (data not shown), it was sufficient to ablate completely the influence of 435-A23pos cells on the invasive behavior of 435-A23neg cells (Figures 5f and g, P < 0.01). Consistently, the transwell migration of 435-A23pos/GFP cells increased by 60% when they were cocultured with 435-A23neg/RFP cells (39 cells per mm2) compared with 435-A23neg/shNOS3 cells (24 cells per mm2; Figure 5h, P < 0.01).

These results demonstrate that NO production is upregulated in A23pos cells and NO is a key messenger mediating the invasive crosstalk between 435-A23pos and 435-A23neg cells.

**DISCUSSION**

Here we showed, for the first time, that ADAM23 silencing can elicit a phenotypic switch from a proliferative to an invasive phenotype in tumor cells. Whereas A23pos tumor cells exhibit a fast-proliferating phenotype, high tumorigenicity and a lower invasive potential, A23neg tumor cells display a highly invasive phenotype, but an increased susceptibility to the trophic effect of the environment for optimal tumor growth. We also demonstrated, at both the mRNA and protein level, that ADAM23 expression is downregulated during breast cancer progression and ADAM23-ITH is a common characteristic of invasive breast carcinomas. ADAM23-ITH was observed in topographically distinct invasive areas of undifferentiated IDCs, with invasive components being frequently composed by mosaic clusters of A23pos tumor cells coexisting in close proximity with A23neg cells.

Surprisingly, however, we observed that A23neg cells are not sufficient to seed metastasis owing to a severe deficiency to proliferate. Instead, the rise of A23pos cells within an

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**Table 1. Metastatic potential of 435-A23pos, 435-A23neg and 435-A23het tumors**

<table>
<thead>
<tr>
<th>Tumor Type</th>
<th>Lung Metastasis</th>
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<tr>
<td>435-A23pos</td>
<td>2/12 (16%)</td>
</tr>
<tr>
<td>435-A23neg</td>
<td>5/14 (36%)</td>
</tr>
<tr>
<td>435-A23het</td>
<td>11/23 (48%)</td>
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Abbreviation: NS, nonsignificant. 435-A23pos or 435-A23neg cells or a 1:1 mixture of both (435-A23pos) were suspended in the Matrigel and injected into the mammary fat pad of female Balb/c nude mice (10² cells in 0.05 ml of the Matrigel). Primary tumors were surgically removed and, after 8 weeks, animals were killed and the number of metastasis in lungs, kidneys and bladders were assessed by macroscopic observation at × 10 magnification.

*P ≤ 0.05 using a χ² test.
Figure 5. NO mediates cooperative invasion in ADAM23-heterotypic environments. Quantification of NO production was performed using 4-amino-5-methylamino-2,7′-difluorofluorescein diacetate (DAF-FM) as described in Materials and methods. (a) Representative histograms of the quantification of NO production in 435-A23pos and 435-A23neg cells. The gray histogram represents the background fluorescence of cells without DAF-FM. (b) NO production of 435-A23pos and 435-A23neg cells (n = 9 per group). *P < 0.01 using Student’s t-test. (c) Migration assays were carried out in the presence of the increasing concentrations of the slow-releasing NO donor S-nitroso-N-acetylpenicillamine (SNAP) (n = 2 per SNAP concentration). **P < 0.01 using one-way analysis of variance (ANOVA). (d) 435-A23neg cells were stably transduced with two different NOS3 shRNAs sequences (shNOS3 nos. 1 or 2) or with control shRNA (shCtrl) and NOS3 mRNA expression levels relative to cells transfected with shCtrl and normalized to hydroxymethylbilane synthase (HMBS) endogenous control gene were determined by RT-qPCR (n = 3). *P < 0.05 using one-way ANOVA. (e) NO production in 435-A23neg/shNOS3 or 435-A23neg/shCtrl cells was measured as described above (n = 4 per group). **P < 0.01 using Student’s t-test. (f) Representative images of the invasive behavior of 435-A23pos/GFP cells in 435-A23pos-MS or in 435-A23neg-MS composed of either 435-A23neg/shCtrl or 435-A23neg/shNOS3 cells. Red arrows highlight the distances traveled by 435-A23pos/GFP cells in different environments. (g) Invasion speed of 435-A23pos/GFP cells in 435-A23pos-MS, 435-A23neg/shCtrl-MS or 435-A23neg/shNOS3-MS (n = 6 MS per group). **P < 0.01 and ***P < 0.001 using one-way ANOVA. (h) Number of 435-A23pos/GFP migrating cells when cocultured in the presence of either 435-A23neg/shCtrl or 435-A23neg/shNOS3 unlabeled cells (n = 5). **P < 0.01 using Student’s t-test. Error bars indicate s.e.m. in all experiments. NS, nonsignificant.

ADAM23-positive tumor generates ADAM23-ITH that drives tumor growth and metastasis by actively enhancing the proliferative and invasive behavior of adjacent highly tumorigenic ADAM23pos cells. Moreover, we demonstrated that LG4 and NO are the key factors produced by A23neg tumor cells enhancing proliferation and invasion, respectively, of adjacent ADAM23pos cells in heterotypic tumors.

It has been proposed that ITH may drive tumor progression by creating an ecological niche in which tumor cells with different genotypes interact through paracrine signals and cooperate to promote tumor growth and dissemination.7 Identifying the key molecules mediating the cellular cooperation is thus promising avenues to reveal new approaches for the effective treatment of metastatic disease. Interactions between LGI and ADAM proteins are known to promote proliferation and differentiation of neuronal precursors cells and adipocytes.42,43 However, the findings reported here indicate that LGI/ADAM interactions are also relevant for tumorigenesis and ablation of LG4 production in A23neg cells significantly attenuates proliferation and tumor growth in the A23-heterotypic environments.

Curiously, changing the ratio of A23pos and A23neg cell subpopulations in A23het-MS significantly altered the proliferative differences between them. One explanation for these observations is that both subpopulations are competing for LG4-dependent proliferative signals, and ADAM23 expression confers an inherent advantage in this competitive environment. Moreover, considering that MDA-MB-435 cells do not express ADAM11 mRNA, but do express ADAM22 mRNA (Supplementary Figure 13), we speculate that the residual LG4-dependent proliferation of 435-A23neg cells is supported by LG4/ADAM22 interactions.

The potential of NOS3 as a therapeutic target has recently been reconsidered44 and could also be particularly useful in the context of an ADAM23-heterotypic environment. Unusual NO production has been detected in different types of tumors, altering the cell redox state and signaling. Tumor-derived NO has been shown to enhance migration and invasion of cancer cells.39–41 Notably, NOS3-ITH has been observed in primary breast tumors, and higher levels of NOS3 were detected in undifferentiated tumor zones than in differentiated areas.30 In conclusion, based on our previous findings26,27 and on the work described here, we suggest that ADAM23 silencing, observed during breast cancer progression as a result of tumor epigenetic instability, generates a subpopulation of ADAM23-negative clones within ADAM23-positive tumors and that metastatic spread and malignancy is in fact driven by the coexistence and interaction between ADAM23-negative and -positive subpopulations in the primary tumor. In this scenario, it is tempting to speculate that the previously observed association between ADAM23 hypermethylation and poor clinical outcome27 is indeed associated with ADAM23-ITH and to propose that additional studies addressing the association between ADAM23-ITH in primary tumors, metastatic disease and clinical outcome are worth pursuing.

MATERIALS AND METHODS

Laser microdissection

Tumor cells were laser captured using the Pix Cell-II LCM System (Arcturus-Engineering Inc., Mountain View, CA, USA) as described previously.45 
Samples were free of stromal cells contamination. Breast DCIS samples are composed of tumor cells captured from ducts of pure DCIS lesion. IDC samples were divided geographically into two components: in situ and invasive components. In situ components are composed of tumor cells captured from mammary ducts and invasive components are composed of infiltrative tumor cells captured outside from the ducts. These experiments were conducted according to ethic standards and approved by the Ethics Committee in Human Research from FAP-AC Camargo (1143/08).

Reverse transcription and quantitative PCR
Total RNA was extracted using Trizol (Life Technologies, New York, NY, USA). Reverse transcription (RT) was carried out with 2 μg DNA-free total RNA using Superscript II reverse transcriptase (Life Technologies). Quantitative PCR (qPCR) was performed using a 7300 Real-time PCR System and SYBR Green Life (Life Technologies). Relative expression levels were calculated as described. Hydroxymethylbilane synthase or hypoxanthine guanine phosphoribosyltransferase were used as endogenous control genes for expression data normalization.

Immunohistochemistry
Frozen sections of tumor biopsies were fixed for 30 min in 4% paraformaldehyde, followed by 5 min denaturation in cold acetone. Endogenous peroxidase activity was depleted by incubation with 3% hydrogen peroxide solution. Sections were permeabilized for 1 h with 0.1% Triton X-100 and incubated overnight at 4 °C with anti-ADAM23 (HPA012130; Sigma-Aldrich Co., St Louis, MO, USA). ADAM23 immunostaining were visualized using a Tyramide Amplification Kit (T2092; Life Technologies) and 3,3′-diaminobenzidine substrate (Vector Laboratories, Burlingame, CA, USA). The tissue sections were counterstained with hematoxylin, dehydrated and covednised with Permount (Fisher Scientific, Waltham, MA, USA).

Cell lines and cell culture
The MDA-MB-435 tumor cell line was obtained from the ATCC (Manassas, VA, USA), the SK-Mel-37 human melanoma and CMS5a murine sarcoma cell lines were donated by Dr. T J. Old and Dr. B G. Ritter (Memorial Sloan-Kettering Cancer Centre, New York, NY, USA). All cells were maintained at 37 °C, under 5% CO2, in a complete medium: RPMI 1640 (Life Technologies) supplemented with 10% fetal bovine serum (Cultilab, Campinas, SP, Brazil) and 1% L-glutamine (Sigma-Aldrich Co.).

Ablation of ADAM23, LG4 and NOS3 genes by shRNAs and ADAM23 overexpression
shRNA ADAM23 ablation was performed as described previously. For the sake of simplicity, results obtained for the wild-type cells and cells transfected with a control shRNA vector were grouped throughout the text and presented as the A23gene, whereas results obtained for two independent ADAM23-shRNA clones (shA23 nos. 1 and 2) were grouped and presented as A23neg. LG4 (shLG4) and NOS3 (shNOS3) ablations were obtained by lentiviral infection using pLKO.1 vector containing two different shRNA sequences obtained from Sigma-Aldrich Co. shRNAs to GFP sequence was used as control (shCtrl) (Sigma-Aldrich Co.). Viral production was carried out using Lipofectamine-mediated transfection of human embryonic kidney 293T cells. CMV5a cells were transfected with pCDNA3.1 empty vector or pCDNA3.1-ADAM23-HA (splicing isosform alpha) kindly donated by Dr S Cal. Membrane isolation protocol
A total of 5 × 10^6 cells were washed with ice-cold phosphate-buffered saline and scraped into the membrane extraction buffer (50 mM Tris–HCl (pH 7.4), 10% sucrose and 5 mM EDTA) containing protease inhibitors and were incubated at 4 °C under agitation for 30 min. Cells were disrupted by passing through a 27G needle and cell membranes were precipitated by centrifugation at 18 000 g for 75 min. Pellets were suspended in NP40 buffer (20 mM Tris–HCl (pH 8.0), 137 mM NaCl, 10% glycerol, 1% Nonidet P-40 and 2 mM EDTA) containing protease inhibitors for 6 h and cleaned at 18 000 g for 30 min.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis/western blot
Quantified membrane extracts were resolved on a 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel, transferred to polyvinylidene fluoride membrane (GE Healthcare Biosciences, Pittsburgh, PA, USA) and probed with antibody to ADAM23 (HPA012130; Sigma-Aldrich Co.) or to av-integrin (AB91930; Millipore Co., Billerica, MA, USA) antibodies. Membranes were incubated for 60 min at room temperature in 0.1% Tween-20 with the secondary HRP-conjugated antibody. Reactive proteins were visualized with the ECL Western Blotting Detection System (GE Healthcare Bioscience) according to the manufacturer's recommendations.

Generation of MS
Semiclone confluent monolayers cultures were suspended in the complete medium and 4 × 10^4 cells per well were plated in 48-well plates (Corning Life Sciences, Tewksbury, MA, USA) coated with 1% agarose in RPMI. After 4 days, a single MS spontaneously aggregated in each well. MS were readily handled with a sterile Pasteur pipette for subsequent experiments.

Proliferation rates by BrdU nuclear labeling
Four-day-old MS were incubated for 6 h at 37 °C in a complete medium containing 10 μM of BrdU (Pharmingen, San Diego, CA, USA). After this period, MS were dissociated with Trypsin-EDTA and single-cell suspensions were fixed, permeabilized and incubated with fluorescein isothiocyanate-conjugated anti-BrdU antibody in accordance with the manufacturer's protocol (BD Pharmingen). BrdU-labeled nuclei were analyzed in 10^5 cells per sample on a FACSCalibur cytometer (BD Pharmingen).

Clonogenic anchorage-independent assays
Single-cell suspensions were mixed to 500 μl of 0.6% agarose diluted in the complete medium and were plated in culture dishes coated with 5 mm of 1% agarose. Plates were incubated at 37 °C and 5% CO2 for 2 weeks to allow colony formation. The number of colonies formed and the colony area (mm²) were determined under the light microscope using the ImageJ software (NIH, Bethesda, MD, USA).

Subcutaneous tumorigenesis assays
In all, 10^3 tumor cells suspended in 100 μl of phosphate-buffered saline were inoculated subcutaneously in the flank of immunodeficient female BALB/c nude mice (4–10 weeks old). Tumor growth was monitored weekly and tumor sizes were determined by measuring two perpendicular tumor diameters through the tumor long axis using a caliper. All experiments were conducted according to ethic standards and approved by the Animal Experimentation Ethics Committee of the Medical and Research Centre at Hospital AC Camargo (043/09).

Invasion assays into three-dimensional collagen matrices
MS were embedded in 1.5–3 mg/ml of reconstituted rat tail type-1 collagen (BD Pharmingen) and plated in 48-well plates coated with 5 mm of 1% agarose. After polymerization, collagen matrices were overlaid with the complete medium. Average invasion speed was obtained dividing the distances, from the MS border, traveled by ‘leader’ cells (80–100 tumor cells per spheroid) by time. Invasion was determined under the bright field or fluorescence microscopy using the ImageJ software (NIH).

Spontaneous metastasis assay
Metastatic assays were performed as described previously. ADAM23- heterotypic tumors were generated by inoculation of a single-cell suspension of A23pos and A23neg cells at a 1:1 ratio. Primary tumors were surgically removed when they reached a mean diameter of 1 cm tumors and skin incisions were closed. After 8-week interval, animals were killed and autopsied. These experiments were conducted according to ethic standards and approved by the Animal Experimentation Ethics Committee from FAP- AC Camargo (043/09).

Haptotactic migration assay
Migration assays were carried out using transwell plates (Corning Life Sciences) with 8 μm polycarbonate filters precoated with 20 μg/ml rat tail.
collagen type-1 (BD Pharmingen). A total of 4 x 10^6 cells were plated in the upper chamber of the transwell and allowed to migrate for 8 h at 37°C. At the end of the experiment, the top side of the filters were scraped with cotton swabs and migrating cells were fixed and stained with 4',6-diamidino-2-phenylindole (Sigma-Aldrich Co.). Cells were counted at x100 magnification in 20 different optical fields/insert.

Fluorometric NO quantification

The cell-permeant fluorophore DAF-FM (4-aminomethyl-2',7'-difluorofluorescein diacetate) was used in accordance with the manufacturer's protocol (Life Technologies). Fluorescence of 2 x 10^4 cells per sample was measured on a FACS Calibur cytometer (BD Pharmingen) and expressed as arbitrary manufacturer's protocol (Life Technologies). Fluorescence of 2 × 10^4 cells seeing the wood for the trees.

**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

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