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Identification of Three Molecular and Functional Subtypes in Canine Hemangiosarcoma through Gene Expression Profiling and Progenitor Cell Characterization

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Canine hemangiosarcomas have been ascribed to an endothelial origin based on histologic appearance; however, recent findings suggest that these tumors may arise instead from hematopoietic progenitor cells. To clarify this ontogenetic dilemma, we used genome-wide expression profiling of primary hemangiosarcomas and identified three distinct tumor subtypes associated with angiogenesis (group 1), inflammation (group 2), and adipogenesis (group 3). Based on these findings, we hypothesized that a common progenitor may differentiate into the three tumor subtypes observed in our gene profiling experiment. To investigate this possibility, we cultured hemangiosarcoma cell lines under normal and sphere-forming culture conditions to enrich for tumor cell progenitors. Cells from sphere-forming cultures displayed a robust self-renewal capacity and exhibited genotypic, phenotypic, and functional properties consistent with each of the three molecular subtypes seen in primary tumors, including expression of endothelial progenitor cell (CD133 and CD34) and endothelial cell (CD105, CD146, and $\alpha_v\beta_3$ integrin) markers, expression of early hematopoietic (CD133, CD117, and CD34) and myeloid (CD115 and CD14) differentiation markers in parallel with increased phagocytic capacity, and acquisition of adipogenic potential. Collectively, these results suggest that canine hemangiosarcomas arise from multipotent progenitors that differentiate into distinct subtypes. Improved understanding of the mechanisms that determine the molecular and phenotypic differentiation of tumor cells *in vivo* could change paradigms regarding the origin and progression of endothelial sarcomas. *(Am J Pathol 2014, 184: 985–995; http://dx.doi.org/10.1016/j.ajpath.2013.12.025)*

Similar to most cancers, sarcomas are classified based on their histologic appearance, which presumably reflects the cells of origin and their capacity for differentiation. These morphologic diagnoses are likely complicated by multiple genomic alterations, microenvironmental differences, and recruitment of nonneoplastic cells into the tumor microenvironment. As a

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result, the phenotype of the tumor bulk may not reflect the tumor progenitor population, a possibility that has clinical implications in terms of diagnostic criteria and therapeutic approaches.

Such morphologic heterogeneity is a feature of canine hemangiosarcoma, a frequent and highly metastatic tumor in dogs that can arise in any organ but that shows predilection for the spleen, right atrium/auricle, and skin or subcutis.[1] The histologic appearance of hemangiosarcomas ranges from the classic cavernous tumor containing neoplastic endothelial-like cells to solid lesions that cannot be distinguished from other soft-tissue sarcomas without the aid of immunohistochemical analysis.[2] Recent findings have challenged the presumed endothelial ontogeny of canine hemangiosarcomas and the histologically similar human angiosarcomas, suggesting instead that these tumors arise from bone marrow progenitor cells that can transit to peripheral vascular sites.[3–5] Therefore, a more precise identification of hemangiosarcoma progenitors may provide a better understanding of disease progression toward the observed endothelial lineage phenotype.

The low incidence and large phenotypic and genetic diversity of human sarcomas hampers understanding of their cellular ontogeny. However, because domestic dogs develop sarcomas spontaneously and with high incidence, the study of canine tumors provides a powerful model in which tumor heterogeneity is maintained. Furthermore, the similarities between human and canine sarcomas make dogs a valuable resource for therapeutic development[6] and investigations into sarcoma cellular ontogeny. Although it has been suggested that mesenchymal stem cells (MSCs) are the cells of origin for sarcoma,[7,8] there is ongoing debate regarding the potential for other cells to give rise to sarcomas and other tumor types.[9] Thus, knowledge of progenitor cell populations capable of giving rise to a particular tumor type is useful to positively impact therapeutic design and clinical outcomes.

For this study, we tested the hypothesis that hemangiosarcomas arise from multipotent hematopoietic progenitors and that this multipotency is associated with the observed tumor heterogeneity. We identified three distinct molecular subtypes of hemangiosarcoma associated with angiogenesis or endothelial cell development and function (group 1), inflammation and myeloid differentiation and function (group 2), and adipogenesis and lipid transport pathways (group 3). Furthermore, we demonstrate that a subset of cells derived from hemangiosarcoma cell lines show the capacity to recapitulate each of these patterns *in vitro*. Thus, these results suggest that hemangiosarcoma progenitors contribute to tumor complexity and reflect the array of histologic phenotypes seen. These results also alter the paradigm regarding the origin and progression of canine hemangiosarcoma and suggest that diagnostic and therapeutic approaches addressing progenitor cell biology, rather than the morphologic or histologic appearance of the tumor, need to be developed for this disease.

Materials and Methods

Pathologic and Immunohistochemical Analyses

Samples from 58 pet dogs with a histologic diagnosis of hemangiosarcoma were included in this study (Supplemental Table S1). Samples were obtained from surgical or necropsy specimens with the owner's consent; representative areas of gross tumor were fixed in 10% neutral buffered formalin for <72 hours. Diagnoses were made from routine H&E-stained slides using standard histopathologic criteria[10] by board-certified veterinary pathologists at the Masonic Cancer Center Comparative Pathology Shared Resource (University of Minnesota, Minneapolis, MN) or at other reference laboratories. Hemangiosarcomas were generally characterized by poorly demarcated and nonencapsulated proliferation of atypical ovoid to spindyloid cells. The cells proliferated as solid sheets but often broke apart to form rudimentary and tortuous vascular channels. The cells also were markedly invasive into adjacent parenchyma. Samples from 20 cases that were included in the microarray analysis were available for reassessment and classification into cavernous or solid morphology based on the number and size of vascular spaces (Supplemental Table S1). As we have reported previously,[5,10,11] there was intertumor and intratumor heterogeneity regarding tumor size, cellularity, and the extent of hemorrhage, necrosis, and inflammation. Individual tumor cells were characterized by scant to moderate eosinophilic cytoplasm, and moderately pleomorphic, euchromatic nuclei with medium-sized nucleoli. There usually was significant organizing hemorrhage in the adjacent, noninvolved parenchyma, and occasional central necrosis. Inflammation was observed in association with areas of hemorrhage and necrosis, but, in some instances, it was present in perivascular spaces and intermixed with tumor parenchyma. Whenever the diagnosis was in doubt, confirmation was sought by routine immunohistochemical analysis to assess expression of CD31 and vWF (Factor VIII–related antigen) as described elsewhere.[10,11] Protocols and procedures for sample procurement were reviewed by the Institutional Animal Care and Use Committee of the University of Minnesota.

Microarray and RNA-seq Analysis

Microarray analysis was used to identify molecular signatures in canine hemangiosarcoma, and the molecular signatures were verified by RNA-seq analysis. RNA prepared from 59 tumor tissue samples collected at surgery or necropsy was quantified and assessed for quality as described elsewhere.[5,12] Briefly, total RNA was quantified using a fluorimetric Quanti-IT RiboGreen RNA assay kit (Life Technologies, Carlsbad, CA), and total RNA integrity was assessed using capillary electrophoresis in the 2100 Bio-Analyzer system (Agilent Technologies Inc., Santa Clara, CA) to generate RNA integrity numbers. Samples passed a quality control step if they contained >1 μg with an RNA integrity

number >6.5 (array hybridization) or >8 (RNA-seq). Total RNA from 24 whole-tissue samples and 18 cell line samples (12 monolayer-cultured cell lines including 2 samples in duplicate and 6 sphere-cultured cell lines including 1 sample in triplicate) were labeled using Agilent's microarray one-color low-input quick amp labeling kit, hybridized to Agilent canine 4 × 44,000 feature gene chips according to Agilent's protocol version 6, and read using an Agilent array scanner (Agilent Technologies Inc.). Total RNA from 47 samples (12 overlapping microarray analyses) was converted to Illumina sequencing libraries using the TruSeq RNA sample preparation kit (Illumina Inc., San Diego, CA). PolyA RNA was enriched from 1 μg of total RNA using oligo-dT−coated magnetic beads, was fragmented, and was reverse transcribed into cDNA. The cDNA was fragmented, blunt-ended, ligated to bar-coded adaptors, and amplified using 15 cycles of PCR. Final library size distribution was validated using capillary electrophoresis and was quantified using the PicoGreen assay (Life Technologies) and by quantitative PCR. Indexed libraries were normalized, pooled, and size selected to 320 bp ± 5% using a Caliper LabChip XT system (PerkinElmer, Waltham, MA). TruSeq libraries were then hybridized to a paired end flow cell, and individual fragments were clonally amplified by bridge amplification using the cBot system (Illumina Inc.). Once clustering was complete, flow cells were loaded onto a HiSeq 2000 sequencing system and were sequenced using sequencing by synthesis chemistry (Illumina Inc.). On completion of read 1, a 7-bp index read was performed. The library fragments were resynthesized in the reverse direction and sequenced from the opposite end of the read 1 fragment, thus producing the template for paired-end read 2. Each sample was sequenced to a targeted depth of approximately 20 million paired-end reads. Base call (.bcl) files for each cycle of sequencing were generated by real-time analysis software (version 1.13, Illumina Inc.). Primary analysis and demultiplexing were performed using CASAVA software version 1.8.2 (Illumina Inc.) to verify the quality of the sequence data. The end result of the CASAVA workflow was demultiplexed into FASTQ files for analysis. Bioanalyzer quality control, RNA labeling, microarray hybridization and reading, and RNA-seq were performed at the University of Minnesota Genomics Center. Microarray data are available from the Gene Expression Omnibus (*http://www.ncbi.nlm.nih.gov/geo;* accession number GSE53219).

Bioinformatic Analyses

Agilent array values for each probe for each experiment were compiled into a matrix of samples by probe. These data were then examined for quality control and were quantile-normalized using Genedata Analyst software version 7.5 (Genedata AG, Basel, Switzerland). Of 45,220 features on each array, 35,676 that had annotations to known genes were used for analysis. Unsupervised hierarchical clustering was based on average linkage using Gene Cluster software version 3.0 for Mac OS X, and a heat map of two defined groups was visualized using Java TreeView software version 1.1.6. Biological functions and canonical pathways of differently expressed genes between the unsupervised groups were defined by Ingenuity Pathway Analysis software version 8.6 (Qiagen, Redwood City, CA) using BH multiple testing corrections to evaluate significance. FASTQ files were mapped to the canine reference genome (canFam3), and the resulting BAM files were summarized to fragments per kilobase of exon per million fragments mapped values using CUFFDIFF.

The values for genes differentially expressed between the tumor subtypes were identified in the RNA-seq data set, and the similarity between overlapping mean centered sample matrices was assessed by calculating Pearson correlation r^2 values between the data matrices. To visually show the similarity, the two full matrices including unique samples were visualized as a heat map.

Cell Culture and Sphere Formation

Nonadherent spheres were derived from early passages of hemangiosarcoma cell lines to generate cell cultures enriched for potential hemangiosarcoma progenitor cells. The hemangiosarcoma cell lines SB-HSA (SB), Frog, and Emma were cultured as described elsewhere.[4,13,14] Sphere formation was supported using conditions reported to favor nonadherent cell growth.[15,16] Cells were plated at a concentration of 5 × 10^4 cells/mL in ultra-low-binding dishes (Corning, Lowell, MA) in Dulbecco's modified Eagle's medium/F-12 medium containing L-glutamine and sodium pyruvate (Life Technologies) and supplemented with 10 ng/mL of basic fibroblast growth factor (PeproTech, Rocky Hill, NJ), 20 ng/mL of epidermal growth factor (PeproTech), 5 μg/mL of insulin (Sigma-Aldrich, St. Louis, MO), and 0.4% bovine serum albumin (MP Biomedicals, Solon, OH). Cells were maintained at 37°C in 5% CO_2 atmosphere, and medium was replaced every 2 to 3 days.

Spheres were dissociated enzymatically once per week into single-cell suspensions for culture maintenance. For this procedure, spheres were transferred from ultra-low adherent dishes to 50-mL conical tubes and were allowed to settle to the tube bottom for 10 minutes at room temperature. The spheres were washed once with 1× PBS without Ca^{2+} or Mg^{2+} and were centrifuged at approximately 240 × *g* for 5 minutes. The cell pellet was resuspended in 1 mL of Accutase solution (Sigma-Aldrich) and incubated for approximately 10 minutes at room temperature with gentle agitation every 2 to 3 minutes. The cells were passed through a 200-μL gel-loading tip (Wheaton UK Ltd., Rochdale, UK) 50 to 100 times to break up the spheres. Cells were checked visually using a hemacytometer and a light microscope at ×10 magnification to ensure dissociation of the spheres.

Images of cells were taken using an Olympus IX71 microscope with an Olympus DP70 cooled digital camera (Leeds Precision Instruments, Golden Valley, MN) and Olympus DP Controller software version 3.3.1 (Olympus

America Inc., Center Valley, PA). Images were converted from RGB to grayscale, and contrast was increased to 15% using Adobe Photoshop CS5 (Adobe Systems Inc., San Jose, CA). No other processing was performed.

Immunophenotyping

The primary antibodies used were anti−CD14-RPE and anti−CD115-RPE [colony-stimulating factor 1 receptor (CSF-1R)] (AbD Serotec, Raleigh, NC); anti−CD51/61-FITC ($\alpha_v\beta_3$ integrin; BD Pharmingen, San Diego, CA); anti−CD117-PE (c-kit) and anti−CD34-PE (eBioscience, San Diego, CA); anti−CD133/AC133-PE (Miltenyi Biotech, Auburn, CA); anti−CD105-FITC (endoglin; Southern Biotech, Birmingham, AL); and anti−CD146-FITC (MCAM; EMD Millipore, Billerica, MA).

For detection of cell surface markers, single-cell suspensions were washed with 200 μL of fluorescence-activated cell sorting staining buffer [PBS containing 2% fetal bovine serum (Atlanta Biologicals, Atlanta, GA) and 2 mmol/L EDTA (Sigma-Aldrich)]. Fc receptors were blocked with normal mouse or rat serum (Jackson ImmunoResearch Laboratories, West Grove, PA) depending on the host species of primary antibody used. Cells were stained with one test volume (according to the manufacturer's recommendations) of primary antibodies for 30 minutes on ice, washed twice in staining buffer, and then fixed with 10% formalin. Fifty thousand events per sample were collected using a BD FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) and then were analyzed using FlowJo software version 10.0.7 (Tree Star Inc., Ashland, OR). To determine the percentage of positive cells, $FL2^+$ regions (<0.5%) were drawn on isotype control plots and then were overlaid onto progenitor marker−stained plots. Isotype controls were used for each of the monolayer and sphere cell populations.

Adipocyte Differentiation Assay

Monolayer or sphere cells (1×10^5) were cultured in control medium or under conditions to induce adipocyte differentiation using a StemPro adipogenesis differentiation kit (Life Technologies). Medium was changed every 3 days. Lipid droplets were visualized as round refractile bodies in the cells after 3 to 6 days. After 10 days of incubation in differentiation medium, cells were fixed with fresh 4.0% paraformaldehyde and were stained with 0.3% Oil Red O in a 60% isopropanol solution (Millipore, Temecula, CA) for 50 minutes.

Phagocytosis Assay

Monolayer and sphere cells were plated in triplicate using 5000 cells per well in 100 μL of culture medium. The next day, fluorescein isothiocyanate−conjugated, rabbit IgG-coated latex beads (Cayman Chemical Co., Ann Arbor, MI) were added to the cells. No beads were added to negative control wells. The emission at 535 nm was measured for each well after 24 hours using a Wallac 1420 VICTOR[2] multilabel counter (PerkinElmer). Relative phagocytosis for each cell line was determined by dividing the emission at 535 nm of the wells with beads by that of the respective negative controls.

Statistical Analysis

All *in vitro* assays, including the progenitor staining and the phagocytosis assay, were performed at least twice, with triplicates in each experiment. Representative results are depicted in this report. Blank values were subtracted from the mean value of each sample where indicated. Data are presented as these adjusted means ± SD. Comparisons between monolayer and spheres were made using a Student's *t*-test. A $P \leq 0.05$ was considered statistically significant.

Results

Genome-Wide Expression Analysis Identifies Three Distinct Molecular Subtypes in Hemangiosarcoma

Using cell lines, we previously demonstrated that inflammation and angiogenesis are important to the pathogenesis of canine hemangiosarcoma.[5] Herein, we profiled intact whole tumors to determine whether we could identify similar patterns and whether additional molecular subtypes could be observed. For this analysis, we used 24 samples obtained from visceral hemangiosarcomas of 20 dogs (12 from spleen, 7 from heart, 4 from liver, and 1 from lung; 2 cases had paired metastases, and 3 sites were examined from a single case); the sample demographic characteristics are shown in Supplemental Table S1. Principal component analysis of the data set using genes with variance >0.5 across all 24 samples identified three distinct subtypes (groups 1 to 3) (Figure 1A). Figure 1B illustrates the unique genes whose overexpression defined each group based on a greater than threefold average increase over the mean with a $P < 0.001$ as determined by analysis of variance with correction for multiple testing. This analysis yielded 1436 differentially expressed genes. Results from Ingenuity Pathway Analysis highlighting enriched functional pathways and upstream activators associated with each group are shown in Supplemental Tables S2, S3, S4, S5, S6, and S7. The groups were characterized by enrichment of genes associated with functions of blood vessel development, angiogenesis, vasculogenesis, endothelial cell development, and migration (group 1, angiogenesis); immune cell differentiation, homeostasis and development, and migration (group 2, inflammation); and lipid and cholesterol transport and fatty acid, cholesterol, and steroid metabolism (group 3, adipogenesis or lipogenesis). The separation of these subtypes was not driven by breed (Figure 1C), location of the primary tumor (Figure 1D), animal of origin (Figure 1E), or morphologic subtype of the tumor (solid or cavernous) (Figure 1F). The average linkage hierarchical clustering

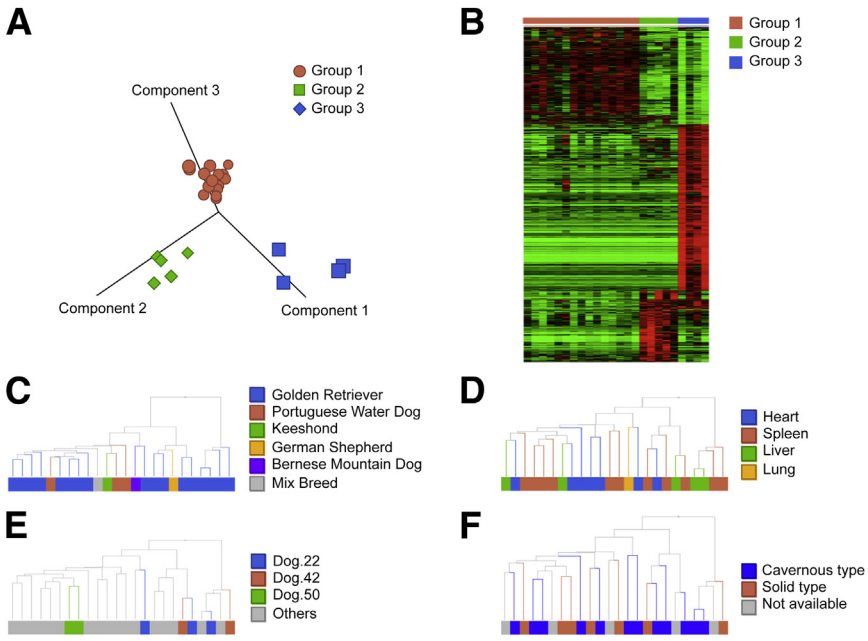

Figure 1 Genome-wide expression analysis identified three molecular subtypes in hemangiosarcoma. **A:** Genes with variance >0.5 across 24 samples were used to generate principal component analyses and hierarchical clustering. Samples were assigned to one of three groups by unsupervised clustering to identify genes with significantly different expression between groups (analysis of variance $P < 0.001$ and an average fold change >3 between groups). **B:** This analysis is shown as a heat map illustrating the 1436 differentially expressed genes among the three groups. Annotation of clustering patterns were not driven by breed (**C**), location of tumor (**D**), animal of origin (**E**), or morphologic features (**F**). Results from Ingenuity Pathway Analysis showing functional pathways and upstream activators associated with these gene signatures are listed in Supplemental Tables S2, S3, S4, S5, S6, and S7.

using positive correlation of all genes from these samples and corresponding to the principal component analysis grouping is shown in Supplemental Figure S1.

We validated the three signatures in 47 samples using RNA-seq analysis, which provided an independent method with greater density of annotation. The demographic features of this cohort are shown in Supplemental Table S1. Unsupervised hierarchical clustering showed a comparable separation of samples into three groups (Supplemental Figure S2A), with 12/12 overlapping samples being assigned to the same group by both analysis methods (Supplemental Figure S2B), suggesting that the molecular identification of canine hemangiosarcoma was robust. Thus, it seems that canine hemangiosarcomas can be segregated into three distinct categories based on molecular genotype rather than histologic evaluation.

These results confirmed earlier findings from cell lines showing that angiogenesis and inflammation are important drivers in the pathogenesis of most of these tumors, but they also highlighted a novel finding that adipogenic or lipogenic pathway genes seem to be highly up-regulated in a smaller subset of tumors. Furthermore, two lines of evidence supported the idea that hemangiosarcomas are derived from common progenitors that can differentiate into one of three possible downstream forms: the lack of an association between gene expression patterns and anatomical tumor location or histologic subtype and the presence of more than one subtype in tumors derived from individual dogs ($n = 3$).

Sphere-Cultured Cells Recapitulate Molecular Pathways from Hemangiosarcoma Tissues

We next tested the idea that tumor heterogeneity might result from the presence of a common progenitor cell with the potential to differentiate down multiple lineage pathways. Sphere formation is a characteristic of normal stem cells and has been used to enrich tumor-initiating cells from cancer cell lines.[15–18] Thus, we used anchorage-independent, serum-free cell culture conditions to generate three-dimensional spheres and enrich for cells with progenitor capabilities. Free-floating, self-renewing spheres were generated from three canine hemangiosarcoma cell lines: SB, Frog, and Emma (Supplemental Figure S3, A–C). Sphere formation was observed within 24 hours in all three lines (Supplemental Figure S3, D–F). Autonomous growth and self-renewing capacity were confirmed in the SB cell line through the persistence of free-floating spheres in culture for more than a year, whereas the phenotypic stability of sphere cells generated from the Frog and Emma cell lines was confirmed over several months.

To investigate the transition of the cell lines into the sphere state, we next analyzed the genome-wide gene expression of adherent (monolayer) cells versus sphere-cultured cells using unsupervised methods as described for the intact tissue samples. The groupings generated from this unbiased approach represent monolayer-cultured cells and sphere-cultured cells, with 79 genes differentially expressed between the two groups based on a greater than twofold average increase over the mean (BH-q < 0.05) (Figure 2A). To establish whether the subtypes identified in intact tumors were represented in the sphere cell population, we performed hierarchical clustering of the 1436 genes profiled on the tumor sample microarray platform against the 79-gene signature.[12] This analysis yielded 34 genes that were significantly expressed between groups 1 to 3 and the sphere cells (Figure 2B). Hierarchical clustering of the 34 genes indicated that the differences shared between groups 1 to 3 and the sphere cells suggest that groups 2 and 3 are more

similar to spheres than to monolayer cultures, although all three groups are represented (Figure 2C). Collectively, these data support the idea that sphere cells grown via anchorage-independent growth conditions generate cell populations that overexpress genes associated with the three differentiated hemangiosarcoma subtypes we identified in dogs. We also interpret the present data to suggest that the hemangiosarcoma subtypes are likely to be derived from a common multipotent cell, and cells with progenitor potential can be enriched in the sphere cells derived from hemangiosarcoma cell lines. If so, sphere cells should show phenotypic and functional characteristics associated with each of the three groups.

Enriched Progenitor Populations from Hemangiosarcoma Cell Lines Display Phenotypic and Functional Characteristics of Angiogenic, Inflammatory, and Adipogenic Tumor Subtypes

Based on the array analyses, we sought to further define the enriched progenitor populations and to determine whether these populations displayed concomitant acquisition of phenotypic and functional properties associated with angiogenesis, myeloid cell differentiation, and adipogenesis. Therefore, we validated enrichment of progenitor cells by first assessing the cell surface expression of markers shared by endothelial and hematopoietic progenitor cells (CD34, CD117, and CD133) in monolayer- and sphere-cultured cells. The percentage of cells expressing CD34, CD117, and CD133 increased in sphere cells from the SB and Frog cell lines within 2 weeks (12.4% versus 2.11% for $CD34^+$; 12.5% versus 4.15% for $CD117^+$; and 17.0% versus 6.16% for $CD133^+$) (Figures 3, A and B). This phenotype was stable for up to 12 months in the SB cell line, and the markers were co-expressed by the same cells (Figure 3, C and D), supporting the notion that hemangiosarcomas may develop from a common progenitor.

We then examined the expression of surface markers associated with endothelial differentiation (CD105, CD146, and $\alpha_v\beta_3$ integrin) because these would represent cellular functions consistent with the genotype displayed by the vascular or angiogenic group 1 tumors. Monolayer and sphere cells from all three lines showed stable expression of CD105 and $\alpha_v\beta_3$ integrin (Figure 4). The expression of CD146 was variable, with high levels of expression in SB monolayer and sphere cells and low to moderate expression in Frog monolayer cells (Figure 4). None of the cells expressed CD90 (Thy-1), a marker associated with MSCs and T cells (data not shown).

Next we looked for cellular phenotypes associated with myeloid cell differentiation and/or inflammation (group 2). As noted in Figure 3, A and B, expression of CD34 and CD117 was higher in sphere cells than in monolayers from the SB and Frog cell lines. Both of these molecules are normally expressed by bone marrow–derived myeloid

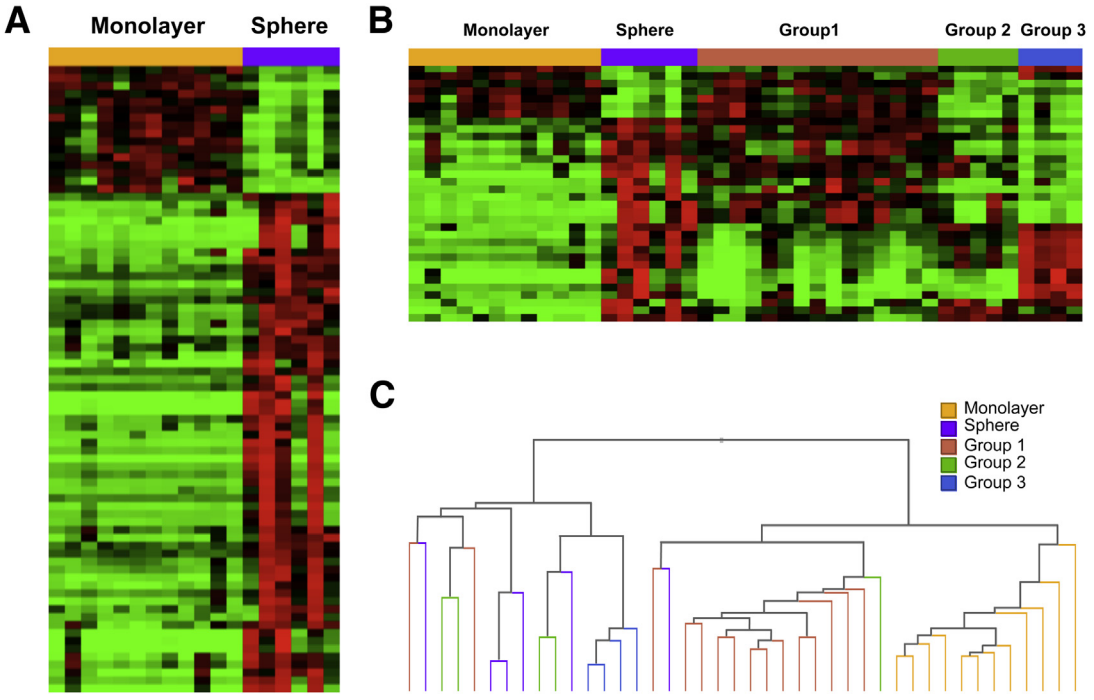

Figure 2 Confirmation of the three molecular phenotypes of hemangiosarcoma in sphere-cultured cells. **A:** Unsupervised hierarchical clustering separates hemangiosarcoma cell line samples into monolayer- and sphere-cultured cells, and a heat map shows that the 79 genes were differentially expressed (average linkage; BH-q < 0.05; fold change > 2). **B:** Heat map of 34 gene expression patterns significant in both the comparisons between the 1436 genes differently expressed in intact tumors and the 79 genes differently expressed by spheres. **C:** Hierarchical clustering of genes is significant to the comparison made in **B**. For **B** and **C**, each data set was independently mean centered.

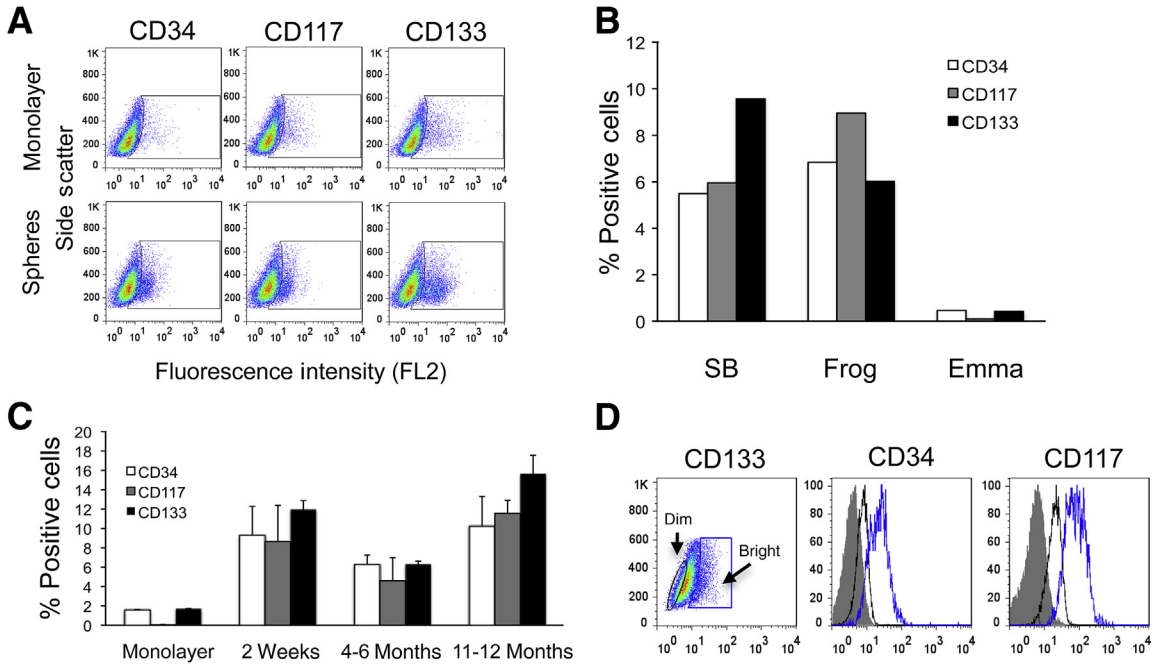

Figure 3 Sphere cells expressed markers for endothelial and hematopoietic progenitors. **A:** Flow cytometric analysis of expression of CD34, CD117, and CD133. **B:** Graphical representation of the percentage of positive cells for each marker in the monolayer cells subtracted from the percentage of positive cells detected in the corresponding sphere cells. **C:** Expression of CD34, CD117, and CD133 as examined in SB sphere cells sustained under nonadherent growth conditions for 2 weeks, 4 to 6 months, or 11 to 12 months. Data shown are the means ± SD of three experiments. **D:** Co-expression of CD133 molecules with CD34 and CD117.

progenitor cells, as are CD14 (a component of the lipopolysaccharide receptor) and CD115 (CSF-1R). The latter two surface proteins were detectable at low levels in SB monolayer cells but not in Frog monolayer cells. However, the proportion of cells expressing both molecules was increased in SB and Frog sphere cells (Figure 5A). Expression of CD14 and CD115 was not observed in the Emma cell line under either culture condition. To increase confidence that the hemangiosarcoma sphere cells had retained or acquired myeloid properties, we determined their

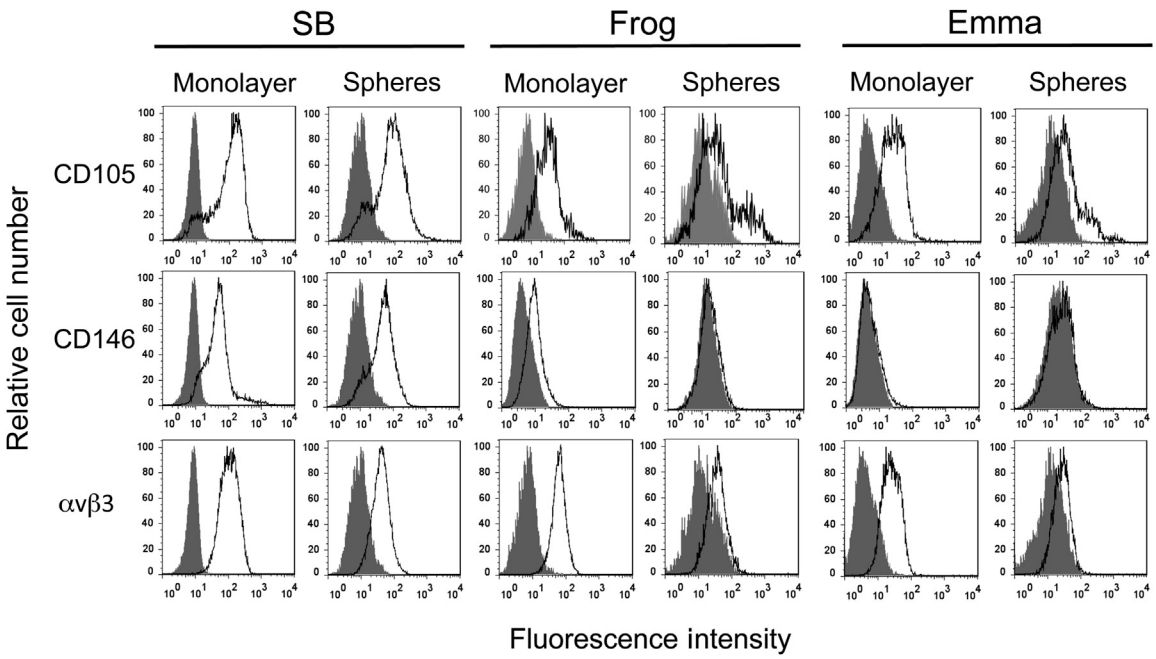

Figure 4 Endothelial (angiogenesis, group 1) markers were expressed by monolayer and sphere cells. Assessment of progenitor and lineage marker expression through flow cytometric analysis of surface expression of CD105, CD146, and $\alpha_v\beta_3$ on monolayer and single-cell suspensions of spheres. Isotype controls or secondary controls, where applicable, are represented as shaded regions.

capacity for phagocytosis. SB sphere cells showed an approximately fivefold increase in phagocytic activity compared with their monolayer counterparts, reaching a level that was comparable with that observed in the mouse macrophage cell line RAW264.7. An even more dramatic enhancement of phagocytic activity (approximately 20-fold) was observed in the Frog sphere cells (Figure 5B). A modest increase also was noted in the Emma sphere cells; however, the phagocytic activity in these cells already was high compared with control RAW264.7 cells (Figure 5B). Taken together, these results suggest that progenitor cells in the hemangiosarcoma population have the potential to differentiate along the myeloid pathway.

Finally, adipogenic/lipogenic potential has not been previously reported in hemangiosarcoma, although progenitor cells from infantile hemangiomas have been shown to undergo endothelial as well as adipogenic differentiation.[19] Lipid droplets were apparent in control human bone marrow–derived MSCs after 3 to 6 days in differentiation medium (Figure 6A), but droplets were not present in MSCs grown under nondifferentiating cell culture conditions (Figure 6B). SB sphere cells elicited lipid after 6 to 9 days in differentiation medium (Figure 6C), whereas SB monolayer cells did not show adipogenic potential (Figure 6D). Similarly, lipid droplets were evident in Frog sphere cells in the sphere structure and around the sphere base, where cells attached to the substrate in the differentiation assay (Figure 6E), but the Frog monolayer cells did not undergo adipogenic differentiation (Figure 6F). Emma sphere cells showed accumulation of lipid in a pattern that was similar to that observed for Frog sphere cells (data not shown), suggesting that hemangiosarcoma sphere cells possess adipogenic

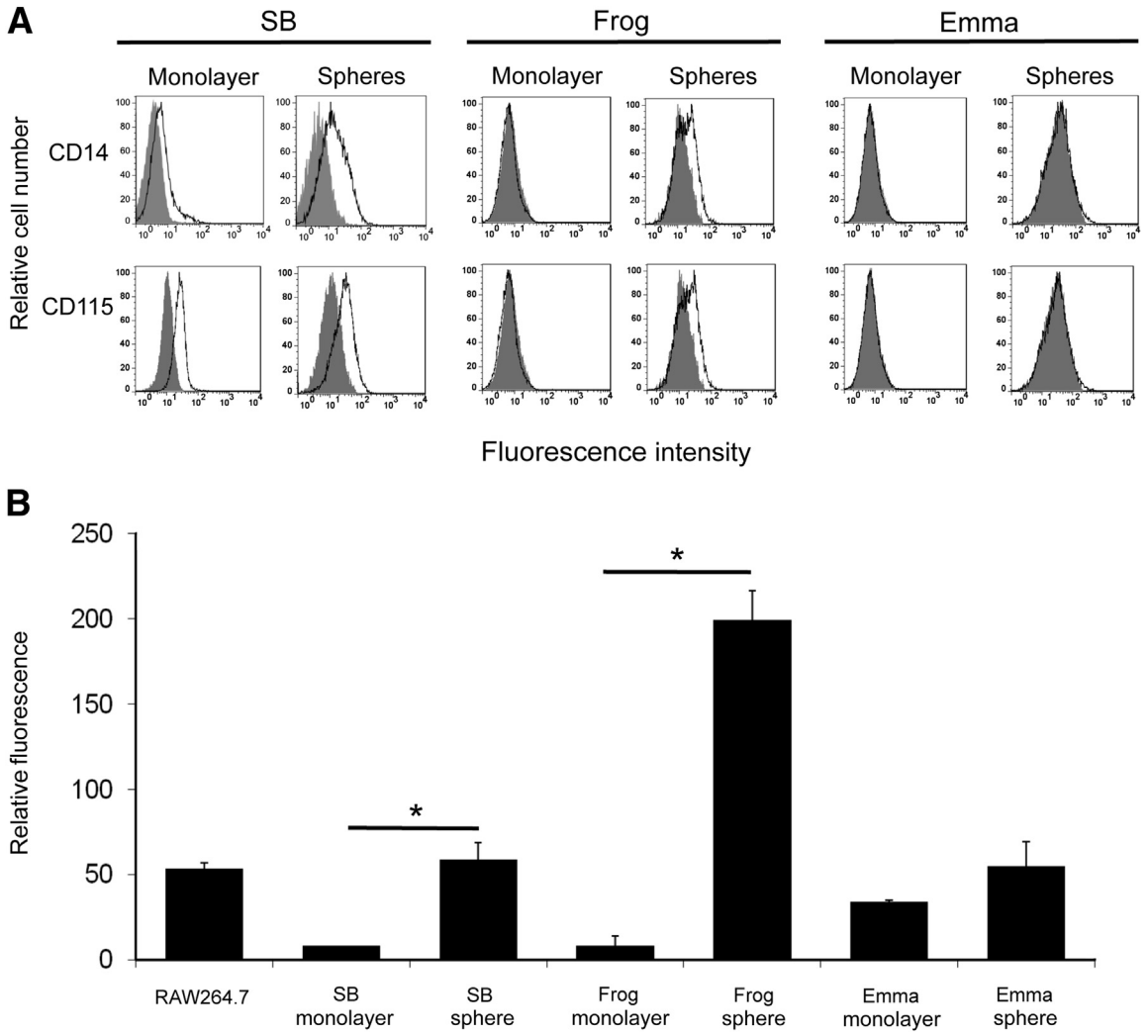

Figure 5 Sphere cells exhibited properties of myeloid progenitors (inflammation/myeloid, group 2). **A:** Cell surface expression of determinant myeloid cell markers CD14 and CD115 (CSF-1R) by monolayer and sphere cell populations. Data shown are representative of three experiments. **B:** Phagocytosis of fluorescein isothiocyanate–conjugated, IgG-coated latex beads by monolayer and sphere cells. Phagocytosis is represented as relative fluorescence intensity by each cell line. The macrophage cell line RAW246.7 was used as a positive control. Data shown are means ± SD representative of at least two independent experiments. The increased phagocytic activity in the SB and Frog sphere cells was significant compared with the activity detected in monolayer cells. $^{*}P \leq 0.05$.

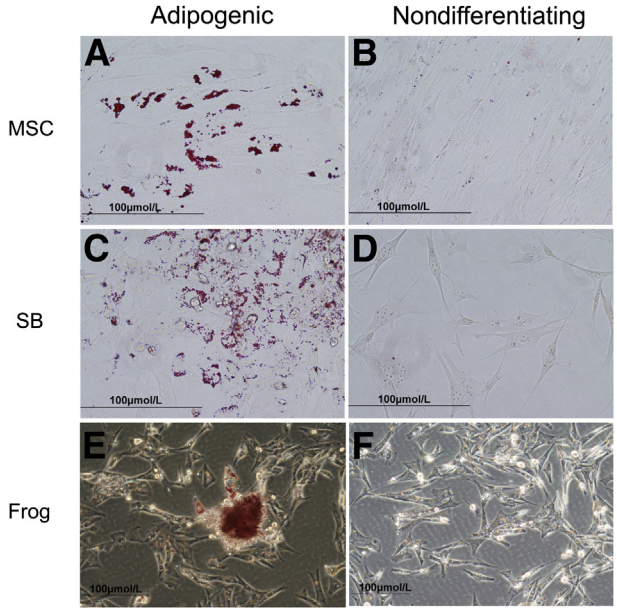

Figure 6 Hemangiosarcoma sphere cells underwent adipogenesis (adipogenesis/lipogenesis, group 3) under differential culture conditions. Human bone marrow–derived MSCs (**A**) and canine hemangiosarcoma SB (**C**) and Frog (**E**) sphere cells were grown in adipogenic medium and were stained with Oil Red O. MSCs (**B**) and SB (**D**) and Frog (**F**) sphere cells were grown in nondifferentiating cell culture medium as controls and were stained with Oil Red O.

properties and/or the capacity to differentiate along an adipogenic pathway. These data provide further support for the idea that nonadherent sphere cells have multipotent progenitor potential because they were able to differentiate into cells that acquired phenotypic and functional characteristics of each of the three hemangiosarcoma subtypes present in dogs.

Discussion

Canine hemangiosarcoma has been classified historically as a tumor of malignant endothelium. We previously proposed that hemangiosarcomas might arise from bone marrow–derived angioblastic progenitors.[4,5] Herein, we expanded this line of investigation by performing an unbiased genome-wide analysis of gene expression in primary tumor samples from spontaneously arising canine tumors, combined with analysis of enriched progenitor cells from hemangiosarcoma cell lines. We identified three ontogenetic subtypes in canine tumors, and these subtypes were reflected phenotypically and functionally in the progenitor populations enriched from hemangiosarcoma cell lines. This suggests that the subtypes are at least partly attributable to the tumor cells themselves and indicate that hemangiosarcomas might arise from cells with multipotency.[4,5] This idea is supported by the co-expression of early endothelial and hematopoietic progenitor cell markers by a subset in the sphere cell population. However, these data should be interpreted cautiously because it is possible that hemangiosarcomas arise from a mix of progenitor cells that each have the capability to undergo lineage-specific differentiation and contribute to tumor progression. Studies using cell sorting of lineage markers or single-cell isolation followed by clonal expansion would distinguish between these two possibilities, but we have not yet undertaken this approach.

Although we do not consider the latter scenario to be likely, the importance of endothelial and myeloid interactions for tumor growth is becoming more apparent. For example, He et al[20] demonstrated that mature mouse endothelial cells provide critical signals for the selective growth and differentiation of macrophages from hematopoietic progenitors. Endothelial-derived CSF-1 was found to be a vital signal for macrophage expansion and survival because blockade of macrophage CSF-1R (CD115) impaired further growth and limited angiogenesis. Thus, the CSF-1R–positive cells found in the sphere cell population might represent differentiated hematopoietic cells that interact with mature endothelial cells to promote angiogenesis and, therefore, contribute to hemangiosarcoma progression. In addition, the classical cell markers for endothelial and myeloid lineages may not adequately inform cell function. Yoder et al[21] described a population of human myeloid cells that co-express hematopoietic and endothelial markers. These cells possessed myeloid progenitor cell activity and differentiated into phagocytic macrophages but failed to form perfused vessels *in vivo*. This myeloid population could be identified by CD45 expression, distinguishing them from a defined population of endothelial cell progenitors. We previously showed that hemangiosarcoma cell lines can show expression of CD45,[4] but rigorous clonogenic and functional assays are needed to validate the identity of the putative malignant hemangioblastic cell(s) in the hemangiosarcoma sphere populations.

In contrast to the myeloid populations described by He et al[20] and Yoder et al,[21] Bailey et al[22] showed that mouse myeloid progenitors give rise to vascular endothelium. Herein, vascular endothelial cells were able to differentiate from common myeloid progenitors and granulocyte/macrophage progenitors, indicating that endothelial cells are an intrinsic component of myeloid-lineage differentiation and underlining the close functional relationship between the vascular and hematopoietic systems. Ritter et al[23] reported co-expression of the dendritic markers CD83 and CD14 along with endothelial cell markers in human infantile hemangioma. Because infantile hemangioma is a clonal disease arising from a single progenitor cell,[23–25] the myeloid-endothelial cell co-expression might represent a step in the myeloid to endothelial cell transition. Further studies are needed to confirm such a mechanism in hemangiosarcoma, including marker co-expression studies and the ability of isolated cell populations to participate in blood vessel formation. Hemangiomas in adults may arise from early endothelial progenitor cells or differentiated endothelial cells,[26] indicating the potential of benign vascular

tumors to arise through multiple mechanisms. Finally, we must bear in mind that at least part of the inflammatory signature observed in the tumor specimens is due to the presence of infiltrating inflammatory cells in the tumor microenvironment. Infiltrating immune cells are a common feature of hemangiosarcoma,[5] and these cells likely contribute to the overall signature. However, we previously demonstrated that an inflammatory signature is intrinsic to hemangiosarcoma cell lines,[5] and this finding is further supported in the present work.

Hemangiosarcomas also arise in mice and humans,[3] and the pathologic appearance of the tumors across species is similar. Recent immunohistochemical analysis of mouse hemangiosarcomas indicated that tumors arise from endothelial progenitor cells expressing CD34, vascular endothelial growth receptor 2, and CD31.[27] In humans, Liu et al[26] determined that hematopoietic stem cells or early endothelial progenitor cells expressing CD34, CD45, and CD117 are involved in tumor formation. CD133 detection was negative in almost all cases, as was staining for CD14. However, as indicated by the authors, CD133 is an early marker for progenitor or initiating cells, and the expression levels may be below the limit of detection by immunohistochemical analysis after cellular differentiation or due to tumor heterogeneity. These results indicate that animal models contribute different aspects to our understanding of vascular sarcomas and that studies in dogs may be especially relevant to understanding the pathogenesis of the human disease. Studies using an extensive panel of early and more differentiated hematopoietic and endothelial markers should be used, and methods to detect rare, early, and mid-progenitor (eg, CD115) cell expression events could be used to more precisely define cellular similarities and differences across species.

To our knowledge, the identification of adipogenesis in hemangiosarcoma development has not been described previously. An adipogenic/lipogenic phenotype might represent a natural differentiation end point of cells that give rise to hemangiosarcoma. In the context of vascular tumors, benign hemangiomas progress through i) endothelial proliferation without defined vasculature; ii) involution, where blood vessel formation becomes defined; and iii) replacement of blood vessels with a fibrofatty residuum, which often leads to spontaneous regression of the tumor. Using CD133-selected and clonally expanded cells from human hemangiomas, Khan et al[19] demonstrated endothelial differentiation of these cells *in vivo* in the form of neovasculature followed by differentiation into adipocytes. The blood vessels and adipocytes were derived from transplanted cells and not from the host (mouse), indicating differentiation of the CD133 progenitors down endothelial and adipogenic lineages. The present results are consistent with the possibility that hemangiosarcoma progenitors undergo adipogenic differentiation, and although it is not clear what triggers adipogenesis in infantile hemangioma, we believe that an epigenetic program, ie, influenced by the microenvironment niche (cell-cell interactions, cytokines, energy, and oxygen), might direct cells toward endothelial and/or adipogenic differentiation.

In addition to bone marrow–derived angioblasts or MSCs, transformation of adipose-derived stem cells (ADSCs) could produce the phenotypes we observed in hemangiosarcoma. Chen et al[28] recently demonstrated that several types of soft-tissue sarcomas originate from malignantly transformed ADSCs and that local hypoxic responses lead to chronic inflammation in adipose tissue.[29] The latter would favor differentiation of ADSCs into multiple lineages, including adipocytes, endothelial cells, and pericytes.[30] In this case, intrinsic or extrinsic inflammation could favor ADSC differentiation toward an angiogenic phenotype and inhibit adipogenic differentiation in tumors. Finally, we must consider that the adipogenic/lipogenic signature in hemangiosarcomas could represent an altered metabolic process or an adaptation to the energetic needs of the tumor.

In summary, these data suggest that hemangiosarcomas exist as different molecular subtypes based on cellular or metabolic processes that favor angiogenesis, inflammation, and adipogenesis. The capacity to generate these phenotypes is retained in a population of progenitor-like cells, ie, enriched when hemangiosarcoma cell lines are cultured as nonadherent spheres. These tumors might arise from single multipotent progenitors that transit through a myeloid/endothelial intermediate and can differentiate down multiple lineages as part of an adaptive process (eg, response to hypoxia and inflammation in the tumor niche), culminating in any of the observed myeloid, endothelial, or adipogenic tumor phenotypes. Alternatively, multiple progenitor lineages might contribute to tumor formation, with one progenitor giving rise to endothelial-like and adipogenic-like cells and another progenitor giving rise to myeloid-like cells that perpetuate the tumor but that are not involved in vascular formation. These two scenarios are not mutually exclusive, and either could explain the heterogeneity associated with hemangiosarcoma and the prominent vascular morphology that characterizes these tumors. However, the observation that metastatic pairs or triads from individual animals showed multiple distinct phenotypes is most consistent with the first possibility. The capability to reproduce the tumor subtypes using enriched progenitor populations from hemangiosarcoma cell lines offers a unique model to study the development and progression of hemangiosarcoma as a means to improve prognosis, prediction, and response to therapy in these tumors.

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Supplemental Data

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