Carbonic Anhydrase IX promotes myeloid-derived suppressor cell mobilization and establishment of a metastatic niche by stimulating G-CSF production

Shawn C. Chafe\textsuperscript{1}, Yuanmei Lou\textsuperscript{1}, Jaclyn Sceneay\textsuperscript{2}, Marylou Vallejo\textsuperscript{1}, Melisa J. Hamilton\textsuperscript{1}, Paul C. McDonald\textsuperscript{1}, Kevin L. Bennewith\textsuperscript{1,4}, Andreas Möller\textsuperscript{2} and Shoukat Dedhar\textsuperscript{1,3,5}

\textsuperscript{1} Department of Integrative Oncology, British Columbia Cancer Research Centre, Vancouver, BC, Canada V5Z 1L3
\textsuperscript{2} Tumour Microenvironment Laboratory, QIMR Berghofer, Herston, Qld 4029, Australia
\textsuperscript{3} Department of Biochemistry and Molecular Biology, University of British Columbia
\textsuperscript{4} Department of Pathology and Laboratory Medicine, University of British Columbia
\textsuperscript{5} To whom correspondence should be addressed:

675 W 10\textsuperscript{th} Avenue
Vancouver, BC,
Canada
V5Z 1L3
Tel: (604) 675-8029
Fax: (604) 675-8099
Email: sdedhar@bccrc.ca

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Abstract

The mobilization of bone marrow-derived cells (BMDCs) to distant tissues prior to the arrival of disseminated tumor cells has been shown preclinically to facilitate metastasis through the establishment of metastatic niches. Primary tumor hypoxia has been demonstrated to play a pivotal role in the production of chemokines and cytokines responsible for the mobilization of these BMDCs, especially in breast cancer. Carbonic anhydrase IX (CAIX, CA9) expression is highly upregulated in hypoxic breast cancer cells through the action of Hypoxia-Inducible Factor-1 (HIF-1). Preclinical evidence has demonstrated that CAIX is required for breast tumor growth and metastasis, however, the mechanism by which CAIX exerts its prometastatic function is not well understood. Here, we show that CAIX is indispensable for the production of G-CSF by hypoxic breast cancer cells and tumors in an orthotopic model. Furthermore, we demonstrate that tumor-expressed CAIX is required for the G-CSF-driven mobilization of granulocytic myeloid-derived suppressor cells (MDSCs) to the breast cancer lung metastatic niche. We also determined that CAIX expression is required for the activation of NF-κB in hypoxic breast cancer cells and constitutive activation of the NF-κB pathway in CAIX-depleted cells restored G-CSF secretion. Together, these findings identify a novel hypoxia-induced CAIX-NF-κB-G-CSF cellular signalling axis culminating in the mobilization of granulocytic MDSCs to the breast cancer lung metastatic niche.
Introduction

Metastasis remains the primary cause of cancer mortality due to the lack of therapies effectively targeting the disease once it has spread beyond the primary tumor (1). Improving our overall understanding of all components involved in metastasis may facilitate the development of new therapeutic strategies. Metastasis is a multi-step program that involves the dissemination of invasive cells from the tumor, entry into the vascular or lymphatic system, transit through the circulation until arrival and arrest in the metastatic organ, followed by extravasation into the organ parenchyma (2). It is known that bone marrow-derived cells (BMDCs) play an important role in every facet of the metastatic cascade by acting in both the tumor microenvironment and at the metastatic site (2). Chemokines and cytokines released by the tumor play a pivotal role in mobilizing these BMDCs to the metastatic organ and therefore play a role in determining organotropism. Furthermore, BMDCs have been found in the metastatic site prior to the arrival of tumor cells creating an environment in the organ parenchyma that is conducive for the survival of disseminated tumor cells known as the “pre-metastatic niche” (3). The recruited cells include myeloid-derived suppressor cells (MDSCs), macrophages, dendritic cells and hematopoietic progenitor cells that act in concert to determine the fate of disseminated tumor cells by controlling survival and proliferation through immune evasion and the release of matrix metalloproteases (MMPs), growth factors and chemokines (2, 4, 5).

We and others have shown that tumor hypoxia has been implicated in driving metastatic niche development (6-8). Many solid tumors are characterized by regions of severe hypoxia which is associated with worse prognosis (9). Exposure to hypoxia leads to a cellular adaptive response mediated by the stabilization of the alpha subunit of the hypoxia-inducible factor (HIF)-1/2 and activation of a transcriptional program mediated by HIF-1/2. This results in a global
rearrangement in the genes expressed by the cells in the hypoxic environment, leading to decreased oxidative phosphorylation, increased glycolytic activity, increased invasive capacity and resistance to radiotherapy and chemotherapy (9). Many downstream targets of HIF-1 are secreted by the tumor, including VEGF, CXCL10, CCL5, CCL2, PlGF, TNFα, resulting in local myeloid cell recruitment (4, 10). However, some of these soluble mediators stimulate the mobilization of myeloid cells to the metastatic site ahead of disseminated tumor cells facilitating the development of the metastatic niche including lysyl oxidase (LOX), LOX-like (LOXL) proteins and CCL2 (6-8). Furthermore, roles for HIF-regulated genes VEGF, PlGF, MMP2, MMP9, CXCL12 and TGF-β have also been described in the metastatic niche (11). In addition, a role for indirect targets of HIF-1 has also been described in the recruitment of myeloid cells to the metastatic niche including the granulocyte colony stimulating factor (G-CSF) (11, 12).

Carbonic anhydrase IX (CAIX) is one of the most highly expressed genes in the hypoxic environment of solid tumors. CAIX facilitates the buffering of intracellular pH through the extracellular hydration of CO₂, producing bicarbonate and protons. The bicarbonate is shunted back into the cell via bicarbonate transporters maintaining an alkaline intracellular pH that is conducive for cell survival, while the protons produced contribute to the acidification of the extracellular space, increasing the migratory and invasive behavior of the tumor (13). In line with this we have demonstrated that CAIX plays a role in the cellular invasion of breast cancer cells (14). Furthermore, CAIX is required for tumor growth and metastasis and maintenance of the stemness phenotype within the hypoxic niche of breast tumors (14-17). CAIX expression has been associated with a worse prognosis in bladder, ovarian, cervical, colorectal, oral, brain and breast cancers (18). However, the pro-metastatic pathways influenced by CAIX function are not well understood.
Herein, we investigated whether CAIX played a role in the early stages of metastasis through the production of chemokines or cytokines required for BMDC recruitment to the metastatic niche. We demonstrated that hypoxia-induced CAIX expression in orthotopic tumors derived from 4T1 breast cancer cells is needed for the production of chemokines and cytokines required for the mobilization of granulocytic MDSCs to a functional metastatic niche in a syngeneic preclinical model of spontaneous breast cancer metastasis. We found that CAIX expression is required for the production of known soluble mediators of breast cancer metastasis, CXCL10, CCL5 and G-CSF, by hypoxic breast cancer cells. Furthermore, we provide evidence that hypoxia-induced CAIX is required for the activation of the NF-κB pathway causing the stimulation of G-CSF production. Moreover, constitutive activation of the NF-κB pathway in hypoxic CAIX-depleted cells restored G-CSF secretion. Thus, we have identified a novel signalling axis in which CAIX, during the early stages of breast cancer metastasis, promotes the development of the breast cancer lung metastatic niche.
Materials and Methods

Cell culture

The murine mammary adenocarcinoma 4T1 cell line (CRL-2539) was obtained from the American Type Culture Collection. Stable cell lines expressing a non-targeting shRNA sequence (shNS) or two separate shRNA sequences targeting the CAIX mRNA (shCAIX1, shCAIX2) have been described previously (15). The cell lines were maintained in DMEM supplemented with 10% fetal bovine serum and non-essential amino acids in a humidified incubator maintained at 37 °C with a 5% CO₂ atmosphere for normoxic studies. For studies done in hypoxia, the cells were maintained at 37 °C in a nitrogen balanced atmosphere of 1% O₂. Cell lines were routinely authenticated for viability, morphology, hypoxia-induced CAIX expression and in vivo tumor growth.

Animal models

All studies were performed in accordance with institutional guidelines (Peter MacCallum Cancer Centre and the British Columbia Cancer Research Centre/University of British Columbia). For conditioned media injection experiments, female 7-9 week old BALB/c mice were injected intraperitoneally (i.p.) with 300 µl conditioned media daily for 7 days. Mice were euthanized by CO₂ asphyxiation and lungs removed for subsequent analysis. For tumor models, experiments were performed as previously described (15). Eleven days post-inoculation the mice were euthanized by CO₂ asphyxiation and tumors, spleens and lungs were removed. Blood was obtained by cardiac puncture immediately following CO₂ asphyxiation and collected into lithium heparin coated plasma collection tubes for plasma recovery (BD Bioscience). In instances where surgery was performed, the mice were anesthetised by i.p. injection with ketamine (75 mg/kg)
and xylazine (10 mg/kg). Mammary tumors were removed while the mice were under anesthesia. Following surgery mice were administered a subcutaneous injection of buprenorphine (0.1 mg/kg). Mice were monitored twice daily and were administered subcutaneous saline once per day to maintain adequate hydration prior to intravenous cell injections (5x10^5 4T1 shCAIX_Luc cells/mouse in 100 μl). Luciferase activity was detected two weeks post cell injection by bioluminescent imaging as previously described (19).

**Statistical Analysis**

Statistical analyses were performed in Excel and GraphPad using the Student’s t-test and Dunnett’s multiple comparison tests. Data was deemed to be statistically significant if \( P<0.05 \). In vitro data reported are representative of 3 independent experiments performed in triplicate each time. Error bars indicate standard error of the mean unless otherwise indicated.

The remaining detailed methods can be found in the supplementary material.
Results

CAIX is required for the production of soluble mediators required for CD11b^+Gr1^+ cell mobilization to the lung

We have previously identified an essential role for the activity of CAIX in hypoxic breast tumor growth and metastasis (14-17). The hypoxic regions of the tumor, where CAIX is upregulated, have also been implicated in the production of chemokines and cytokines involved in myeloid cell recruitment to both the tumors and distant metastatic organs (6, 7, 20). To assess whether CAIX is involved in the metastatic cascade beyond its role within the tumor microenvironment, we investigated the possibility that CAIX expression played a role in preconditioning the metastatic lung. Utilizing previously established 4T1 murine breast cancer cell lines which stably express shRNA that effectively silence CAIX expression (Figure 1A), we investigated leukocyte recruitment in immunocompetent, tumor-free mice injected with hypoxic cell-free conditioned media (6, 7, 15). After daily intraperitoneal injection of hypoxic conditioned media for 7 days, the mice were sacrificed and the lung tissue was harvested and assessed for leukocyte recruitment by flow cytometry. Analysis of lung leukocytes revealed that cell populations from both the innate and adaptive arms of the immune system were recruited to the lungs of mice injected with conditioned media obtained from CAIX-expressing (shNS) and CAIX-depleted (shCAIX) 4T1 cells grown in both oxygen tensions (Figure 1B). However, the recruitment of macrophages, dendritic cells, B cells, CD4^+ and CD8^+ T cells or NK cells was unaffected by the decrease in CAIX expression. In agreement with a previous report, the injection of media from hypoxic breast cancer cells stimulated an increase in the homing of CD11b^+Gr1^+ myeloid cells to the lungs (Figure 1C) (6). Notably, similar to normoxic control levels, the media from hypoxic, CAIX depleted cells failed to reach similar frequencies, indicating that CAIX expression plays a
role in promoting the production of soluble mediators required for their mobilization (Figure 1C).

Since the CD11b^+Gr1^+ population can be subdivided into granulocyte and monocyte subpopulations based on expression of Ly6G and Ly6C epitopes, we analyzed the composition of the CD11b^+Gr1^+ cells recruited to the lungs (Figure 1D) (21). Analysis of the lungs of mice injected with hypoxic media recovered from both shCAIX cell lines contained fewer granulocytes (Ly6G^+Ly6C^{low/-}). These data indicate that CAIX expression by hypoxic breast cancer cells promotes the production of soluble mediators required for the mobilization of granulocytic cells to the lung metastatic niche in tumor-free mice.

CAIX expression is required for the production of G-CSF

We next sought to identify the secreted factors present within the hypoxic conditioned media whose production is stimulated by CAIX expression and leads to the recruitment of granulocytic cells. We assessed the levels of chemokines and angiogenic cytokines in the conditioned media recovered from normoxic and hypoxic 4T1 cells by antibody array (Supplemental Figure S1A and S1B). Conditioned media obtained from shNS and shCAIX cells contained numerous chemokines and angiogenic cytokines. The media obtained from hypoxic 4T1 shNS cells contained increased levels of CXCL10 (black box) and CCL5 (red box) compared to the media obtained from the CAIX-depleted cells. Both CXCL10 and CCL5 have previously been implicated in breast cancer metastasis through the recruitment of bone marrow derived mesenchymal stem cells, while a role for CCL5 in MDSC recruitment has also been suggested (10, 22, 23).
To assess whether the observed changes in the levels of secreted chemokines was due to reduced gene expression, we assessed mRNA levels of the CXCL10 and CCL5 genes, along with the CXCL10 receptor CXCR3. Also included in the analysis were molecules not contained within the array, but were previously implicated in metastatic niche development; placental growth factor (PIGF) and granulocyte colony stimulating factor (G-CSF) (Figure 2A) (3, 12). Under normoxic growth conditions, the 4T1 cells express detectable levels of all genes assessed, while growth in hypoxia for 24 h resulted in a substantive increase in the amount of CAIX, PIGF and CXCL10 expressed by these cells (Figure 2A). The reduction in CAIX expression with shRNA resulted in significant decreases in CCL5 and CXCL10 mRNA levels explaining the CAIX-dependent decreases observed by antibody array (Figure 2A and Supplemental Figure S1A and S1B). In addition, the reduction in CAIX expression also caused a decrease in the amount of CXCR3 expressed by these cells. Furthermore, the reduction in CAIX expression in hypoxic cells resulted in a 50% decrease in G-CSF gene expression (Figure 2A). Also, the expression of PIGF mRNA appeared to trend towards a decrease along with CAIX expression although the variability in the hypoxic induction of PIGF may have masked the statistical significance associated with this. Notably, the observed decreases in mRNA expression were specific as control genes, including interleukin 1-alpha, were unaffected by the reduction in CAIX mRNA levels (Figure 2A). These data suggest that the loss of CAIX expression by hypoxic breast cancer cells is influencing the activity of a unique transcription factor and is not causing a global cellular decrease in transcriptional output. Furthermore, the reduction in G-CSF expression accompanying CAIX-depletion may explain the decreased recruitment of granulocytic MDSC to the lungs observed in mice injected with hypoxic conditioned media obtained from these cells.
G-CSF has previously been shown to promote breast cancer metastasis through the recruitment of CD11b+Ly6G+Ly6C+ granulocytes to the lungs of mammary tumor-bearing mice (12). Furthermore, increased G-CSF levels have been detected in the conditioned media recovered from hypoxic MMTV-PyMT breast cancer cells and in the plasma obtained from tumor-bearing mice treated with antiangiogenic agents implicating a role for hypoxia in the production of G-CSF (6, 24). To quantify the reduction in secreted G-CSF and CXCL10, we assessed the levels of the individual proteins in the hypoxic conditioned media recovered from 4T1 shNS or shCAIX cells by ELISA (Figure 2B and C). 4T1 cells were found to produce large quantities of both CXCL10 and G-CSF (Figure 2B & 2C, Supplemental Figure S1C & S1D). Moreover, in agreement with both the antibody array and qPCR data, when CAIX expression was reduced by shRNA, G-CSF and CXCL10 secretion was significantly decreased (Figure 2B & 2C, Supplemental Figure S1C & S1D). These data indicate that CAIX expression is necessary for the production of G-CSF and CXCL10 by hypoxic breast cancer cells. Tumor-derived G-CSF has previously been implicated in breast cancer metastasis through the recruitment of granulocytes to the metastatic niche (12). Furthermore, our previous gene expression analysis identified G-CSF overexpression in the highly metastatic, CAIX expressing 4T1 tumors but not in the corresponding isogenic non-metastatic 67NR tumors, which do not express CAIX (19) (Gene Expression Omnibus database # GSE11259). Based on these observations and our findings thus far, we decided to focus on determining whether CAIX elicits its prometastatic function within the hypoxic niche through the regulation of G-CSF production.

**Over-expression of shRNA-resistant human CAIX restores G-CSF Production**

To investigate the specificity of the reduction in G-CSF secretion achieved by shRNA-mediated CAIX depletion, we attempted to restore G-CSF secretion through expression of the shRNA-
resistant human CAIX (hCAIX) in CAIX-depleted cells. We have previously demonstrated that this cell line which expresses the full length, catalytically active hCAIX is able to functionally complement the loss of murine CAIX and restore tumor growth in vivo (15). Analysis of lysates obtained from 4T1 cells grown in normoxic and hypoxic conditions by Western blot revealed that CAIX was produced by hypoxic cells expressing a non-specific shRNA, but was reduced to normoxic levels in those cell lines expressing a CAIX-specific shRNA (Figure 2D, top panel, compare lanes 1 and 2 with 3-6). Expression of the human full length CAIX transgene was confirmed in lysates obtained from CAIX-depleted cells using an antibody specific to the human CAIX isoform (middle panel). We verified the activity of the full length human CAIX transgene using an established cell based enzymatic activity assay which confirmed the functional complementation of the 4T1 shCAIX cell line (Supplemental Figure S2).

To assess the ability of human CAIX to restore the secretion of G-CSF, we recovered hypoxic conditioned media from 4T1 cells expressing the WT hCAIX, as well as control and CAIX-depleted cells and measured G-CSF by ELISA (Figure 2E). Expression of the human CAIX was sufficient to restore G-CSF secretion in media recovered from these cells. Thus, these data demonstrate that CAIX activity is important for G-CSF production by hypoxic breast cancer cells in vitro.

CAIX is required for G-CSF mediated granulocyte mobilization to the lungs of tumor-bearing mice

To investigate the role that CAIX plays in the G-CSF-dependent formation of the lung metastatic niche in vivo, we utilized the syngeneic 4T1/BALB/c model. This model has previously been shown to recruit CD11b^+Gr1^+ cells to the “pre-metastatic” lungs of mice bearing tumor volumes
of 250 mm\(^3\) (12). We inoculated mice orthotopically with 4T1 or shCAIX cell lines (shCAIX1 and shCAIX2). We have previously demonstrated in this model that mice inoculated with shCAIX cell lines readily form tumors that grow for 13 days prior to regressing and ultimately disappearing by 50 days post-inoculation (15). Therefore, in order to assess metastatic niche formation prior to tumor regression in this model, the mice were allowed to form tumors for 11 days before being euthanized to assess CD11b\(^+\)Gr1\(^+\) cell recruitment to the lungs and spleens. As we have shown previously, tumor growth over 11 days for the 4T1 shNS and shCAIX cell lines in the syngeneic model results in similar growth kinetics producing tumors with volumes of ~80 mm\(^3\) (15). Furthermore, the loss of CAIX expression in the shCAIX cell line abrogated the ability of the tumors formed with this cell line to metastasize (15). In concordance with data on the leukemoid reaction elicited by the 4T1 model, we observed that growth of 4T1 tumors in the syngeneic model for 11 days promotes the expansion of the CD11b\(^+\)Gr1\(^+\) population in the spleen (Figure 3B) (25). Analysis of the CD45\(^+\) leukocyte population that homed to the lungs and spleens revealed that the 4T1, shCAIX1 and shCAIX2 cell lines all produced tumors that stimulated the mobilization of CD11b\(^+\)Gr1\(^+\) cells (Figure 3A & B). The leukocyte population within the lungs of mice bearing CAIX-expressing tumors was predominated by the presence of CD11b\(^+\)Gr1\(^+\) cells. However, the inability to upregulate CAIX in the tumor resulted in a 50% reduction of CD11b\(^+\)Gr1\(^+\) cells in the lungs of mice bearing tumors formed with the shCAIX cell lines (Figure 3B). Furthermore, this decrease was also observed in the spleens.

We next assessed the relative contribution of the granulocytic and monocytic subpopulations to the recruited CD11b\(^+\)Gr1\(^+\) population. The 4T1 tumor model has been shown to predominantly expand and mobilize granulocytic cells (CD11b\(^+\)Ly6G\(^+\)Ly6C\(^{low/-}\)) in tumor-bearing mice (Figure 3C) (21, 26). Upon assessing the contribution of the individual populations to the total CD11b\(^+\)
population present in the lungs (top) and spleens (bottom) of 4T1 or 4T1 shCAIX tumor-bearing mice we identified that tumors depleted of CAIX mobilized significantly less Ly6G$^+$Ly6C$^{low/-}$ and Ly6G$^-$Ly6C$^{med}$ cells to the lungs and spleens than mice bearing tumors containing substantive CAIX expression (Figure 3D). Notably, the Ly6G$^-$Ly6C$^{high}$ monocytic population was unaffected in the lungs by the loss of CAIX expression in the tumors which is consistent with a role for G-CSF mediated expansion and mobilization of granulocytes and not monocytes (Figure 3D, right).

The reduction in lung and spleen of granulocyte levels suggests that there may be an inability for this cell population to expand and mobilize from the bone marrow, consistent with a decrease in the levels of a cytokine responsible for their proliferation and egress. To assess whether the decreased mobilization correlated with a reduction in circulating G-CSF, we measured plasma G-CSF levels by ELISA (Figure 3E). Naïve mice contained very little steady-state G-CSF in their blood plasma (Figure 3E). In contrast, growth of 4T1 tumors for 11 days resulted in a considerable increase in the plasma G-CSF concentration. Furthermore, plasma recovered from mice bearing 4T1 shCAIX tumors contained G-CSF concentrations similar to levels observed in naïve, tumor-free mice, indicating that the primary source of G-CSF detected in the plasma was tumor-derived and dependent upon CAIX expression. The reduction in G-CSF plasma concentration appears to be CAIX specific as CXCL10 levels remained constant across all three groups of mice irrespective of CAIX levels present in the tumors (Figure 3F). These data suggest that CAIX expression is required for the production of G-CSF in breast tumors and that tumor derived G-CSF is responsible for the mobilization of granulocytes to the lungs of tumor bearing mice, observations that are consistent with previous reports demonstrating that G-CSF levels are critical for CD11b$^+$Gr1$^+$ mobilization (12, 27-30).
The CAIX-dependent mobilization of granulocytic MDSCs initiates a functional metastatic niche

To assess whether the CAIX-dependent mobilization of granulocytes to the lungs of mice bearing orthotopic 4T1 mammary tumors resulted in a functional metastatic niche we evaluated the ability of this tumor-primed setting to enhance the metastatic ability of the poorly metastatic shCAIX cell line (15). Mice were inoculated with 4T1 or shCAIX cells orthotopically and allowed to form tumors for 11 days before undergoing surgical excision. Three days post-surgery the mice were then challenged with luciferase positive CAIX-depleted cells (shCAIXLuc) intravenously and metastasis was monitored by bioluminescent imaging (Figure 4A). In agreement with our earlier findings (Figure 3D), investigation of the lung leukocyte composition of both the 4T1 and shCAIX groups after 11 days of tumor growth confirmed that the lungs were primed with granulocytes and that this population was again reduced in the mice bearing shCAIX tumors (Figure 4B). The expression of both CD11b and Gr1 describes a generic MDSC phenotype in mice (31). MDSCs have previously been implicated in breast cancer metastasis through their prometastatic and immunosuppressive roles in both the tumor microenvironment and metastatic niche (6, 31-33). To this point we have immunophenotypically classified these cells as MDSCs based on their expression of CD11b and Gr1. However, since these markers can be expressed on additional cell types (26), which have been shown to have tumoricidal activity in the premetastatic niche (34), we assessed their immunosuppressive activity toward T cells. We isolated Gr1+ cells from the lungs of mice bearing 4T1 and shCAIX tumors and observed that this population was equally immunosuppressive in both groups, irrespective of the CAIX levels in the tumor and are thus herein referred to as MDSC (Figure 4C). When we compared the metastatic ability of the shCAIX cell line, metastases could be detected two weeks after
intravenous injection of shCAIX\textsuperscript{Luc} cells in the lungs of mice that initially had 4T1 primary tumors, however, when CAIX levels were reduced in the primary tumor, these mice did not contain any detectable metastases (Figure 4D & E). These data demonstrate the power of the primary tumor in initiating a functional metastatic niche and indicate that CAIX plays a significant role in promoting its establishment.

**CAIX expression is required for activation of NF-κB in hypoxia**

We next sought to identify the cellular signalling axis stimulated by CAIX expression in the hypoxic environment which was promoting G-CSF production. An established regulator of chemokine transcription is NF-κB and multiple stimuli have been reported to activate NF-κB activity including pH and hypoxia (35-37). Based on the critical role that CAIX plays in the regulation of intra- and extracellular pH of cancer cells in hypoxia, we sought to assess whether CAIX expression stimulated NF-κB activity \textit{in vitro} (Figure 5A). Introduction of the exogenous reporter construct into 4T1 cells resulted in the detection of a basal level of NF-κB activity under normoxic conditions. Incubation of the 4T1 shNS cell line in hypoxia for 48 h resulted in a 2.5 fold increase in NF-κB activity. However, hypoxic, CAIX-depleted cells failed to activate NF-κB transcriptional activity above basal levels, suggesting that CAIX expression is required for increased NF-κB transcriptional activity in hypoxia. Since IKK-β-dependent NF-κB transcriptional activity is dependent upon translocation of the heterodimeric p65/p50 transcription factor to the nucleus following IκB\textalpha degradation, we assessed whether the decreased transcriptional activity of NF-κB was due to reduced levels of p65/RelA protein in the nucleus (Figure 5B & C) (35). Immunocytochemical staining of 4T1 cells cultured in normoxic conditions contained p65 in 7\% of the nuclei assessed, whereas hypoxia stimulated the 3-fold increase in nuclear translocation of p65 in the cells analyzed (Figure 5C). Interestingly, p65 was
localized predominantly in the cytoplasm under both normoxic and hypoxic conditions in CAIX-depleted cells indicating that the decreased NF-κB transcriptional activity detected was due to altered p65 nucleocytoplasmic dynamics.

To assess the ability of CAIX to restore NF-κB transcriptional activation in CAIX-depleted cells, we monitored NF-κB activity in cells expressing human CAIX (Figure 5D). Incubation of the 4T1 shNS cell line in hypoxia for 48 h resulted in increased NF-κB activity above the basal level observed in both normoxic shNS and hypoxic CAIX-depleted cells. Constitutive expression of the WT hCAIX in shCAIX cells incubated in hypoxia restored the hypoxia-stimulated activation of NF-κB activity to levels observed in hypoxic cells expressing endogenous levels of CAIX. These data demonstrate that the activity of CAIX is required for the hypoxia-stimulated increase in NF-κB transcriptional activity.

To demonstrate the direct link between NF-κB activity and G-CSF production in CAIX-expressing breast cancer cells, we directly inhibited NF-κB signalling with the IKKβ inhibitor, BAY11-0782, and then assessed G-CSF levels in the conditioned media by ELISA. Treatment with the NF-κB inhibitor reduced the level of G-CSF secreted relative to the vehicle treated cells (Figure 5E and Supplemental Figure S3). Thus, this demonstrates that G-CSF production is downstream of both CAIX and NF-κB signalling.

**Constitutive activation of NF-κB restores G-CSF production in CAIX-depleted cells**

NF-κB heterodimers have previously been shown to bind to the G-CSF promoter to regulate G-CSF transcription (38). To demonstrate that the reduction in G-CSF production in CAIX-depleted cells was due to decreased NF-κB activity in these cells, we restored NF-κB activity through expression of the constitutively active IKKβ S179E S181E (IKKβ EE) mutant isoform
and assessed G-CSF secretion (39). To confirm that IKKβ EE expression resulted in NF-κB pathway activation, we assessed the subcellular location of p65/RelA in hypoxic 4T1 shNS cells, CAIX-depleted cells (shCAIX EV) and CAIX-depleted cells expressing the IKKβ EE mutant (shCAIX IKKβ EE) by immunocytochemistry. Similar to the hypoxic 4T1 control cells, expression of IKKβ EE in the CAIX-depleted cells resulted in nuclear translocation of p65; however, nuclear p65 was not detected in the same cells transfected with the empty vector (Figure 6A). Western blot analysis of lysates obtained from cells grown under identical conditions confirmed the expression of the IKKβ EE transgene in CAIX-depleted cells (Figure 6B). To verify that the nuclear accumulation of p65 was transcriptionally active, we measured the activity of the NF-κB response element driven luciferase reporter in cells incubated in hypoxia (Figure 6C). Once again, CAIX expressing cells demonstrated significantly greater levels of NF-κB activity under hypoxic stimuli than the CAIX-depleted cells. Constitutive activation of the NF-κB pathway in shCAIX cells through the introduction of the IKKβ EE isoform resulted in significantly greater NF-κB promoter activity than cells expressing the empty vector, thus confirming restoration of a transcriptionally active NF-κB pathway.

Upon confirming that expression of the constitutively active IKKβ EE mutant restores NF-κB activity in our CAIX-depleted cells, we assessed whether restoration of NF-κB activity in CAIX-depleted cells would result in the concomitant restoration of G-CSF production. Conditioned media was recovered from hypoxic shCAIX-IKKβ EE cells and G-CSF production monitored by ELISA. Growth of 4T1 shNS cells in hypoxia resulted in increased G-CSF production over cells grown in normoxia (Figure 6D). G-CSF levels were reduced over 5-fold in media assayed from CAIX-depleted cells (mock transfected) and this was unaffected by introduction of the empty expression vector used to introduce the IKKβ EE gene. Interestingly, constitutive activation of
the NF-κB signalling cascade resulted in partial restoration of G-CSF secretion, which was significantly greater than the concentration detected in the media from cells with reduced NF-κB activity. To determine whether the increased G-CSF production upon restoration of NF-κB activity was due to increased activity in the G-CSF promoter region, we assessed the activity of a G-CSF promoter driven firefly luciferase reporter under these same conditions (Figure 6E). Similar to what we observed for NF-κB activity, a basal level of G-CSF promoter activity was detected in the 4T1 cells, consistent with the significant amount of secreted G-CSF detected in the conditioned media obtained from this cell line under normoxic conditions. Upon incubation of the cells in hypoxia for 48 h, the CAIX depleted cells were unable to stimulate G-CSF promoter activity above basal levels detected in normoxia, whereas the cells capable of upregulating CAIX expression in hypoxia contained 50% more transcriptional activity at the G-CSF promoter. Since the G-CSF proximal promoter region contains binding sites for numerous transcription factors, we assessed the contribution of NF-κB to the increased activity of the G-CSF promoter observed in hypoxia by utilizing a G-CSF promoter containing a mutated NF-κB binding site (40). 4T1 shNS and shCAIX cells were transfected with the NF-κB mutant G-CSF promoter and reporter activity was measured. As detected with the wild type G-CSF promoter, basal transcriptional activity was detected under normoxic and hypoxic conditions in the shCAIX cell line. However, the increase in G-CSF promoter activity observed in hypoxia in the shNS cells with the wild type reporter was abrogated upon altering NF-κB binding to the promoter and was reduced to promoter levels observed in shCAIX cells. These data confirm the importance of the NF-κB pathway in G-CSF production. Furthermore, they identify that the CAIX-dependent influence on G-CSF production is a result of CAIX-dependent stimulation of NF-κB activity in hypoxia.
Discussion

Primary tumor hypoxia, through the activity of HIF-1, has been shown to play a pivotal role in the development of the metastatic niche (6-8). We demonstrated here for the first time that, in addition to its prosurvival role within the hypoxic niche of the primary tumor, the hypoxia-induced CAIX promoted the production of chemokines and cytokines involved in the formation of a metastatic niche. We determined that 1) CAIX expression and activity are required for the production of G-CSF and that CAIX-dependent G-CSF production was necessary for the mobilization of granulocytic MDSC (G-MDSC) to the metastatic niche, an environment that enhanced the metastatic propensity of a poorly metastatic shCAIX cell line, 2) CAIX expression and activity were required for the stimulation of NF-κB transcriptional activity in hypoxia, and 3) CAIX acts through NF-κB to stimulate G-CSF production. Our data identified a novel mechanism by which CAIX promotes the metastatic capability of breast cancer cells through the development of the lung metastatic niche via the NF-κB-dependent production of G-CSF.

The prevailing body of preclinical evidence suggests that in tumor-bearing mice, granulocytes, which include G-MDSC, are found in the metastatic niche where they assist in the establishment of metastases (6, 12, 29, 30, 32, 33, 41-43). However, it has also been suggested that mobilized neutrophils have anti-metastatic activity in the lung premetastatic niche (34). The reason for the discrepancy in the role of these cells in the lungs in the premetastatic phase between our two systems is not known. Multiple investigators have utilized the same syngeneic system to establish a prometastatic role for granulocytic cells, while others have established this in other syngeneic systems. Most recently, a requirement for granulocytes in the extravasation of circulating cancer cells into the lung has been shown for the premetastatic phase (43). Utilizing previously defined criteria to distinguish G-MDSC from neutrophils (4, 26) our data agrees with
the former but provides further novelty in demonstrating that hypoxia-induced CAIX in the primary tumor is involved in the mobilization of G-MDSC to the lungs early in the metastatic cascade. Specifically, we show that CAIX stimulates G-CSF expression and secretion via the canonical NF-κB pathway. Moreover, when CAIX levels are diminished within the primary tumor, circulating G-CSF levels are near those observed in naive mice resulting in a concomitant reduction in G-MDSC detected in the lung. Based on our observations identifying reduced plasma G-CSF in mice with CAIX-depleted tumors it is likely that the observed CAIX-G-CSF axis is one mode by which CAIX activity promotes the metastasis of hypoxic breast cancers, by influencing the G-CSF-granulocytic MDSC axis (44). These important findings are our contribution to this field.

G-CSF has been widely used for treating chemotherapy induced neutropenia and for the mobilization of hematopoietic stem cells following hematopoietic cell transplantation due to its key role as a myeloid growth factor (45). G-CSF may not be well known for its chemoattractant properties, however recent studies have demonstrated that treating tumor-free mice with recombinant G-CSF alone results in the accumulation of MDSC in organs where G-CSF expressing tumors are likely to metastasize (12, 29). Furthermore, blocking the action of circulating G-CSF or its receptor with antibodies is sufficient to reduce the accumulation of these cells in the lungs of tumor-bearing mice, and in fact attenuate metastasis formation (12, 30). It remains possible that G-CSF triggers production of chemoattractant molecules in these tissues (12), however, this and the identity of these molecules requires further characterization.

G-CSF expression has been detected in the tumors of many solid malignancies including breast cancer (46-49) and tumors that express G-CSF have been found to be more aggressive and associated with increased likelihood of recurrence (46). G-CSF can induce T-cell tolerance and
has been shown to drive MDSC development from human hematopoietic stem cells cultured in vitro (31, 49). Including our data, there is an increasing list of preclinical studies implicating G-CSF in tumor progression and metastasis (12, 24, 27, 29, 30, 42, 50), and it is plausible that immune modulation is a contributing factor. Together, these studies suggest further investigation into the G-CSF-driven MDSC expansion in cancer patients is needed.

The metastatic niche conditions the metastatic organ parenchyma to become receptive for tumor growth (11). Here we demonstrated the power of the metastatic niche by enhancing the metastatic capacity of a non-metastatic, CAIX-depleted cell line. Central to this finding was that the strength of the niche relied upon G-MDSC mobilization in response to tumor derived factors secreted by a hypoxic, CAIX-expressing tumor. Notably, tumors with reduced CAIX expression were incapable of this metastasis enhancing feat in the same period of time due to the reduced mobilization of MDSC, reinforcing the critical role played by CAIX in stimulating the production of molecules driving the formation of the metastatic niche. A previous study has demonstrated that administration of exogenous G-CSF to mice was sufficient to enhance the metastatic ability of a non-metastatic cell line (12). Also, our data agrees with previous observations (7) demonstrating the ability of the tumor-driven metastatic niche to enhance the metastatic capability of an otherwise poorly metastatic cell line and suggest that G-CSF is instrumental in this setting. Our data also suggest that additional factors produced by the tumor, irrespective of CAIX or G-CSF levels present, stimulate the immune suppressive activity of MDSC. Moreover, although our data demonstrates that G-CSF plasma levels in mice with shCAIX tumors were comparable to those observed in tumor-free mice, we cannot exclude the possibility that additional tumor derived factors contributed to the mobilization of MDSC (51).

We observed decreases in gene expression for PlGF, CXCL10, and CCL5 in cells where CAIX
expression had been reduced. Of these, only PIGF has an established role in the initiation of the lung metastatic niche (3). CXCL10 and CCL5 have both been demonstrated to be involved in breast cancer metastasis through paracrine signalling with mesenchymal stem cells within the tumor microenvironment (10, 22) and it remains to be seen whether the loss of CAIX in the tumor microenvironment has altered this tumor-stroma communication. The concentration of CXCL10 in plasma was unaffected by CAIX levels \textit{in vivo}, and because of this it is unlikely that CXCL10 is contributing to the enhanced metastatic capability of the CAIX-depleted cell line. The decrease in CXCL10 observed in the cell autonomous setting \textit{in vitro} when CAIX levels were depleted is a reflection of the reduction of NF-κB activity in these cells. The discordance between our \textit{in vitro} and \textit{in vivo} data on CXCL10 is likely a reflection of the high baseline concentration present in naïve mice and may be indicative of the CXCL10 contributed by host immune cells in response to the implanted tumors (52).

NF-κB has previously been implicated in the regulation of G-CSF production (38). We show here that CAIX expression is required for the hypoxia-mediated stimulation of NF-κB activity and G-CSF production. NF-κB activity has been shown to be stimulated by hypoxia and by decreased pH in culture, a known consequence of hypoxia induced glycolytic activity of cancer cells (35-37, 53). Decreased extracellular pH is often observed in hypoxic areas of tumors and arises as a consequence of increased metabolic activity (13). We have previously demonstrated that decreased production of CAIX in a hypoxic environment decreases the ability of cells to acidify the medium (15). Therefore, it is possible that CAIX activity impacts the hypoxia induced activity of NF-κB by maintaining the optimal intracellular pH to allow for maximal NF-κB activity and studies are currently underway to test this. We cannot ignore the influence of additional transcription factors in maintaining basal levels of G-CSF production observed in
these cells in the absence of CAIX, nor can we conclude that NF-κB is acting alone since activation of NF-κB in CAIX-depleted cells only partially restored G-CSF secretion. However, in agreement with the observations of others (40) disruption of NF-κB binding to the G-CSF promoter abrogated G-CSF production, irrespective of the presence of CAIX, reinforcing that NF-κB activity is a requirement for G-CSF expression, and is downstream of CAIX.

Here we have demonstrated that CAIX plays an integral role in the recruitment of G-MDSC to the breast cancer lung metastatic niche. In addition to the preclinical evidence surrounding the role of MDSC in numerous cancer models, MDSC have been shown to be correlated with clinical stage and metastatic burden in breast cancer patients (54). Consequently, efforts to specifically target MDSC as a cancer therapy are ongoing (31). The recent development of highly selective small molecule inhibitors of CAIX has advanced research into targeting CAIX in vivo (18). Our findings indicate that CAIX inhibition in the primary tumor alters the systemic mobilization of myeloid cells that contribute to tumor progression and metastasis, which may aid normalizing the immune response to the tumor (15). Therefore, these findings reinforce the clinical utility in targeting CAIX activity for the treatment of hypoxic breast tumors.

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References


Figure legends

**Figure 1.** CAIX expression is required for the production of soluble mediators required for CD11b^+Gr1^+ cell recruitment to the lungs. **A** Western blot analysis of the indicated 4T1 lysates grown in hypoxia (1% O_2) for 72 h demonstrating CAIX knock-down by two independent shRNA sequences. β-actin confirms equal loading across all lanes. **B** Analysis of CD45^+ leukocytes in lungs obtained from mice following daily injection (7 days) with the indicated cell-free 4T1 conditioned medium by flow cytometry identifying the proportions of macrophages (CD11b/F4/80), dendritic cells (CD11c/MHC II), B cells (CD19/B220), T cells (CD3/CD4 and CD3/CD8) and NK cells (CD3/NK1.1) recruited, n=6 mice/group. **C** Analysis of CD11b^+Gr1^+ and **D** the granulocytic (Ly6G^+Ly6C^low^-) populations recruited to the lungs of mice injected with the indicated cell-free 4T1 conditioned media. Error bars indicate +/- SEM, *P <0.05 as assessed by Dunnett’s multiple comparison test.

**Figure 2.** CAIX expression is required for the production of G-CSF. **A** qPCR analysis was performed to determine the relative expression level of the indicated genes in control (shNS) and CAIX-depleted 4T1 cells (shCAIX1 and 2) grown in serum free media under normoxic and hypoxic conditions for 24 h. Data are represented as fold change in mRNA level for each individual gene relative to mRNA levels detected in the shNS normoxic sample (assessed a value of 1) from 3 individual experiments. Error bars indicate +/- SEM. *P < 0.05 as assessed by student’s t-test. Analysis of CXCL10 **B** and G-CSF **C** levels in conditioned media recovered from control (shNS) and CAIX-depleted (shCAIX) 4T1 cells incubated in serum-free media in hypoxia for 24 h by ELISA. Values are normalized to total protein content of the conditioned media. Bars indicate average protein concentration +/- SEM. Data are representative of 3 individual experiments performed in triplicate. **P < 0.01 as assessed by student’s t-test unless otherwise indicated. **D** Lysates from normoxic and hypoxic 4T1 shNS, shCAIX and shCAIX +WT hCAIX (+ WT hCAIX) cells were analyzed by Western blot with the indicated antibodies. Hypoxic conditioned media recovered from 4T1 shNS, shCAIX and shCAIX +WT hCAIX cells was assessed for G-CSF levels by ELISA. Bars indicate average protein concentration +/- SEM. Data are representative of 3 individual experiments performed in triplicate. *P < 0.05, relative to the shNS sample, # P<0.05 relative to the shCAIX sample, as assessed by Dunnett’s multiple comparison test.

**Figure 3.** CAIX is required for G-CSF mediated granulocyte recruitment to the lungs of tumor-bearing mice. **A** Representative flow cytometry plots depicting tissue CD11b^+Gr1^+ recruitment 11 days after orthotopic inoculation of 4T1, 4T1 shCAIX1 and 4T1 shCAIX2 cells. **B** Quantification of CD11b^+Gr1^+ recruitment to the lungs (top) and spleen (bottom) of individual mice for each group as a proportion of the infiltrating CD45^+ leukocyte population, n=5 mice per group. **C** Representative flow cytometry plots depicting analysis of Ly6G^+Ly6C^[high], Ly6G^+Ly6C^[Med] and Ly6G^+Ly6C^[low/-] CD11b^+Gr1^+ subsets. **D** Quantification of subset frequencies within the lungs (top) and spleen (bottom) of individual mice as a proportion of the
CD11b+ population. Plasma G-CSF (E) and CXCL10 (F) levels of individual mice were measured by ELISA. The naïve sample represents the plasma level of each cytokine prior to tumor cell inoculation. The plasma was pooled from saphenous vein bleeds from 5 mice 3 days before the study began. Bars represent mean plasma chemokine levels +/- SEM, n=5 mice per group. * P<0.05, ** P<0.01, *** P<0.001 relative to 4T1 samples as assessed by students t-test.

Figure 4. A functional metastatic niche is developed in mice bearing CAIX expressing mammary tumors. A Experimental schema. B and C The lungs were removed from a subset of mice (n=3) from both groups 11 days post orthotopic implantation of each cell line. B The MDSC subset composition of the CD11b+ cells recruited to the lungs after 11 days was assessed by flow cytometry. C Gr1+ cells were isolated from these lungs and assessed for their ability to suppress stimulated T cell proliferation. Splenocytes stimulated with αCD3/αCD28 (Responder Control cells, RC) were mixed with Gr1+ cells at a 1:1 ratio for 72 h and 3H-thymidine incorporation over the final 18 h was determined and compared to unmixed RC cells. Bars represent mean +/- SD. **P<0.01 relative to the proliferative rate of RC cells by student’s t-test. D Representative bioluminescent images of mice 14 days post intravenous injection of shCAIXLuc cells; Depicted are n=4 of 6 for the 4T1 primary tumor group and n= 4 of 8 for the shCAIX primary tumor group. E Quantification of total photon flux detected in mice with a luciferase signal above a threshold of 5x10^3 photons/s. *P<0.05 relative to 4T1 samples as assessed by student’s t-test. PT: primary tumor, i.v.: intravenous.

Figure 5. CAIX expression is required for activation of NF-κB in hypoxia. A Analysis of NF-κB activity in control (shNS) and CAIX-depleted (shCAIX2) 4T1 cells incubated in normoxia (black bars) or hypoxia (white bars) for 48 h. Data are expressed as a ratio of firefly luciferase levels to Renilla luciferase levels. Bars indicate mean +/- SEM and are representative from 3 individual experiments performed in triplicate. * P<0.05 relative to hypoxic shNS sample, # P<0.05 relative to normoxic shNS sample. B shNS and shCAIX2 cells grown in normoxia or hypoxia for 48 h were assessed for p65/RelA cellular localization by immunofluorescence microscopy. White boxes indicate regions of interest zoomed in on and depicted in insets. Arrows indicate nuclear accumulated p65/RelA. Data are representative images captured from 3 separate experiments where 5 hpf were assessed in each. Scale bars, 10 µm. C Quantification of the percentage of the total cell population displaying nuclear p65 accumulation. * P<0.05 relative to normoxic shNS sample, ** P<0.01 relative to hypoxic shNS sample. D Analysis of NF-κB activity in lysates obtained from shNS, shCAIX2 and hCAIX restored (+ WT hCAIX) 4T1 cells incubated in normoxia or hypoxia for 48 h. Data are represented as described for A. Bars represent mean +/- SEM and are representative of 3 individual experiments performed in triplicate. *P<0.05 relative to shNS hypoxic samples, #P <0.05 relative to shCAIX2 hypoxic samples as assessed by student’s t-test. E ELISA measuring G-CSF levels in the conditioned media recovered from hypoxic 4T1 cells after 24 h treatment with the IKKβ inhibitor BAY11-0782 (10 μM). Bars denote average G-CSF concentrations +/- SEM and are representative of 3 individual experiments performed in triplicate. *** P<0.001 as assessed by student’s t-test.
**Figure 6.** Constitutive activation of NF-κB activity in CAIX-depleted cells rescues G-CSF production.  

**A** Immunofluorescent microscopy performed on hypoxic 4T1 shNS (top panel), shCAIX + empty vector (EV) and shCAIX + IKKβ S179E S181E (IKKβ EE) cells depicting the cellular location of p65/RelA after 48 h in hypoxia. Boxes indicate regions of interest zoomed in on and displayed in insets. Images are representative of 3 separate experiments assessing 5 hpf in each. Scale bars, 10 µm.  

**B** Western blot analysis of the indicated lysates from cells cultured under identical conditions as (A).  

**C** Analysis of a NF-κB activity in lysates obtained from the indicated cell lines after 48 h incubation in hypoxia. Bars indicate relative activity expressed as the ratio of firefly luciferase to Renilla luciferase +/- SEM and are representative of 3 individual experiments performed in triplicate.  

**D** Conditioned media recovered from the indicated cell lines grown in normoxia (black bar) or hypoxia (white bars) for 24 h in serum-free media was analyzed for G-CSF production by ELISA. Shown is a representative experiment from 3 independent experiments performed in triplicate. Values are normalized to protein concentration of conditioned media. Bars represent the average protein concentration +/- SD.  

**E** Analysis of G-CSF promoter activity in the indicated cell lines grown in normoxia or hypoxia for 48 h. Data are represented as described in C. Error bars represent the SEM of 3 individual experiments unless otherwise stated. *P< 0.05.
Figure 1
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Figure 6
Carbonic Anhydrase IX promotes myeloid-derived suppressor cell mobilization and establishment of a metastatic niche by stimulating G-CSF production

Shawn C Chafe, Yuanmei Lou, Jaclyn Sceneay, et al.

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