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H460 non-small cell lung cancer stem-like holoclones yield tumors with increased vascularity

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Abstract

Cancer stem-like cells were isolated from several human tumor cell lines by limiting dilution assays and holoclone morphology, followed by assessment of self-renewal capacity, tumor growth, vascularity, and blood perfusion. H460 holoclone-derived tumors grew slower than parental H460 tumors, but displayed significantly increased microvessel density and tumor blood perfusion. Microarray analysis identified 177 differentially regulated genes in the holoclone-derived tumors, of which 47 were associated with angiogenesis. The dysregulated genes include several small leucine-rich proteoglycans that may modulate angiogenesis and serve as novel therapeutic targets for inhibiting cancer stem cell-driven angiogenesis.

Keywords

cancer stem-like cell; H460 holoclone; angiogenesis; microarray; small leucine-rich proteoglycan

1. Introduction

The cancer stem cell hypothesis postulates that solid tumors are supported by a sub-population of cancer stem-like cells (CSLCs) that are required for tumor initiation, tumor growth and tumor proliferation, and which differentiate into the bulk cells of the tumor [1]. CSLCs are resistant to radiation [2] and chemotherapeutic drugs [3] and have increased metastatic ability [4]. CSLCs have been isolated from solid tumors and tumor-derived cell lines, including brain [2], breast [5], colon [6], lung [7], melanoma [8], and prostate cancers [9]. Studies of CSLCs may help elucidate factors that stimulate tumor growth, identify biomarkers of drug response and drug resistance, and facilitate the development of new therapies.

Several approaches have been used to isolate and characterize CSLCs from human tumor tissue and cultured tumor cell lines. CSLCs can be identified by the expression of cell

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Author's contributions: EMJr and DJW conceived and designed the study, analyzed the microarray data, and prepared the manuscript for publication. EMJr carried out all of the laboratory studies, organized the data for presentation, and performed DAVID and IPA analysis and other statistical analysis. DJW oversaw the overall design and execution of the project. Both authors read and approved the final manuscript.

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surface markers [10; 11] and by their ability to efflux drugs and drug-like dyes, in a manner similar to hematopoietic stem cells, which were first isolated by flow cytometry as a side population of cells that efflux the dye Hoechst 33342 [12] *via* drug transporter pumps [13; 14]. Spheroid assays can be used to identify CSLCs based on their ability to grow in anchorage-independent conditions [15]. CSLCs can also be identified in populations of tumor cells grown in cell culture based on their characteristic holoclone morphology [16]. Many CSLCs can be grown as holoclones, which are comprised of tight round colonies, and have strong proliferative and self-renewal potential [17]. Another, distinct colony morphology, termed paraclone, is characterized by loosely associated cells that divide slowly and have little or no proliferative capability. A third cell morphology, meroclone, displays characteristics intermediate between holoclones and paraclones, and is associated with some ability to differentiate and with limited self-renewal capacity.

Holoclones correspond closely to stem cells, while meroclones and paraclones are considered early and late transit-amplifying cells, respectively [16]. A large and growing body of literature has established that cancer cell line-derived holoclones are, in fact, CSLCs. Holoclones with CSLC properties have been isolated from breast, melanoma, ovarian, colon, prostate, head and neck squamous cell carcinoma and pancreatic cancer cell lines [9; 17; 18; 19; 20; 21; 22; 23; 24]. For example, PC3 prostate cancer holoclones form spheres, have reduced sensitivity to 4-OOH-cyclophosphamide, and form tumors when seeded at low cell densities [9; 25]. U251 brain tumor holoclones show increased expression of vimentin, nestin and CD44, and form spheroids that differentiate when placed in non-spheroid media [18]. BxPC3 holoclones self-renew, form tumors at low density, and are chemoresistant compared to paraclones, while BxPC3 meroclones and paraclones are incapable of initiating tumor growth [17]. BxPC3 holoclones show increased expression of the stem cell marker CXCR4 and decreased expression of CD24, while paraclones display the opposite pattern [17]. Collectively, these studies establish that tumor cell line-derived holoclones are cancer stem-cell enriched/derived, as validated by their cell surface marker expression, spheroid and colony formation capacity, and *in vivo* tumorigenicity [9; 19; 23].

As solid tumors grow beyond $\sim 1 \text{ mm}^3$ in size they become hypoxic, leading to changes in the tumor microenvironment [26]. Hypoxia stabilizes HIF-1 α , which increases HIF1 α -dependent activation of downstream gene targets, including the pro-angiogenic factor *VEGFA* [27; 28]. Tumor blood vessels induced under these conditions are often leaky and tortuous, which facilitates tumor cell extravasation and increases the likelihood of metastasis. Tumors seeded with CSLCs derived from glioma, prostate cancer, and renal cell carcinoma tumors show increased angiogenesis [9; 25; 29; 30] by a mechanism that may involve release of microvesicles rich in pro-angiogenic mRNAs and microRNAs in the case of renal carcinoma [30]. However, it is unclear whether increased tumor angiogenesis is a general property of CSLCs, if it is restricted to CSLCs derived from specific tumor types, or if drug selection or exposure to hypoxia is required to manifest this increase. Presently, we investigate these questions using a panel of tumor cell line-derived holoclones. Our findings show that tumors derived from H460 CSLCs, isolated as holoclones, but not those derived from Colo-205 or A549 holoclones, are consistently more highly vascularized and have increased blood perfusion compared to parental H460 cell-derived tumors. Further, we identify a network of genes encoding both pro-angiogenic and anti-angiogenic factors that are dysregulated in H460 CSLC-derived tumors compared to parental H460 cell-derived tumors. We also identify a link between extracellular matrix proteins and angiogenesis, suggesting that targeting extracellular matrix proteins may be a useful strategy for inhibiting tumor angiogenesis.

2. Methods

2.1 Chemicals and antibodies

Crystal violet and formaldehyde were purchased from Sigma-Aldrich (St. Louis, MO). HPLC grade methanol was purchased from J.T. Baker (Phillipsburg, NJ). Paraformaldehyde solution (32%; methanol-free) was purchased from Electron Microscopy Sciences (Hatfield, PA). Fetal bovine serum (FBS) was obtained from Atlanta Biologicals (Lawrenceville, GA). Normal horse serum, avidin/biotin blocking kit, biotinylated horse anti-mouse antibody (BA-2000), Vectastain Elite ABC Kit, Impact VIP and VIP peroxidase substrates, and VectaMount were purchased from Vector Laboratories (Burlingame, CA). DMEM and RPMI 1640 culture media were purchased from Invitrogen (Carlsbad, CA). MEM and McCoy's 5A culture medium were purchased from American Type Culture Collection (ATCC) (Manassas, VA).

2.2 Cell lines

Human tumor cell lines were authenticated by and obtained from the following sources: FaDu (head and neck) and HT29 (colon), from ATCC; and A549 (lung), H460 (lung), MDA-MB-231 (breast), and Colo-205 (colon), from Dr. Dominic Scudiero (National Cancer Institute, Bethesda, MD). FaDu cells were grown in Eagle's MEM containing 10% FBS. HT-29 cells were grown in McCoy's 5A medium supplemented with 10% FBS. All other cell lines were grown in RPMI containing 7% FBS. All cells were grown at 37°C in a 5% CO₂ atmosphere and were supplemented with penicillin/streptomycin.

2.3 Isolation of holoclones, meroclones and paroclones

The clonal composition of each tumor cell line was determined by limited-dilution assay in 96-well plates [23; 31]. For plating by limited dilution, each well of a 96-well plate was seeded with 100 µl culture medium containing a calculated 10 cells/ml [9; 31]. Cells were grown overnight and on the following day wells containing single cells were circled with a marker and then used in further studies as described below. Individual clones that yielded colonies within 6 – 21 days were designated holoclones, meroclones or paroclones based on their colony morphology [9; 23]. Colonies were grown to confluence and transferred to 6-well plates where they were maintained until near confluent, at which time they were: 1) plated to assay their capacity for self-renewal in a colony formation assay (see below); or 2) replated in 10 cm dishes and propagated under conditions where they retain their holoclone morphology and CSLC properties [9]. Specifically, cells were split 1:6 and passaged every 3 days. Cells were grown to ~ 80% confluence and frozen for cell preservation prior to further propagation for inoculation into mice as xenografts.

2.4 Colony formation assay

Holoclonal cells were plated at low density (1×10^3 cells seeded in a 6-well plate, or 8×10^3 cells seeded in a 10 cm dish) to achieve an initial density of 50 to 200 cells per cm² [23]. Six to 21 days later, colonies were photographed at 4x and 10x magnification under a light microscope. Wells were then rinsed with 1x PBS and stained with crystal violet [32]. Examination of the stained colonies confirmed that the holoclones could self-renew, as indicated by their ability to reform holoclones at high frequency (~75% for A549, ~90% for FaDu and HT29, and 95% for H460 holoclones). Results were not quantified for Colo-205 holoclones, and MDA-MB-231 holoclones did not reform holoclones. High-resolution images were taken at 4.2x magnification with an Olympus FXS100 Bio Imaging Navigator microscope.

2.5 Tumor xenograft studies

Mice were housed in the Boston University Laboratory of Animal Care Facility in accordance with approved protocols and federal guidelines. H460, A549 and Colo-205 parental tumor cells and holoclones derived from each cell line were implanted s.c. in 6 wk old (24–26 g) male Fox Chase ICR *scid* mice (Taconic Laboratories, Germantown, NY). Autoclaved cages containing food and water were changed once a week and body weights were measured every 3–4 days. H460, Colo-205 and A549 cells were inoculated at 2, 5 and 6×10^6 cells/injection site, respectively, s.c. on the rear flanks of each mice (2 sites/mouse, for $n=3-5$ mice, corresponding to 6 to 10 tumors per parental tumor cell line and 6 to 10 tumors per holoclone line for each tumor cell line). On the day of tumor cell inoculation, tumor cells at 70–80% confluence were trypsinized and resuspended in serum-containing medium, centrifuged at 1,000 rpm for 5 min, then resuspended in serum-free culture medium and centrifuged again. The resulting cell pellet was resuspended in serum-free media in a volume that permitted delivery of the specified number of cells in a volume of 0.2 ml using an insulin syringe (29G). Tumor sizes were measured twice a week using digital calipers (VWR International, West Chester, PA) and tumor volumes were calculated as $(3.14/6) \times (\text{length} \times \text{width})^{3/2}$. Mice were euthanized and tumors were collected for further analysis when the tumors reached a vol of $\sim 1,000-1,500 \text{ mm}^3$.

2.6 CD31 immunohistochemistry

Freshly isolated tumors were divided into three pieces. One piece was fixed in dry-ice cooled 2-methylbutane for 1 min and transferred to -80°C for storage prior to cryosectioning. A second piece was snap-frozen in liquid N_2 and stored at -80°C prior to RNA isolation. A third piece was immersed in 4% paraformaldehyde at 4°C overnight, then stored in 70% ethanol at 4°C prior to paraffin embedding. Tumor cryosections ($6 \mu\text{m}$, 3–4 sections/slide) were prepared using a Leica 1800 cryostat. Cryosections were rinsed with PBS for 30 sec and fixed with 1% paraformaldehyde in PBS at room temperature for 30 min. Following a PBS wash (5 min), slides were incubated with permeabilization solution (1% Triton X-100 (v/v) and 1% sodium citrate (w/v) in 1x PBS) for 5 min on ice followed by a second PBS wash (5 min). Slides were blocked with PBS containing 2% normal horse serum for 20 min at room temperature. Slides were stained with rat anti-mouse CD31 antibody (BD Pharmingen, 1:1000 dilution) for 1 hr at room temperature, followed by incubation with biotinylated rabbit anti-rat secondary antibody (Vector Laboratories, Inc., Burlingame, CA, 1:200 dilution) for 1 hr at room temperature, with 3×5 min rinses with 1X PBS after each antibody incubation. The tumor sections were then incubated with ABC complex and stained with peroxidase substrate (Vector Laboratories). Slides were dehydrated and sealed with VectaMount. Stained tumor sections were examined using an Olympus FSX100 Bio Imaging Navigator microscope at 4.2x magnification, and 3–15 high resolution images were captured for each of 3–5 separate regions of each tumor. Vascular area (percentage of CD31 stained area in each image) was quantified using NIH ImageJ software and expressed as a mean value for each tumor $\pm$ S.E. for each treatment group. Paraffin sections ($6 \mu\text{m}$, 3 sections/slide) prepared using a Leica RM2255 microtome were baked for 50 min at 60°C and dewaxed using BioGenex dewax solution (2×10 min, 10 dips in dd H_2O , and 10 dips in OptiMax supersensitive wash buffer). Samples were washed with PBS (2×5 min at room temperature) then incubated for 10 min in a solution of 3% H_2O_2 (final concentration) dissolved in aqueous solution containing 80% methanol. After two more washes with PBS, antigen retrieval was carried out by steaming for 30 min in 1 mM citric acid buffer, pH 6.0, containing 0.05% Tween 20. Blocking was carried out as described above. Slides were stained for 1 hr at room temperature with mouse anti-human CD31 antibody (Leica Microsystems, Cat. No. NCL-CD31-1A10, 1:50 dilution) followed by incubation for 1 hr at room temperature with biotinylated horse anti-mouse secondary antibody (Vector Laboratories, Inc., Burlingame, CA, 1:200 dilution), with 3×5 min rinses

with 1x PBS after each antibody. The tumor sections were then incubated with ABC Elite complex and stained with Impact VIP peroxidase substrate (Vector Laboratories). Slides were dehydrated, sealed, and analyzed for the presence of human blood vessels.

2.7 Hoechst 33342 staining

Hoechst 33342, 16 mM in PBS, was prepared and stored in the dark. Mice were injected with 15 mg Hoechst 33342/kg body weight using a 29G insulin syringe and euthanized 1 min later. Tissue samples were collected and processed for cryosectioning as described above. Samples were analyzed using an Olympus FSX100 Bio Imaging Navigator microscope. Background fluorescence was determined by imaging tissue from a mouse that was not injected with Hoechst dye. At least 3–15 images, covering 3–5 separate tumor regions, were taken per tumor (minimum of 1 image per tumor section). Staining intensity was quantified using NIH Image J software. Data are presented as relative percent positive staining area, mean $\pm$ S.E. based on $n = 6$ –8 tumors per group. Quantitative data presented generally based on triplicate assays, as specified in each figure. Statistical significance of differences was assessed by Student's *t*-test using GraphPad Prism 4.0 software, with statistical significance indicated by $p < 0.05$.

2.8 Microarray analysis

Global transcriptome profiling was performed for tumor xenografts derived from four independent H460 holoclones, selected based on their unambiguous holoclone morphology and self-renewal capabilities, and designated H460/2E1, H460/2E7, H460/3F1 and H460/2H3. Results were compared directly to parental H460 tumor xenografts in a two-color microarray design. (RNA isolated from H460/2F1 tumor was partially degraded and was excluded from these analysis). Tumors ($n = 6$ to 10) were grown from each of the four H460 holoclones and from parental H460 cells. Total RNA was prepared from each individual tumor, and two independent pools of RNA for each holoclone-derived tumor or parental cell-derived tumor were prepared by combining equal amounts of RNA from each tumor ($n = 3$ to 5 tumors per pool). Tumors were grouped to give similar average tumor volumes in each group, ranging from 1,070–1,565 mm³. All RNAs had an RNA integrity number >7.0 , as determined using an Agilent Bioanalyzer 2100 instrument (Agilent Technologies Inc., Santa Clara, CA). cDNAs transcribed from pools of RNA were labeled with Alexa 647 dye or Alexa 555 dye in a fluorescent reverse pair (dye swap) design. Samples were hybridized to Agilent Whole Human Genome v2 Microarrays (platform G4845A; 4 $\times$ 44K slide format; Agilent Technology, Palo Alto, CA), which include 41,000 human DNA probes, each comprised of a single 60-nucleotide sequence. Sample labeling, hybridization to microarrays, scanning, analysis of TIFF images using Agilent's feature extraction software, calculation of linear and LOWESS normalized expression ratios and initial data analysis and *p*-value calculation using Rosetta Resolver (version 5.1, Rosetta Biosoftware) were carried out at the Wayne State University microarray facility (Detroit, MI) as described [33; 34]. The Rosetta error model provides a gene-specific estimate of error by incorporating two elements: a technology-specific estimate of error and an error estimate derived from replicate arrays [35]. The technology-specific component utilizes an intensity-dependent model of error derived from numerous self-self hybridizations. Two arrays, based on independent pools of biological replicates, were used for each array comparison. By including the technology-specific estimate, the Rosetta error model avoids false positives that occur from under-estimation of error when a small number of replicate arrays are available, thus resulting in an increase in statistical power equivalent to that which would be obtained with at least one additional replicate. Furthermore, a log-ratio error estimate was derived in the Rosetta error model from the individual error estimates of each sample (color) used in the co-hybridization. Then, for each feature an average log ratio and associated *p*-value was obtained from replicate measurements (arrays) using the Rosetta

error model error-weighted averaging method, which weighs the ratio of each sample inversely proportional to the variance of that sample. This gives an averaged ratio with the smallest possible error. The Rosetta error model has superior accuracy in detecting and quantifying relative gene expression when compared to other statistical methods commonly used in microarray analysis, as shown by validation with spike-in experiments [36].

To identify microarray probes (genes) that show statistically significant and reproducible differences in expression between holoclone and parental H460 tumor xenografts, the four separate array comparisons (one for each holoclone) were filtered to obtain a list of 274 probes that met the combined criteria of $p < 0.005$ and fold-change > 2 for at least 3 of the 4 individual H460 holoclones compared to parental H460 tumors. After removal of redundant probes (two or more probes mapping to the same gene) as well as genes that showed inconsistent, i.e., opposite changes in expression in one holoclone compared to the other three, we identified 177 genes showing differential expression in tumors derived from H460 holoclones compared to H460 parental tumors. 141 of the 177 genes were down regulated in at least 3 of the 4 holoclones compared to parental cells and 36 genes were up regulated. Functional annotation clustering was carried out for this gene set using DAVID version 6.7 [37]. Gene names, fold change and p -values for the 177 differentially regulated genes were analyzed using Ingenuity Pathway Analysis (IPA) software (www.ingenuity.com). Each gene was mapped to its corresponding object in the Ingenuity® Knowledge Base to obtain a set of Network Eligible Molecules (i.e., genes), which were overlaid onto a global molecular network developed from information contained within the Ingenuity Knowledge Base. Networks based on these genes were generated algorithmically based on their connectivity. The dataset was also overlaid over the angiogenesis pathway in the Ingenuity Knowledge Base. Additionally, the Upstream Regulator Analysis module of IPA was used to predict upstream regulators associated with the 177 differentially expressed genes, and whether each regulator is in an activated or inhibited state. For each upstream regulator, an activation Z-score was calculated by IPA by comparing the known effect (activation or suppression) of the regulator on each target gene to the observed changes in gene expression. Based on the concordance between them, an activation Z-score was assigned by IPA, inferring whether a potential upstream regulator was in an activated (Z-score > 2), or inhibited (Z-score < -2) or uncertain state. Upstream regulators with $|z\text{-score}| \geq 2$ were deemed significant. Overlap P -values were calculated by IPA using Fisher's Exact Test to determine the significance of the overlap between the known targets of each upstream regulator and our set of 177 genes; overlap P values < 0.01 were considered significant. Upstream regulators that were exogenous chemicals were excluded from further consideration and not presented.

2.9 RNA isolation and qPCR analysis

Total RNA was isolated from frozen solid tumor tissue excised from *scid* mice using TRIzol reagent (Invitrogen; Carlsbad, CA) according to manufacturer's instructions. Replicate pools of RNA (1 μ g) in diethylpyrocarbonate-treated water that were used for microarray analysis were reverse transcribed in a vol of 20 μ l using the High Capacity cDNA Reverse Transcription kit (Cat. #4368814; Applied Biosystems, Foster City, CA). cDNA was diluted 1:50 in 50 ng/ μ l yeast tRNA (Invitrogen). Amplification of cDNA was carried out in qPCR reactions in a total vol of 16 μ l that contained 8 μ l of Power Syber (2x), 1 μ l of forward and reverse primers (5 μ M each), 1 μ l ddH₂O and 4.8 μ l cDNA. cDNA for 18S was diluted 1:10,000 in yeast tRNA and was amplified in reactions similar to the other genes except that 4.4 μ l of ddH₂O and 1.6 μ l of cDNA was used. Triplicate 4 μ l real-time PCR mixtures were loaded onto a 384-well plate. Samples were incubated at 95°C for 10 min followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min in an ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems). Results were analyzed using the $\Delta\Delta$ Ct method normalizing to 18S rRNA content of each sample. H460 parental tumors were averaged and

presented as fold change 1, to which the other 4 holoclones were compared. Data is presented as mean $\pm$ S.D. of 2 replicate pools. Statistical analyses were carried out by 1-way ANOVA with multiple comparisons using GraphPad PRISM software version 4. qPCR primers were designed using Primer Express software (Applied Biosystems) and are listed in Additional file 1 and analyzed using BLAT and BLAST to ensure human-specificity.

3 Results

3.1 Holoclone, meroclone and paraclone populations in human tumor cell lines

Tumor cell lines plated under limiting dilution conditions (1 cell/well) produced holoclones, meroclones, and paraclones after 6–21 days in culture. Representative clones are shown for six human cancer cell lines representing four tumor types (Figure 1A). For each cell line, the holoclones were round in shape and highly compact. Cells comprising paraclones were sparsely spaced, and meroclones exhibited intermediate features of holoclones and paraclones. Colo-205 paraclones were only visible as single cells and did not divide, even after 30 days culture with continuous media replacement, suggesting they require cell-cell communication for growth. The frequency of holoclones ranged from 12% (MDA-MB-231) to 69% (HT-29) across the six cell lines (Table 1). The poorly differentiated FaDu and HT-29 tumor cell lines [38; 39; 40] formed holoclones at the highest frequencies, consistent with observations in other poorly differentiated tumor cell lines [41].

3.2 Self-renewal capacity of holoclones

The ability of the holoclones to self-renew was assessed by a colony formation assay carried out with cell replated at low density (see Methods) and grown for 6–14 days. H460 holoclones primarily reformed holoclones (H460 holoclone 2H3 at 85% frequency, all other H460 holoclones at >90%), whereas H460 parental cells produced a mixture of colony morphologies (Figure 1B). Holoclones derived from the other tumor cell lines also exhibited self-renewal capacity, with the exception of MDA-MB-231 holoclones, which were not stable under adherent conditions and did not reform holoclones upon replating (data not shown). Given this instability of the MDA-MB-231 holoclones and given the high frequency of FaDu and HT-29 holoclones (60–70% of the total parental cell population; Table 1), which may decrease the likelihood of there being major differences between holoclone-derived and parental tumor cell line-derived tumors in the case of those two lines, our follow up studies were focused on holoclones derived from the other three tumor cell lines (H460, A549 and Colo-205).

3.3 Holoclone-derived tumor growth rates and angiogenesis

Tumor xenografts seeded with holoclones grew more slowly than parental tumor cell-derived xenografts, as seen for H460 holoclones (Figure 2A) and for A549 and Colo-205 holoclones (Additional file 2), and as was seen previously for tumors grown from prostate cancer PC3 holoclones [9]. This differential tumor growth rate was seen for all five independent H460 holoclones, with holoclone-derived tumors H460/2E1 and H460/3F1 showing the slowest growth rates (Figure 2A). Tumor growth had no negative impact on body weight gain (Figure 2B).

Given the increase in angiogenesis in tumors derived from prostate, glioma and renal CSLCs [9; 25; 29; 30], we investigated whether tumor xenografts grown from holoclones are more vascularized than those grown from the corresponding parental tumor cell populations. Tumor vascularity (microvessel density) was determined by immunostaining for the endothelial cell marker CD31. No consistent difference in vascular density was seen in the Colo-205 and A549 holoclone-derived tumors compared to the corresponding parental tumors (Additional file 3). In contrast, tumors from 4 of the 5 independent H460 holoclones

showed increased microvessel density compared to parental H460 tumors (Figure 3A; Additional file 4), with the differences being statistically significant for the H460/2E1, H460/2E7 and H460/2F1 tumors (Figure 3C). A significant increase in tumor blood flow was seen in all five sets of H460 holoclone-derived tumors (i.e., increased uptake of Hoechst 33342 dye; Figure 3B, 3D; Additional file 5), which indicates an increase in tumor blood vessel functional capacity. Thus, increased angiogenesis and increased blood flow are general characteristics of tumors grown from H460 holoclones. Using a human CD31-specific antibody, we saw no staining of H460 parental or H460 holoclone tumor sections, as compared to strong positive staining of human tonsil and human liver (positive controls), indicating that the blood vessels in these CSLC-seeded tumors are from the mouse host (data not shown).

3.4 Transcriptional profiling of H460 holoclone-derived tumors

Microarray analysis was carried out to characterize the gene expression profiles of H460 holoclone-derived tumors compared to parental H460 tumors, to discover H460 holoclone-associated CSLC markers and to identify factors that may contribute to the increased angiogenesis of the holoclone-derived tumors. DAVID analysis indicated that the genes dysregulated in each of the H460 holoclone-derived compared to parental H460 cell-derived tumors showed greatest enrichment for secreted proteins, transmembrane, cell adhesion, extracellular matrix, plasma membrane, wound healing, synaptic transmission, carbohydrate binding and angiogenesis (Table 2). 177 genes were consistently dysregulated, with 36 genes up regulated and 141 genes down regulated in at least 3 of the 4 independent holoclone-derived tumor lines. Moreover, 39 of the 177 genes showed a consistent pattern of altered expression in all four holoclone tumors, with 38 genes down regulated and one gene up regulated (Additional file 6).

At least 47 of the 177 differentially regulated genes (27%) have functions related to angiogenesis (Table 3; Additional file 6), indicating a substantial enrichment for this biological pathway. Several well-characterized mediators of angiogenesis, including VEGF and VEGFR, did not, however, show altered expression in the holoclone-derived tumors. 19 of the 47 angiogenesis-associated genes showed a pattern of expression consistent with the observed higher vascularity of the holoclone-derived tumors. Thus, 12 of 14 anti-angiogenic factors were down regulated in the holoclone-derived tumors, while 7 of 29 pro-angiogenic factors were up regulated. Examples include lumican (LUM), which is anti-angiogenic and was strongly down regulated in the holoclone-derived tumors (mean of 82-fold decrease), and haptoglobin and matrix metalloproteinase 3 (MMP3), which are pro-angiogenic and were up regulated (9-fold and 4.3-fold, respectively). Four of the 47 genes have been associated with both pro-angiogenesis and anti-angiogenesis activity (ALOX15, ANGPT1, TNFSF11, and DCN (decorin)) and were down regulated (Table 3). The apparent lack of correlation between the increase in H460 holoclone-derived tumor vascularity and the direction of change in expression of the other 24 angiogenesis-related genes may reflect the activation of compensatory, feedback inhibitory pathways, which is common in angiogenesis.

Ingenuity Pathway analysis (IPA) was used to identify networks based on known interactions of the 177 differentially expressed genes. Gene networks that were highly differentially regulated between the holoclone-derived and parental H460 tumors include: 1) cellular development, hematological system development and function; 2) cellular development, cellular growth and proliferation, hematological system development and function; 3) hair and skin development and function, embryonic development, organ development; and 4) hereditary disorder, skeletal and muscular disorders, cell cycle (Additional file 7). The third network includes 16 of the 177 holoclone differentially expressed genes, all but 3 of which were down regulated in the H460 holoclone-derived tumors (Figure 4), and 11 of which are associated with angiogenesis (Additional file 6).

Three of the 16 genes encode small leucine-rich proteoglycans (SLRPs) that play roles in extracellular matrix remodeling and collagen assembly, namely, decorin (DCN), lumican (LUM) and fibromodulin (FMOD).

3.5 H460 holoclone tumor-derived upstream regulators

We used IPA to identify putative upstream regulators of holoclone-specific patterns of gene expression associated with the set of 177 differentially expressed genes. Six upstream regulators were identified based on the significance of their activation scores ($Z > 2$ or $Z < -2$), combined with the significance of enrichment of genes downstream of the regulator, independent of the gene expression direction of change (overlap p -value < 0.01). A seventh factor, VEGF, just missed the p -value cutoff ($P = 0.012$). All 7 factors were predicted to be inhibited in the H460 holoclone-derived tumors based on the patterns of differential expression shown by their target genes (Additional file 8). One of the upstream regulators, angiotensinogen (AGT), is an anti-angiogenic factor [42] whose predicted decrease in activity is consistent with the observed increase in angiogenesis in the holoclone-derived tumors. Another upstream regulator, EP300, is a co-activator of HIF1A (hypoxia-inducible factor 1 alpha) [43], and plays a role in the stimulation of hypoxia-induced genes such as VEGF, and has been implicated in activation of VEGF-responsive enhancers and in VEGF-induced transcriptional responses [44].

3.6 DCN^{low}/LUM^{low}/FMOD^{high} is a molecular profile of H460-derived holoclones

qPCR analysis of selected genes that were significantly dysregulated in the H460-holoclone-derived tumors was used to validate the microarray analysis and to validate molecular signatures associated with the observed increase in angiogenesis (Figure 5). Examination of the three SLRPs identified by microarray analysis revealed that DCN and LUM were consistently down regulated while FMOD was consistently up regulated in the holoclone-derived tumors, in accord with the microarray results. Other genes that were significantly down regulated in H460 holoclone-derived tumors were FGF13, regenerating islet-derived 1-alpha (REG1A), hemoglobin subunit epsilon (HBE1) and interleukin 11 (IL11), with the extent of down regulation generally greater in H460 holoclone 2H3- and 3F1-derived tumors than in holoclone 2E1- and 2E7-derived tumors (Figure 5).

4 Discussion

Studies of CSLCs and their impact on tumor gene expression may help elucidate tumor-stromal cell interactions as they affect tumor growth and angiogenesis. Previous work has shown that tumors grown from prostate, brain and renal CSLCs exhibit increased vascularity [9; 25; 29; 30]. Presently, we show that increased tumor blood vessel density and increased tumor blood flow is a characteristic of CSLCs derived the lung cancer cell line H460, and we characterize global differences in gene expression between tumors grown from these CSLCs and parental H460 cell populations, most notably genes associated with angiogenesis.

CSLCs can be identified and isolated by a variety of methods, including their ability to grow with a characteristic holoclone morphology with retention of the capacity that CSLCs identified by other methods have for self-renewal, spheroid formation, tumor initiation and marker gene expression, as shown in a large number of tumor models [9; 17; 18; 19; 20; 21; 22; 23; 24]. Holoclone morphologies were originally identified in keratinocytes and associated with stem cells, in contrast to differentiated cells, which are comprised of meroclones and paraclones [16]. Tumor-derived meroclones and paraclones have little or no ability to initiate tumor growth on their own, but may cooperate with the tumor-initiating holoclone cells to facilitate tumor growth [9; 19; 23]. We isolated CSLC holoclones from six

different human tumor cell lines at frequencies ranging from 12% to 69%. The high frequency of CSLCs in the FaDu and HT-29 cell lines (~60–70%) was unexpected, given that CSLC holoclones typically constitute a smaller fraction in other tumor cell lines (typically up to ~10%) and an even smaller population in tumor cell populations in other models [12; 45].

Five of the six tumor cell lines studied here yielded holoclones that showed self-renewal capacity – all except for the MDA-MB-231-derived holoclones. Previous studies showed that CSLCs selected from MDA-MB-231 cells using repeated cycles of hypoxia and re-oxygenation are stable when grown as spheroids [46], suggesting these cells require hypoxia to maintain a stem-like state, as seen in other tumor models [47; 48]. MDA-MB-231-derived holoclones can be grown as spheroids [24] but may not be stable when grown under adherent conditions [22]. In the present study, paraclones isolated from each of the cancer cell lines generally grew slowly and did not yield holoclones during further cell culture. Most of the paraclones could be passaged several times, however, Colo-205 paraclones did not advance beyond the single cell stage, even after 30 days in culture, and may be terminally differentiated cells that require interactions with other cells for cell division.

H460 non-small cell lung carcinoma is an aggressive tumor characterized by its high vascularity [49], invasiveness, and ability to metastasize [50]. H460 side population cells with CSLC properties have been isolated based on their ability to efflux Hoechst 33342 dye via drug transporters [7; 45; 51]. H460 CSLCs selected based on resistance to doxorubicin or cisplatin yield spheroids [7, 52], overexpress stem cell markers and form tumors that are highly invasive and metastatic and show elevated levels of growth factors and angiogenic factors, including VEGF and bFGF [7]. While these studies have implicated drug-selected H460 CSLCs in tumor growth and angiogenesis, it is unclear whether these properties are a consequence of drug selection, or whether they reflect the CSLC characteristics of these cells. CSLCs isolated based on drug resistance and Hoechst dye exclusion are a mixed cell population and may be comprised of a subset of CSLCs that overexpress drug transporter pumps. In contrast, the H460 holoclones characterized here were isolated by holoclone morphology, and may constitute a more representative stem cell population. Indeed, we identified H460 holoclones with a frequency of 24%, substantially higher than the 3.5–5.6 % reported for side population H460 cells [7; 45; 51].

Tumor xenografts grown from H460, A549, and Colo-205 holoclones grew more slowly than tumors from the corresponding parental tumor cell lines when using large numbers of cells to seed the tumors ($>2 \times 10^6$ cells/tumor) (Figure 2, Additional file 2). The same observation was made previously with tumors derived from PC3 holoclones [9]. The slower growth of holoclone-derived tumors may at first seem counterintuitive, given the established role of CSLCs in tumor growth initiation, and the increased vascularity of the resultant tumors. Two possibilities may explain this slower overall growth rate for the holoclone-seeded tumors, despite their increased vascularity. First, the parental cell population is comprised of a sub-population of many independent CSLC holoclones, which when present together may cooperate to initiate tumor growth at a rate that is more rapid than that achieved by any single holoclone. Figure 2A supports the conclusion that individual holoclones initiate tumor growth at different rates. Second, the slower growth of the holoclone-derived tumors can be explained by the need for the holoclones to differentiate into supporting cells that promote tumor growth. Thus, tumors seeded with pure holoclones can be expected to grow slowly until sufficient numbers of differentiated supporting cells appear. In contrast, tumors seeded with a complete parental cell population grow more rapidly, as they already contain holoclones as well as the full complement of differentiated tumor cell types, including supporting differentiated cells that promote tumor growth. However, when tumors are seeded at a very low inoculation density, as is typically done in

in vivo tumorigenicity/limiting dilution assays, tumor take rates are much higher when using holoclones compared to meroclones and paraclones, as only holoclones contain the stem-like cells required to initiate tumor growth and also the ability to differentiate into the required supporting cells [9; 17; 19]. Tumors seeded with meroclones or paraclones are devoid of CSLCs, and cannot differentiate into CSLCs, and are thus unable to initiate robust tumor growth.

Tumor xenografts grown from H460 holoclones exhibited increased vascularity compared to parental H460 tumors, indicating that H460 CSLCs promote angiogenesis, in agreement with earlier findings with renal [30], prostate [9; 25] and brain CSLCs [29]. Further, we observed an even larger increase in tumor blood perfusion, as monitored by Hoechst dye staining, suggesting that vascular patency is increased to a greater extent than vascular density in the H460 holoclone-derived tumors. Increased expression of the pro-angiogenic factors VEGF, bFGF and IL6, and other cytokines, chemokines and growth factors, was previously seen in tumors grown from drug-selected H460 CSLCs compared to parental cell-derived H460 xenografts [7]. Glioma spheroids form xenografts characterized by increased blood flow and angiogenesis that is partly mediated by VEGF and stromal derived factor 1 α (SDF-1 α) [52], as was also seen with the drug-selected H460 CSLC xenografts [7]. While the increased angiogenesis shown here for H460 holoclone-derived tumors could be due to differentiation of the holoclones into tumor blood vessels, as was reported for brain tumor CSLCs [53; 54], our findings favor the alternative possibility that the H460 CSLCs secrete factors that stimulate angiogenesis (Additional file 6, Table 3). Indeed, using a human CD31-specific antibody, we could not detect the vascular endothelial cell marker CD31 in H460 holoclone-derived tumors, indicating that in this case the increased tumor angiogenesis is not due differentiation of the human CSLCs into human tumor blood vessels; rather, it likely reflects interactions between the CSLCs and the mouse host leading to increased formation of mouse vasculature.

Using a human microarray platform, we identified a set of 177 genes that consistently showed differential expression between H460 holoclone-derived tumors and parental cell-derived H460 tumors. Although all of the H460 holoclones studied here were derived from the original parental H460 cell population, that population is heterogeneous and comprised of many individual holoclones, as well as meroclones and paraclones; thus, it is to be expected that tumor xenografts derived from the parental tumor cell line will have a different gene expression pattern than tumors derived from a single, individual holoclone. Analysis of the holoclone-specific gene expression profile showed that 47 of the 177 differentially regulated genes have functions related to angiogenesis. The 47 angiogenesis-related genes include 19 genes whose altered expression was consistent with the observed increase in angiogenesis and 24 genes whose altered expression was in the opposite direction of that anticipated based on the increased angiogenesis of the holoclone-derived tumors, i.e. pro-angiogenic genes that were down regulated and anti-angiogenic genes that were up regulated. Four other dysregulated genes have both pro- and anti-angiogenic properties (Table 3). Some of these differences could result from discrepancies between differential regulation of RNA vs. protein levels – a potential limitation of all expression microarray and other transcriptomic analyses – although overall, global correlations between gene and protein expression data are quite good [55; 56]. Alternatively, feedback inhibition mechanisms activated in response to the increased angiogenesis of the holoclone-derived tumors may account for the observed up regulation of anti-angiogenic factors and down regulation of pro-angiogenic factors, and for the apparent inhibition of several upstream regulators associated with VEGF-induced transcriptional responses, notably EP300 and VEGF itself (Additional file 8). IPA upstream regulator analysis also identified angiotensinogen (AGT) as a putative upstream regulator whose activity is inhibited in the H460 holoclone-derived tumors. AGT is an anti-angiogenic serpin peptidase inhibitor that

inhibits tumor growth [42; 57], suggesting that its inhibition contributes to the observed increase in vascularity of the H460 holoclone-derived tumors.

IPA analysis identified a network (network 3, “hair and skin development and function, embryonic development, and organ development”) that includes 16 genes differentially expressed in H460 holoclone-derived tumors, 11 of which are associated with angiogenesis (Figure 4, Table 3). Four of the 11 genes are anti-angiogenic factors that were down regulated in the holoclone-derived tumors (LUM, ACE2, PRL, NOG), two are pro-angiogenic factors that were up regulated (FMOD, MMP3), and two are down regulated factors that show both pro- and anti-angiogenic activities (ALOX15, DCN). MMP3 activates MMP9 [58], which is required for the angiogenic switch [59]; however, MMP9 RNA levels were not elevated in the H460 holoclone-derived tumors. PRL (prolactin), which is anti-angiogenic and blocks pericyte recruitment by endothelial cells and interferes with PDGFR-B signaling [60], was down regulated in all four H460 holoclone tumors compared to parental H460 tumors, as were LUM, NOG, and DCN (Additional file 6).

Three of the angiogenesis-related genes of network 3 are SLRPs that bind to type I collagen type and play roles in collagen fibrillogenesis [61]: DCN, FMOD, and LUM. DCN is anti-angiogenic *in vivo* [62]; it inhibits VEGF secretion, as well as tumor microvessel sprouting and neovascularization. DCN also suppresses the pro-angiogenic factor HIF-1 α and up regulates the anti-angiogenic factors TIMP3 and thrombospondin 1 in HIF-1 α -overexpressing MDA-MB-231 cells [63], and its loss could contribute to the increased angiogenesis in the holoclone-derived H460 tumors. DCN can also promote angiogenesis in wound healing, postnatal angiogenesis, corneal angiogenesis, and inflammation-induced angiogenesis, and it regulates endothelial cell-matrix interaction through endothelial cells [64]. Decreases in expression of DCN and LUM, as seen in our H460 holoclone-derived tumors, are associated with poor prognosis and shorter time to tumor progression in breast cancer [65]. LUM and FMOD both bind to the same site on collagen I fibrils [66] and compete for a common low-affinity binding site [67]. The observed changes in these two factors may be mechanistically interrelated, as suggested by the inverse relationship between these two factors [68]. LUM inhibits endothelial cell angiogenesis mediated by MAPK signaling *in vitro*, and it inhibits angiogenesis and blood perfusion in matrigel plug assays *in vivo* [69]. LUM also inhibits angiogenesis in lung metastases derived from melanomas [70]. FMOD can increase neovascularization in the injured cornea when DCN is ablated [71]; it also increases vascularization in embryonic retinas and was proposed as a treatment for macular degeneration [72]. FMOD promotes angiogenesis in HUVEC cells and in chick embryo chorioallantoic membrane assays [73]. While treatment of tumor xenografts with FMOD siRNA slows tumor growth [72], its effects on tumor vascularity were not investigated. Together, these findings support the hypothesis that the loss of DCN and LUM, coupled with the increase in FMOD in the holoclone-derived tumors contribute functionally to the increase in tumor angiogenesis.

5 Conclusions

We demonstrate that the proportion of holoclones in cancer cell lines varies widely in different tumor models. Tumors derived from H460 holoclones exhibit increased angiogenesis and tumor blood perfusion associated with many changes in gene expression. These changes include dysregulation of several SLRPs, which may play a role in tumor angiogenesis and tumor progression, as well as decreased activity for angiotensinogen, which was identified as an upstream regulator whose inhibition may contribute to the increase in vascularity of the H460 holoclone-derived tumors. Given the contributions of extracellular matrix proteins to angiogenesis [74; 75], targeting extracellular matrix proteins may serve as a useful approach for inhibition of tumor growth and angiogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of Abbreviations

CSLC	cancer-like stem cell
SLRP	small leucine-rich proteoglycan

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Figure 1. Heterogeneity of holoclone, meroclone and paraclone morphology of six human tumor cell lines

A) Shown are representative clonal morphologies of HT-29, FaDu, A549, Colo-205, H460, and MDA-MB-231 cells plated at 1 cell/well in 96 well plates after growth for 6–21 days. Images taken at 10X magnification. B) H460 holoclone self-renewal assay. H460 parental and H460 holoclone cells were plated in 10 cm plates at 8,000 cells/plate. After 6 days, the plates were rinsed with 1x PBS, and then stained for 10 min with crystal violet solution (1.25 g crystal violet, 50 ml paraformaldehyde, 450 ml methanol). Excess stain was removed by submerging the plates in cold water. Holoclones yielded tight colonies while parental cells ('wt') produced all three colony morphologies.

Figure 2. H460-derived tumor growth (A) and body weight profiles (B) in *scid* mice
Male *scid* mice were inoculated s.c. on both flanks with 2×10^6 H460 parental or holoclone 2E1, 2F1, 2H3, 3F1, or 2E7 cells/site. Tumor volumes are mean $\pm$ S.E. values for $n = 8-10$ tumors per group and body weights are mean $\pm$ S.E. values for 4-5 mice per group.

Figure 3. Increased microvessel density and tumor blood perfusion in H460 holoclone tumors compared to parental tumors

Tumor microvessel density based on CD31 immunostaining and tumor blood perfusion determined by Hoechst dye staining were assayed as described in Materials and Methods. A) Representative CD31 staining of H460 parental tumors and H460 holoclone-derived tumors corresponding to the average values show in C. High resolution images taken at 4.2X with a FSX 100 Bio Imaging Navigator microscope. B) Representative Hoechst 33342 staining of H460 parental tumors and H460 holoclone derived tumors corresponding to the average values show in D. High resolution images taken at 4.2X with a FSX 100 Bio Imaging Navigator microscope with background fluorescence subtracted from non-Hoechst injected mice. C) Quantification of immunohistochemical staining of mouse CD31- positive vascular area (NIH ImageJ quantification, mean $\pm$ S.E. for n=6–10 parental H460 tumors and holoclone-derived H460 tumors/group, based on 3–5 separate regions for each tumor and 3–15 images per slide). * $p < 0.05$, ** $p < 0.005$. D) Quantification of Hoechst 33342 positive staining (NIH ImageJ quantification, mean $\pm$ S.E. for n = 6–10 parental H460 tumors and holoclone-derived H460 tumors/group, based on 3–5 separate regions for each tumor and 3–15 images per slide). * $p < 0.05$, ** $p < 0.005$. The large error bar for H460/2E7 reflects one tumor sample with very low staining.

Figure 4. Network associated with hair and skin development and function, embryonic development, and organ development from Ingenuity Pathway Analysis (IPA) in H460 holoclones compared to parental H460 tumors

This network was identified by Ingenuity Pathway Analysis with an IPA score of 27 (Network 3, Additional file 7). This network includes 16 genes that were significantly dysregulated in the H460 holoclone xenografts compared to H460 parental xenografts, with 11 of these genes related to angiogenesis (Additional file 6). Genes up regulated in holoclones are shown in *red*, and genes down regulated are shown in *green*, with the color intensity indicating the relative extent of up or down regulation. Solid lines indicate direct interactions, and dashed lined signify indirect interactions.

Figure 5. qPCR analysis of H460 parental and H460 holoclone-derived tumors

Total RNA was prepared from 2 pools of individual tumors for each of the four indicated holoclone-derived tumors and for H460 parental cell-derived tumors ('WT'). Shown are relative expression levels for each gene, mean $\pm$ S.D. values after normalization to levels of 18S RNA in each sample. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$ for comparison of H460 parental tumors to each holoclone.

Table 1
Colony forming efficiency of human tumor cell lines

Cell Line	Holoclone	Meroclone	Paraclone	Ambiguous morphology
	<i>percentage</i>			
HT-29	69.4 ± 0.9	19.4 ± 0.9	5.6 ± 0.9	5.6 ± 0.9
FaDu	63.6 ± 5.0	23.5 ± 9.5	6.5 ± 3.7	6.5 ± 3.7
A549	35.0 ± 7.1	30.0	35.0 ± 7.1	
H460	23.9 ± 5.3	24.3 ± 3.7	51.8 ± 1.7	
Colo-205	32.1 ± 7.1	19.7 ± 3.01	48.2 ± 9.0	
MDA-MB-231	11.7 ± 7.6	28.6 ± 18.3	33.7 ± 20.2	

Tumor cells were seeded in 96 well plates at 1 cell/well and grown overnight. Wells containing single cells were identified and colonies counted 6–21 days later. Colony forming efficiency (CFE) is defined as the total number of each clone type divided by the total number of clones × 100. Data presented are mean ± S.E. values based on of 2–4 independent experiments and represent % of clones observed. Clones that bordered on being holoclone/meroclone or meroclone/paraclone, or were too difficult to assign were designated ambiguous.

Table 2
Enrichment Scores for DAVID analysis of H460 holoclone-derived tumors compared to parental H460 tumors

Gene Category	Enrichment Score 2E1	Enrichment Score 2H3	Enrichment Score 3F1	Enrichment Score 2E7	Mean fold-enrichment
Secreted Protein	28.2	27.4	34.3	25.3	28.8
Transmembrane	5.5	5.4	19.5	8.1	9.6
Cell Adhesion	9.1	7.9	11.1	10.1	9.6
ECM	9.4	11.1	6.5	9.9	9.2
Plasma Membrane	6.8	4.9	11.5	8.3	7.9
Wound Healing	5.2	5.3	10.8	4.4	6.4
Synaptic Transmission	2.0	11.1	–	2.6	5.2
Carbohydrate Binding	4.1	4.5	6.1	6.0	5.2
Angiogenesis	3.5	5.7	6.5	2.2	4.5
Homeostasis	1.8	11.1	2.3	1.9	4.3
Cell Motility	3.6	3.7	5.1	4.6	4.2
Basement Membrane	4.0	4.7	3.9	–	4.2
Inflammation	4.8	2.4	4.3	5.1	4.2
Blood Coagulation	3.4	4.7	4.9	2.0	3.8

DAVID analysis of expression data for tumors derived from each individual H460 holoclone (2E1, 2H3, 3F1 and 2E7) compared to H460 parental cell-derived tumors by functional clustering analysis to identify gene expression categories across the holoclones. Enrichment Scores shown indicate the fold likelihood of a gene being overexpressed by chance. The last column is the average Enrichment Score for each category.

Table 3
Angiogenesis related genes differentially expressed in H460 holoclone compared to H460 parental xenografts

Shown are the 47 angiogenesis-related genes that show consistent differential expression in H460 holoclone-derived tumors compared to parental H460 cell-derived tumors. MVD, microvessel density. See Additional file 6 for further details, including references.

Gene	Mean Fold Change	Pro- or Angiogenic, or Both	Notes
LUM	-82.0	Anti	Decreases tube formation and angiogenesis, decreases MMP2 and MMP9 mRNA; increases Fas mediated endothelial cell apoptosis
SSTR1	-31.8	Anti	SSTR1 agonists reduces VEGF and ERK signaling
ACE2	-12.7	Anti	Blocks VEGFa production
PF4V1	-7.7	Anti	Inhibits angiogenesis and tumor growth: blocks endothelial cell proliferation, migration, and tube formation
GC	-7.1	Anti	Reduces MVD; inhibits endothelial cell proliferation and VEGF signaling
FST	-7.0	Anti	Reduces MVD
SPON1	-6.7	Anti	Blocks endothelial integrin alphavbeta3
PRL	-5.3	Anti	Reduces tumor angiogenesis
NOG	-5.1	Anti	BMP2 antagonist, decreases blood vessel formation
REN	-5.1	Anti	Reduces sca-1/VEGFR2 positive pro angiogenic cells in blood
TFPI2	-3.2	Anti	Reduces tube formation and decreases MVD
SEMA3E	-3.1	Anti	Reduces vessel formation, inhibits VEGF signaling
CD36	1.6	Anti	Reduces tumor growth and vascularity, blocks endothelial cell migration and tube formation
SHROOM 2	5.1	Anti	Reduces branching and sprouting angiogenesis
ESM1	-26.3	Pro	Increases MVD and metastasis, expression induced by VEGFA
NTS	-16.8	Pro	Overexpression linked with VEGF and MMP9; overexpressed in CD133+ liver CSLCs with CXCL1, increased tumor angiogenesis
OLFM3	-10.9	Pro	Increases tumor angiogenesis, endothelial cell migration and pericyte coverage, binds to BMP4
PIK3CG	-10.1	Pro	Required for HUVEC migration, Endothelial cell proliferation
KIT	-10.0	Pro	Promotes endothelial cell proliferation, migration, and tube formation; increases VEGF expression and CD31 staining
ITGA4	-8.7	Pro	Mediates attachment of monocytes to vascular endothelium; promotes adhesion between endothelial cells and pericytes, promotes angiogenesis in presence of proangiogenic stimuli
EPHA3	-7.8	Pro	Correlates with increased microvessel density and VEGF expression
RXFP2	-7.4	Pro	Increases NO production, which leads to increased circulation of bone marrow-derived endothelial cells
ABCB1	-6.9	Pro	Increases endothelial cell migration, tube formation; overexpressed in brain microvascular endothelial cells
PRKG1	-5.9	Pro	iNOS induces angiogenesis, promotes angiogenesis and vasculogenesis
GUCY1A 3	-5.7	Pro	Linked to overexpressed cGMP and VEGF
PAEP	-5.7	Pro	Promotes HUVEC migration and tube formation, induces β -catenin nuclear translocation
GPC5	-4.8	Pro	Indirect effect, via increases in FGF2 and HGF

Gene	Mean Fold Change	Pro- or Angiogenic, or Both	Notes
PTGFR	-4.7	Pro	Increased mRNA expression in tumor endothelial cells, and in blood vessels
IL6	-3.0	Pro	Induced u-PA and VEGF in prostate cancer; increases tumor angiogenesis and metastasis
FZD4	-3.0	Pro	Promotes retinal angiogenesis
NOX4	-2.6	Pro	Induced by hypoxia, with subsequent induction of endothelial cell proliferation, migration and tube formation; increases eNOS expression; increases VEGF mRNA
FGF12	-2.6	Pro	Overexpressed in activated HUVECs
THPO	-2.5	Pro	Increased release of CXCR4+ VEGFR1+ hemangiocytes; increases HUVEC migration
OSM	-2.1	Pro	Increases u-PA and VEGF, increases metastasis; induces VEGFA and ID1 expression
PDGFA	-1.7	Pro	Overexpressed with other proangiogenic factors in neuroblastomas, found in late stage disease
WTAP	-1.5	Pro	Promotes smooth muscle cell proliferation, Induces MMP3 and EGFR in glioblastoma
SEMA3C	2.3	Pro	Increases MVD, increases migration and tube formation
FMOD	2.4	Pro	Increased HUVEC spreading and tube formation, Increases VEGF and Ang2
SUSD2	2.6	Pro	Observed in breast cancer cells, interacts with galectin-1, which promotes angiogenesis
TFF3	4.2	Pro	Promotes tube formation, Correlates with CD31 and CD34 staining
MMP3	4.3	Pro	Linked to vascular invasion; Promotes MMP9 cleavage
IGF2	5.6	Pro	Increases endothelial progenitor cell homing; decreases VEGF expression
HP	9.0	Pro	Promotes endothelial progenitor cell angiogenesis and tube formation; increased in systemic vasculitis
ANGPT1	-8.3	Both	Promotes endothelial cell sprouting and angiogenesis; Decreases vessel density and vascular permeability
ALOX15	-4.2	Both	Promotes VEGF secretion and angiogenesis; Blocks VEGFA and PlGF induced angiogenesis in muscle and blocks VEGFR2
DCN	-3.9	Both	Reduces HIF1A, VEGFA; reduces MMP2 and MMP9; increases tube formation; increases CD31 staining during inflammation
TNFSF11	-1.6	Both	Linked to increased VEGF expression in myeloma