Tumors modulate fenestrated vascular beds and host endocrine status

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Abstract
Allograft and xenograft transplantation into a mouse host is frequently utilized to study cancer biology, tumor behavior, and response to treatment. Preclinical studies employing these models often focus solely upon the intra-tumoral effects of a given treatment, without consideration of systemic toxicity or tumor–host interaction, nor whether this latter relationship could modulate the toxicologic response to therapy. Here it is demonstrated that the implantation and growth of a range of human- and mouse-derived cell lines leads to structural vascular and, potentially, functional changes within peripheral endocrine tissues, a process that could conceivably ameliorate the severity of anti-angiogenic-induced fenestrated vessel attenuation. Observations suggest a multifactorial process, which may involve host- and tumor-derived cytokines/growth factors, and the liberation of myeloid-derived suppressor cells. Further investigation revealed a structurally comparable response to the administration of exogenous estrogen. These findings, in addition to providing insight into the development of clinical anti-angiogenic “adaptation,” may be of significance within the “cancer-cachexia” and cancer-related anemia syndromes in man.

KEYWORDS
anti-angiogenic, cancer-cachexia, endocrine, tumor–host, vascular modulation

1 | INTRODUCTION

The past decades have witnessed huge advances in the understanding of cancer growth and progression, allowing the development of multiple targeting modalities in preclinical oncology research (Seebacher et al., 2019). Much of this work has utilized allograft and xenograft transplantation in mice. Although this has led to breakthroughs in new treatments, these efficacy and pharmacodynamic studies have until recently experienced limited utility in the field of toxicology research, that is, the safety testing of drugs within a diseased population (Kim & Sharpless, 2012; Morgan et al., 2017, 2013).

In the field of vascular endothelial growth factor (VEGF)-inhibiting and other anti-angiogenic therapies, preclinical animal models have been used to identify treatment-related microvascular lesions, which may contribute to a range of dose-limiting and potentially severe toxicities, clinically, for example, hypertension, proteinuria, hemorrhage/thrombosis, gastro-intestinal perforation, and endocrine dysfunction (Brinda et al., 2016; Chen & Cleck, 2009; Hanna et al., 2020; Hayman et al., 2012; Izzedine et al., 2009; Kamba et al., 2006; Kamba & McDonald, 2007; Pena-Hernandez et al., 2019; Syrigos et al., 2011; Touyz et al., 2018; Yang, Zhang, et al., 2013; Zhang et al., 2016).

A complete understanding of the factors responsible for the de novo formation of tumor-associated blood vessels in cancer is
currently lacking. Additionally, the question remains as to whether similar factors could impact the vasculature in host tissues, via comparable mechanisms, and whether or not this could also influence the vascular response during therapy (Grunewald et al., 2006; Lugano et al., 2020; Zuazo-Gaztelu & Casanovas, 2018).

In addition to providing insight into the pathogenesis of cancer-endocrine comorbidity, an enhanced understanding of such processes could aid in the prediction of how a given vascular modulating agent (VMA)-treated cancer patient may respond systemically to angiogenic inhibition.

We have previously demonstrated that both the nude mouse and the C57Bl immunocompetent mouse respond similarly to taxicologic modification of systemic endocrine vasculature via the administration of VMAs (Hargreaves et al., 2017).

During these preliminary investigations, we determined that the transplantation and growth of a Calu-6 xenograft tumor, in the absence of any treatment, could potentially increase resident vascular density within host endocrine tissues (Figure S1). The aim of this manuscript was to test a hypothesis that this observation within fenestrated endocrine vascular beds may occur in association with other tumor types, of variable lineage, and with differential expression of angiogenic signaling mediators.

Here we further demonstrate that the implantation and growth of a range of human- and mouse-derived xenografts/allografts leads to structural vascular and, potentially, functional changes within peripheral endocrine tissues. Observations suggest a multifactorial process, which may involve host- and tumor-derived cytokines/growth factors, and the liberation of myeloid-derived suppressor cells (MDSCs).

2 MATERIALS AND METHODS

2.1 Tumor-bearing animal models

In order to examine the systemic vascular and endocrine effects of tumor burden, a series of studies were conducted in non-tumor-bearing and tumor-bearing, untreated, mice.

All mice were supplied by the Rodent Breeding Unit (AstraZeneca, Alderley Park, UK) and housed in negative pressure isolators with 12-h light/dark cycles and provided with sterilized food (supplied by Special Diet Services, Alderley Park, UK) and water ad libitum. The mice weighed approximately 25 g and were at least 8 weeks of age at study commencement. Animals were randomized into either control non-tumor-bearing or tumor-bearing groups (n = 6 in each) (Table 1).

CT26 (syngeneic colorectal cell line) tumors were established by subcutaneous injection of 5 × 10^5 CT26 cells suspended in 0.1 ml of phosphate-buffered saline (PBS) into the flank of female BALB/c (inbred) mice; PC3 (human-derived prostate cell line) xenografts were inoculated by subcutaneous injection of 5 × 10^5 cells suspended in Matrigel TM into the flank of female athymic nude (Swiss nu/nu genotype) mice; 4T1 (syngeneic breast cell line) tumors were established by the orthotopic implantation of 5 × 10^5 cells suspended in 0.1 ml of PBS into the fourth inguinal mammary fat pad of female BALB/c mice; and BT474c (human-derived estrogen receptor [ER]-positive breast cell line) xenografts were inoculated by subcutaneous injection of 5 × 10^5 cells suspended in Matrigel TM into the flank of female athymic nude (Swiss nu/nu genotype) mice.

For BT474c studies, mice were supplemented with 0.36 mg/60 day release 17β estradiol pellets (Innovative Research of America, Florida, USA), 1 day prior to cell implantation. Two control groups, with and without estradiol supplementation, were included within this study cohort.

Following study completion, all mice were culled by terminal narcosis with 5:1 CO2/O2 mixture.

All procedures were conducted in accordance with Home Office (UK) and local ethical review committee guidelines and complied with the Animals Scientific Procedures Act 1986.

2.2 Tissue harvesting, processing, and vascular/cell quantification

Following the in life phase, selected endocrine organs (right adrenal gland, thyroid gland, and pancreas) were removed from all animals (the adrenal gland weighed). Tissues were fixed, embedded, and processed for CD31 immunohistochemical (IHC) staining and 3D fiberlength density analysis, using the Definiens XD image analysis platform (version 2.0.4 Definiens AG), with Tissue Studio and Developer XD, as described previously (Hargreaves et al., 2017). For CD11b and GR-1 (MDSC) expression, variations in slide preparation were as follows. For CD11b expression, slides were incubated overnight at 4°C with the primary antibody, rat anti-mouse CD11b (clone M1/70; BD Biosciences, New Jersey, USA), diluted at 1:40. For GR-1 expression, slides were incubated overnight at 5°C with the primary antibody, rat anti-mouse GR-1 (clone RB6-8C5, eBioscience, California, USA), diluted at 1:80. For both protocols, rat IgG2b was used to establish the appropriate isotype control. Discovery Red (#760-228; F. Hoffmann-La-Roche, Basel, Switzerland) and Discovery Yellow (#760-239) chromogens were used to visualize the expression of CD11b and GR-1, respectively. Due to the inherent paucity of CD11b and GR-1-positive cells within the systemic endocrine tissues, a total

| Tumor group                                      | Host mouse | Study duration (days) |
|-------------------------------------------------|------------|-----------------------|
| CT26 (syngeneic colorectal tumor)               | BALB/c     | 28                    |
| PC3 (human-derived prostate tumor)              | Nu/nu      | 28                    |
| 4T1 (syngeneic breast tumor)                    | BALB/c     | 28                    |
| BT474c (human-derived estrogen receptor [ER]-positive breast tumor) | Nu/nu | 28 |
cell number, per tissue section, determined via light microscopy by a board-certified anatomic pathologist, was deemed an accurate method by which to determine the overall cellularity.

2.3 Quantitation of potential endocrine signaling changes in the circulation

To evaluate the endocrinological response of the mouse host to the presence of tumor burden, serum samples for adrenocorticotrophic hormone (ACTH) and thyroid-stimulating hormone (TSH) were quantified using the Milliplex mouse pituitary/thyroid magnetic bead panel (MPTMAG-49K; EMD Millipore, Massachusetts, USA).

In addition, to assess the induction of pro-angiogenic signaling, a series of xenograft (human; angiopoietin-2, endothelin-1, fibroblast growth factor [FGF]-1, FGF-2, granulocyte colony stimulating factor [G-CSF], interleukin-8 [IL-8], leptin, placental growth factor [PIGF], and VEGF-A) or host (mouse; endothelin-1, FGF-2, G-CSF, IL-6, leptin, PIGF-2, prolactin, tumor necrosis factor alpha [TNFa], and VEGF-A)-derived cytokines/growth factors were quantified using Milliplex MAP kits (HAGP1MAG-12K and MAGPMAG-24K; EMD Millipore, Massachusetts, USA), on a LumineX 200™ System, using xPONENT software (LumineX Corporation, Texas, USA).

Whole blood samples were taken from the tail vein prior to study termination. Samples were placed in a covered test tube, allowing the blood to clot at room temperature for 30 min. Clot separation was undertaken by centrifuging the samples at 10,000 rpm, for 10 min at 4°C, in a refrigerated centrifuge (Eppendorf, Thermo Fisher Scientific, Massachusetts, USA). The serum was then placed into clean polypropylene tubes using a Pasteur pipette, before immediate analysis, as per the manufacturer’s guidelines (PROTOCOL_FOR_00037988MAN_MPTMAG-49K; PROTOCOL_HAGP1MAG-12K; PROTOCOL_MAGPMAG-24K; Merck Millipore, Massachusetts, USA).

2.4 Fluorescence-activated cell sorting bone marrow analysis

In order to analyze the distinct lineages of bone marrow-derived cells, a combination of the differential expression of leukocyte common antigen (CD45, MCA103F; Bio-Rad Serotec, Oxfordshire, UK) and transferrin receptor (CD71, MCA1033PE; Bio-Rad Serotec, Oxfordshire, UK) was coupled with side scatter analysis and the DNA stain LDS-751 (Molecular Probes, L-7595; Thermo Fisher Scientific, Massachusetts, USA), the latter separating immature versus mature red cells. For further analysis of endothelial precursor cells (EPCs), amongst control and estrogen-supplemented animals within the BT474c cohort of the investigation, phycoerythrin (PE)-conjugated CD202b (Biolegend, California, USA) monoclonal antibodies (5 μl) were additionally incorporated into the protocol.

Fresh bone marrow suspensions were prepared by obtaining femoral samples from all non-tumor-bearing and tumor-bearing animals at termination, cleaning residual tissue with a scalpel, and removing the diaphyseal portion. A 27-gauge needle on a 3-ml syringe was used to place 2 ml of PBS + 50% fetal bovine serum (FBS) through the bone and into a 12 × 75-mm tube. The expelled solution was drawn up through the bone and back into the syringe, the process repeated five times. The cell suspension was filtered through a 100-μm nylon mesh filter before underlaying with 1-ml FBS, centrifuging at 3000 rpm for 5 min at 4°C (Eppendorf, Thermo Fisher Scientific, Massachusetts, USA), and resuspending the pellet in 4 ml of PBS + 0.5% bovine serum albumin (BSA).

Fluorescein isothiocyanate (FITC)-conjugated CD45 (5 μl) and PE-conjugated CD71 (10 μl) monoclonal antibodies (Bio-Rad Serotec, Oxfordshire, UK) were added to 100 μl of the bone marrow cell suspension and incubated on ice in the dark for 20 min. Cells were washed with PBS containing 0.5% BSA and re-centrifuged at 3000 rpm for 5 min at 4°C. The resulting cell pellet was resuspended in 0.5-ml PBS + 0.5% BSA, and 20 μl of LDS-751 staining solution (Molecular Probes, L-7595; Thermo Fisher Scientific, Massachusetts, USA). Samples were then placed in the dark for 20 min, prior to flow cytometric analysis, incorporating negative control samples (adding only 10-μl PBS/BSA; 5-μl CD45:FITC; 10-μl CD71:PE; and 20-μl LDS-751, to four wells, respectively, before analogous processing). Analysis was performed using a Becton Dickinson FACSCanto II, with FACSDiva software (BD Biosciences, New Jersey, USA), as per the manufacturer’s instructions, generating scatter plot cyograms.

2.5 Statistics

For statistical analysis, data were examined for normal distribution using the Shapiro–Wilk test. All comparisons containing normally distributed data groups were subject to unpaired t test analysis with Welch’s correction (with assumed unequal variance). Comparisons of any groups found to have a non-Gaussian distribution were completed using Mann–Whitney non-parametric analysis. The significance level for all tests was 0.05.

3 RESULTS

Tumor growth was well-tolerated in all animals over the duration of study, with no premature decedent animals, nor clinical signs indicative of systemic morbidity. No abnormal macroscopic findings were present within any harvested endocrine tissues, there were no tumor-related variations in adrenal weight, and the light microscopic appearances of the selected endocrine organs from CT26, PC3, 4T1, and BT474c tumor-bearing mice showed no differences in hematoxylin and eosin (H&E) staining characteristics, when compared with non-tumor-bearing control animals.

With the application of CD31 IHC, there were no visually observable differences in endocrine tissue vascularity between non-tumor-bearing and tumor-bearing mice, in any group, with the exception of non-tumor-bearing and BT474c tumor-bearing.
estradiol-treated, animals. When compared with non-estradiol-treated, non-tumor-bearing, nu/nu mice, animals in the estradiol-treated groups had an observable increase in sinusoidal vascularity within the adrenal gland (Figure 1).

3.1 | CT26 tumors

Automated analysis of 3D fiberlength density did not reveal increases in peripheral endocrine organ vascularity amongst CT26 tumor-bearing mice, when compared with the cohort of non-tumor-bearing BALB/c animals. The serum concentrations of ACTH and TSH, as assayed via the multiplex serology, also remained within normal limits; there were tumor-associated serum elevations in mouse-derived G-CSF and IL-6 only. Fluorescence-activated cell sorting (FACS) analysis of the bone marrow revealed comparable blood cell lineage ratios between non-tumor-bearing and tumor-bearing animals. These findings suggested that the CT26 syngeneic colorectal tumor did not alter the vascularity of the distant endocrine tissue (Figure 2).

3.2 | PC3 tumors

Automated analysis of 3D fiberlength density revealed tumor-related increases in the vascularity of thyroid interstitial tissue amongst PC3 tumor-bearing mice, when compared with the thyroid glands from non-tumor-bearing nu/nu animals. These animals also showed a decrease in TSH serum concentration. Multiplex serology also displayed a significant release of xenograft (human)-derived angiopoietin-2, G-CSF and IL-8, and FACS analysis of the bone marrow revealed reductions in erythrocyte and lymphocyte cell lineage numbers, with a concomitant increase in myeloid cells, leading to an increased myeloid:erythroid ratio, amongst PC3 prostate xenograft tumor-bearing animals (Figure 3).

3.3 | 4T1 tumors

Automated analysis of 3D fiberlength density revealed increases in the vascularity of the adrenal gland and thyroid interstitial tissue, amongst 4T1 tumor-bearing mice, when compared with non-tumor-bearing BALB/c mice. There was also a decrease in serum concentrations of both ACTH and TSH amongst these mice. Multiplex serology revealed a significant increase in the serum concentrations of murine G-CSF, IL-6, PIGF-2, and prolactin.

As with the PC3 xenograft, FACS analysis of the bone marrow from the mice carrying the 4T1 syngeneic breast tumor showed an increase in the myeloid:erythroid ratio. However, the increase was even greater than that seen with the PC3 mice, due to a greater decrease in the immature nucleated red blood cell component (Figure 4).

CD11b/GR-1 IHC-labeling of endocrine tissues from non-tumor-bearing and 4T1 tumor-bearing animals revealed a significant infiltration of dual positive cells into the adrenal cortical sinusoids, pancreatic islets, and thyroid interstitium of implanted mice. The observation of this cell type was rare amongst the tissues of non-tumor-bearing mice (Figure 5).

FIGURE 1  Low-power (A–C; original objective magnification 10×, scale bar = 500 μm) and high-power (D–F; original objective magnification 40×) CD31-stained sections of adrenal gland from non-tumor-bearing and BT474c tumor-bearing mice; (A/D) adrenal gland from control, non-estradiol-treated, non-tumor-bearing, mouse, (B/E) adrenal gland from control, estradiol-treated, non-tumor-bearing, mouse, (C/F) adrenal gland from estradiol-treated, BT474c tumor-bearing, mouse. Note the apparent increased cortical vascularity, amongst the estradiol-treated specimens, notably in that bearing a BT474c tumor. Vascular profiles are indicated with arrows. ZR, ZF, and ZG; regions of the zona reticularis, zona fasciculata, and zona glomerulosa, respectively
Automated analysis of 3D fiberlength density showed that those mice implanted with estrogen pellets showed increased vascularity of the adrenal glands, pancreatic islets, and thyroid interstitial tissue, when compared with the non-estrogen-supplemented animals, irrespective of whether or not the mice were implanted with tumors. The serum levels of ACTH and TSH were decreased in mice-bearing BT474c tumors. In addition, there was an apparent decrease in circulatory TSH amongst non-tumor-bearing nude mice subject to supra-physiological estrogen exposure alone. In mice implanted with the BT474c breast xenograft, there was a significant increase in the serum concentration of xenograft (human)-derived IL-8. In addition, there was an increase in mouse-derived G-CSF and prolactin concentration within the serum of tumor-bearing, estrogen-supplemented, animals. G-CSF and prolactin increases within the serum were also apparent with estrogen-supplementation in isolation, albeit to a lesser extent. Finally, in mice implanted with estradiol, but without a tumor, there were increased serum concentrations of leptin. FACS analysis of the bone marrow revealed a dramatic reduction in progenitor/precursor cells of all lineages, amongst estrogen-supplemented mice, regardless of tumor burden; this was not associated with an increase in CD202b-positive cells at the terminal time-point (Figure 6).
4 | DISCUSSION

The term “tumor–host interaction” has traditionally encapsulated a range of clinically measurable effects resulting from the perturbation of distant organs by the effects of cancer. Examples have included the occurrence of acromegaly in the presence of growth hormone-secreting pituitary neoplasia and the production of hypercalcemia as a paraneoplastic effect associated with gynecologic malignancy. However, mounting evidence has suggested that such phenomena may only represent a small, albeit conspicuous, cohort of molecular interactions occurring between a tumor and the tissues of its host (McAllister & Weinberg, 2010; Melmed, 2009; Zagzag et al., 2018). Other interactions may be paramount in, for example, the coordination of tumor–host stroma synergy (Mahadevan & Von Hoff, 2007; Nakasone et al., 2012; Obacz et al., 2019; Poljak et al., 2009; Vennin et al., 2018), facilitating the growth of secondary or metastatic tumors at distant sites, via the utilization of cytokine/endocrine signaling (Apte et al., 2006; Chen et al., 2018; Talmadge &
Fidler, 2010; Tao et al., 2017), or clinical manifestations of cancer anorexia–cachexia, a complex of multiple pathologic processes leading to decreases in body weight, anorexia, and anemia (Aoyagi et al., 2015; Ezeoke & Morley, 2015; Graul et al., 2016; Ramos et al., 2004).

Preliminary investigations utilizing the Calu-6 xenograft tumor, in the development of structural biomarkers of vascular modulation, led to our hypothesis that additional “tumor–host interactions” may be responsible for structural and, potentially, functional changes within peripheral endocrine tissues, notably those with a fenestrated endothelial vascular plexus (Kamba et al., 2006; Kamba & McDonald, 2007).

Here it is demonstrated that the implantation and growth of a range of mouse- and human-derived allografts/xenografts in mice not only lead to structural vascular changes within the peripheral endocrine tissues but may also impart functional consequence.

**FIGURE 4** Plot showing that automated estimation of 3D fiberlength density detected significant increases in adrenal gland and thyroid interstitial vascularity amongst 4T1 tumor-bearing animals, when compared with non-tumor-bearing control mice (A,B). Pancreatic islet vascular densities were similar between the two groups. This was associated with increased serum concentrations of murine granulocyte colony stimulating factor (G-CSF), interleukin (IL)-6, PIGF-2, and prolactin (C–F), and with an apparent decrease in circulating adrenocorticotropic hormone (ACTH) and thyroid-stimulating hormone (TSH) (G). Fluorescence-activated cell sorting (FACS) analysis (H) revealed a significant shift in myeloid:erythroid ratio (MER) (increased) amongst 4T1 tumor-bearing mice. Dot plot showing total nucleated cell (TNC) and red blood cell (RBC) distribution from a non-tumor-bearing control BALB/c mouse (I) and a 4T1 tumor-bearing mouse (J). This demonstrates a sharp reduction in nucleated cells of erythroid lineage, with a concomitant increase and wider distribution of precursor/progenitor cells of myeloid lineage, in the tumor-bearing animal. Red = red blood cells, blue = nucleated erythroid cells, pink = lymphoid cells, green = myeloid cells. Median ± 10th–90th percentile for six animals per group. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
Histogenetically diverse subtypes of tumor appeared stratified in their ability to influence peripheral endocrine vascular bed density. Specifically, CT26 (syngeneic colorectal) tumors were not associated with structural vascular alteration amongst peripheral endocrine tissues, nor with serum changes in ACTH/TSH. These findings were associated with a relatively "weak" release of the murine pro-angiogenic cytokines/growth factors analyzed and the absence of a notable myeloid reaction.

PC3 (human-derived prostate) tumors were associated with an apparent increase in thyroid interstitial vascularity, with concomitant reductions in serum pituitary TSH liberation. These changes were associated with a relatively "weak" release of the murine pro-angiogenic cytokines/growth factors analyzed and the absence of a notable myeloid reaction.

4T1 (syngeneic breast) tumor growth was associated with enhanced adrenal cortical and thyroid interstitial vascularity, and decreased ACTH and TSH serum concentrations. The increased serum concentrations of murine G-CSF, IL-6, PIGF-2, and prolactin were consistent with the production of a series of growth factors/hormones, with varying angiogenic potency (Corbacho et al., 2002; De Falco, 2012; Gopinathan et al., 2015; Nagasaki et al., 2014; Nejabati et al., 2017; Reuwer et al., 2012). However, as this was a syngeneic model, it was unclear, within the context of this study, as to the exact source of these proteins, that is, whether liberated from host tissues (inclusive of murine intra-tumoral stromal/inflammatory cells) or tumor derived (Cheteh et al., 2020; Clapp et al., 1998; Lonnroth...
et al., 1994; Yan et al., 2013; Yang, Meyer, & Friedl, 2013; Yonekura et al., 1999; Zhao et al., 2008).

The 4T1 implantation and growth also induced a marked shift in myeloid:erythroid ratio within the bone marrow, in part due to a dramatic decrease in nucleated erythroid cell production. Interestingly, the role of IL-6 in the suppression of erythropoiesis is well documented; the serum concentrations of this cytokine were significantly elevated within the 4T1 study, recapitulating the known role of this
protein in the pathogenesis of anemia (Ershler, 2003; Macciò et al., 2005; Madeddu et al., 2018). IHC for CD11b and GR-1 indicated a significant influx of cells with morphologic and staining characteristics consistent with MDSCs into the peripheral endocrine sinusoidal vasculature of tumor-bearing mice. The contribution of bone marrow-derived cells, to the promotion of neoplastic angiogenesis, is well-established (Cousens & Werb, 2002; Gabrilovich, 2017; Hsu et al., 2019; Murdoch et al., 2008; Varner & Schmid, 2010; Wesolowski et al., 2013). Interestingly, the liberation of MDSCs has also been documented in myriad other non-neoplastic disease contexts, such as trauma, endotoxemia, radiation, thermal injury, myocardial infarction, and hepatic fibrosis (Liné et al., 2013; Song et al., 2018; Suh et al., 2012; Thanasegaran et al., 2015; Van Rompaey & Le Moine, 2011; Wu et al., 2014; Yao et al., 2015). Although the cellular source of murine G-CSF was not designated within the context of these studies, previous work has demonstrated an enhanced liberation of CD11b⁺/GR-1⁺ MDSCs, via the intra-tumoral production of G-CSF, in the development of tumor refractoriness to anti-VEGF therapy in mice, and highlighted this cytokine as a potential therapeutic target (Li et al., 2016; Morales et al., 2010; Shojaei & Ferrara, 2008; Shojaei et al., 2009). Here we demonstrate that a potentially comparable phenomenon may occur within tumor-bearing host peripheral endocrine tissues. Further work is required, both to clarify the precise cellular source of this and the other measured proangiogenic cytokines/growth factors and also to elucidate the role of such observations in the development of refractoriness to the induction of systemic vascular toxicity by anti-VEGF/VMA therapeutics.

Experiments incorporating the use of the BT474c (human-derived ER-positive breast) xenograft demonstrated that enhanced systemic endocrine vascularity may not only be observed in the presence of cancer but that a similar phenomenon may hold true with the administration of supra-physiological quantities of exogenous estrogen. Not only did the implantation of 17β estradiol pellets elicit an apparent widespread increase in endocrine vascular density but this was also associated with decreased serum concentrations of ACTH and TSH in tumor-bearing animals. Although there were measured increases in xenograft (human)-derived IL-8 and mouse-derived G-CSF and prolactin serum concentrations, amongst tumor-bearing animals, these signaling proteins did not appear to confer additive effect upon endocrine vascular structure or, potentially, function, over and above those observed with estrogen administration alone. Whether these findings relate to the relative “strength” of endocrine versus cytokine/growth factor influence remains to be determined. The involvement of MDSCs in these events appeared doubtful, in light of the marked suppression of all progenitor/precursor cell lineages, amongst estrogen-supplemented animals; a well-documented, albeit relatively poorly understood, effect of supra-physiological estrogen administration (Abid et al., 2017; Farris & Benjamin, 1993; Gaunt & Pierce, 1985; Jilka et al., 1995; Macneil et al., 2011; Sontas et al., 2009). There is a well-established association between ER activation and the promotion of angiogenesis, and/or enhanced blood flow, within many physiological and pathological disease states, including cancer (Groothuis et al., 2005; Iorga et al., 2017; Krause et al., 2006; Losordo & Isner, 2001; Seo et al., 2004; Sohrabji, 2015). Importantly, blocking the ER cascade has also recently emerged as a modality by which to potentially abrogate the development of antiangiogenic resistance (Gu et al., 2020). Within this study, we were unable to demonstrate the involvement of CD202b⁺ EPCs within these observations. However, this cell type may only be transiently released with estrogenic stimulation; such events may have preceded the time-point at which bone marrow aspirates were harvested (Akwii et al., 2019; Masuda et al., 2007; Rajoria et al., 2011; Suriano et al., 2008). Further investigation would be needed to delineate the functional and structural sequelae involved in this observation of steroid-mediated sinusoidal “hy pervascularity.”

When linked to an apparent effect upon host endocrine status, the presence of a tumor burden appeared to be associated with a variable but relative decrease in host terminal ACTH and TSH serum concentrations. This suggested that direct glandular stimulation, via the pituitary gland, may not be causal to any observed increases in endocrine vascularity. Previous investigation amongst critically ill patients, in studies of sepsis, and in the analysis of tumor burden as a model of cancer anorexia–cachexia, have documented adrenal insufficiency. Interference in ACTH synthesis and release and/or changes in cortisol/corticosterone metabolism have been suggested as contributory, with exaggerated adrenal blood flow, a finding consistent with the structural vascular findings presented here, also reported (Boonen et al., 2013; Boonen et al., 2015; Kanczkowski et al., 2015; Kanczkowski et al., 2017; Lang et al., 1984; Peeters et al., 2015). Similarly, depressed thyroid gland function has also been previously documented in experimental models of cancer anorexia–cachexia. However, as in the clinical scenario, confounding effects of decreased food intake and/or weight loss have been variably linked to the observed decreases in thyroid action (Costelli & Baccino, 2000; Persson et al., 1985; Svaninger et al., 1986). Weight loss and inappetence were not recorded during the studies outlined here. Within this study, both ACTH and TSH serum concentrations were measured at the terminal sacrifice time-point. Although a direct and potentially vascular-mediated action upon the adrenal and thyroid glands is possible as a novel mechanism of cancer-induced host endocrine modulation, further work would be required to delineate these findings in greater detail. This would ideally incorporate the sequential monitoring of both pituitary stimulation (ACTH/TSH), and end-organ function (corticosteroid/thyroxine production), in addition to further exploration around other confounding factors that may influence these signaling axes (De Vries et al., 2016; Gong et al., 2015; Louis et al., 2017; Romanò et al., 2019; Wondisford, 2018).

## 5 | CONCLUSION

Here we demonstrate that the implantation and growth of a range of human- and mouse-derived tumors leads to structural vascular and, potentially, functional signaling changes within host mouse endocrine tissues. Tumor- and host-derived cytokines/growth factors, and MDSC liberation, are implicated in this phenomenon, and these
observations may be of significance within the development of clinical anti-angiogenic “adaptation.”

Further study will expand knowledge of the potential for cancer and supra-physiological steroid administration to directly influence the vascular structure and function of systemic endocrine tissue, elucidate the role of these findings within the multifaceted “cancer-cachexia” and cancer-related anemia syndromes, and clarify the clinical significance regarding on-target endocrine vascular toxicity, and apparent modification in the presence of varying tumor types, in man.

CONFLICT OF INTEREST
The authors have no conflicts of interest to declare.

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SUPPLEMENTARY FIGURE
During preliminary method development investigations (Hargreaves et al., 2017), automated analysis of 3D fiberlength density revealed an increase in adrenal cortical and pancreatic islet vascularity amongst Calu-6 tumor-bearing animals, when compared with the cohort of non-tumor-bearing animals. There was no significant increase in thyroid interstitial vascularity (Figure S1).

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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