The Antidepressant Desipramine and \( \alpha_2 \)-Adrenergic Receptor Activation Promote Breast Tumor Progression in Association with Altered Collagen Structure

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Abstract

Emotional stress activates the sympathetic nervous system (SNS) and release of the neurotransmitter norepinephrine to promote breast tumor pathogenesis. We demonstrate here that the metastatic mammary adenocarcinoma cell line 4T1 does not express functional adrenergic receptors (AR), the receptors activated by norepinephrine, yet stimulation of adrenergic receptor in vivo altered 4T1 tumor progression in vivo. Chronic treatment with the antidepressant desipramine (DMI) to inhibit norepinephrine reuptake increased 4T1 tumor growth but not metastasis. Treatment with a highly selective \( \alpha_2 \)-adrenergic receptor agonist, dexametomidine (DEX), increased tumor growth and metastasis. Neither isoproterenol (ISO), a \( \beta \)-AR agonist, nor phenylephrine, an \( \alpha_1 \)-AR agonist, altered tumor growth or metastasis. Neither DMI- nor DEX-induced tumor growth was associated with increased angiogenesis. In DMI-treated mice, tumor VEGF, IL-6, and the prometastatic chemokines RANTES, M-CSF, and MIP-2 were reduced. Tumor collagen microstructure was examined using second harmonic generation (SHG), a nonabsorptive optical scattering process to highlight fibrillar collagen. In DMI- and DEX-treated mice, but not ISO-treated mice, tumor SHG was significantly altered without changing fibrillar collagen content, as detected by immunofluorescence. These results demonstrate that \( \alpha_2 \)-AR activation can promote tumor progression in the absence of direct sympathetic input to breast tumor cells. The results also suggest that SNS activation may regulate tumor progression through alterations in the extracellular matrix, with outcome dependent on the combination of adrenergic receptor activated. These results underscore the complexities underlying SNS regulation of breast tumor pathogenesis, and suggest that the therapeutic use of adrenergic receptor blockers, tricyclic antidepressants, and adrenergic receptor agonists must be approached cautiously in patients with breast cancer.

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Introduction

In patients with cancer, chronic emotional stress or other negative psychological factors such as depression or lack of social support promote tumor growth and progression (1, 2). The sympathetic nervous system (SNS) is an important pathway by which stress can facilitate tumor growth (3–6). The SNS neurotransmitters norepinephrine and epinephrine activate \( \alpha \)- and \( \beta \)-adrenergic receptors (AR). In animal models using \( \beta \)-AR–expressing cancer cell lines, stressor exposure or \( \beta \)-AR stimulation increased tumor growth and/or metastasis by mechanisms such as increased tumor angiogenesis and density of tumor associated macrophages (7, 8). SNS activation can also target \( \beta \)-AR-expressing host cells residing in the tumor or in metastatic sites to promote tumor growth and metastasis (6, 9). These studies provide compelling evidence that norepinephrine and AR-expressing tumor cells or host stromal cells modulate tumor pathogenesis.

Despite progress in understanding the molecular mechanisms underlying sympathetic regulation of tumor progression, several critical questions remain. First, the role for \( \alpha \)-AR has not been carefully examined despite the fact that in human breast cancer, \( \alpha \)-AR expression has been linked to poor prognosis (10). Second, variation in breast cancer cell line adrenergic receptor expression (11, 12) is recapitulated in human breast tumors that display heterogeneity in \( \alpha \)- and \( \beta \)-AR expression (10). The functional consequences of such heterogeneity have yet to be systematically explored. It is reasonable to assume that when breast cancer cells express no or low levels of adrenergic receptor, host stromal adrenergic receptor would be the direct targets of elevated norepinephrine. Stromal cells, including cells of the immune system, endothelial cells, and fibroblasts, express \( \alpha \)-AR and \( \beta \)-AR normally and in tumors (8, 13, 14). We propose that...
SNS activation can promote tumor pathogenesis by acting on stromal cells to alter the tumor extracellular matrix. To test this hypothesis, we have used multiphoton laser scanning microscopy and second harmonic generation (SHG) to visualize a component of the tumor stroma, fibrillar collagen. SHG is an endogenous optical signal produced when 2 excitation photons combine to produce one emission photon, "catalyzed" by a non-centrosymmetric structure, such as ordered collagen triple helices (15). Tumor collagen fiber microstructure, as revealed by SHG, is of great interest because several studies have suggested that it influences tumor progression, specifically tumor metastasis. It is important to note that not all collagen fibers produce detectable SHG (16), and that tumor cells can migrate toward blood vessels via SHG+ fibers, locomoting along such fibers more efficiently than cells moving independently. Interestingly, the extent of SHG-associated disease-free survival, independent of tumor grade, size, and the University of Rochester Committee on Animal Resources. The University of Rochester Animal Resource is fully accredited by AAALAC International.

Cell lines and tissue culture
4T1 (mammary adenocarcinoma; CRL-2539), MDA-MB-231 (MB-231; HTB-26), and human foreskin fibroblast-1 cells (HFF; SCRC-1041) were acquired from American Type Culture Collection (ATCC) within the last 2 years. ATCC authenticates cell lines using short tandem repeat analyses. Upon acquisition, cells were expanded for no more than 3 passages and frozen. All cell lines were used within 3 months of thawing. For passaging, 4T1 was grown in RPMI containing penicillin/streptomycin and 10% fetal calf serum. MB-231 and HFF were grown in Dulbecco’s modified Eagle medium containing l-glutamine and supplemented with penicillin/streptomycin and 10% fetal calf serum. All media and media components were purchased from Gibco (Invitrogen Inc.). All cells were regularly tested for mycoplasma contamination.

In vitro culture of 4T1 cells to measure proliferation and cytokine production was described previously (11). Proliferation was assessed using CyQuant NF Proliferation Assay kit (Invitrogen) following the manufacturer’s instructions. Fluorescence was detected with a 485 nm excitation filter and 530 nm emission filter using a multi-well plate reader (Biotek). For cytokines, cell-free supernatants were harvested after 72 hours in culture and stored at −80°C.

Drug treatment
Drug treatments commenced 2 days before tumor cell injection and continued for the duration of the experiment. For DMI treatment, continuous release pellets (Innovative Research of America) were implanted subcutaneously under ketamine/xylazine anesthesia (90/9 mg/kg). The adrenergic receptor agonists ISO (Sigma-Aldrich), phenylephrine (Sigma-Aldrich), and DEX (Pfizer) were dissolved in sterile saline. Mice were injected intraperitoneally (i.p.) daily for the duration of the experiment. ISO and phenylephrine doses were chosen based on ISO-induced increased tumor pathogenesis reported in other murine tumor models (7, 8) and on pilot toxicity studies. Two doses of DEX were tested: 10 µg/kg elicited no apparent sedative effects and 25 µg/kg elicited mild and transient anesthetic effects (slowed movements following injection that were no longer apparent 1 hour after injection). DMI, ISO, and phenylephrine treatment elicited early and transient decreases in body weight (≤10%) that recovered over time.

Materials and Methods

Mice
Female BALB/cByl mice (6–8 weeks of age; The Jackson Laboratories) were housed 3 to 4 per cage with food and water ad libitum, and used experimentally 2 weeks after arrival. All animal procedures were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the University of Rochester Committee on Animal Resources and the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the University of Rochester Committee on Animal Resources.

Tumor implantation, growth, and tissue harvest
4T1 cells (1 × 10⁵ in sterile saline) were injected into the third mammary fat pad (MFP) under ketamine/xylazine anesthesia. Tumors were measured with calipers every 2 to 3 days without knowledge of experimental group. Mice were sacrificed by pentobarbital overdose (200 mg/kg, i.p.) followed by cervical dislocation to harvest tumor, spleen, and lungs. Tumors and spleens were weighed and divided. For
catecholamine and cytokine analyses, tissue was immediately placed on dry ice and stored at −80°C. Tumor volume (V) was calculated using the equation \( V = \frac{1}{2} \times \text{length} \times \text{width}^2 \). Tumor growth is presented either as the raw tumor volume over time or as normalized tumor growth. Normalized tumor growth was calculated by dividing an individual’s tumor volume at a given time point by its volume at the earliest time point all tumors were detected (day 3 or 5 post-4T1).

**Norepinephrine/normetanephrine and cytokine analyses**

For norepinephrine and normetanephrine, tissue was homogenized in 0.01 M HCl at 10% volume (mL) by tissue weight. Norepinephrine and normetanephrine were determined by ELISA (Rocky Mountain Diagnostics) following the manufacturer’s instructions. For cytokines, tissue was homogenized in radioimmunoprecipitation assay buffer containing protease inhibitors (Pierce). Cytokines were measured using mouse-specific ELISAs (R&D Systems) following the manufacturer’s instructions.

For multiple analyte analysis, a Milliplex mouse cytokine/chemokine magnetic bead panel kit (Millipore; catalogue #MCYTOMAG-70K) was used following the manufacturer’s instructions. A Luminex 200 plate reader equipped with xPOnent software (University of Rochester Flow Cytometry Core) was used to determine median fluorescence intensity for each analyte. The concentration of each analyte was calculated using the corresponding standard curve fit to a 5-parameter logistic equation.

For all ELISAs, absorption was measured at 450 nm using a multiwell plate reader (Synergy HT; Biotek Instruments Inc.). Curve fitting and sample concentration calculations were conducted with Gen5 software (Biotek). Concentrations were normalized to total protein in homogenates as determined with a BCA protein assay (Pierce).

**Immunohistochemistry, SHG, and image analysis**

Tumors were fixed in 4% paraformaldehyde for 72 hours, followed by incubation in 10% sucrose and 30% sucrose for 24 hours each. Three adjacent tumor sections (20-μm-thick) were collected every 100 μm. Standard immunohistochemical techniques were used to detect blood vessels using rat-anti CD31⁺ antibody (diluted 1:20; Abcam), proliferating cells using polyclonal rabbit-anti Ki67 (1:500; Abcam) or collagen I using polyclonal rabbit anti-collagen type I antibody (1:200; Abcam). Species-appropriate Alexa-Fluor-594-conjugated secondary antibodies (Invitrogen) were used to detect the primary antibodies. Fluorescein isothiocyanate (FITC)-conjugated anti-F4/80⁺ antibody (Abcam) was used to detect F4/80⁺ macrophages.

For immunofluorescent and SHG imaging of collagen, tumor sections were imaged using multiphoton microscopy. Five random fields of view per tumor section were imaged by a blinded observer using a 0.8 NA 20× water immersion objective lens and electronic zoom at 1×, one section per tumor. SHG and immunofluorescence emission was collected simultaneously under constant imaging conditions in each sample, including excitation wavelength (810 nm), laser power (8 mW at the sample), and photomultiplier tube voltages. To detect immunofluorescence, fluorophore emission was collected using bandpass filters 520/40 (for FITC) and 635/30 (for Alexa-Fluor 594). SHG signal was separated from fluorescence by a 475 nm long-pass dichroic (Semrock) and detected through a bandpass 405/30 emission filter.

All images were analyzed by personnel blinded to group using custom algorithms in ImageJ (NIH Freeware). To quantify CD31 and Ki67 immunofluorescence, a threshold that excluded autofluorescence was determined in adjoining tissue sections stained with secondary antibody alone, and the percentage of pixels above threshold was calculated. Average blood vessel area was calculated and normalized to cell density based on 4′,6-diamidino-2-phenylindole nuclear staining. To quantify SHG and anti-collagen immunostaining, a common threshold was determined for all samples by determining background pixels averaged from 2 tissue-free images from each channel. The average background intensity was subtracted from all images, then common SHG and immunostained thresholds were applied to distinguish SHG or immunostained pixels from background pixels. Two calculations were used to represent SHG or immunohistochemical collagen signal: the percentage of pixels above threshold in each image and the average intensity of those pixels above threshold. The SHG and collagen immunostaining values from 5 regions of interest from each tumor section were averaged for each animal.

**Lung metastases**

Lungs were fixed in 10% formalin and paraffin embedded. Five-μm-thick sections were collected every 100 μm through the entire lung. Tissue sections were stained using standard hematoxylin and eosin (H&E) techniques. Metastases were visualized using a ×4 objective lens and counted in each tissue section by a blinded observer.

**β-AR Expression and Intracellular Cyclic AMP**

A standard radioligand-binding assay was used to determine specific binding of [125I]-cyanopindolol (NEN Radiochemicals) to whole cells to quantify β-AR expression, as described previously (11). The procedure to measure intracellular cAMP was described previously (11). Cyclic AMP content was measured by ELISA (R&D Systems) following the manufacturer’s instructions.

**Statistical analyses**

Statistical analyses were conducted with GraphPad PRISM software with \( P < 0.05 \) considered statistically significant. When 2 groups were compared, an \( F \) test for variance was conducted to compare variance. If variance was similar, an unpaired two-tailed Student t test was used. If variance differed, group comparisons were conducted using the nonparametric Mann–Whitney U test. To compare more than 2 groups, one- or two-way ANOVA was used. When variance differed significantly between groups, the nonparametric Kruskal–Wallis test was used, with post
hoc analysis by Dunn’s multiple comparison test. Tumor growth over time was analyzed using repeated measures two-way ANOVA. Significant interactions or main effects were analyzed by simple effects or by Holm–Sidak multiple comparison test.

**Results**

4T1 tumor cells do not respond to norepinephrine in vitro or signal via adrenergic receptor

To determine if norepinephrine directly regulates 4T1 tumor cell functional responses, norepinephrine or selective adrenergic receptor agonists were incubated with 4T1 cells in vitro. Norepinephrine did not alter 4T1 proliferation (Fig. 1A) or VEGF production (Fig. 1B) under in vitro conditions that altered VEGF production and proliferation in MB-231 and other β-AR–expressing breast cancer cell lines (11). Furthermore, specific binding of the β-AR ligand $^{125}$I-cyanopindolol was readily detectable with MB-231 cells, but not 4T1 cells (Fig. 1C), and isoproterenol (ISO), a β-AR agonist, elevated intracellular cAMP in MB-231 cells but not in 4T1 cells (Fig. 1D). Together, these results demonstrate that 4T1 cells do not possess cell surface β-AR. In terms of α-AR, the α$_1$-agonist phenylephrine did not significantly alter 4T1 proliferation or VEGF production (Fig. 1A and F). The α$_2$-agonist DEX had no effect except at the highest concentration tested (42 µmol/L), where 4T1 proliferation was reduced and VEGF production was increased (Fig. 1G and H; Supplementary Fig. S1A and S1B). However, yohimbine, an α$_2$-AR antagonist, did not block these DEX-induced effects (Supplementary Fig. S1A and S1B), and there was no evidence for α$_2$-AR signaling by cAMP inhibition (Supplementary
Fig. S1C; ref. 25), as demonstrated here in a human fibroblast line (Supplementary Fig. S1D). We conclude that 4T1 cells do not express functional α1-AR, α2-AR, or β-AR and therefore norepinephrine cannot directly affect 4T1 function. We next tested if elevated synaptic norepinephrine altered 4T1 tumor pathogenesis in vivo.

DMl, a norepinephrine reuptake inhibitor, increases 4T1 tumor growth, but not metastasis

DMl inhibits norepinephrine reuptake through the norepinephrine transporter and thereby increases synaptic norepinephrine (23, 24). To assess alterations in synaptic norepinephrine in the periphery, norepinephrine and its metabolite normetanephrine were measured in the densely innervated spleen. Norepinephrine concentration primarily represents norepinephrine synthesized and stored intraneuronally, but normetanephrine is produced by an extraneuronal enzyme (catechol-O-methyltransferase), and reflects norepinephrine released and metabolized in the synapse (26). In mice implanted subcutaneously with 21-day continuous release pellets containing DMI (10 mg) or placebo, DMI increased splenic norepinephrine 2-fold and normetanephrine 4-fold relative to placebo in association with reduced spleen mass 3 days after pellet implantation (Supplementary Fig. S2A–S2C). The increased normetanephrine is evidence of effective norepinephrine uptake blockade, and elevated normetanephrine (but not norepinephrine) was detected in the spleen up to 7 days after DMI implantation (data not shown). In comparison to daily intraperitoneal injection of DMI, subcutaneous pellets elicited greater magnitude and longer lasting elevation in splenic norepinephrine and normetanephrine (data not shown).

To assess tumor growth, mice were implanted with pellets containing DMI or placebo 2 days before 4T1 injection, a treatment regimen similar to the chronic pharmacological β-AR activation that elicited increased tumor growth/metastasis in β-AR-expressing tumor models (7–9). Using pellets containing 5, 7.5, or 10 mg DMI in pilot studies (data not shown), we determined the 10 mg dose increased tumor growth most effectively. DMI treatment (10 mg) increased tumor volume (mm3; Fig. 2A; see figure legends for statistical analyses) and growth rate (volume normalized; Fig. 2B), in association with significantly increased tumor weight by day 14 post-4T1 injection (Fig. 2C). Despite the increase in tumor growth, metastasis to the lungs was not altered in DMI-treated mice (Fig. 2D and E). DMI treatment significantly reduced tumor VEGF, a key proangiogenic cytokine (Fig. 2F), and transiently decreased tumor IL-6, a proinflammatory cytokine with proangiogenic activity (Fig. 2G); however, DMI treatment did not alter CD31+ blood vessel density (Supplementary Fig. S3). Tumor norepinephrine was not altered by DMI treatment (Fig. 2H). A transient increase in tumor normetanephrine was detected at day 12 post-4T1 injection (Fig. 2I).

α2-AR activation increases breast tumor growth and metastasis

To determine if selective activation of adrenergic receptors can increase tumor growth, mice were injected daily with 5 mg/kg ISO (nonselective β-AR agonist), 10 mg/kg phenylephrine (α1-AR), or 10 and 25 μg/kg DEX (α2-AR) beginning 2 days before 4T1 injection and continuing until sacrifice. Neither ISO nor phenylephrine treatment altered normalized tumor growth, tumor weight, or lung metastases (Fig. 3A–D). Tumor VEGF and IL-6 did not differ between phenylephrine or ISO treatment and saline controls at sacrifice (Fig. 3E and F). Daily treatment with the highly selective α2-AR agonist DEX (10 and 25 μg/kg) increased the rate of tumor growth and the number of metastasis in the lung compared with saline controls (Fig. 3G and H). Immunohistochemistry using anti-Ki67 to detect proliferating cells revealed an increase in proliferating cells in the 10 μg/kg DEX group compared with saline and 25 μg/kg groups (Fig. 3I). DEX treatment did not alter tumor VEGF (Fig. 3J) or IL-6 (Fig. 3K) at sacrifice.

Potential cytokine/chemokine mechanisms underlying DMI- and DEX-induced tumor pathogenesis

To further probe the mechanisms underlying differences in DMI versus DEX-induced tumor progression, additional tumor cytokines and chemokines were measured by multiplex analysis. In DMI-treated mice, the proinflammatory cytokine TNF-α was nonsignificantly increased (Fig. 4A; Mann–Whitney, P = 0.07). A similar trend was detected in mice treated with 10 μg/kg DEX (Fig. 4B; ANOVA, P = 0.052) but not in phenylephrine- or ISO-treated mice (data not shown). TNF-α added directly to 4T1 cells did not alter proliferation in vitro (Supplementary Fig. S4), indicating that elevated TNF-α cannot directly increase 4T1 tumor growth. Furthermore, neither DMI nor DEX treatment altered the proinflammatory cytokine IL-1β, the T-cell-associated cytokines IL-2 and IFN-γ, or the anti-inflammatory cytokine IL-10 (data not shown). However, several tumor chemokines that promote tumor metastasis and regulate macrophage activity including RANTES (CCL-5), M-CSF (CSF-1), and MIP-2 (CCL-2) were decreased by DMI treatment (Fig. 4A), but not altered by DEX treatment (Fig. 4B). Neither DMI nor DEX treatment significantly altered the density of F4/80+ tumor macrophages (Supplementary Fig. S5).

DMl- and DEX-induced tumor growth is associated with altered SHG-producing tumor collagen

Structural alterations in fibrillar collagen, uniquely visible via SHG imaging, are associated with tumor cell proliferation and motility (18, 27). Our laboratory has demonstrated that stromal TNF-α knockout, or depletion of macrophages, reduced tumor growth and metastasis and was associated with alterations in SHG (28). These findings, combined with the association between DMI- and DEX-induced tumor growth and trends toward increased tumor TNF-α, led us to explore a matrix-based mechanism underlying the increased tumor growth.

We analyzed collagen in 4T1 tumors from DMI-, DEX-, and ISO-treated mice using 2 methods: standard immunohistochemical analysis and SHG imaging. Figure 5A shows 2 representative images from a 4T1 tumor with SHG-producing collagen (in blue) and collagen type I.

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Figure 2. DMI treatment increased tumor growth but not metastasis. Mice were implanted with 10 mg DMI or placebo continuous release pellets 2 days before 4T1 inoculation. Tumor growth expressed as volume (A) or normalized (B) and as tumor weight (C). Lung metastasis (D) were measured 14 days post-4T1 injection. E, representative H&E-stained lung with metastatic lesion indicated by arrow. ×4 magnification; scale bar = 200 µm. A, D, results represent 1 of 2 experimental replications at each time point. For (H) and (I) results from 2 experimental replications were normalized relative to the respective placebo controls. Results are expressed as mean ± SEM, n = 6–7 mice per group for day 7 and 12, n = 9–10 per group day 14 post-4T1 injection. Statistical analyses: (A) main effect of DMI treatment, \( P = 0.047; \) treatment \( \times \) time interaction, \( P < 0.0001; \) main effect of time, \( P < 0.0001; \) B, main effect of DMI treatment, \( P = 0.0005; \) treatment \( \times \) time interaction, \( P = 0.0001; \) C, tumor weight: main effect of DMI, \( P = 0.02; \) DMI \( \times \) time interaction, \( P = 0.04; \) D, lung metastasis: Student t test, \( P = 0.06); \) F, VEGF: DMI treatment, \( P = 0.0006; \) no interaction or effect of time; G, IL-6: main effect of DMI treatment, \( P = 0.004; \) no DMI \( \times \) time interaction, main effect of time, \( P = 0.0001; \) H, tumor normetanephrine (NMN): DMI \( \times \) time interaction, \( P = 0.02); \) Asterisks indicate significant differences versus corresponding placebo control group by Holm–Sidak’s multiple comparisons test (\( P < 0.05\)).

Discussion

Adrenergic receptor activation promotes tumor growth or metastasis in several animal models of cancer in which the tumor cells express functional adrenergic receptor (7, 8, 29). We demonstrate here that the mammary adenocarcinoma cell line 4T1 lacks \( \alpha \)-AR and \( \beta \)-AR expression and signaling capacity and is unable to directly respond to norepinephrine. To our knowledge, no studies have investigated the in vivo impact of sympathetic activation and norepinephrine detected by immunohistochemistry (in green) in the same section. Image analysis revealed that in 4T1 tumors from DMI-treated mice, the SHG-emitting pixel intensity was increased (Fig. 5B) without a change in the density of SHG-emitting collagen as determined by the number of SHG pixels above a common threshold (Fig. 5C). No change in total collagen was detected by anti-collagen I immunohistochemistry (Fig. 5D). However, DEX treatment did not alter the intensity of SHG pixels above threshold (Fig. 5E), but it increased the number of SHG pixels above threshold (Fig. 5F) with no change in total collagen (Fig. 5G). The \( \beta \)-agonist ISO produced no change in SHG intensity (Fig. 5H) and a nonsignificant increase in SHG pixel number (Fig. 5I; \( P = 0.07)\). By immunohistochemistry, ISO also produced a nonsignificant reduction in total collagen pixel intensity (Fig. 5I; \( P = 0.15\)). These alterations in SHG indicate changes in tumor collagen microstructure with DMI, DEX, and ISO treatment, and each treatment uniquely correlates with augmentation of primary tumor growth (DMI), tumor growth and metastasis (DEX), or no alteration (ISO; summarized in Table 1).
stimulation on tumor pathogenesis when the tumor cells cannot directly respond to norepinephrine. Doing so removes the influence of tumor adrenergic receptors and thus allows the investigation of adrenergic receptor stimulation of adrenergic receptor-expressing host stromal cells, effects that may be masked when tumor cells express functional adrenergic receptor. Under these conditions, we demonstrated that inhibition of norepinephrine reuptake to elevate synaptic norepinephrine promotes 4T1 tumor growth, and α2-AR activation can drive tumor growth and metastasis. The distinct alterations in SHG emission from tumor collagen accompanying elevated norepinephrine and adrenergic receptor stimulation suggests that SNS activation may modify the tumor extracellular matrix to regulate pathogenesis. Together, these results reveal novel pathways by which SNS activation can drive tumor growth and metastasis despite the inability of the tumor cells themselves to respond to norepinephrine.

**A role for α2-AR and host stromal cells in tumor growth and metastasis**

The finding that DMI treatment elevated norepinephrine and increased 4T1 tumor growth is consistent with other reports demonstrating augmentation of tumor growth and/or metastasis associated with sympathetic innervation and β-AR–expressing tumor or stromal cells (6–8). However, our
results differ in several important ways from these reports. We found no evidence that increased tumor growth was associated with β-AR activation or with increased angiogenesis or proangiogenic cytokines. Furthermore, in this adrenergic receptor-negative tumor model, β-AR activation with ISO did not increase lung metastasis or elevate M-CSF (also known as CSF-1; data not shown), as reported by Sloan and colleagues. We contend a β-AR–induced reduction in CSF-1 or other stromal-derived cytokines/chemokines may be obscured if tumor cytokine production (such as CSF-1) is increased by β-AR activation. In this scenario, whether or not the tumor cells can respond to β-AR stimulation may dictate directionally opposite tumor outcomes.

Our results are consistent with a small number of reports of increased proliferation by α2-AR–expressing breast tumor cell in vitro and increased tumor growth in vivo (13, 29, 30), but we have been unable to detect functional α2-AR in several breast cancer cell lines (data not shown). In fact, DEX at supra micromolar concentrations may act on imidazoline receptors (31) to reduce 4T1 cell proliferation and increase VEGF production in vitro (Fig. 1G and H). This concentration of DEX, orders of magnitude above the Ki for α2-AR (1.08 nmol/L; ref. 32), is unlikely to be achieved in vivo at the doses used here. Instead, we propose that DEX ligation of α2-AR did not affect VEGF, IL-6, RANTES, and other prometastatic cytokines, DMI-induced elevation of synaptic norepinephrine reduced them, presumably via adrenergic receptors other than α2, thus providing an antimetastatic counter to the prometastatic effects of α2-AR stimulation by elevated norepinephrine. One way to test this possibility is to selectively block adrenergic receptor in DMI-treated mice, but we found the combination of DMI and β-blocker treatment was associated with a high level of mortality; we therefore tested direct stimulation of β-AR and α1-AR. Although we did not detect significant alterations in 4T1 tumor growth and metastasis with ISO or phenylephrine treatment, in ISO-treated mice, a trend toward reduced tumor growth (Fig. 3A) and chemokine production was noted (data not shown), and we are further exploring β-AR activation in this tumor model.

We have yet to identify the stromal targets of norepinephrine and α2-AR stimulation or their location. DMI only transiently increased synaptic norepinephrine in the tumor, as indicated by the norepinephrine metabolite normetanephrine. It should be noted that at no time point post-DMI treatment was splenic or tumor norepinephrine or normetanephrine content reduced, indicative of sympathetic nerve depletion because of chronic elevation of norepinephrine. Splenic norepinephrine and normetanephrine were dramatically increased early after DMI implantation, an effect that subsided but was still apparent as the tumor developed (data not shown). The DMI-induced increase in
Splenic norepinephrine points to the potential targeting of adrenergic receptor–expressing cells in extratumoral organs, such as spleen and bone marrow, that play a role in 4T1 pathogenesis (34). Similarly, DEX treatment may target α2-expressing cells within the tumor and the lung to promote metastasis. Furthermore, we cannot rule out these drugs acting at the level of the central nervous system. For example, the DEX-induced increase in tumor Ki67⁺ proliferating cells (Fig. 3I) and TNF-α (Fig. 4B) at 10 μg/kg, but not 25 μg/kg, may indicate distinct mechanisms underlying DEX treatment involving the mild sedation (central nervous system effects) observed with 25 μg/kg DEX. Nonetheless, our results demonstrate that the elevated tumor growth associated with DEX and DMI treatment is driven by changes apparent within the tumor, including the extracellular matrix.

**A novel mechanism for SNS regulation of tumor progression: collagen microstructure and SHG imaging**

Tumor stromal cells, including macrophages and fibroblasts, regulate tumor collagen structure. The detected SHG

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<th>Table 1. Summary of DMI, DEX, and ISO-induced tumor matrix alterations</th>
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<td><strong>Treatment (AR-selectivity)</strong></td>
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signal from a collagen fiber is sensitive to the amount of collagen (15), as well as the diameter of the fibrils that form fibers (35, 36), their spacing (36), and the order versus disorder in fibril packing (37). Here, we define a change in one or more of the latter 3 parameters (fibril diameter, fibril spacing, and order) as a change in collagen fiber ‘microstructure.’ Hence, the signal produced by SHG differs from immunofluorescent detection of collagen, which is sensitive to epitope concentration and reports primarily the amount of collagen in a given region of interest. The 2 readouts [SHG and immunofluorescence (IF)] from a given collagen fiber can be compared with gain insight into the extent of changes in fibril microstructure (primarily alters SHG) versus changes in collagen content (alters both SHG and IF; see refs. 28, 38, and 39).

As revealed by image analysis, tumor SHG-emitting collagen was increased by both DMI and DEX treatment in subtly different ways. In DMI-treated mice, tumor SHG pixel intensity above threshold increased without an increase in the percentage of pixels above threshold, representing a change in SHG+ fiber microstructure (Fig. 5B and C). However, increased SHG emission from tumors from DEX-treated mice was because of an increase in the percentage of pixels above threshold without an increase in the intensity of those pixels above threshold (Fig. 5E and F), suggesting an increase in SHG+ fiber content relative to control tumors. Neither of these types of increased SHG was associated with altered total collagen as measured by IF (Fig. 5D and G), indicative of an alteration in the structure of the tumor collagen and not collagen deposition. Interestingly, ISO elicited a trend toward decreased total collagen as measured by IF (Fig. 5I), and yielded no change in tumor growth or metastasis despite the trend toward increased SHG+ fiber content (Fig. 5I). Based on the evidence that microstructural changes in tumor collagen, as detected by SHG, may drive tumor cell proliferation, local invasion, and metastasis (21, 40, 41), we contend that the treatment-dependent changes in SHG-emitting collagen shown here reflect a stromal-based mechanism by which adrenergic receptor activation may promote tumor progression. Studies are underway to understand the adrenergic receptor mechanisms that alter SHG+ collagen microstructure and to demonstrate that such changes lead to DEX- or DMI-induced tumor progression. These results illustrate the potential power of using SHG imaging in the 4T1 model to distinguish overlapping and opposing effects of increased norepinephrine with sympathetic activation consistent with our proposal that norepinephrine elicits effects via mixed adrenergic receptor signaling that can oppose each other.

**Clinical implications**

An important aspect of this work is that DMI and DEX are used clinically. A retrospective study examining the clinical use of antidepressants and breast cancer development found an association between DMI and increased breast cancer risk (42), consistent with the protumor growth effect shown here. DEX is used as a sedative to treat cancer pain, adding urgency to delve further into the mechanisms underlying chronic DEX treatment and increased tumor growth and metastasis (43, 44). Finally, our results with DMI and elevated synaptic norepinephrine imply a balance between prometastatic effects of α2-AR and antimetastatic β-AR with increased norepinephrine release. If true, caution should be applied in the clinical adjuvant use of β-blockers, as proposed by others (3, 45).

In summary, our results strongly implicate α2-AR activation as a promoter of tumor pathogenesis in the absence of direct sympathetic input to the tumor cells. The results suggest a unique, matrix-based mechanism whereby norepinephrine can facilitate breast tumor growth through regulation of the tumor extracellular matrix, specifically collagen microstructure. Further investigation into this mechanism is critical in part because of the possibility of using SHG imaging to detect SNS-induced alterations in the tumor matrix as a marker of a more aggressive tumor phenotype. Our results suggest that norepinephrine can elicit directionally opposing effects within the tumor that must be carefully investigated to understand the impact of stress-induced SNS activation in a disease as molecularly heterogeneous as breast cancer.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors' Contributions**

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