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Arteriogenic expansion of extratumoral macrovessels – impact of vascular ageing

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The critical role of the vasculature in cancer progression is predominantly studied at the capillary level, and often equated with angiogenesis. However, the mechanisms that ensure the supply of increasing blood volume to the expanding tumor microcirculation remain presently unclear. Here we used established mouse tumor models to document the enlargement (arteriogenesis), of macroscopic feeding vessels at considerable distances upstream from the malignant lesion, but not contralaterally. These changes are not affected by the procoagulant host tissue factor (TF), but are modulated by vascular ageing and atherosclerosis in ApoE-/- mice. Moreover, arteriogenic growth involves infiltration of bone marrow derived (YFP-labeled) cells and changes the gene expression profiles in the vessel wall. Thus, our observations suggest that in addition to local angiogenesis tumors influence distant remodeling of regional macroscopic blood vessels in a manner that is modulated by certain vascular comorbidities.

Key words: cancer, angiogenesis, arteriogenesis, ageing, atherosclerosis

The vascular system is profoundly involved in progression of human malignancies and their systemic manifestations [1-4]. This includes the implicit role of the circulation in metabolic supply of cancer cells, their metastatic dissemination [5], thrombosis [6] and the recruitment of bone marrow derived cells into the tumor microenvironment [7, 8]. These systemic events are often attributed to the ability of cancer and stromal cells to release soluble factors, cytokines and extracellular vesicles (EV) into the general circulation [9-11]. An enabling event in this context is the onset of tumor angiogenesis controlled by factors released locally from transformed, hypoxic, activated or inflammatory cells present within the milieu of the emerging malignant lesion [12, 13].

These considerations led to the notion of therapeutic targeting of tumor angiogenesis [14], and resulted in a wealth of mechanistic insights as to the regulation of this important process [13, 15]. This evidence also resulted in the development and approval for anticancer use of several agents capable of blocking angiogenic pathways, among which the key role is attributed to vascular endothelial growth factor (VEGF) and its receptors [16, 17]. Indeed, antiangiogenesis has become a standard of care in several human malignancies, including metastatic renal cell (mRCC), colorectal (mCRC), and hepatocellular carcinoma (HCC), as well as non-small cell lung cancer (NSCLC), gastrointestinal stromal tumors (GIST) and glioblastoma (GBM) [18]. In some cases, such as RCC, the molecular pathogenesis of the malignant process is strongly linked to copious production of VEGF, which is also commonly upregulated by hypoxic cancer cells [16]. However, in spite of this compelling rationale the clinical efficacies of antiangiogenic agents are often variable, transient, or nil, a circumstance attributed to several mechanisms of acquired therapeutic resistance [13, 16, 19, 20].

On the other hand, it is also increasingly recognized that capillary angiogenesis and sprouting of endothelial cells are not the only relevant events involved in formation of the tumor microcirculation, which entails a spectrum of tumor-vascular interactions (vascular co-option, vasculogenesis, transdifferentiation) as well as recruitment of mural and inflammatory cells with diverse structural and regulatory functions [13]. Formation of blood vessels is also closely linked with the activation of the coagulation system and affected by its main trigger known as tissue factor (TF) or thromboplastin [21, 22]. In fact tumor vasculature is structurally and biologically heterogeneous and is presently thought to contain at least 6 types of blood vessels generated by processes resembling

angiogenesis and 'arterio-venogenesis' [12]. Thus, targeting larger and diverse intratumoral vessels and their modulating bone marrow derived cell populations has recently attracted considerable interest [23].

One circumstance that is infrequently considered in the context of tumor neovascularization and therapy is the state of vessels that supply blood to the expanding tumor microcirculation but lie outside of the tumor boundaries. Indeed, the growing volume of intratumoral micro-vasculature and medium sized vessels must be matched by the volume of 'external' macro-vasculature that links the tumor to the general circulation [24]. By analogy, the capillary growth within ischemic limbs or developing organs must be matched by the corresponding circumferential growth of upstream or collateral arteries that lie outside of the hypoxic, angiogenic, healing or expanding tissues, and satisfy the perfusion demands of the capillary networks downstream. This enlargement process, is often referred to as 'arteriogenesis', and fundamentally differs from angiogenesis, in that it is thought to be driven by a retrograde recruitment of inflammatory cells, release of nitric oxide (NO), specific growth factors (FGF2) and chemokines (MCP-1), all of which orchestrate circumferential remodeling of the vessel wall [25, 26]. Arteriogenesis also differs from formation of larger vessels within the tumor mass, such as arterioles or angiogenic 'mother vessels', which are of microscopic sizes and in direct contact with cancer cells and stroma [12, 27, 28].

Interestingly, intratumoral microvasculature responds to systemic cardiovascular conditions such as atherosclerosis and vascular ageing [27, 29], which are also potent modulators of angiogenesis and arteriogenesis associated with cancer-unrelated tissue ischemia [30]. What remains unknown, however, is how closely such changes in different segments of the vascular tree become involved in tumor neovascularization, and whether there are equivalents of the 'collateral' macrovessel growth in the context of aggressive malignancies.

Here we provide evidence of macrovascular remodeling associated with three different models of transplantable tumors in mice. This process affects vascular segments remote to the tumor mass and involves recruitment of bone marrow cells and changes in gene expression within the vascular wall. Some of these changes are also modulated by vascular ageing and hyperlipidemia associated with the ApoE-/- phenotype. We postulate that mechanisms of this extratumoral arteriogenesis process may be of interest as therapeutic targets in cancer.

Materials and methods

Cells and culture conditions. Human clear cell RCC cell line (786-O) is deficient for VHL tumor suppressor gene and overproduces VEGF, and along with U87 glioma and mouse Lewis Lung Carcinoma (LLC) cells were obtained from American Type Culture Collection (ATCC; Manassas, VA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Burlington, Ont, CANADA) supplemented with 10% fetal bovine serum (Wisent ,St-Bruno, Que, Canada) and 50 U/ml penicillin and 50 μg/ml streptomycin (Life Technologies, Burlington, Ont. Canada).

Tumor generation and analysis. Single cell suspensions of 786-O, U87 or LLC cells were prepared from subconfluent cultures and injected subcutaneously into the flank region of YFP/SCID immune deficient mice (maintained in house)[31], C57BL/6 and C57BL/6/ApoE-/- mice purchased from Harlan Laboratories (USA) and Taconic Farms (USA), respectively. The SCID strain of mice hypomorphic for TF was generated as previously described [32]. These mice are viable due to residual (1%) of the wild type TF activity but are prone to hemorrhage and fibrosis when challenged by pregnancy or ageing, respectively [33]. Tumors were generated in either homozygotes (TF-deficient), or else in heterozygotes or wild type mice (TF-proficient). Whenever indicated, the mice were used as tumor recipients at different ages, either at 9 – 10 weeks (designated as "young") or at 52 – 53 weeks (designated as "old"). The following numbers of cancer cells were injected per mouse to achieve a consistent tumor take 5 x 10^6 cells for 786-O and U87 cells; 0.5 x 10^6 for LLC . Tumor growth was monitored by frequent measurements using vernier caliper. In selected cases the subcutaneous vasculature within and around the tumor was imaged using high frequency ultrasound system (VisualSonics), and the diameters of the left and right mammary artery and adjacent peritumoral large vessels, as well as blood velocity were measured as indicated, at transmission of frequency of 40MHz, field of view 6x6 mm and at 40 MHz, depth of 6.2 mm, angle 59 deg and doppler gain 5.0 db, respectively. Once tumors reached experimental endpoint (upward of 5 – 10 mm in diameter) and no later than upon reaching the clinical endpoint the animals were humanely euthanized and autopsy was carried out to expose anatomically, and image, photograph and measure with calipers and rulers the subcutaneous macroscopic blood vessels on both the tumor side and contralateral side of the same mouse. These measurements were also verified histologically by imaging crossectional dimensions of tumor-related and contralateral subcutaneous large vessels using morphometry software installed on the Zeiss AXIO microscope. All *in vivo* experiments were conducted according to protocols approved by the Animal Care Committee at our Institution (MCH; RI MUHC) and in accordance with the guidelines of the Canadian Council of Animal Care (CCAC).

Histology. Tumor masses and adjacent skin were carefully excised at autopsy and fixed in 4% phosphate buffered paraformaldehyde (PFA). The specimens were dissected to isolate fragments containing tumor mass from those with tumor feeding vessels. Segments of the latter with surrounding skin approximately 5 mm away from the tumor mass were prepared, and similar preparation was carried out for contralateral anatomically corresponding skin fragments. Tissues were processed in ethanol, xylene and paraffin embedded before 5 um sections were cut and mounted on slides.

For tissue visualization by hematoxylin and eosin (H&E) staining rehydrated slides where washed and then incubated with Hematoxylin, 1.5% Acid Solution, pH 2.5, washed in water and then put in Blueing Solution. Slides where then partially dehydrated (from 50% to 80% ethanol) before being dipped in Eosin solution followed by three five-minute washes in 99% ethanol and Xylene. For immunostaining, antigen retrieval was first carried out using Vector Antigen Unmasking Solution heated to 95°C for 15 minutes. For immunostaining the following antibodies were used: CD105 (AF1320, R&D Systems) and rabbit anti-Ki-67 (NCL-Ki-67 -MM1, Novocastra Laboratories, Leica Microsystems Inc., Concord, Ont. Canada.) to highlight endothelial and dividing cells, respectively. Correspondingly, the Alexafluor, chicken anti-goat 488 (A21467) and Alexafluor donkey anti-rabbit 594 (A21207) conjugated secondary antibodies (Life Technologies, Burlington, Ont. Canada) were used to visualize the respective signals. For YFP staining the chicken anti-GFP antibody (06-896, Upstate Technologies, EMD Millipore, USA) and Alexafluor goat anti-chicken 488 (A11039, Life Technologies, Burlington, Ont., Canada) secondary antibodies were used in a similar manner. Antibody concentrations were as recommended by the respective suppliers and detailed previously [34].

Gene expression profiling. At autopsy the tumor feeding of contralateral arteries from young and old mice, as indicated, were exposed and carefully excised within the sheath of surrounding connective tissues, drained of blood and snap frozen. The pools of 2-3 preparations were extracted with Trizol, mRNA samples were prepared and applied to SABiosciences PCR Mouse Endothelial Cell Biology (PAMM-015) gene expression arrays (Qiagen, Mississauga, ON) and processed as recommended by the manufacturer. The signal was calculated as ratio (fold change) between samples prepared from tumor feeding vessels and corresponding preparations of contralateral vessels. It should be noted that while care was taken to prepare vascular structures the surrounding stromal cells likely contributed to the signal.

Analysis of bone marrow cell recruitment. We used YFP/SCID mice as donors of the bone barrow (BM), which was introduced into syngeneic C56BL/6 recipients subsequently inoculated with LLC tumor cells to monitor the feeding vessel enlargement. C56BL/6 mice were irradiated in a Gamma Cell cesium-137 irradiator to receive the dose of 9 Gy, for a complete myeloablation [35, 36]. Prophylaxis with antibiotics (trimethoprim sulfa) was introduced in drinking water to prevent infection. BM donor mice were sacrificed and two femurs were used to flush out BM cells (approximately 10,000,000 cells in total) to repopulate each recipient mouse. For this purpose BM cells were suspended at 5 x 10^7 cells per mL and injected i.v. within 8 hours of irradiation. The vast majority (90%) of bone marrow recipients recovered fully within 3-4 weeks, after which the mice were confirmed for hematopoietic repopulation by FACS analysis of YFP positivity in fresh cell suspensions isolated from peripheral blood, spleen and BM. Remaining BM chimera were injected with LLC cells (s.c.) and analysed for YFP immunostaining for BM-derived cells in tissues surrounding tumor feeding vessels.

Data analysis. Numerical data were presented as the mean value (±SD) derived from several independent data points (mice, tissue culture wells). Five mice were allocated to each experimental group for in vivo experiments, unless otherwise indicated. Student t test was carried out where indicated and the p value > 0.05 was chosen as criterion of statistical significance.

Results

Enlargement of regional macrovessels in response to tumor growth in mice. To explore tumor-related vascular events under well controlled conditions we first employed the 786-O model of human RCC, in which natural loss of the VHL tumor suppressor gene leads to constitutive activation of the hypoxia pathway, exuberant production of VEGF and expression of highly pro-angiogenic phenotype [37]. We observed that subcunateous xenotransplantation of 786-O cells into immune deficient (YFP/SCID) mice leads to a rapid formation of tumor masses, which on gross examination exhibited red colouration and a network of superficial large caliber vessels. Notably, vascular changes were not limited to the tumor bed and affected the supplying regional arteries (mammary artery), which became visibly extended and engorged, not only in the proximity of the tumor, but in their entire length, 20-30 mm away from the tumor mass (Fig. 1A). These changes were more prominent in the case of larger tumors but not necessarily proportional to the measurements of the tumor volume. Moreover while in most cases a single feeding artery (mammary artery) was involved, larger tumors sometimes triggered enlargements of additional peritumoral vessels. Such changes were not observed on the contralateral side of the abdomen where mammary arteries maintained an appearance similar to that typical of tumor-free mice (Fig. 1B).

Cross sectional microscopic analysis of skin tissues collected approximately 5 mm from the edge of the tumor and from the corresponding contralateral region revealed the enlargement of the tumor feeding vessel that reached over 300 um in diameter (or more) versus 50-60 um of the corresponding segments of the contralateral artery (Fig. 1CD). Moreover, in live mice tumor feeding vessels were detectable using high frequency unltrasound probe, which confirmed their size range and existence of a rapid blood flow (15-16 mm/sec). These observations indicated that these vessels function as supply arteries for the growing tumor and carry an increased volume of blood (Fig. 1E-G).

We reasoned that the enlargement of mammary arteries induced by growth of 786-O tumors might involve either passive distension of the vascular wall, or active remodeling that would be associated with endothelial cell proliferation. In the latter case endothelial cell division could be visualized similarly to processes observed during capillary angiogen-

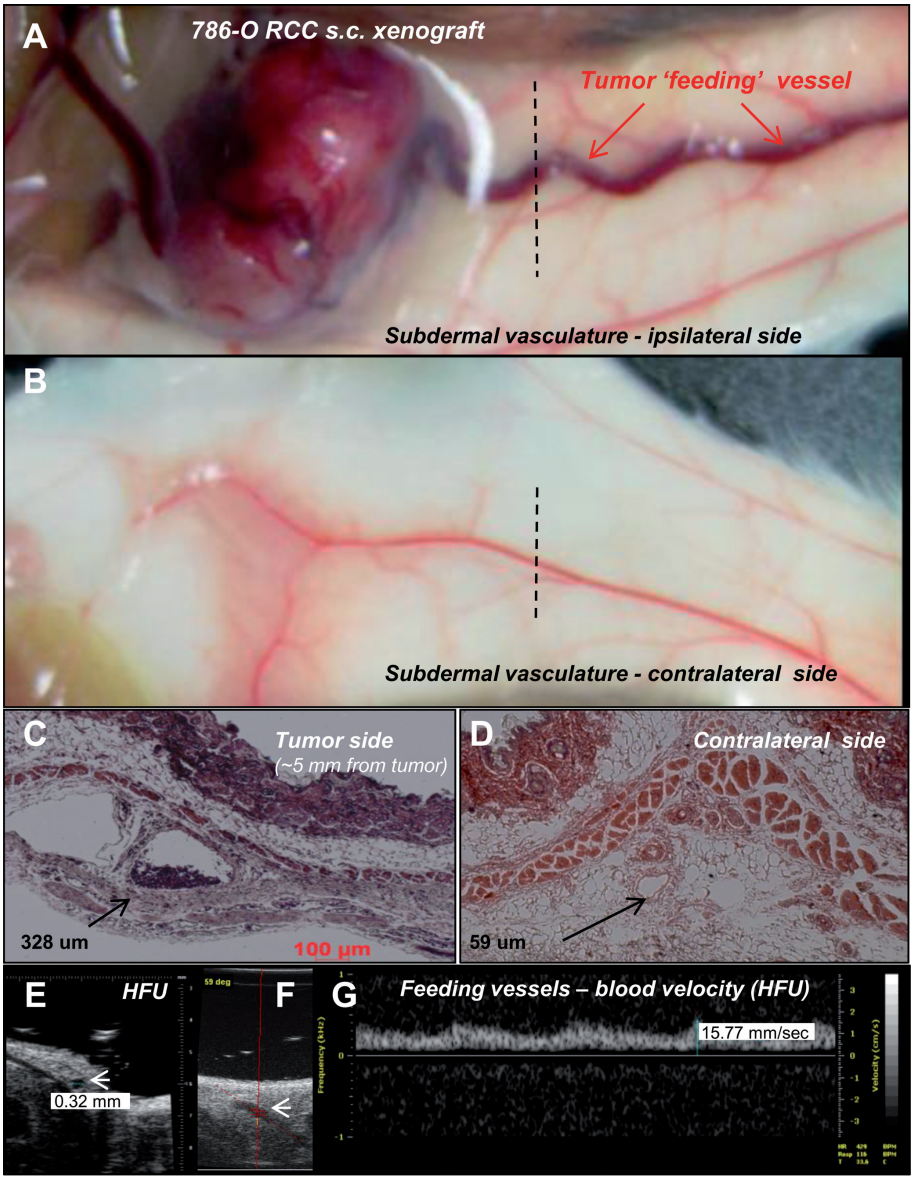

Figure 1. Formation of extra-tumoral feeding vessels in response to growth of 786-O human renal cell carcinoma xenograft. Tumor cells were injected subcutaneously (s.c.) at 5 x 10^6 per mouse and once the tumor was established the surrounding macrovasculature was imaged and photographed. A-B. Extensive enlargement of tumor related mammary artery (but not contralateral vessel) under gross pathological examination. C-D. Histological evidence for 5-6 fold increase in mammary artery diameter 5 mm away from the tumor plane (dashed line in A-B). H&E staining was performed on a full thickness skin cross-sections. E-G. Imaging of the tumor feeding artery in live mice using high frequency ultrasound (HFU). E. Confirmation of the feeding vessel diameter of approximately 300 um as also observed histologically (C-D). F. Detection of the feeding vessel for flow measurements. G. Measurement of blood velocity using VisualSonics Doppler analysis (15.77 mm/sec)

esis. To explore these questions in more detail we prepared sections of 786-O tumors and remote segments of their corresponding feeding vessels, and the tissues were co-stained for endothelial marker antigens (CD105) and markers of cellular proliferation (Ki67). Double positive cells (proliferating endothelium) were readily observed in vascular sprouts and, to a lesser extent, in enlarged intratumoral vessels, as well as in feeding vessels albeit to a lesser extent (Fig 2). Indeed, fewer and mostly single CD105+/Ki67+ cells were noted in the latter context.

Thus, angiogenic tumor growth is closely linked with pathological enlargement of regional arterial macrovessels, including their segments located at large distances from the tumor mass, and in a manner that involves low level endothelial cell proliferation. By analogy to arteriogenic vascularization of ischemic tissues, and in contrast to intratumoral or tumor-adjacent

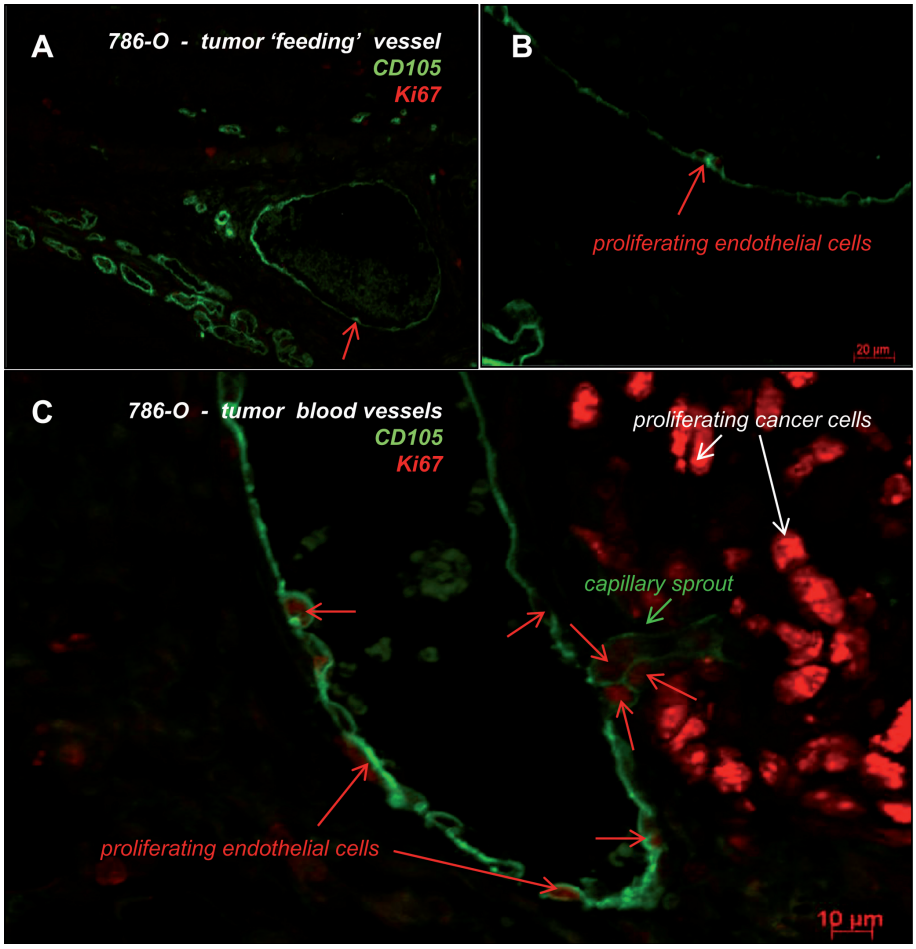

Figure 2. Immunofluorescence-based evidence for endothelial cell proliferation in arteriogenic and angiogenic tumor related blood vessels. 786-O xenografts containing capillary networks and their feeding arteries were stained for endothelial (CD105 – green) and proliferation markers (Ki67 – red). A-B. Scarce CD105+/Ki67+ proliferating endothelial cells in feeding arteries located outside of the tumor mass. C. Robust Ki67 signal in proliferating clusters of cells within tumor mass, including proliferating cancer cells (white arrows), sprouting microvascular endothelial cells (green arrows) and in their related dilated larger capillary vessels of origin (red arrows). Size bars 100, 20 and 10 um, respectively, as indicated.

arterio-veno-genesis [12], we refer to this distant process as extra-tumoral arteriogenesis.

Extratumoral arteriogenesis in glioma xenograft model is not affected by the tissue factor status. To verify features of arteriogenic growth in an independent animal model we generated subcuateneous xenografts of U87 human glioma cells in immune deficient (SCID) mice and observed similar expansion of tumor feeding vessels (Fig. 3). Since other forms of tumor-induced vascular regulation, such as developmental and malignant angiogenesis and inflammatory changes [34] may depend on the function of the coagulation system and the tissue factor (TF) activity [21, 38] we examined this question more closely using mice with variable TF expression levels. To accomplish this we employed as U87 tumor recipients the previously characterized strain of SCID mice harboring the hypomorphic TF transgene (low-TF/SCID mice)[32], a genetic modification which results in a marked, albeit not lethal, TF deficiency (1% of the wild type levels)[33]. However, the comparison of extratumoral arteriogenic growth between these TF-deficient mice (homozygous carriers of the hypomorphic TF allele) and their TF-proficient (heterozygous or wild type SCID) counterparts revealed similar features of tumor feeding vessels (Fig. 3). Summarizing, these results suggest that the robust extratumoral arteriogenic growth can be detected in at least two different human tumor models, and that wild type TF levels are not required for these processes to occur.

Impact of vascular ageing and atherosclerosis on extratumoral arteriogenesis. In human cancer processes of vascular growth within and outside of the tumor mass may be affected by vascular ageing and comorbidities, including atherosclerosis [29]. Because inflammatory and lipidemic alterations associated with this latter condition cannot be recapitulated in SCID mice we employed the LLC tumor model in immune

competent C57BL/6 mice harbouring null mutation of the *ApoE* gene, which controls lipid uptake and susceptibility to atherosclerosis [39]. Indeed, C57BL6/ApoE-/- mice exhibit a dramatic hyperlipidemia at the early age, which in older mice (over one year of age) develops into a full blown vascular atherosclerosis, known to affect intratumoral neovascularization [29].

Once again, we observed that formation of LLC tumors leads to enlargement of their feeding vessels in both young and old C57BL6/ApoE-/- mice, and in their wild type ApoE+/+ control counterparts (Fig. 4A-D and data not shown). We also conducted measurements of the external width of extratumoral arteriogenic vessels, which were found to be larger in the case of the old ApoE+/+ mice relative to their young counterparts, but were less extensive in the case of old ApoE-/- (atherosclerotic) tumor recipients. While tumor feeding vessels in young ApoE-/- mice were somewhat smaller then in the age-matched ApoE+/+ recipients this difference was relatively small and not significant. Thus, extratumoral arteriogenic growth occurs in several tumor settings, in both immune competent and immune deficient mice, and is modulated by vascular ageing and the related vascular wall comorbidities, such as full blown atherosclerosis, but not by the incipient hyperlimpidemic phenotype alone.

Gene expression changes in the extratumoral arteriogenic macrovessels. To gain insight into the transcriptional programs associated with extratumoral arteriogenesis we carefully excised the feeding or control vessels (including the attached connective tissue membranes) and subjected them to targeted gene expression analysis focusing on transcripts related to angiogenesis. This was executed using a commercially available PCR-array platform, as described earlier (SAB Arrays)[31]. The signal was quantified and normalized to that of pooled contralateral normal vessels isolated from the same mice.

For comparison we included C57BL/6 mice harbouring LLC tumors at either young (6-12 weeks) or old (approximately 52-72 weeks) age to assess approximately how this change may impact molecular correlates of the extratumoral arteriogenesis (Fig. 5). As expected, several genes related to vascular growth processes were found to be upregulated in tumor feeding vessel tissues of young mice, including: PECAM1 (CD31), SERPINE1 (PAI-1), IL6 (interleukin 6), TYMP (tymidine phosphorylase). In similar settings the most prominently downregulated genes included: SELE (E-selectin) and CXCL2 (MIP-2a). This profile differed somewhat in tumor feeding vessels of old mice, in that SERPINE1, IL6, IL1B and SELE were markedly upregulated in this setting, while IFNB1 and NOS2 were suppressed, relative to samples collected from contralateral arteries. While these results need to be confirmed using other methods they suggest that extratumoral arteriogenesis is associated with changes in gene expression patterns may change as a function of vascular ageing.

Detection of bone marrow derived cells in the wall extratumoral arteries. The observed changes in the gene

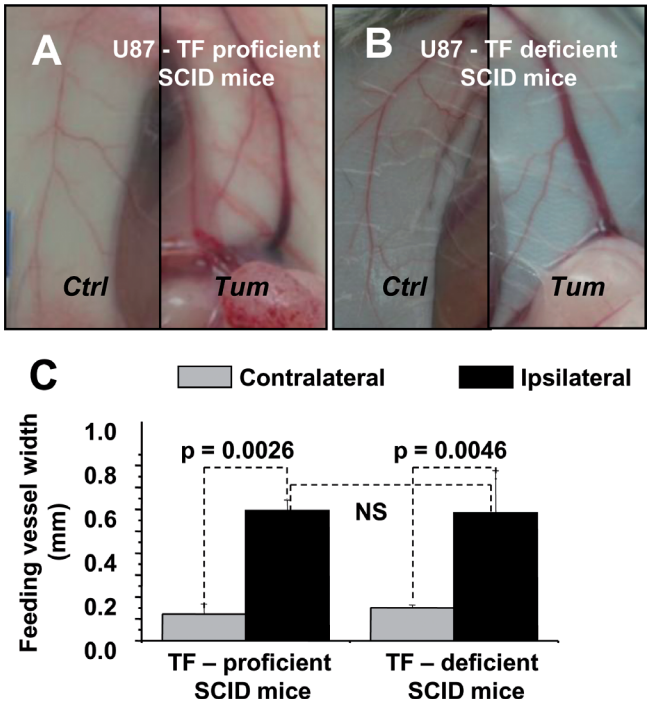

Figure 3. Tumor-related arteriogenesis in the model of human glioma remains unaffected by the levels of host tissue factor expression. U87 human glioma xenografts trigger arteriogenic growth of tumor feeding blood vessels in TF-proficient (A) and deficient (low-TF/SCID; B) immune deficient mice. The status of TF does not alter the extent of arteriogenic remodeling (C); p values as indicated; blood vessels were calculated through several measurements in each of 3 mice in each group, mean +/- SD are shown.

expression pattern could originate from either differential cellular response to arteriogenic stimuli, or from variation in cellular composition within the expanding vessel, or both. In this regard, the ischemic arteriogenesis is often associated with (and controlled by) the recruitment of myeloid cells that accumulate within the wall of vessels exposed to increased shear force and other factors acting in a retrograde manner [25]. To explore this possibility we replaced the endogenous bone marrow of C57BL/6 mice with bone marrow (BM) tagged with yellow fluorescent protein (YFP) obtained from YFP/SCID mice. The degree of BM chimerism was confirmed by FACS using bone marrow aspirates and blood cell preparations (Fig. 6AB). It is of note that this manipulation resulted in immunologically suppressed (SCID-BM) and otherwise C56BL/6 compatible mice that were used as recipients of LLC tumor transplants.

Upon generation of LLC outgrowths in chimeric mice we prepared sections of tumor feeding and contralateral vessels and stained them for YFP and CD105 to visualize BM-derived cells and endothelial lining, respectively (Fig. 6CD). Indeed, YFP+ (BM-derived) cells were predominantly detected in tumor related feeding vessels and not in their contralateral counterparts of the same mice. This pattern resembles a *bona*

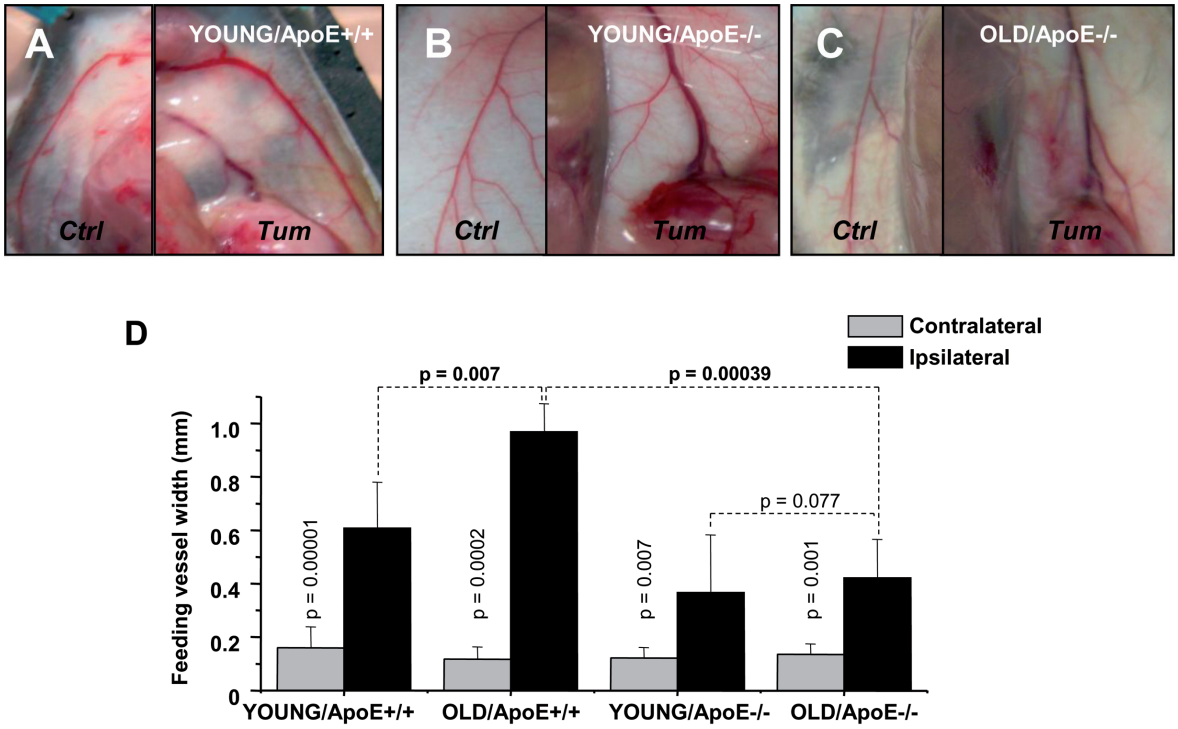

Figure 4. Tumor arteriogenesis in immune competent mice susceptible to atherosclerosis. A-C. Appearance of LLC tumor feeding vessels. LLC tumor cells were inoculated subcutaneously at 5 x 10^5 cells per mouse into syngeneic C57BL6 or C57BL/6/ApoE-/- recipients of different age. Tumor-related arteriogenesis and contralateral vessels in young C57BL/6 (control mice) young ApoE-/- mice (hyperlimidemic; 71 day old) and old ApoE-/- mice (atherosclerotic, 363 days old) harbouring LLC tumors were imaged and analysed. D. Measurements of vessel diameters either related to the tumor (T) or contralateral (C), in wild-type (ApoE+/+) or ApoE-/- mice of different ages. While young ApoE-/- are hyperlimidemic they are usually free of atherosclerosis which affects these mice at the older age. The data indicate age-related increase, and atherosclerosis/hyperlipidemia-dependent decrease in feeding vessel size (details in the text).

fide arteriogenic process, and suggests that the influence of the tumor extends along the supplying artery and results in the recruitment or selective capture of regulatory BM cells from the circulation. Naturally, this observation does not explain the mechanisms of such recruitment or shed light on the nature, phenotype or function of YFP+ positive cells. Therefore, further analysis is required to confirm and extend this finding using molecular and cellular methods. Overall, however, our data suggest that extratumoral arteriogenesis may be a common occurrence, distinct from angiogenesis and intratumoral arteriogenesis, and involving local and systemic (BM) cellular and molecular responses.

Discussion

It is increasingly clear that vascular responses to solid tumor growth are more complex than originally thought, and are not restricted to the sprouting angiogenesis at the capillary level [13, 40]. Indeed, macroscopic vascular structures are often observed on the surface and around growing tumor masses during gross pathology inspection, angiography or anatomical imaging [41]. Although this is often referred to as a sign of tumor 'angiogenesis' the caliber of such vessels exceeds that of angiogenic capillaries by orders of magnitude and therefore must result from a different cancer-related biological process, which deserves a more in depth study [27].

In this regard our present report points to several novel observations. First, we characterized a process of retrograde enlargement of major arteries that supply blood to the tumor microvascular bed in three different mouse models of cancer. This tumor-induced remodeling of large blood vessels occurs remotely, outside of the boundaries of the tumor mass and throughout the length of the ipsilateral mammary artery, but not systemically (contralateral vessels are not affected). TF-dependent vascular processes do not appear to be involved in this remodeling. Second, this process operationally resembles arteriogenic formation of collateral circulation adjacent to regions of tissue ischemia, and therefore we refer to it as extratumoral arteriogenesis [24, 27]. Third, this form of macrovascular growth is modulated by human-like vascular ageing conditions and atherosclerosis that we modeled in old ApoE-/- mice. Fourth, tumor feeding vessels tissues express altered gene expression profiles, which could be further modulated by vascular ageing. Fifth, extratumoral arteriogenesis is associated with recruitment of bone marrow-derived cells to the vessel wall and to the perivascular space, again a feature reminiscent of ischemic arteriogenesis.

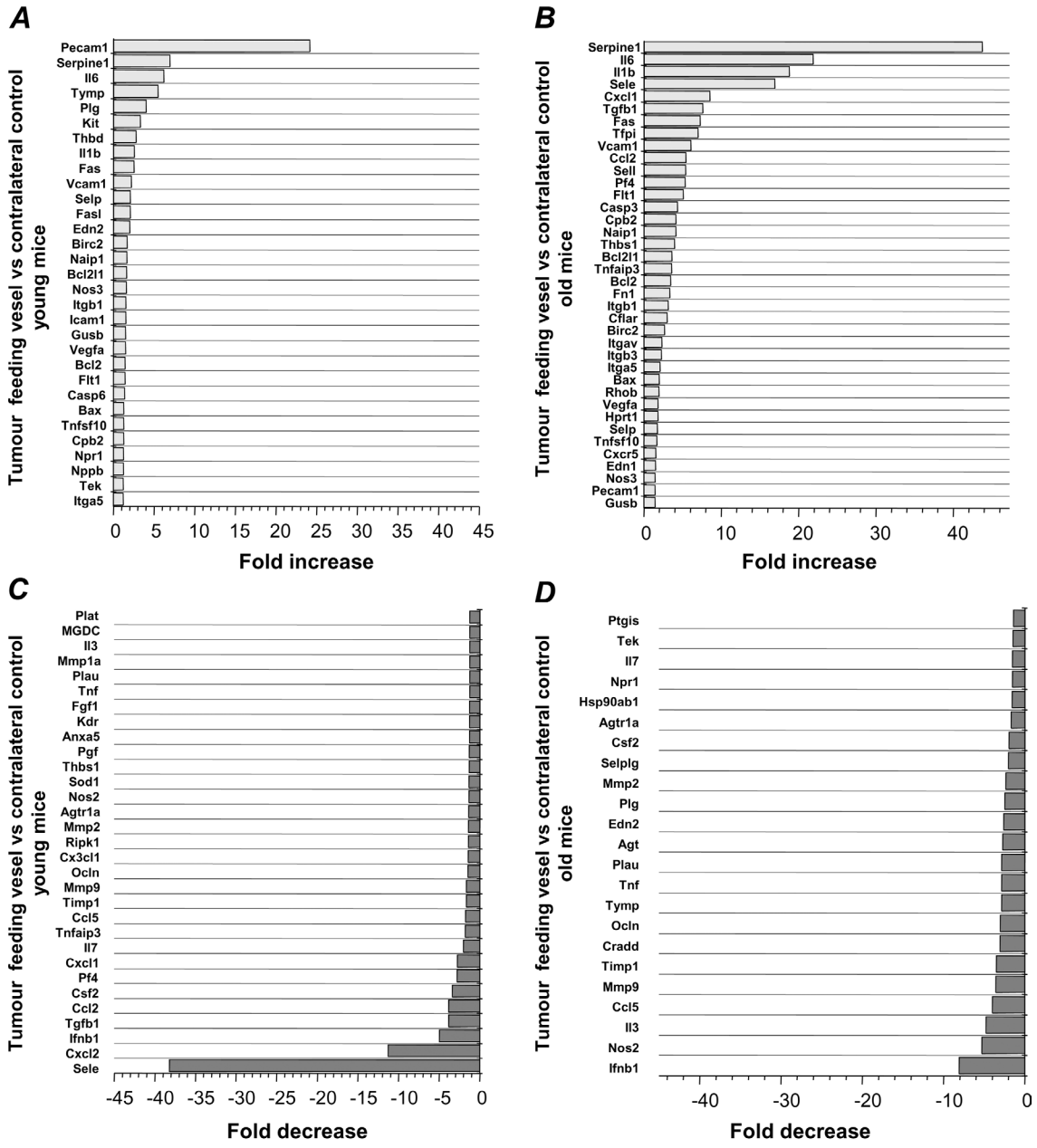

Figure 5. Targeted profiling of gene expression in preparations of tumor feeding vessels of young and old mice. SAB-Arrays containing 88 angiogenesis related genes were used to profile the expression of transcripts in micro-excised LLC tumor feeding vessels in young (A, C) and old (C, D) C57BL/6 mice. The data are expressed as the increase (A, B), or decrease (C, D) in signal intensity compared to that of contralateral vessels in the same mice. This analysis documents the possible changes in the tumor-induced microvasculature. The comparison between age groups is suggestive of changes in gene expression but is also indirect, and requires further validation.

Our present work complements the evolving understanding of the anatomical and functional continuity of the vascular system in health and disease, including cancer (Fig. 7) [12]. Indeed, formation of vascular structures and their interface with surrounding tissues is regulated by several populations of resident cells, such as endothelium, pericytes, stroma and inflammatory cells, but also by regional and systemic events, including regulatory effects of several populations of bone marrow derived cells (BMDCs), myeloid effectors and progenitors [8, 13]. This circuitry also includes elements of the coagulation system, platelets and connective tissue [42, 43], and they must include spatial information across all facets and segments of the vasculature.

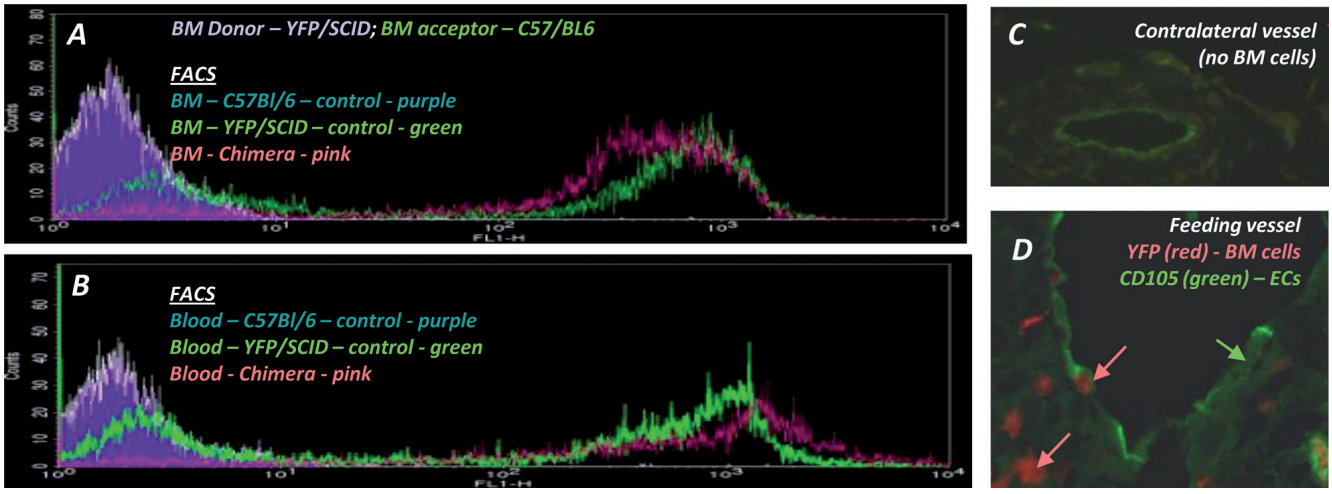

Figure 6. Detection of bone marrow cells in the wall of arteriogenic tumor feeding vessels. A-B. Repopulation of irradiated C57BL/6 recipient mice with YFP-tagged bone marrow results in detection of the YFP signal in bone marrow (A) and peripheral blood (B) of transplant recipients. A residual content of YFP- cells suggests incomplete BM replacement, with sufficient content of YFP+ cells to enable their positive identification in target tissues. C-D. Bone marrow-derived (YFP+) cells are detected by immunistaing within the walls of tumor feeding vessels (D – red), but not the corresponsing contralateral arteries (C).

The hierarchical anatomy and hemodynamic functionality of the vascular system is coupled with the ability of different vascular segments to mount coordinated responses to emerging perturbations [44]. For example, sprouting angiogenesis resulting in formation of new capillary structures is often preceded by adaptive enlargement of activated capillaries ('mother vessels') and is linked to remodeling of precapillary arterioles and venules to accommodate the increasing flow [12, 13]. In growing or regenerating organs these microscopic events are associated with macroscopic enlargement of sup-

Vascular hierarchy *Vessel caliber* *Measurement*	•*capillaries* •*8-20 μm* •*MVD*	•*pre-capillaries,* •*20 – 100 μm* •*MVD, VMI*	• ***feeding neoarteries (veins)*** • *>100 μm*
Driving processes *Driving stimulus* *Molecular effectors* *Type of growth*	•*angiogenesis* *vasculogenesis* •*hypoxia,oncogenes* *inflammation* •*VEGF, FGF, GFs* •*Sprouting*	• *angiogenesis,* *dilation* • *hypoxia,oncogenes* *GF gradients* •*VEGF, FGF, GFs* •*Remodeling*	• ***neo-arteriogenesis*** • *bone marrow cells (?)* • *FGF2, MCP1 other factors* • *Circumferential enlargement*
Available therapy	•*anti-angiogenics*	•*VDAs*	• ***unknown***

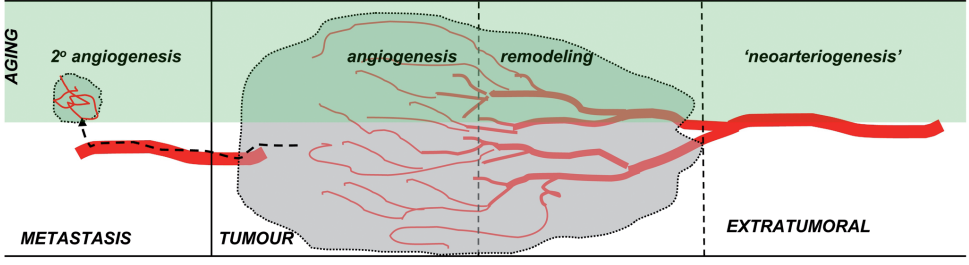

Figure 7. The continuum of vascular responses to progressive tumor growth – a model. As indicated in the text several facets and segments of the vascular system respond to the cues originating from the growing malignancy. This includes formation of angiogenic microvessels at the primary (orthotopic) and metastatic (ectopic) tumor sites (secondary angiogenesis), as well as remodeling of various calibers of intratumoral pre- and post-capillary vessels. The present study points out that these processes extend beyond the boundaries of the tumor mass and include arteriogenic growth of large vessels that supply blood to the tumor microcirculation. At different levels of this vascular hierarchy the regulatory events are likely different, and so are the influences of natural (ageing, atherosclerosis) and exogenous factors (therapy). Implications of this model are discussed in the text.

ply arteries, while revascularization of ischemic sites triggers formation of large collateral vessels outside/upstream of the affected region [25]. Thus, signals that trigger capillary growth reverberate throughout the vascular tree to result in adaptation of blood supply to the affected tissue. While signals that initiate this cascade are usually related to hypoxia or repair responses (inflammation), the upstream propagation of vascular changes requires a different nature of regulatory stimuli. Those are thought to result from shear stress to the vessel wall and recruitment of cells that may trigger the circumferential responses of resident endothelial and mural cells [25].

Similar responses of large supplying vessels could be expected in the case of cancer, though they remain virtually unstudied. This is surprising in view of the enormous progress in our understanding of tumor neovascularization, angiogenesis, and the emerging data on remodeling of larger intratumoral vessels [12, 27, 28, 40]. Indeed, hemodynamic challenges posed by the expanding tumor compounded with vascular and flow abnormalities and systemic regulatory responses would likely exert a retrograde effects on large vessels. Some of the characteristics of these influences are described in our present study.

While we document morphological, histological and molecular evidence of responses of large vessels to the growing tumor mass the mechanisms of these effects and their mutual linkages remain to be studied in more depth. Notably, cancer-related vessel dilatation has also been reported to occur within the lymphatic system via a mechanism involving VEGF-D, and is linked to lymph node metastasis [45-47]. In this case vascular remodeling occurs down stream from the tumor.

In contrast, feeding blood vessels become enlarged upstream of the tumor, which suggests (but does not prove) that the regulatory stimuli are retrograde (sheer stress, resistance) or systemic (circulating cells) in nature. further studies are required to understand the mechanisms of circumferential growth of tumor feeding blood vessels, as well as the role and characteristics of bone marrow cell recruitment associated with these processes. Of note is the fact that while our work concentrates on the arterial (upstream) part of the macrovascular growth induced by the tumor, the analogous enlargement ('extratumoral venogenesis') could also occur at the opposite (downstream) side of the tumor vascular tree, and the mechanism of these processes are likely very different.

Since we observed an active but modest mitogenesis of endothelial cells lining arteriogenic macrovessels it is possible that their enlargement is gradual and entails cell migration and repositioning in addition to some proliferation. Further gene expression profiling and in situ studies would be required to understand the significance of the related functional and molecular changes, and which cells contribute to these alterations.

Our earlier studies pointed to the role of age and age-related comorbidities in shaping the intratumoral vascular architecture and dynamic [27, 29]. Therefore it is reasonable to ask whether these processes also impact the formation of extratumoral feeding vessels. Moreover, a recent experiments pointed to the role of vascular ageing in differential involvement of angiogenesis and arteriogenesis in revascularization of ischemic limbs [48]. In this regard our present results suggest that tumor feeding vessels are larger in old mice, even though we reported earlier that in such mice the density and mitogenic activity of capillary microvessels is markedly reduced [29]. Again, future studies may illuminate the mechanisms of these processes in greater detail.

Atherosclerosis appears to have the most pronounced and broad impact on tumor neovascularization. Thus, in our present study we observed a reduction in the caliber of tumor feeding vessels in old ApoE-/- mice, while previously we documented that also the capillary bed and circulating bone marrow cell populations are diminished in this setting [29]. It is tempting to speculate that alterations in formation of smaller vessels in old mice could lead to changes in shear stress, blood velocity, or other hemodynamic events that could impact the response of the vessel wall upstream. These responses could be curtailed in the context of atherosclerotic pathology, that affects multiple vessel wall components, such as endothelial, mural and inflammatory cells, as well as the global bone marrow fitness [29, 49].

Extratumoral arteriogenesis is likely essential for efficient perfusion of the tumor mass and its extent could be informative as to the underlying vascular events in a broader sense. Indeed, new treatment opportunities may arise in this context such as combining therapies directed at intratumoral microvessels (angiogenesis, vascular disrupting agents) with approaches aimed to curtail their external blood supply arteries. It could also be envisaged that genes selectively expressed in arteriogenic macrovessels or bone marrow cells recruited to their walls could become therapeutic targets in the future.

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