An Integrated Gene Expression Landscape Profiling Approach to Identify Lung Tumor Endothelial Cell Heterogeneity and Angiogenic Candidates

Highlights
- We single-cell RNA-sequenced 56,771 endothelial cells (ECs) from human, mouse, and cultured lung tumor models
- Tip ECs were resolved into migratory and basement-membrane remodeling phenotypes
- Capillary and venous ECs expressed immunoregulatory gene signatures
- Integrated analysis identified collagen modification as an angiogenic pathway

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In Brief
Goveia et al. use single-cell RNA sequencing to provide an inventory of tumor endothelial cell (TEC) phenotypes from human and mouse non-small cell lung cancer and validate them functionally. Specific TEC phenotypes are associated with prognosis and response to anti-angiogenic therapy.

Data Resources
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An Integrated Gene Expression Landscape Profiling Approach to Identify Lung Tumor Endothelial Cell Heterogeneity and Angiogenic Candidates

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SUMMARY

Heterogeneity of lung tumor endothelial cell (TEC) phenotypes across patients, species (human/mouse), and models (in vivo/in vitro) remains poorly inventoried at the single-cell level. We single-cell RNA (scRNA)-sequenced 56,771 endothelial cells from human/mouse (peri)-tumoral lung and cultured human lung TECs, and detected 17 known and 16 previously unrecognized phenotypes, including TECs putatively regulating immune surveillance. We resolved the canonical tip TECs into a known migratory tip and a putative base-membrane remodeling breach phenotype. Tip TEC signatures correlated with patient survival, and tip/breach TECs were most sensitive to vascular endothelial growth factor blockade. Only tip TECs were congruent across species/models and shared conserved markers. Integrated analysis of the scRNA-sequenced data with orthogonal multi-omics and meta-analysis data across different human tumors, validated by functional analysis, identified collagen modification as a candidate angiogenic pathway.

INTRODUCTION

During vessel sprouting in a retinal vascularization model (Blanco and Gerhardt, 2013), endothelial cells (ECs) exhibit heterogeneity: a navigating tip EC leads, while proliferating stalk ECs elongate the sprout. Tip and stalk ECs are not genetically pre-determined fixed states, but dynamically interchangeable phenotypes (Blanco and Gerhardt, 2013). In tumors and eye diseases, therapeutic targeting of endothelial cells (ECs) to block tumor angiogenesis is clinically approved but suffers resistance. Here we surveyed EC phenotypes in human and murine lung cancer using single-cell RNA sequencing in combination with orthogonal bulk-omics approaches to provide a molecular atlas of tumor EC (TEC) phenotypes. Classical angiogenic tip and proliferating ECs comprised only a minority of TECs, and we identified distinct subpopulations expressing gene signatures related to basement-membrane breaching, immune cell recruitment, and semi-professional antigen presentation. Integrated analysis revealed previously overlooked tip cell markers, including transcription factors, matricellular proteins, and collagen cross-linking enzymes. Functional analysis confirmed novel angiogenic candidates and highlights the potential of our resource to revisit anti-angiogenic strategies.
the vasculature is structurally disorganized and functionally abnormal (Carmeliet and Jain, 2011a), but the transcriptome heterogeneity of tumor ECs (TECs) at the single-cell level across patients, species (human versus mouse), and models (freshly isolated versus cultured), analyzed in a single study, has not been inventoried.

In the tumor angiogenesis field, some single-cell RNA (scRNA) sequencing (scRNA-seq) studies reported a descriptive list of previously known TEC phenotypes (Lambrechts et al., 2018; Zhao et al., 2018), but did not identify previously unknown functionally validated angiogenic candidates. It can even be questioned if a single scRNA-seq study of one tumor type is sufficiently powerful at all to prioritize angiogenic candidates. Indeed, tip-like TECs in 2 human colon carcinoma xenograft models in a single study already expressed different markers (Zhao et al., 2018), but did not identify previously unknown functionally validated angiogenic candidates.

RESULTS

Single-Cell Atlas of EC Phenotypes in Human Lung Cancer

Focusing on human non-small cell lung cancer (NSCLC), we profiled freshly isolated human tumor ECs (hTECs) and (paired from the same patient) human (peritumoral) non-tumor pulmonary ECs (hNPECs) from 1 large cell carcinoma, 4 squamous cell carcinomas, and 3 adenocarcinoma treatment-naive patients (Table S1). Single-cell suspensions, magnetic-activated cell sorting (MACS)-depleted for CD45+ leukocytes and enriched for CD31+ ECs (Figure 1A), were subjected to scRNA-seq using a 10x genomics-based single-tube protocol. After quality filtering for the number of detected genes and mitochondrial read counts, duplicates were assessed and unique transcripts were normalized for total read depth (STAR Methods; Table S2).

ECs were in silico selected (Figures S1A and S1B), and up to 12,323 hTEC and 8,929 hNPEC transcriptomes from 8 patients were pooled, batch-corrected, clustered, and visualized using t-distributed stochastic neighbor embedding (t-SNE) plots (Figures 1B, 1C, and S1C; Table S3). To ensure that batch correction did not remove relevant biological features, we also analyzed each sample separately. Clusters detected in batch-corrected data were largely similar to those obtained without batch correction (not shown); any relevant differences are indicated below. To assess cluster reproducibility (Tasic et al., 2018), we performed hierarchical clustering and bootstrap analysis (Figure S1D). For biologically relevant subclusters that were not resolved by bootstrapping (e.g., lymphatic ECs from tumor and peritumoral tissue), we ascertainment that they were statistically separable using pairwise differential analysis (Innes and Bader, 2018) (Figure S1D, Table S4). Each of the 13 phenotypes is numbered as in Figure 1C (H1, H2, etc).

Clusters were biologically annotated based on the relative abundance of top-ranking marker genes in hNPECs and hTECs, and differences in marker gene expression levels shown in display items were statistically quantified to support the qualitative biological annotation (Figures 1D–1G and S1E; Tables S4–S6).
Figure 1. Construction of hTEC and hNEC Taxonomy

(A) Study design. IF, immunofluorescence; NEC, normal EC; TEC, tumor EC.

(B) t-SNE plot color-coded for ECs from peritumoral non-malignant lung (hPNEC; gray) and tumor tissue (hTEC; red).

(C) t-SNE plot of hTEC and hPNEC transcriptomes, color- and number-coded for the 13 phenotypes identified by graph-based clustering.

(D) t-SNE plots, color-coded for expression of indicated marker genes (red arrowheads).

(E) Gene expression levels of top-ranking marker genes in different EC phenotypes. In this and all further heatmaps depicting marker genes, colors represent row-wise scaled gene expression with a mean of 0 and an SD of 1 (Z scores).

(F) Relative abundance of each phenotype in hTECs and hPNECs.

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and S5). To obtain a more robust interpretation, we focused on clusters defined by gene set signatures rather than single genes (Table S4), and based cluster annotation on the expression of canonical gene signatures (e.g., arterial, capillary, venous, lymphatic, tip, and immature stalk phenotypes; listed in Table S3). To improve the generalizability of our analysis, we discuss all clusters at least once but focused mainly on clusters that were reproducible in multiple patients (Table S3). A detailed description of previously documented roles of the top-ranking markers, used to infer the putative biological role of each cluster, is provided in Tables S4 and S5.

Arterial ECs (cluster H1) expressed genes involved in vascular integrity, homeostasis, and vasotonus (Figure 2A), while postcapillary venous ECs (H2) expressed genes implicated in leukocyte recruitment, tissue perfusion, and pulmonary blood pressure (Figure 2B). We identified a type I (H3) and type II (H4) alveolar capillary EC phenotype, characterized by differential expression of von Willebrand Factor (VWF, upregulated in type II compared with type I; fold change = 3.63, adjusted p value = 2.36 × 10^{-7}) and endomucin (EMCN, upregulated in type I compared with type II; fold change = 2.89, adjusted p value = 1.22 × 10^{-6}), which might be involved in, respectively, vasoregulation and anti-microbial defense (Figures 2C–2E). Compared with other clusters, capillary ECs expressed a signature of genes involved in MHC-II-mediated antigen presentation and processing at higher levels than other phenotypes (Figure 2F), but the co-stimulatory molecules CD80 and CD86 were not detectable (not shown), suggesting a function as semi-professional antigen presentation cells (APCs). We also identified 2 novel capillary phenotypes that might be induced by tumor-derived cytokines: scavenging capillaries (H5) were by bootstrap analysis similar to type II capillary ECs, but upregulated scavenging receptors and genes associated with macrophages and antigen processing (Figures 2G, 2H, and S1D), while activated capillaries (H6) expressed EC activation markers (Figure 2I). An intermediate capillary EC phenotype (H7) resembled activated capillaries, but consisted mostly of cells derived from a single patient (Figure S1D; Table S3).

Traditional angiogenic phenotypes, such as tip and proliferating ECs (presumed targets of anti-angiogenic therapy [AAT]), were detectable only in hTECs (Figures 3A, S1E, and S2A; Table S3). Tip cells (H8), expressing gene signatures associated with EC migration, matrix remodeling, and vascular endothelial growth factor (VEGF) signaling (Figure 3B), comprised only a minority (<10%) of hTECs (Figure S1E; Table S3). Notably, tip hTECs showed highly restricted expression of the disease-specific molecule Pgf (placental growth factor; PGF [adjusted p value = 2.41 × 10^{-14}]) (Table S4). We also identified an immature hTEC phenotype (H9), which was similar to tip cells, but upregulated genes involved in the maturation of newly formed vessels, vessel barrier integrity, and Notch-signaling, possibly resembling stalk-like ECs (Figures 3C and S1D). Proliferating hTECs were only detected in a subset of patients, representing <1% of hTECs, and were no longer detected as a separate cluster after batch correction (Figure S2A). In hTECs, an activated postcapillary vein phenotype (H10) upregulated immunomodulatory factors and ribosomal proteins (Figure 3D), features of high endothelial venules (HEVs) in inflamed tissues (Girard and Springer, 1995). In contrast to the extensive phenotypic heterogeneity of blood vascular ECs, the gene expression signatures of tumor and peritumoral lymphatic ECs (LEC; H11, H12) were highly similar, and LEC subpopulations were not detected (Figures 3E and S1D; Table S4). An ambiguous phenotype, expressing markers of tip and arterial ECs (Figures 1C and S1D), predominantly consisted of cells from a single patient (patient 5, H13) and is not discussed.

To explore the generalizability of our NSCLC EC taxonomy, we used top-ranking marker genes to train a machine-learning algorithm (Kiselev et al., 2018) to automatically annotate 574 hNECs and 638 hTECs, in silico selected from a 52,698-cell catalog from tumors of 5 treatment-naive NSCLC patients (Lambrechts et al., 2018). The majority of ECs were annotated with high confidence (similarity threshold >0.5) (Figure 3F), indicating that our taxonomy is an externally valid resource that comprises all major EC phenotypes detectable in 13 lung cancer patients.

We validated the taxonomy using orthogonal techniques. Immunostaining combined with quantitative RNAscope to count transcript numbers confirmed that Pgf levels were upregulated in CD31/VWFhigh tip hTECs, while triple immunostaining showed co-localization of CXC4 and PIGF in TECs (Figures 3G and 3H; S2B). We also confirmed by immunostaining of (1) SELP the signature of activated postcapillary vein ECs (Figures S2C and S2D); (2) VWF and EFNB2, respectively, the venous and arterial phenotype (Figures S2E–S2H); and (3) the HEV-specific MECA-79 antigen the signature of the HEV phenotype (Figure S2I). RNAscope, combined with staining for the EC marker CD31, revealed that arterial (EFNB2) and venous (ACKR1) marker transcripts did not colocalize in the same hTECs (Figures S3A and S3B), while activated postcapillary vein hTECs expressed the marker CCL14 (Figure S3C). Further, transcripts of the scavenging capillary markers CD52 and CD68, and of the type I capillary markers EDNRB and IL1RL1 co-localized in the same hTECs (Figures S3D and S3E).

We used time-of-flight mass cytometry (CyTOF) to quantify protein levels of marker genes in single cancer, EC, and stromal cells, freshly isolated from NSCLC samples, using metal-conjugated antibodies against 26 preselected markers of these cells. Unbiased clustering and t-SNE visualization of the CyTOF single-cell data revealed separate clusters of ECs, stromal, and cancer cells (Figures 3I and S4A–S4D). Consistent with scRNA-seq, we detected type I (VWFlow) and type II (VWFhigh) alveolar capillary ECs, postcapillary vein ECs (ACKR1high/VWFhigh), and lymphatic hNEC and hTEC phenotypes (PROX1high) (Figure S4E). A phenotype expressing the highest CXC4 levels (tip cell marker)
but downregulating the expression of capillary markers (CD36, CA4, HLA-II proteins) likely represented angiogenic hTECs. We also detected a poorly resolved cluster, putative activated capillaries that, compared with type I and type II capillaries, expressed reduced capillary markers (CA4, CD36, HLA-II), but increased levels of the vein TEC marker VWF and the tip hTEC marker CXCR4 (Figures 3J and S4E). CyTOF confirmed the increased levels of HLA and CD36 in capillary ECs and their downregulation in hTECs (Figures 3J and S4F). To validate the observation that the populations identified using scRNA-seq and CyTOF coincided, we performed hierarchical clustering using key surface markers (STAR Methods) (Figure 3K). LECs and capillary ECs clustered together, while vein ECs clustered with angiogenic ECs. Notably, multiscale bootstrap analysis revealed that scRNA-seq resolved type I and type II capillary ECs as most similar to their CyTOF counterparts, cross-validating the presence of 2 distinct capillary phenotypes at the mRNA and protein levels.

**Possible Clinical Implications**

Despite all patients having early-stage treatment-naive disease, we observed inter-patient heterogeneity in the relative fraction of the different EC phenotypes (Figure 4A; Table S3). In hPNECs, arterial, capillary, venous, and lymphatic ECs were detected at variable proportions, especially for the capillary hPNEC phenotypes. In hTECs, the number of capillary ECs was reduced (Table S3), a finding confirmed by immunohistochemistry (Figure 4B),

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**Figure 2. Quiescent and Activated Normal Pulmonary EC Phenotypes**

(A and B) Expression levels of (A) arterial and (B) postcapillary venous EC markers. (C and D) Expression levels of the indicated genes of (C) capillary EC phenotypes and (D) the type I alveolar capillary EC phenotype. A.U., arbitrary units. (E–I) Expression levels of markers of (E) type II alveolar capillary ECs, (F) genes involved in MHC-II antigen presentation, (G) scavenging EC markers (scavenging receptors in red), (H) defense marker genes (complement, cathepsins and cystatins), and (I) genes associated with activated alveolar capillary ECs. See also Table S4.
Figure 3. Pathological Tumor EC Phenotypes and Validation by CyTOF

(A) Relative abundance of EC phenotypes in hTECs and hNECs, weighted by the number of cells per patient.

(B–E) Expression levels of marker genes of (B) tip, (C) immature, (D) activated postcapillary vein, and (E) lymphatic ECs.

(F) Assignment of hTEC and hNEC phenotypes identified in the taxonomy to hTECs and hNECs freshly isolated from an independent cohort of 5 NSCLC patients (publicly available data; Lambrechts et al., 2018).

(G) Number of CXCR4 and PGF mRNA transcripts per cell in NSCLC tumor vessels as determined by RNAscope (n = 3 patients). CXCR4 and PGF positively correlate (Spearman r = 0.584; two-sided p value <0.0001).

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although not to the same extent in each patient. Statistically, immature hTECs were more abundant in adenocarcinoma than squamous cell carcinoma patients (Table S3), although larger cohorts are required for meaningful interpretation (see below).

To correlate expression of EC phenotype-specific gene signatures with NSCLC patient survival, we took advantage of a large bulk RNA-seq dataset of 1,024 NSCLC patients of the Cancer Genome Atlas (TCGA). After identifying EC-specific marker gene sets for each phenotype utilizing a publicly available resource (Lambrechts et al., 2018) (STAR Methods), we used gene set variation analysis (GSVA) to score the enrichment of these signatures in each of the 1,024 NSCLC patients. Notably, (H) RNAscope images of an NSCLC tumor vessel probed for **CXCR4** (white) and **PGF** (green) mRNA. ECs are immunostained for CD31 (red). Nuclei are stained with Hoechst (magenta). Dotted lines denote (part of) the vessel; boxed area is magnified on the right. Green and white arrowheads indicate **PGF** and **CXCR4** mRNA transcripts, respectively. Scale bar, 20 μm.

See also **Figures S2–S4** and **Tables S3 and S4**.

![Figure 4. Translational and Possible Therapeutic Implications of the NSCLC EC Taxonomy](image-url)

(A) EC phenotype composition in tumor and normal tissue from individual patients. Left: Relative contribution of each phenotype scaled to 100%. Right: Contribution of each phenotype in absolute numbers; total number of analyzed ECs on the right. Asterisks indicate the significantly increased fraction of immature TECs in adenocarcinoma compared with squamous cell carcinoma patients (n = 4 squamous and n = 3 adenocarcinoma patients, p < 0.05 by two-tailed unpaired t-test).

(B) Representative micrograph of human peritumoral tissue (upper) and NSCLC tumor (lower) section immunostained for CD31 and CD36. Nuclei are stained with Hoechst. Images to the right are magnifications of the respective boxed areas. Scale bar, 50 μm. Right: Quantification of the CD36 signal intensity (mean ± SEM; n = 3 patients, p < 0.05 by two-tailed unpaired t test).

(C) Overall survival of 502 lung squamous cell carcinoma patients selected from the TCGA dataset and stratified by the indicated gene set expression signature scores. See also **Table S3**.
Figure 5. Construction of mTEC and mNEC Taxonomy

(A) Experimental design.
(B) t-SNE plot, color-coded for mNECs from healthy lung (gray) and mTECs from tumor-bearing mice (red).
(C) t-SNE plot, color-coded for the pulmonary mNEC and mTEC phenotypes.
(D) t-SNE plots of mNECs and mTECs, color-coded for the expression of indicated marker genes (red arrowheads).
(E and F) Expression levels of (E) tip and (F) breach mTEC marker genes.
(G) Differentially expressed genes in breach cells versus tip cells.
(H) Relative composition of mTEC phenotypes upon treatment with control immunoglobulin (Ig)G, DC101, or PTK787. *p < 0.05 by Dunnett’s method; #p < 0.05 by two-tailed unpaired t test.
(I) Expression level of the 18-gene disorganization signature (mean ± SEM; n = 3, *p < 0.05 by one-tailed unpaired t test).

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squamous (but not adenocarcinoma [not shown]) NSCLC patients, who expressed high levels of gene set signatures of angiogenic tip, immature, activated postcapillary, or lymphatic TECs, had shorter overall survival, presumably because these signatures reflect active angiogenesis and lymphatic spread (Figure 4C).

**Lung Tumor EC Phenotypes in Other Species and Models**

To compare lung TEC taxonomies across species and models for identification of common angiogenic TEC phenotypes and targets in an integrated congruency analysis, we used a similar strategy to construct 2 additional lung TEC taxonomies, one of 29,007 murine mNECs and mTECs micro-dissected from Lewis lung carcinoma (LLC) (another species), treated or not with anti-VEGF, and another of 6,512 human cultured hcTECs from human lung tumor (another model) (Tables S2, S3, and S4). We used the LLC model, since it relies on angiogenic vessel sprouting (the target of AAT), unlike other preclinical mouse NSCLC tumor models (Eldridge et al., 2016).

As in human lung, we identified mNEC-enriched phenotypes, expressing typical markers of arterial (M1), alveolar type I and II capillary (M2, M3), venous (M4) and lymphatic (M5) ECs (Figures SA–SD and SSA–SSG; Tables S4 and S6). Type II alveolar capillary ECs expressed the highest levels of genes involved in MHC-II antigen presentation, processing, and loading (Figures SSB and SSH), but did not express the co-stimulatory genes Cd80 and Cd86 (not shown). As in NSCLC, the fraction of capillary ECs was underrepresented in tumors (Figure SSA; Table S3), and mTECs downregulated the typical capillary gene signature, including MHC-II expression (Figure SSH), mTECs also downregulated Cd36 (a gene involved in fatty acid uptake), except in one capillary TEC phenotype (adjusted p value = 7.81 × 10^{-26}; M6) (Figure 5D). Unlike in human lung, the vein EC phenotype in murine lung expressed resident endothelial stem cell markers (Cd200, adjusted p value = 4.61 × 10^{-26}; Bst1, adjusted p value = 2.80 × 10^{-29}), previously identified in large pulmonary vessels (Wakabayashi et al., 2018) (Figure S5I).

Proliferating (M7) and tip (M8) mTECs were also detected (Figures SC and SE); proliferating mTECs were more abundant than in human lung tumors, consistent with the faster growth of murine lung tumors and a possible different type of tumor vascularization in NSCLC (Figures SD and SE). An immature mTEC phenotype (M9), lacking a strong transcriptome signature, was also recognized (Figure SC). Consistent with human ECs, we observed high venous plasticity and detected TEC-enriched phenotypes including large (M10) and postcapillary (M11) vein ECs (Figure SC). The postcapillary vein mTECs upregulated HEV markers (Figures SSJ and SSK), confirmed by immunostaining (Figures SSA and SSB).

The remaining murine phenotypes were predominantly detected in mTECs (Figures SC and SSF; Table S3). A neo- phalanx mTEC phenotype (M12) expressed capillary and arteriole markers, while activated artery mTECs (M13) upregulated neo-arteriogenesis markers. Notably, only mTECs, not mNECs, exhibited a phenotype characterized by the expression of an interferon (IFN) response gene signature and chemokines, involved in immune cell recruitment and angiostasis (M14) (Figure SSC; Tables S3 and S4). We also identified a previously unrecognized EC phenotype, which we termed breach cells (M15), and their putative precursors, pre-breach cells (M16) (Figures SE–SG). Breach cells upregulated expression of not only tip TEC markers, but also of genes previously involved in VEGF-induced podosome rosette-mediated basement-membrane (BM) and collagen remodeling (Tables S4 and S6) (Seano et al., 2014). Immunostaining confirmed the expression of signature markers of arterial, capillary, venous, and tip mNEC and mTEC phenotypes at the protein level (Figures S6C–S6J).

In cultured hcTECs, the in vivo artery, capillary, and vein EC phenotypes were no longer detectable (Figures S7A and S7B; Tables S2, S3, and S4). It was therefore surprising that the typical tip EC phenotype (C1), which is a plastic transient phenotype (Blanco and Gerhardt, 2013), was detectable in hcTECs. As expected for propagating ECs in culture, proliferating hcTECs (C2) were detected. Perhaps related to the culture conditions (presence of transforming growth factor β, an inducer of endothelial-to-fibroblast transition in serum), a phenotype (C3) exhibiting a signature of endothelial-to-mesenchymal transition (TAGLN, SERPINE1, FN1, CD44, adjusted p value < 5 × 10^{-4} for all genes) (Dejana et al., 2017) was identified. We also detected an intermediate transitioning phenotype (C4) that upregulated ribosomal genes, suggesting EC phenotypes on their way to adopting another phenotype (Figure S7B).

**Effect of VEGF Blockade on TEC Phenotypes**

**Differential Sensitivity of TEC Phenotypes**

Using the mTEC taxonomy, we explored if specific EC phenotypes were differentially sensitive to anti-VEGF AAT (VEGFR2 antibody [DC101], VEGFR tyrosine kinase inhibitor [PTK787]) (Figure 5H). These compounds were tested at doses that inhibit pathological angiogenesis and reduce EC numbers in tumors (Liu et al., 2005), and inhibited tumor growth (Figure S7C). Notably, control- and AAT-treated ECs were composed of the same clusters (Figure 5H; Table S3), suggesting that AAT did not alter the global transcriptome signatures of the different EC phenotypes. However, quantification of individual EC phenotypes revealed that tip and breach TECs were most sensitive, consistent with human colon carcinoma xenografts showing tip cell sensitivity to aflibercept (Zhao et al., 2018). Postcapillary vein and proliferating TECs were less sensitive to VEGF blockade, while capillary TECs were less sensitive to PTK787 treatment (Figure 5H). Whether this is due to the tumor switching from vessel sprouting to vessel cooption is unknown.

**Molecular Signature of Tumor Vessel Disorganization and Effect of VEGF Blockade**

Tumor vessels are structurally disorganized and functionally abnormal (Carmeliet and Jain, 2011b; Jain, 2005, 2014), but a detailed unbiased molecular footprint is lacking. Traditionally,
AAT is considered to prune angiogenic ECs, although VEGF blockade can also normalize the abnormal tumor vasculature (“tumor vessel normalization”) (Jain, 2005). We explored if VEGF blockade tuned ECs by inducing more subtle gene expression changes. We therefore first constructed “normal” and “tumor vessel disorganization” gene signatures by comparing angiogenic versus non-angiogenic phenotypes in a pairwise differential analysis to identify genes that were significantly (adjusted p value <0.01) differentially expressed from an effective fold change threshold of ±1.5. This revealed 18 and 28 genes that were significantly higher expressed in disorganized (among which tip, breach, and immature EC marker genes)
and normal (including capillary marker genes) phenotypes, respectively (Figures 5I, 5J, and S7D and Table S4).

GSVA using the 18 disorganization gene signature revealed that tumor vessel disorganization was partially reversed by VEGF blockade (Figures 5I and 5J). Gene set enrichment analysis further showed that both VEGFR inhibitors reduced the expression of tip EC gene signatures (glycolysis; De Bock et al., 2013; BM remodeling), while increasing the expression of signatures associated with mature homeostatic functions (antigen presentation, barrier, and blood vessel development) (Figures S7E and S7F). Thus, VEGF blockade not only pruned but also tuned TECs to promote a more quiescent and mature tumor vasculature with homeostatic functions, suggesting partial molecular tumor vessel normalization.

Identification of Conserved TEC Phenotypes and Tip Cell Markers

Anti-VEGF therapy efficacy is limited by resistance due to upregulation of alternative pro-angiogenic signals (Ebos and Kerbel, 2011). To identify alternative angiogenic candidates, we hypothesized that TEC phenotypes, conserved across species and models, and congruently overexpressed genes might be stronger and more robust angiogenic candidates. We thus performed an integrated combined single-cell transcriptomics, bulkomics, and transcriptomics meta-analysis to identify such candidates (Figure S8A).

We used the scRNA-seq data to assess similarity of angiogenic EC phenotypes across species and models using pairwise Jaccard similarity coefficients of marker gene sets and applied principal component analysis (PCA) for visualization (Figure 6A and S8A). The in vivo artery, capillary, and vein phenotypes were lost in hTECs in vitro; in contrast, the tip and breach EC transcriptome signature of in vivo hTECs and mTECs was conserved in cultured hTECs. ECs with a proliferation signature were only detected in cultured and murine TECs, and in hTECs from some NSCLC patients (see above). Freshly isolated human vein and murine immature TECs, and intermediate hTECs highly expressed ribosomal genes, suggesting plastic phenotypes, transitioning to other angiogenic EC phenotypes. All other phenotypes were model- or species-specific. Since tip TECs expressed a similar marker gene signature across all species and models tested, we focused on identifying congruent tip TEC markers.

To construct a ranked list of tip TEC markers, we first selected 296 conserved tip cell-enriched genes that were consistently highest expressed in tip TECs across species and models. Second, for each conserved gene, we defined a rank score by calculating the product of the marker gene rank in each of the 3 datasets (in this list, genes that are consistently among the most upregulated genes in tip cells rank the highest) (Figure 6B). For subsequent analyses, we focused on the top-ranking 50 enriched markers (adjusted p value < 5 × 10⁻³). Validating our approach, we confirmed the inclusion of tip EC markers (ANGPT2, APLN, FSCN1, PFG, PLXND1, ADM, PDGFβ, CXCR4, others), previously detected in tip ECs but not necessarily further characterized at the expression pattern and functional levels (del Toro et al., 2010; Strasser et al., 2010; Zhao et al., 2018). Half of the 50 top-ranking genes were not previously described as tip TEC markers (not detected in transcriptomics studies of ECs in tumor or physiological angiogenesis). Congruent tip TEC markers were associated with the migratory tip EC phenotype, including laminins (LAMA4, LAMC1, LAMB1), matricellular proteins (SPARC, Lxn), cytoskeleton-associated genes (Vim, MARCK5, Myh9, Myo1B), and cell adhesion molecules (CD93, MCAM, ITGA5). We also identified novel tip TEC-enriched transcription factors (TCF4, SOX4, SMAD1).

The disorganized vascular architecture in tumors impedes precludes topographical identification of tip TECs: Figure S6J shows heterogeneous expression of a tip cell marker, but its position at the forefront of the tumor vessel sprout and its expression in morphological tip cells cannot be unambiguously identified. We, therefore, used the postnatal retinal angiogenesis model to validate the expression of Lxn (Latexin) and FSCN1 (Fascin) in tip ECs at the vascular front (Figure S8B). To document their functional role, we examined if silencing these tip cell markers affected tip cell competitiveness (De Bock et al., 2013). We silenced Lxn or FSCN1 expression in human umbilical vein ECs (HUVECs; >70%–80% silencing efficiency; Figure S8C) and generated mosaic spheroids containing a 1:1 mixture of control wild-type HUVECs (red) and HUVECs silenced for Lxn or FSCN1 (green). Silenced cells were less often at the tip position, confirming the tip cell role of these markers (Figures 6C and 6D).

Integrated Analysis Highlights Collagen Modification as an Angiogenic Pathway

Genes encoding collagens (COL4A1, COL4A2, COL18A1) ranked in the top 10 most congruent tip cell markers, while other collagen-modifying (cross-linking) enzymes (PLOD1, PLOD2) ranked in the top 50 (Figure 6B). Bulk RNA-seq analysis of 13 independent NSCLC patients (Figure S8A; Table S3) and confirmatory RT-PCR analysis showed that the expression of genes involved in collagen cross-linking (LOXL2; fold change = 2.5; p = 5.15 × 10⁻³) and hydroxylation (PLOD1-3; fold change 1.24–1.74; p < 0.05 for all genes) was higher in TECs than NECs (Figures 6E and 6F). Procollagen-lysine, 2-oxoglutarate 5-dioxygenase (PLOD) isoenzymes (known as lysyl hydroxylases) intracellularly hydroxylate lysine residues in collagen, while lysyl oxidase (LOX), or its homologue LOXL2, extracellularly catalyze the first step in collagen crosslink formation (Gilkes et al., 2014). To explore upregulation of collagen-modifying enzymes in TECs from other tumor types, we performed a meta-analysis of 6 publicly available NEC versus TEC bulk transcriptomics datasets, freshly isolated from patients with 5 different tumor types (Table S4). Also in this analysis, transcripts encoding collagen-modifying enzymes were enriched (p < 0.05 for all genes) and ranked among the top 1%–5% most consistently upregulated genes in TECs (Figure 6G).

To explore upregulation of collagen-modifying enzymes in TECs at the protein level, we performed proteomics analysis on an in-house-generated cohort of 144 prospectively collected TEC and NEC samples from lung, kidney, and colorectal (CRC) tumors and colorectal liver metastasis (CRLM) (Figure S8A; Table S3). In TECs from NSCLC patients (n = 27 patients), LOXL2 was the highest upregulated protein (>4-fold; p = 6.89 × 10⁻⁷), while PLOD1 and PLOD2 were upregulated 1.7-fold (p = 5.52 × 10⁻³) and 1.9-fold (p = 4.39 × 10⁻⁴), respectively (Figure 7A). Meta-analysis across all 4 tumor types identified 288
We also identified Notably, TECs represent the first-line defense contact for HEVs or semi-professional APCs in tumor immune surveillance. Highly powered single-cell transcriptomics studies now allow characterization of EC phenotypes in more detail and enable revision of the traditional outlook on the tumor endothelium. Our integrated scRNA-seq and multi-omics approach showed that only the tip TEC phenotype was conserved across species and models. This was surprising, given that the tip EC phenotype is not a genetically predetermined state (Blanco and Gerhardt, 2013), but may be due to the presence of VEGF in the culture medium, an inducer of the tip EC phenotype (Siemerink et al., 2012).

In agreement with the above GO analysis, migration, proliferation, and vessel sprouting were higher in TECs than NECs (Figures 8A–8C). Silencing of PLOD1, PLOD2, or LOXL2 in HUVECs (>70%–80% silencing efficiency; Figure S8E) reduced EC migration (Figure 8D) and impaired vessel sprouting in HUVEC spheroids (Figures 8E and 8F). Minoxidil, a pharmacological PLOD blocker (Shao et al., 2018), reduced EC migration in vitro (Figure 8G). Administration of minoxidil or the lysyl oxidase inhibitor beta-aminopropionitrile (BAPN) (Rodriguez et al., 2010) inhibited corneal angiogenesis in vivo (Figure 8H). Overall, functional validation revealed that targeting genes identified by integrated analysis inhibits vessel sprouting.

**DISCUSSION**

Previous bulk analysis-based transcriptomics studies revealed only a limited number of EC phenotypes (Coppello et al., 2015; Marcu et al., 2018; Nolan et al., 2013; Sabbagh et al., 2018). Highly powered single-cell transcriptomics studies now allow characterization of EC phenotypes in more detail and enable revision of the traditional outlook on the tumor endothelium. Our data support a possible role for peritumoral (scavenging) capillary NECs and TECs with a transcriptome signature of HEVs or semi-professional APCs in tumor immune surveillance. Notably, TECs represent the first-line defense contact for immune cells in the tumor micro-environment. We also identified ECs that expressed markers of tip cells and an EC phenotype, implicated in the initiation of vessel sprouting by creating an opening in the basement membrane to assist invasive tip cells to sprout (Seano et al., 2014), which we coined breach ECs. We additionally identified vein ECs with a resident endothelial stem cell signature in mice, which might contribute to lung tumor vascularization, although this remains to be further studied.

Extending previous findings (Zhao et al., 2018), our data show that a VEGFR2-selective inhibitor not only affects more sensitively tip but also breach mTECs, while having smaller effects on vein and capillary mTECs. In addition, this study provides an unbiased molecular characterization of disorganized TECs and shows that VEGF blockade induces partial molecular tumor vessel normalization, including the induction of the activated postcapillary vein phenotype (resembling HEVs) and other more quiescent NEC phenotypes. This raises the question if AAT, in addition to pruning the small number of angiogenic and proliferating TECs, might benefit from tuning the more abundant TEC phenotypes contributing to tumor vessel disorganization. A concern is that tip TECs in human lung tumors make up only <10% of all TECs, raising the question if targeting such a small TEC subpopulation suffices to inhibit tumor angiogenesis and if the paucity of angiogenic tip and proliferating TECs in human lung tumors contributes to the insufficient efficacy and resistance to VEGF blockade AAT. Regardless, our study provides initial evidence for a correlation of angiogenic signatures with NSCLC patient survival.

Our integrated scRNA-seq and multi-omics approach showed that only the tip TEC phenotype was conserved across species and models. This was surprising, given that the tip EC phenotype is not a genetically predetermined state (Blanco and Gerhardt, 2013), but may be due to the presence of VEGF in the culture medium, an inducer of the tip EC phenotype (Siemerink et al., 2012). The identification of the tip TEC phenotype in culture raises the opportunity to study tip TEC targets in vitro, in contrast to arterial, venous, capillary, and other in vivo TEC phenotypes that are lost in culture.

To overcome inter-patient heterogeneity and contextual differences in marker gene signatures due to differences in species (human versus mouse), tumor type (NSCLC versus LLC),
and model (freshly isolated versus cultured TECs), we used scRNA-seq in combination with orthogonal multi-omics approaches to identify multiple genes involved in posttranslational collagen modification, illustrating that the discovery of these genes as angiogenic candidates was not accidental. We confirmed LOXL2 as known angiogenic candidate (Baker et al., 2013; Gilkes et al., 2014; Osawa et al., 2013; Zaffryar-Eilot et al., 2013), and identified and functionally validated procollagen cross-linking PLOD isoenzymes. Collagens have been implicated in tumor angiogenesis, albeit contextually (Fang et al., 2014; Sottile, 2004), but PLOD isoenzymes have not been substantially and consistently involved in angiogenesis yet. Hence,
an integrated approach, based on orthogonal multi-omics single-cell and bulk methods, may offer improved chances to identify conserved TEC markers that are biologically and translationally relevant.

This study provides a public resource for data exploration, available at www.vibcancer.be/software-tools/lungTumor_ECTax. While our study shows how this integrated approach can identify highly relevant angiogenic targets, we acknowledge limitations of our work. First, the inferred biological role for each EC phenotype is putative and requires functional validation to probe their biological role. Second, larger numbers of patients must be analyzed to probe inter-patient heterogeneity and identify all possible TEC phenotypes at deep resolution. Third, it should not be surprising that mouse and human TEC taxonomies do not completely overlap, given that tumors in both species grow differently, and patients exhibit larger genetic and environmental heterogeneity than mice.

STAR METHODS

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.ccell.2019.12.001.

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DECLARATION OF INTERESTS

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