Transcriptional and Functional Activity of Canine Hemangiosarcoma to Support Hematopoiesis Demonstrate Bone Marrow Nurse Cell Ontogeny

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Running Title: Bone marrow nurse cell ontogeny of hemangiosarcoma

Key Points:

- Canine hemangiosarcoma supports expansion and differentiation of human hematopoietic progenitors, enabling development of hematopoietic tumors.
- Molecular programs of canine hemangiosarcoma that support hematopoiesis and create a tumor immune niche resemble those of bone marrow nurse cells.

Abstract

Hemangiosarcoma and angiosarcoma are soft tissue sarcomas of malignant blood vessel-forming cells in dogs and humans, respectively. These vasoformative sarcomas are aggressive and highly metastatic, with disorganized, irregular blood-filled vascular spaces. Our objective was to define molecular programs that support the niche, enabling progression of canine hemangiosarcoma and human angiosarcoma. Here, we show that the transcriptional landscape of canine hemangiosarcoma and human angiosarcoma included comparable angiogenic and inflammatory programs. Dog-in-mouse hemangiosarcoma xenografts recapitulated the vasoformative and highly angiogenic morphology and molecular characteristics of primary tumors. Blood vessels in the tumors were complex and disorganized, and they were lined by both donor and host cells, a trait that was not observed in xenografts from canine osteosarcoma and lymphoma. In some cases, the xenografted hemangiosarcoma cells created exuberant myeloid hyperplasia and gave rise to lymphoproliferative tumors of mouse origin. We did not uncover a definitive transmissible etiology, but our data indicate that transcriptional programs of hemangiosarcoma cells resemble those of hematopoietic nurse cells, and these malignant cells support expansion and differentiation of human hematopoietic progenitors. We conclude that canine hemangiosarcoma, and possibly human angiosarcoma, originate from nurse cells that make up the stromal bone marrow niche, and that these cells may also support the growth of hematopoietic tumors.

Keywords: angiosarcoma, bone marrow, hemangiosarcoma, hematopoiesis, tumor immunity, tumor niche, tumor ontogeny.

Introduction

Canine hemangiosarcoma is a vasoformative tumor originating from bone marrow (BM)-derived progenitor cells.[1-4] Unlike human angiosarcoma, which is a rare disease, spontaneous hemangiosarcoma occurs commonly in dogs. The histology and natural history of these tumors is similar in both species: both are comprised of disorganized, tortuous, dilated blood vessels with moderate to high proliferative activity and extremely high metastatic potential.[5,6] Furthermore, canine hemangiosarcoma and human angiosarcoma appear to establish convergent molecular programs despite their genomic complexity.[7]

Angiogenesis and inflammation are key molecular features of canine hemangiosarcoma,[8] and convergent transcriptional programs regulating these processes are also observed in human angiosarcoma.[7] These molecular programs are the foundation for a novel subclassification of canine hemangiosarcoma.[4] However, the contribution of the tumor microenvironment to these programs remains incompletely understood. The microenvironment creates the tumor niche, a complex of cellular and non-cellular components that are essential for tumor cell survival, disease progression, and metastasis.[9] Emerging data suggest that cancer cells re-educate niche cells; and conversely, niche cells modulate the function of cancer cells.[9-11] This effect is especially important for sustaining stemness and self-renewal of cancer stem cells in both solid and hematopoietic tumors.[12-14]

The hematopoietic niche consists of osteoblasts, and various stromal nurse cells, including endosteal endothelial cells, fibroblasts, sinusoidal nestin-positive mesenchymal stromal cells, sinusoidal leptin receptor-positive stromal cells, and CXCL12-abundant reticular (CAR) cells.

Together, these cells support hematopoietic stem and progenitor cells (HSPCs) to proliferate and differentiate into lineage-committed cells, promoting hematopoiesis.[15] Impairment of the regulation of HSPCs can cause numerous blood disorders,[16,17] as well as hematopoietic malignancies such as leukemia and lymphoma.[18-20]

Our goal was to define the molecular origin of canine hemangiosarcoma and human angiosarcoma. Our data indicate that components of the transcriptional programs in these tumors resemble those of bone marrow nurse cells, and that in addition to their capability to form vasoformative tumors, canine hemangiosarcoma cells create a niche for expansion and differentiation of blood cells.

Methods

Canine samples and cell lines

HSA cell lines (SB, COSB, Emma, DD1, JHE, and JLU) were previously established.[1,2,21-24] Canine tissue samples were obtained from surgical removal or biopsy of tumor at the University of Minnesota or private veterinary clinics, and additional HSA cell lines (DHSA-1401, DHSA-1420, and DHSA-1426) were generated and cultured as described previously.[1,2,22] All protocols and procedures for sample procurement were reviewed by the Institutional Animal Care and Use Committee (IACUC; protocols 0802A27363, 1101A94713, 1312-31131A) of the University of Minnesota.

Human and mouse cell lines

Human BM-derived mesenchymal stromal cells (hBM-MSCs) were isolated from whole bone

marrow purchased from AllCells (Emeryville, CA, USA) as previously described.[25-28] M2-10B4 murine bone marrow stromal cells were purchased from the ATCC (Manassas, VA, USA) and maintained as previously described.[26] Human umbilical cord blood (hUCB) samples were obtained from the University of Colorado Cord Blood Bank, ClinImmune Stem Cell Laboratory.[29]

Mice and xenotransplantation

Female NOD-Scid-Gamma (NSG) and Beige-Nude-Xid (BNX) mice were used for adult xenografts. Both male and female NSG mice were used for neonatal xenografts. All mice were housed and handled according to the Research Animal Resources (RAR) husbandry and care protocols. Procedures for breeding animals and for tumor implantation were reviewed by the IACUC of the University of Minnesota (protocols 1006A84813, 1106A00649, 1306-30712A, and 1311-31104A). We performed xenotransplantations in a total of 132 mice as detailed in **Table S1**.

First, we injected cultured-tumor cells from three HSA cell lines (SB, Emma, and JHE) in 150 µL of PBS into irradiated (200gy) NSG mice (N=20): 5 X 10^6 SB cells were injected into the subcutaneous (SQ) space of four mice and 5 or 10 X 10^6 SB cells were injected intraperitoneally (IP) into four mice; 2 X 10^6 Emma cells were injected into four mice each by the SQ and IP routes; and 3 X10^5 JHE cells were injected into four mice by the SQ route. Second, we injected tumor cells from five HSA cell lines (Emma, DD1, JLU, DHSA-1401, and COSB) into NSG neonates (1 or 2 days after birth; N=52): 5 X 10^5 Emma, DD1, JLU, or DHSA-1401 cells, or 6.25 X 10^5 COSB cells were injected IP in 50 µL of PBS. Third, we injected 5 X 10^6 cells from three HSA cell lines (JLU, DHSA-1420, and DHSA-1426) in a mixture of 100 µL of PBS and

100 µL of BD Matrigel™ Basement Membrane Matrix (Growth Factor Reduced; BD Biosciences, Bedford, MA) into the SQ space of BNX mice (N=12). For DHSA-1426, we injected tumor cells from passage-5 and passage-14, independently. We used BNX mice for patient-derived tumor xenograft. Sections of viable tumor from four dogs affected with HSA (DHSA-1413, DHSA-1416, DHSA-1420, and DHSA-1426) were each dissected and implanted into subcutaneous pockets of four mice (N=16). Sections of non-HSA splenic tissues from seven dogs were similarly implanted into 18 mice as controls. The tissues included sections from splenic hematomas (DHSA-1412, DHSA-1417, DHSA-1419, DHSA-1421, and DHSA-1430). Finally, after visible tumors developed in mice, we serially transplanted the tumors by inoculation of cultured tumor cells (N=3 mice); or by direct implantation of single cell suspensions of the tumor (N=8 mice). Mice were sacrificed when they reached a tumor endpoint, including a mass measuring 1.5 cm in the longest diameter, or at the end of a 16-week period after xenotransplantation.

Statistical analysis

Mann-Whitney U test or Welch's (Heteroscedastic) t-test were performed to determine differences of continuous values between two groups. Pearson's correlation coefficient was calculated for correlation between two variables. Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software, Inc., San Diego, CA, USA) or Microsoft Excel. Kaplan-Meier survival analysis and log rank test were performed for survival difference using R programming. P-values are presented without inference of significance, consistent with the American Statistical Association's Statement on P-Values.[30]

Detailed and additional materials and methods are provided in the supplemental Methods.

Results

Canine hemangiosarcoma and human angiosarcoma show parallel transcriptional programs and patterns of immune cell infiltration

Canine hemangiosarcomas can be classified into angiogenic, inflammatory, and adipogenic subtypes based on their transcriptional programs (**Figure S1A**).[4,7] This separation is prognostically significant: of the 43 dogs in this cohort for which we had treatment and outcome data, those with inflammatory hemangiosarcomas accounted for nearly all that showed prolonged survival (>150 days, **Figure S1B**). Intriguingly, the subset of dogs whose hemangiosarcomas had a predominant angiogenic phenotype could be further subdivided into a group with a "pure angiogenic" molecular signature and a group with a "mixed angiogenic and inflammatory" molecular signature. Of these, the dogs whose tumors were purely angiogenic (*i.e.*, had deficient inflammatory signatures), showed the shortest overall survival times (**Figure S1B**).

Consistent with previously reports,[31,32] human angiosarcomas showed highly enriched angiogenic signatures.[7] However, the presence of inflammatory signatures in this tumor has not been thoroughly examined. Using a defined methodology to compare transcriptional signatures in tumors from different species,[33] we identified 588 differentially expressed genes in canine angiogenic and inflammatory hemangiosarcomas and evaluated the expression of their homologs in human angiosarcomas. **Figure S1C** shows that comparable angiogenic, inflammatory, and mixed angiogenic/inflammatory gene expression signatures were present in canine hemangiosarcomas and in human angiosarcomas.

We then used bioinformatics approaches and immunostaining to establish whether the inflammatory gene signatures arose from the malignant cells themselves[4] or from inflammatory cell infiltrates within the tumors. ESTIMATE was used to assign tumor purity, and xCell was used to assign immune scores to each tumor: both tools resulted in consistent scores (**Figure S2A and B**). Predictably, the immune score for canine samples was correlated with the predominant transcriptional phenotype for the tumor; *i.e.*, angiogenic tumors had low immune scores and inflammatory tumors had high immune scores (**Figure S2C and D**). There was a similar correlation between transcriptional signatures and immune scores in human angiosarcomas (**Figure S2E and F**). To further verify that these were the result of immune and inflammatory cells present in the tumors, we stained sections from 11 canine hemangiosarcomas and 10 human angiosarcomas with antibodies against T cells (CD3), B cells (Pax5), myeloid cells (Mac387) and macrophages (Iba1 for canine; CD163 for human) (**Figure 1A and B**). CD3+ T cells, PAX5+ B cells, MAC387+ myeloid cells and Iba1+ or CD163+ macrophages were detectable in both canine and human tumors. Myeloid cells were found most abundantly, while T cells ranged from rare to abundant and B cells were infrequent, and when they were present, the inflammatory cells were diffusely distributed throughout the tumor tissue. There was a direct correlation between xCell immune scores and immunohistochemistry scores for both the canine (Spearman R=0.38; P = 0.255) and human (Spearman R=0.78; P = 0.011) samples examined (**Figure 1C and D**).

We next segregated human angiosarcomas into tumors with high and low immune scores ("immune-high" vs "immune-low") and identified 461 up-regulated genes (FDR P < 0.05) in the

immune-high group compared to immune-low group (**Figure 1E)**. Fifty-eight of these genes were also found in canine inflammatory hemangiosarcomas, and they were associated with T- and B-cell activation (**Figure 1F and G**). The similarity in tissue organization and infiltrating patterns of immune cells between the canine and human samples was striking, yet we were unable to confirm the prognostic value of the immune scores in human angiosarcoma because this sample set was not annotated with outcome data.

Canine hemangiosarcoma cells can recapitulate the disease *in vivo*

We used *in vivo* xenografts to examine the significance of the angiogenic and inflammatory phenotypes in tumor biological behavior.[21,24,34-36] We inoculated mice with canine hemangiosarcoma cell lines or primary tissues as described in Table S1. The engraftment efficiency of canine hemangiosarcoma xenografts was low: in these experiments, only cultured DHSA-1426 cells injected subcutaneously were able to reproducibly generate vasoformative tumors in immunodeficient BNX mice (**Figure 2**), and the tumorigenic potential of this cell line was maintained over multiple passages. These tumors could be serially passaged from cells cultured out of the tumor xenografts and injected into new recipient mice (**Figure S3**).

Xenografts enable quantification of the stromal contribution to the hemangiosarcoma microenvironment

To establish the contribution of stromal elements to the formation of canine hemangiosarcoma, we used fluorescence *in situ* hybridization (FISH) to enumerate canine and mouse cells in the tumors. We selected probes for canine *CXCL8*, since this gene is absent from the mouse genome, and for a unique region of the mouse X chromosome, to identify canine and mouse cells,

respectively. The data show that the hemangiosarcoma xenografts achieved a complex topological organization, with blood vessels lined concomitantly by donor and host cells (**Figure 3A**). The pattern of organization seen in canine hemangiosarcoma xenografts was distinct from that of canine osteosarcoma and canine lymphoma xenografts (**Figure 3B**). The hemangiosarcoma tumors were comprised of a mixture of 50-70% malignant canine cells and 30-50% mouse stromal cells. The cellular composition of orthotopic osteosarcoma xenografts was similar, but none of the malignant canine cells were seen lining blood vessels. The composition of lymphoma xenografts was remarkably different, including fewer than 5% mouse stromal cells. Malignant canine cells were also absent from the blood vessels in these tumors (**Figure 3C)**.

To establish the contribution of stromal elements to the gene expression signatures of canine hemangiosarcoma, we used a bioinformatics method that aggregates and then segregates transcripts by species[37] and identified 459 differentially expressed mouse (stromal) genes in the tumor xenografts (**Table S2**). We name-mapped these genes to the canine genome and examined their expression across all of the 76 samples of primary and metastatic canine hemangiosarcoma tumors. We then selected overlapping genes between the 1,477 subtype-specific genes that were differentially expressed across the three canine hemangiosarcoma subtypes and the 371 mouse-defined stromal genes (**Figure S4A**). This generated a list of 56 genes, whose expression was also examined across the primary and metastatic canine hemangiosarcoma samples **(Figure S4B)**. Strikingly, the results from both analyses showed that the separation of tumors according to their molecular phenotype was retained in its entirety. It suggests that the transcriptional programs of tumor stroma are distinct among different types of hemangiosarcomas, and that

stromal elements are necessary and sufficient to define these different tumor types. However, our experiments do not exclude the possibility that "tumor-education" is responsible for the distinct patterns of gene expression in stromal elements.

Canine hemangiosarcoma cells can induce expansion of hematopoietic cells as well as lymphoma cells *in vivo*

An unexpected series of results helped to inform the relationship between hemangiosarcoma and the presence of inflammation in the microenvironment. We and others showed previously that canine hemangiosarcoma cells could form hemangiosarcomas in NSG mice.[7,21,24,35,36] We also observed that some of the NSG mice inoculated with SB-HSA cells or with another cell line called Emma-brain (EFB), and some BNX mice inoculated with DHSA-1426 canine patient-derived tumor fragments developed what appeared to be round cell tumors. Four mice that received 5 x 10^6 SB-HSA cells intraperitoneally and one of four mice that received 2 x 10^6 EFB cells subcutaneously died acutely two weeks after inoculation. The mice showed evidence of anemia and splenomegaly. The spleens in all five mice were expanded by monomorphic populations of hematopoietic cells (**Figure S5A and B**). Upon further analysis, the cells were determined to be of mouse origin, representing erythroid progenitors (Ter-119^+), with few canine hemangiosarcoma cells admixed in the population (**Figure S5C - F**). We were unable to definitively establish that these cells had undergone malignant transformation.

Three of four mice that received DHSA-1426 tumor fragments (1st-generation canine patient - derived xenograft or CPDX) formed apparent xenograft tumors in multiple organs including spleen, lymph nodes, meninges, cerebrum, and mesentery 12 weeks after implantation. However,

the cellular morphology of these tumors was consistent with that of round cell tumors (**Figure S6A and B)**. We determined that the tumor cells expressed CD45 of mouse origin, B220, and Pax5, without expression of CD3, Ter-119, and MPO, indicating a mouse B-cell origin (**Figure S6C - H**). We verified the mouse origin using flow cytometry based on the tumor cells staining with an antibody directed against mouse CD45, but not with antibodies directed against canine CD45 and human $\alpha V\beta_3$-integrin, which cross reacts with canine, but not with mouse $\alpha V\beta_3$-integrin (**Figure S7**). These B-cells could be serially passaged, forming B-cell lymphomas in recipient BNX mice without the need for supporting canine hemangiosarcoma cells (**Figure S8A**). Intriguingly, we observed a similar result when we inoculated single cell suspensions derived from fresh tumor fragments of canine hemangiosarcoma xenografts into BNX recipients (2nd generation CPDX from a 1st generation cell line tumor). The resulting tumors had similar morphology and were also of mouse B-cell origin and could be serially passaged in BNX mice (**Figure S8B**).

Canine hemangiosarcoma supports expansion and differentiation of CD34+ human umbilical cord blood cells

The potential etiology of these tumors was perplexing. We first examined whether the tumors might be caused by horizontal gene transfer. We used the method described above (in **Figure 3** and **Figure S4**) to identify species-specific transcripts.[37] Canine DHSA-1426 cells expressed only canine genes, whereas canine hemangiosarcoma xenograft tumors expressed a mixture of canine and mouse genes (**Figure S9)**. In contrast, the genes expressed by the B-cell lymphomas arising from the hemangiosarcoma xenograft were almost exclusively of mouse origin.

Next, we sequenced viral RNA from the xenografts and used PathSeq platform to evaluate the potential for a transmissible, infectious agent as the cause of these tumors. Notably, no bacterial or viral sequences with tumorigenic potential were identified in the xenografts as well as in the primary or metastatic canine hemangiosarcoma tumor samples or cell lines. A recent study reported an association between *Bartonella spp* and canine hemangiosarcoma.[38] Ten of 24 dogs tested in our study had detectable *Bartonella spp* sequences, although these were present in low abundance (**Table S3**). Furthermore, four of the samples contained only sequences from *B. bacilliformis, B. grahamii*, or *B. tribocorum*, which infect humans and rats, respectively, and none of which is known to infect dogs as a primary or accidental host.[39,40] Three of the remaining six dogs had sequences for *B. clarridgeiae* and three had sequences for multiple *Bartonella spp.*, including the human and rodent-specific types. Together, the low abundance and the presence of sequences from organisms that do not infect dogs suggest that the *Bartonella spp*. sequences might have been contaminants.

On the other hand, murine leukemia virus (MuLV) reads were reproducibly identified in the mouse B-cell lymphomas arising from the hemangiosarcoma xenografts (**Table S4**). The presence of MuLV sequences was further confirmed in one mouse B-cell lymphoma arising from the hemangiosarcoma xenografts and in two subcutaneous canine hemangiosarcoma xenograft tumors through independent viral RNA sequencing experiments. Moreover, MuLV sequences could be amplified by PCR from normal mouse tissues (liver and spleen), from each of three mouse B-cell lymphomas arising from the hemangiosarcoma xenografts, and from two subcutaneous canine hemangiosarcoma xenograft tumors. MuLV appears to be a promiscuous

virus, and we also found it in SB-HSA cells that had been previously passaged through mice as hemangiosarcomas.

These results suggested that MuLV might be the transforming agent giving rise to the mouse lymphomas, and pointed to a third possibility, that canine hemangiosarcoma cells were able to provide a suitable environment for expansion of these MuLV-transformed B-cells. We thus examined the potential for DHSA-1426 and EFB canine hemangiosarcoma cells to promote and maintain hematopoiesis in long-term culture initiating cell (LTC-IC) assays designed to promote expansion and differentiation of $CD34^+$ human umbilical cord blood hematopoietic progenitor cells (HPCs). Mouse M2-10B4 and human bone marrow derived mesenchymal stromal cells (MSCs) were used as positive controls, and HPCs cultured in the absence of feeder cells were used as a negative control. We found that DHSA-1426 cells promoted expansion of human $CD34^+$ HPCs with at least equal, if not better efficiency than conventional mouse or human feeder cells, and they achieved comparable proportions of hematopoietic cell differentiation *in vitro* across all cell lineages (**Figure 4 and Figure S10**). Similar results were obtained for EFB cells, although the expansion and differentiation were somewhat more limited.

Canine hemangiosarcoma and human angiosarcoma establish transcriptional programs that support hematopoietic expansion and differentiation to create a tumor immune niche

Finally, we evaluated whether hemangiosarcoma cells expressed genes that characterize distinct stromal cells in the bone marrow niche, including sinusoidal $nestin^+$ mesenchymal stromal cells, CXCL12-abundant reticular (CAR) cells, and sinusoidal leptin receptor $(LeptinR)^+$ stromal cells. **Figure 5 and Table S5** show that canine hemangiosarcoma tissues showed enrichment of genes

expressed by CAR cells, sinusoidal stromal cells, endosteal niche cells, endothelial progenitors, and hematopoietic progenitors. The tumors showed higher expression of cytokines such as *CSF3*, *IL6*, *IL11*, and *LIF*, associated with hematopoiesis and expansion of hematopoietic progenitors, whereas expression of *FLT3* and *IL7*, which are also involved in hematopoiesis and lymphoid homeostasis, were lower in hemangiosarcomas than the hematoma tissues. The tumors also showed down-regulation of genes associated with myeloid cells and macrophages. Next, we sought to determine if these gene signatures were also enriched in cultured hemangiosarcoma cells (cell lines) where the tumor microenvironmental factors were depleted. **Figure 6 and Table S6** show that hemangiosarcoma cells up-regulate some genes associated with endothelial progenitors and hematopoietic cytokines such as *PECAM1*, *TIE1*, *KDR*, *CD34*, *CSF3*, *IL6*, *IL11*, and *LIF in vitro*, with significant down-regulation of *NES*. Unlike hemangiosarcoma tissues, the hemangiosarcoma cell lines showed a higher level of genes expressed by myeloid cells and macrophage such as *CSF2RB*, *CSF1R* and *CD68*. DHSA-1426 and EFB cells in particular, showed remarkable enrichment of those genes, consistent with their documented capability to support hematopoietic expansion and differentiation (**Figure 4**).

Discussion

Vasoformative sarcomas are uniformly aggressive tumors with uncertain cellular origin. In humans, angiosarcomas are rare tumors that can arise idiopathically, or they can be secondary to genotoxic exposures from monomeric vinyl chloride, thorium, or radiation therapy.[41,42] In dogs, hemangiosarcomas are common and mostly idiopathic, although heritable risk factors might play a role.[6,43] These tumors are also seen in mice after exposure to certain genotoxic agents, as well as to non-genotoxic agents that cause severe tissue hypoxia,[44,45] and they are seen rarely in other

domestic and non-domestic species. The anatomical organization of these tumors across species is remarkably consistent. Angiosarcomas of humans and hemangiosarcomas of dogs have been proposed to arise from a multipotent bone marrow cell, possibly a hemangioblastic progenitor, while hemangiosarcomas of mice may arise from lineage committed endothelial progenitor cells.[1-4,7]

Angiosarcomas of humans and hemangiosarcomas of dogs are chemoresponsive tumors, but for most human patients and for dogs, the benefits are transient and the prognosis for long-term survival is poor.[46-48] A better understanding of the cell of origin and the mutational spectrum for these tumors would help to inform the development of effective treatments for this group of diseases.

We and others have reported on the mutational spectrum of human angiosarcomas and canine hemangiosarcomas.[7,48-53] Human angiosarcomas and canine hemangiosarcomas have a limited shared mutational spectrum, primarily in the canine visceral forms of the disease and in human breast angiosarcomas.[50] But the tumors in both species, as well as in zebrafish, seem to activate convergent transcriptional programs characterized by deregulation of phosphoinositide 3-kinase pathways.[7,54]

Here, we document additional shared properties of human angiosarcoma and canine hemangiosarcoma. The transcriptional landscape of these vasoformative sarcomas is strongly pro-angiogenic; however, a subset of tumors from both species is characterized by the presence of robust transcriptional immune and inflammatory signatures, which are proportional to the

number of detectable T cells and macrophages. Our results also suggest that at least in dogs, the tumors with such signatures accounts for virtually all of the long-term survivors. Additional work will be necessary to establish if the different outcomes we observed in this cohort is generally representative of canine splenic hemangiosarcoma, where approximately 15% of dogs with localized or regional disease are expected to survive a year or longer when treated with the standard of care.[55,56] If this were the case, our work would provide the first potential set of biomarkers to inform personalized treatment strategies for this disease, with surgery and adjuvant cytotoxic chemotherapy providing adequate tumor control for some dogs with inflammatory hemangiosarcomas, but not for dogs with angiogenic hemangiosarcomas.

The data from our canine hemangiosarcoma xenograft experiments confirm the notion that the chaotic vascular organization is driven by the tumor cells.[1,21,24] The experiments show that the tumor vessels are formed by a combination of malignant tumor cells and non-malignant host endothelial cells. This observation was unique to hemangiosarcoma among the three types of xenografts we examined, and it supports the capacity of hemangiosarcoma cells to adopt endothelial functions. In parallel, it suggests that normal (non-malignant) cells are necessary for the formation of malignant blood vessels in the vasculogenic tumors. Intriguingly, these experiments also show that the stromal contribution to the angiogenic and inflammatory transcriptional signatures is highly conserved in the xenografts, suggesting that the stromal cells in these tumors are heavily conditioned or reprogrammed by the malignant cells. Together, the results suggest that the interactions between the tumor and its microenvironment are rigorously orchestrated in the formation of the hemangiosarcoma niche, highlighting a potential point of vulnerability.

The development of exuberant myeloid and erythroid hyperplasia, as well as of bona fide lymphomas arising from mouse cells in animals with primary or secondary hemangiosarcoma xenografts was initially perplexing. Our results indicate that a transmissible etiology (from dog to mouse) is unlikely to be the cause of these expanded hematopoietic cell populations. Rather, the data indicate that at least a subset of canine hemangiosarcomas is capable of supporting robust expansion and differentiation of hematopoietic progenitor cells *in vitro*, and we interpret that this property is responsible for the expansion of myeloid cells *in vivo*, both in mice and in primary canine tumors. In the case of lymphomas, which occurred repeatedly and independently in animals receiving different tumor preparations, MuLV might have provided the driver events for transformation of residual lymphoid elements within a hyperproliferative environment created by the hemangiosarcoma cells. Our findings are also consistent with a report showing angiosarcoma in the bone marrow of a human patient with tumor-associated myeloid proliferation and extramedullary hematopoiesis.[57] The presence of transcriptional programs seen in hemangiosarcoma cells that resemble those of CAR cells, sinusoidal stromal cells, and endosteal niche cells, is consistent with this interpretation, and it increases the probability that canine hemangiosarcomas – and possibly human angiosarcomas – arise from one or more of these bone marrow nurse cells.

Other unexpected tumors have been reported in xenograft experiments and in pre-clinical models of stem cell transplantation. For instance, transplantation of murine MSCs has been reported to induce tumor formation and tissue malformation, potentially as a result of their possible genetic instability and/or cellular transformation.[58-60] It has also been reported that patient-derived

xenografts of human solid cancers including breast, colon, pancreatic cancer, and rhabdomyosarcoma induce lymphomagenesis or lymphocytic tumors in immunodeficient mice, but in these cases, the tumors were derived from human tumor-infiltrating lymphocytes transformed by Epstein-Barr virus.[61-64] These previously reported tumors were all of donor origin, while the tumors in our study originated from the mouse recipients and were distinct from the donor hemangiosarcomas. We were unable to identify and reports of hematopoietic tumors of recipient origin arising from xenotransplantation experiments using other types of canine cancers in the published literature, and we have not observed such an event in our own studies.[37,65,66] Thus, this finding appears to be unique to canine hemangiosarcoma, and may be due to the ability of hemangiosarcoma cells to support hematopoietic expansion. Our findings also suggest that the normal counterparts of canine hemangiosarcoma cells might contribute to the development of hematopoietic malignancies through the creation of a permissive niche. Additionally, transdifferentiation between lymphoma and sarcoma may occur through cellular reprogramming, potentially initiated by hematopoietic disruption.[67] In this light, it is especially interesting that a shared region of the canine genome was found to be significantly associated with B-cell lymphomas and hemangiosarcomas of golden retrievers.[43]

The capacity to create space for bone marrow transplants and adoptive cell therapies has taught us that the hematopoietic niche is resilient, and that bone marrow stromal cells are highly resistant to chemotherapy and radiation. These intrinsic properties could explain the relatively poor long-term responses of human patients with angiosarcoma and of dogs with hemangiosarcoma to cytotoxic therapies and may open the door to develop more effective treatments. Nevertheless, we must recognize that therapies targeting the hematopoietic niche

might themselves carry the potential for high toxicity.

Our data provide a new model to understand the etiology and cell of origin of canine hemangiosarcomas and possibly human angiosarcomas. We propose that the malignant cells originate from a bone marrow nurse cell, which has the potential to create a niche that favors angiogenic proliferation or hematopoietic expansion, as illustrated by the models in **Figure 7**. The robust inflammation observed in some of these tumors, then, may be intrinsic to the tumor, and not simply due to extrinsic factors associated with tissue disruption. These paths of differentiation may also control the biological behavior of the tumors, with those showing strong angiogenic propensity also having the most aggressive behaviors.[7]

Finally, our data do not support a transmissible etiology for hemangiosarcoma, but they do suggest that the permissive niche created by these cells can lead to the development of hematopoietic tumors driven by leukemia viruses in mice, raising the possibility that the bone marrow niche plays a similar role in viral lymphomas and leukemias of humans.

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Authorship Contributions

J.H.K., and J.F.M conceptualized the study, designed experiments, and supervised the project. J.H.K., A.L.S., N.C., J.A., M.B., and K.L.T. analyzed RNA-seq data and provided computational methodology. S.B., C.S.P., and M.M ran PathSeq algorithms and analyzed data. S.K., and S.M.G. performed PCR experiment from viral RNA and analyzed MiSeq data. M.M., and M.B. contributed to analysis and interpretation of PathSeq and MiSeq data. J.H.K, A.J.S., and A.M.F. performed mouse experiments. M.G.A., T.C.L., D.S.K. performed LTC-IC and CFU assays and analyzed data. A.J.S. performed flow cytometrical analysis. C.L.F. generated histological

samples and performed IHC. A.J.S., and M.L. provided administrative, technical, and material support. J.H.K., I.C., T.D.O., and M.G.O. performed histopathological review and scoring analysis. L.O. conducted FISH experiments. J.H.K., A.J.S., A.L.S., E.B.D., and J.F.M. wrote, reviewed, and revised the manuscript with help from all authors. All authors read the manuscript and approved the final draft.

Disclosure of Conflicts of Interest

No potential conflicts of interest were disclosed.

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Figure legends

Figure 1. Immune cell infiltration and comparative immune signatures between canine hemangiosarcoma and human angiosarcoma. (A and **B)** Representative photomicrographs of H&E and immunohistochemical staining showing histological morphology and immune cell infiltration in canine hemangiosarcoma (**A**) and human angiosarcoma tissues (**B**) using anti-CD3, anti-PAX5, anti-MAC387, and anti-Iba1 (for canine) or anti-CD163 (for human) antibodies for detecting T cell, B cell, and macrophages. H&E = hematoxylin and eosin. Horseradish peroxidase or alkaline phosphate (for Iba1) conjugates were used. Counterstain = hematoxylin. Bar = 100 um. (**C** and **D**) Scatter plots display correlation between transcriptional and immunohistochemical immune score in canine hemangiosarcoma (**C**) and human angiosarcoma (**D**). Spearman's correlation coefficient (R) was calculated. (**E**) 461 upregulated genes were identified in immune-high (N = 8) compared to immune-low (N = 5) groups in human angiosarcomas (FDR P value < 0.05). (**F**) 567 immune gene signatures were identified among three molecular subtypes of canine hemangiosarcomas (N = 76; FDR P value < 0.001; fold change > 3). The heatmaps show up-regulated (red) and down-regulated (green) genes by unsupervised hierarchical clustering (average linkage; mean-centered; $\log^2$ transformed). (**G**) Venn diagram shows 58 common genes associated with signaling pathways of immune cell functions between human and canine tumors.

Figure 2. Establishment of xenografts derived from canine hemangiosarcoma in

immunodeficient mice. (**A**) Schematic illustration depicts process of tumor xenografts in beige-nude-xid (BNX) mice. (**B**) DHSA-1426 hemangiosarcoma cells were established from canine patient-derived tumor fragments. DHSA-1426 cells formed tumors histologically classified as hemangiosarcoma. Left panel: H&E stain was done in xenograft tumor tissue. Right panel: Immunohistochemistry was performed for detection of CD31 protein (alkaline phosphatase conjugates; counterstain = hematoxylin). Top panel: 100X magnification (Bar = 100 um); Bottom panel: 200X magnification (Bar = 50 um).

Figure 3. Organization of tumor and stromal cells in mouse xenografts of canine hemangiosarcoma, osteosarcoma, and lymphoma. (**A**) Fluorescence *in situ* hybridization images using canine-specific (*IL8*, red) and mouse-specific (X chromosome, green) probes in a canine hemangiosarcoma (HSA) xenograft, a canine osteosarcoma (OS) xenograft, and a canine lymphoma xenograft transplanted into receptive immunodeficient female mouse hosts. Red and green arrows point to representative xenograft canine tumor cells and mouse stromal cells, respectively, to aid in identification. (**B**) Schematic representation of A, illustrating the organization of HSA, OS, and lymphoma xenografts. (**C**) Individual points on graph represent relative quantity of donor (dog) and host (mouse) cells in each tumor type. 10-12 fields of pictures at high magnification (400X) per slide were acquired. A total of approximately 1,000 cells in individual xenograft tumor was counted, and the percentages for each species-cells are presented.

Figure 4. Canine hemangiosarcoma cells support expansion and differentiation of CD34+ human umbilical cord blood (hUCB) cells. (**A**) Flow cytometric data show populations of cells

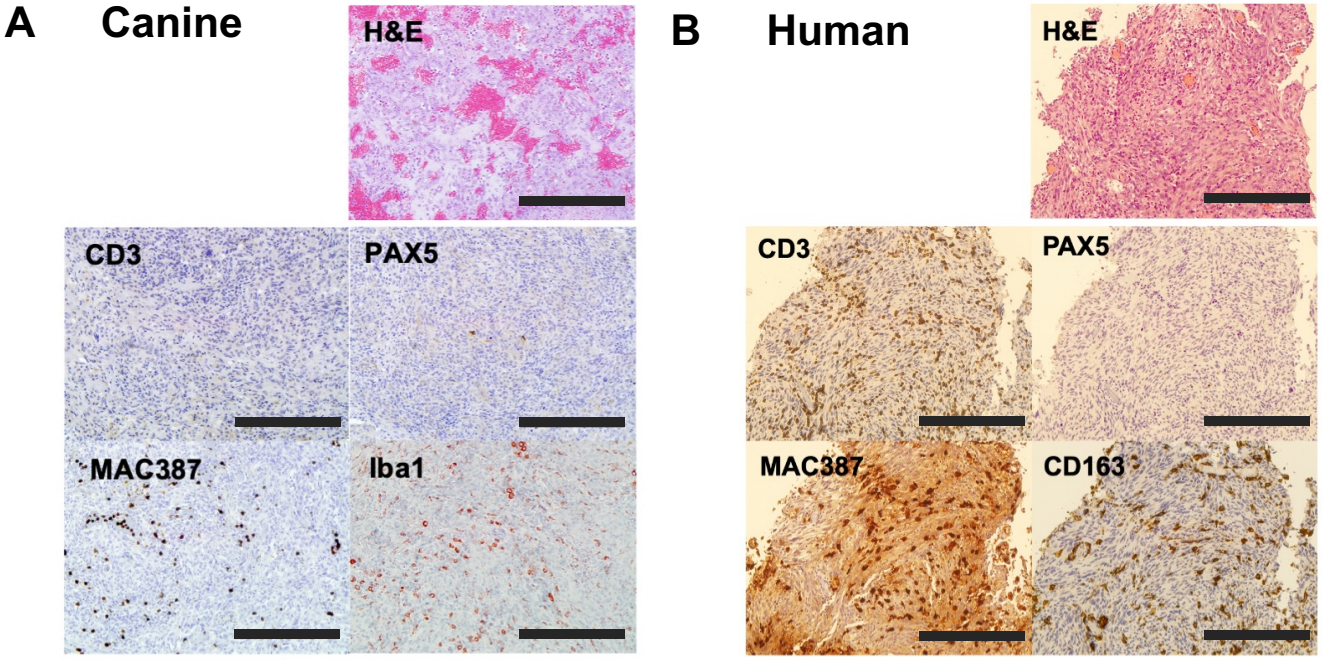
expressing CD43 and CD45 differentiated from CD34+ hUCB cells. CD34+ hUCB cells were pooled from two patients. M2-10B4, hBM-MSCs, and canine HSA cells (DHSA-1426 and EFB) were seeded on gelatin-coated 24-well plates at a density of $1x10^5$ cells/well. Gelatin-coated wells without stroma served as a negative control. Surface antigens of CD34, CD43, and CD45 were analyzed at week 5. (**B** and **C**) Bar graphs show number of different colonies formed by hUCB CD34+ cells co-cultured with feeder cells. Both DHSA-1426 and EFB canine hemangiosarcoma cell lines expanded hUCB CD34+ cells similar to the M2-10B4 and hBM-MSC positive control lines, while gelatin-coated wells alone failed to support expansion. Burst-forming unit-erythroid (BFU-E), CFU-Erythroid (CFU-E), CFU-Granulocyte/Macrophage (CFU-GM), CFU-Macrophage (CFU-M), and CFU-Granulocyte/Erythroid/Macrophage/Megakaryocyte (CFU-GEMM) were determined for colony-forming unit assay.

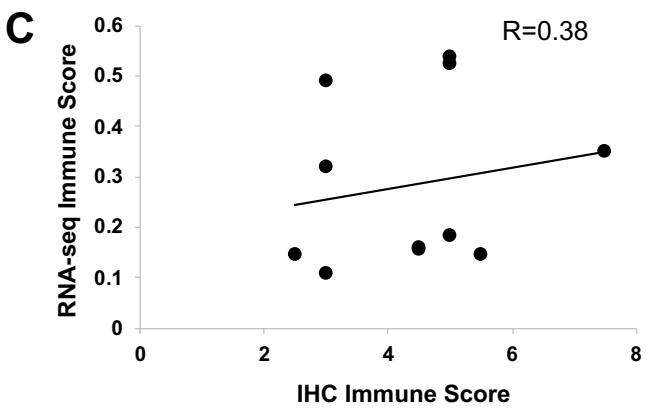
Figure 5. Gene expression signature of hematopoietic and immune cell function enriched in hemangiosarcoma tissues. Bar graphs show representative gene signature enrichment associated with bone marrow niche cells, endothelial and hematopoietic progenitor, myeloid and macrophage, signaling network, and hematopoietic cytokines between hemangiosarcoma (N = 76) and hematoma tissues (N = 10). Expression values represent count per million reads calculated by RNA-seq transcriptomic data. CAR = CXC chemokine ligand 12 (CXCL12)-abundant reticular; MSC = mesenchymal stromal cell; LepR = Leptin Receptor. P values and statistical significance were determined by Mann-Whitney U test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

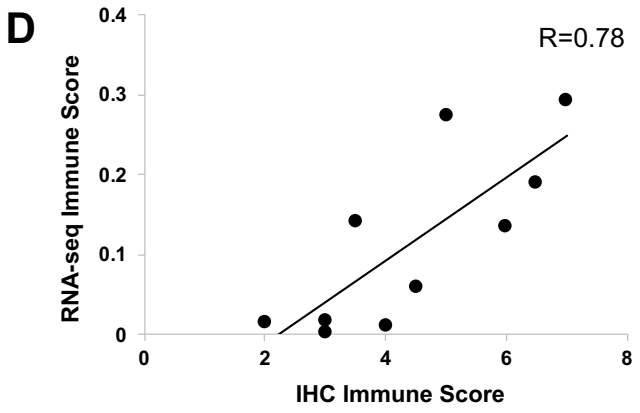
Figure 6. Gene expression signature of hematopoietic and immune cell function enriched in hemangiosarcoma cells. Bar graphs representative gene expression associated with bone marrow niche cells, endothelial and hematopoietic progenitor, myeloid and macrophage, signaling network, and hematopoietic cytokines between hemangiosarcoma cell lines (N = 11) and hematoma cells (N = 4). Expression value represents count per million reads calculated by RNA-seq transcriptomic data. CAR = CXC chemokine ligand 12 (CXCL12)-abundant reticular; MSC = mesenchymal stromal cell; LepR = Leptin Receptor. P values and statistical significance were determined by Mann-Whitney U test. *, P <0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.

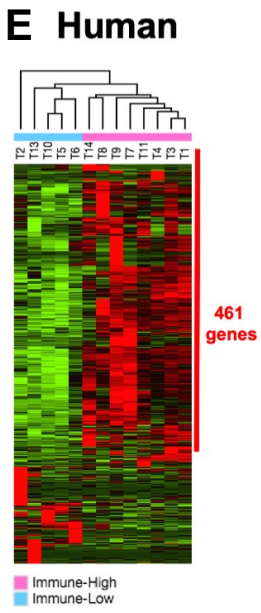
Figure 7. Hypothetical models for establishment of distinct molecular phenotypes of canine hemangiosarcoma. Non-mutually exclusive models illustrate discreet cells of origin for distinct molecular subtypes of hemangiosarcoma. Hemangiosarcoma may progress from bone marrow nurse cells that create a niche for hematopoietic expansion and inflammation, to a transitional pro-angiogenic state and full progression to a pure angiogenic state, or to malignant transformation with an inflammatory phenotype.

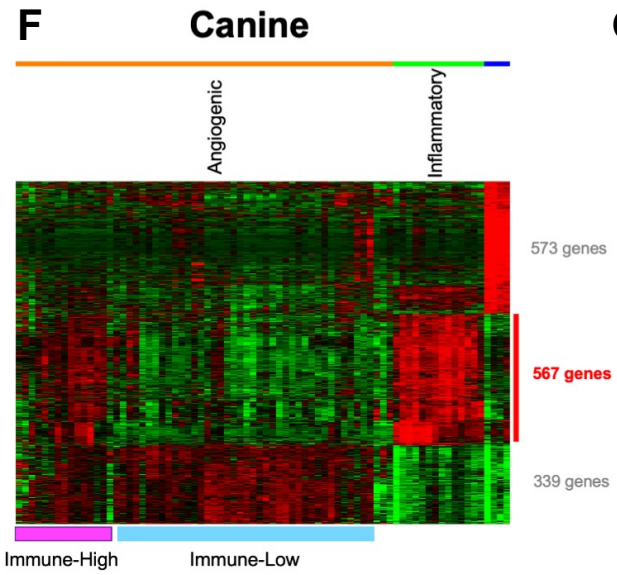
Figure 1

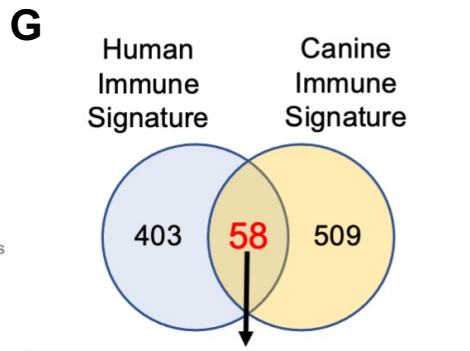












Pathways	P value	FDR P
B cell activation	3.3E-09	5.5E-07
T cell activation	2.2E-08	1.8E-06
Inflammation mediated by chemokine/cytokine signaling	1.5E-06	8.3E-05
Toll receptor signaling	1.0E-03	3.3E-02

Fisher's Exact test

Figure 2

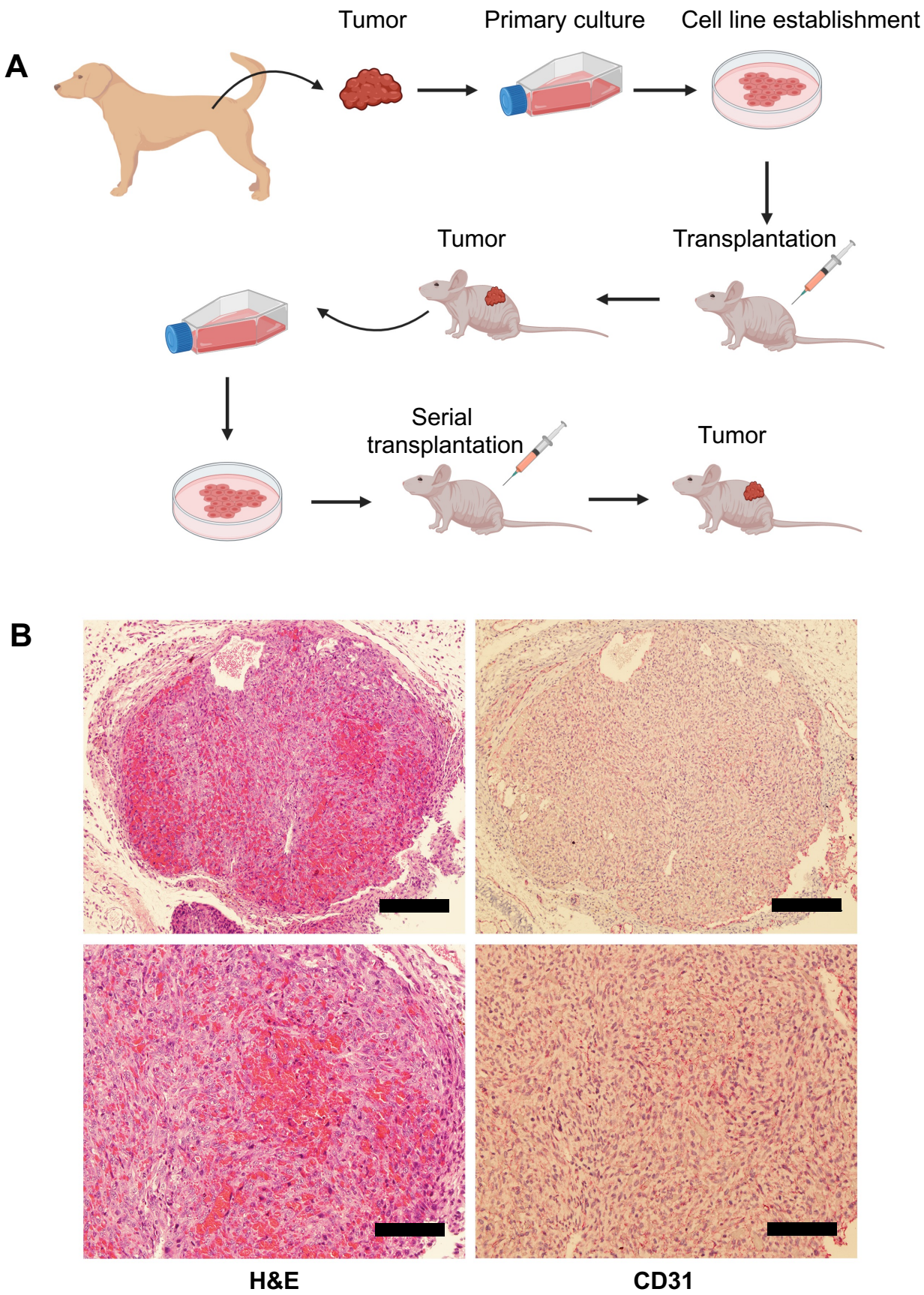

A
Tumor
Primary culture
Cell line establishment
Transplantation
Tumor
Serial transplantation
Tumor
B
H&E
CD31

Figure 3

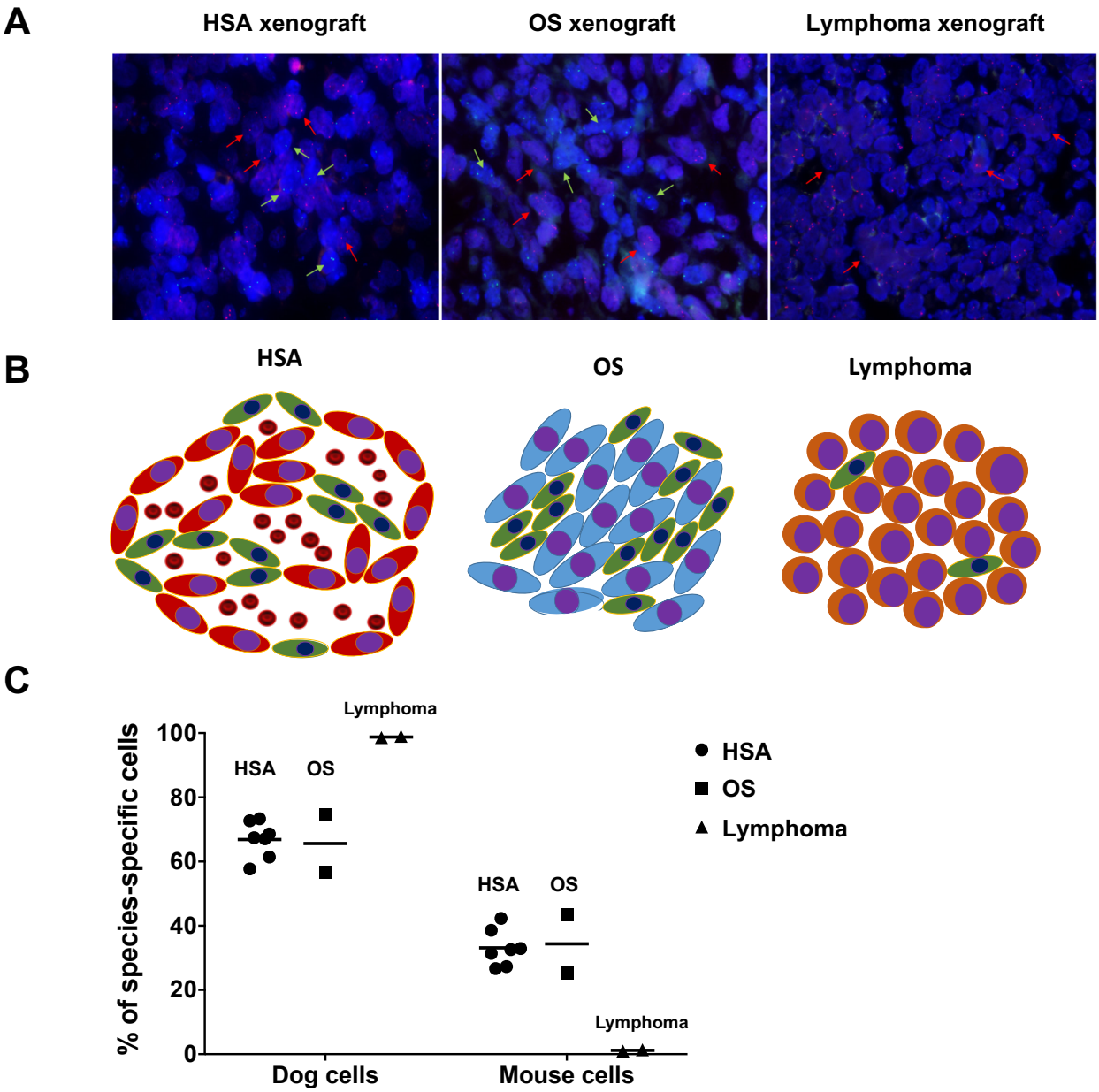
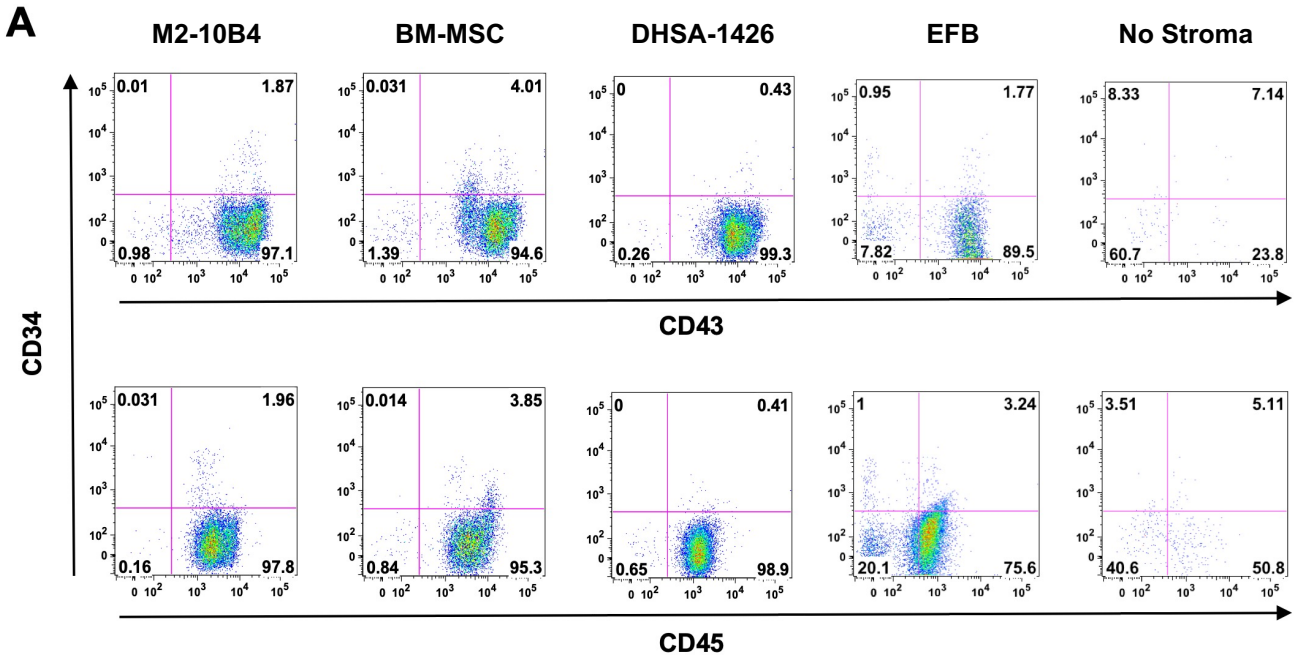
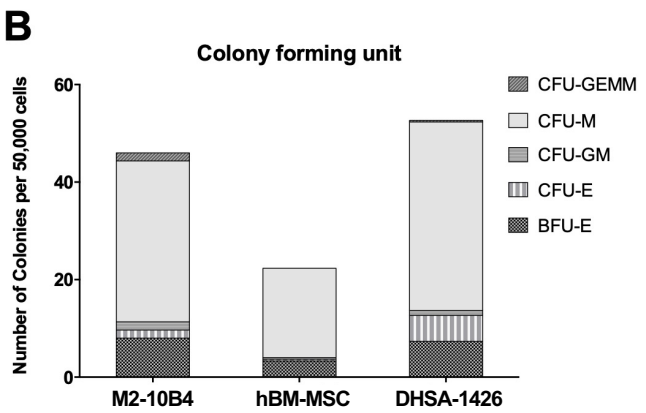

A
HSA xenograft
OS xenograft
Lymphoma xenograft
B
HSA
OS
Lymphoma
C
% of species-specific cells
100
80
60
40
20
0
HSA
OS
Lymphoma
HSA
OS
Lymphoma
Dog cells
Mouse cells
HSA
OS
Lymphoma

Figure 4


A
M2-10B4
BM-MSC
DHSA-1426
EFB
No Stroma
CD34
CD43
CD45
0.01
1.87
0.98
97.1
0.031
4.01
1.39
94.6
0
0.43
0.26
99.3
0.95
1.77
7.82
89.5
8.33
7.14
60.7
23.8
0.031
1.96
0.16
97.8
0.014
3.85
0.84
95.3
0
0.41
0.65
98.9
1
3.24
20.1
75.6
3.51
5.11
40.6
50.8


B
Colony forming unit
Number of Colonies per 50,000 cells
60
40
20
0
M2-10B4
hBM-MSC
DHSA-1426
CFU-GEMM
CFU-M
CFU-GM
CFU-E
BFU-E

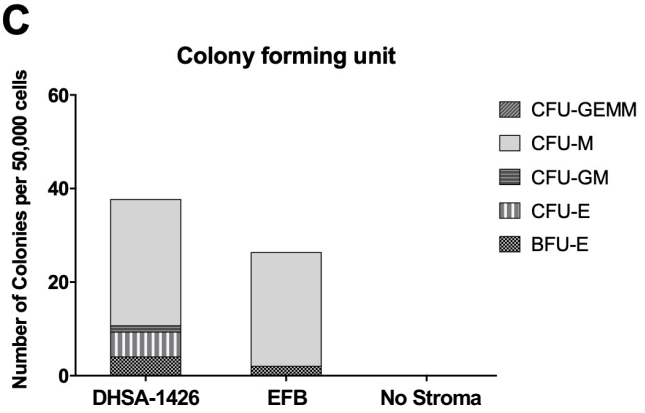

C
Colony forming unit
Number of Colonies per 50,000 cells
60
40
20
0
DHSA-1426
EFB
No Stroma
CFU-GEMM
CFU-M
CFU-GM
CFU-E
BFU-E

Figure 5

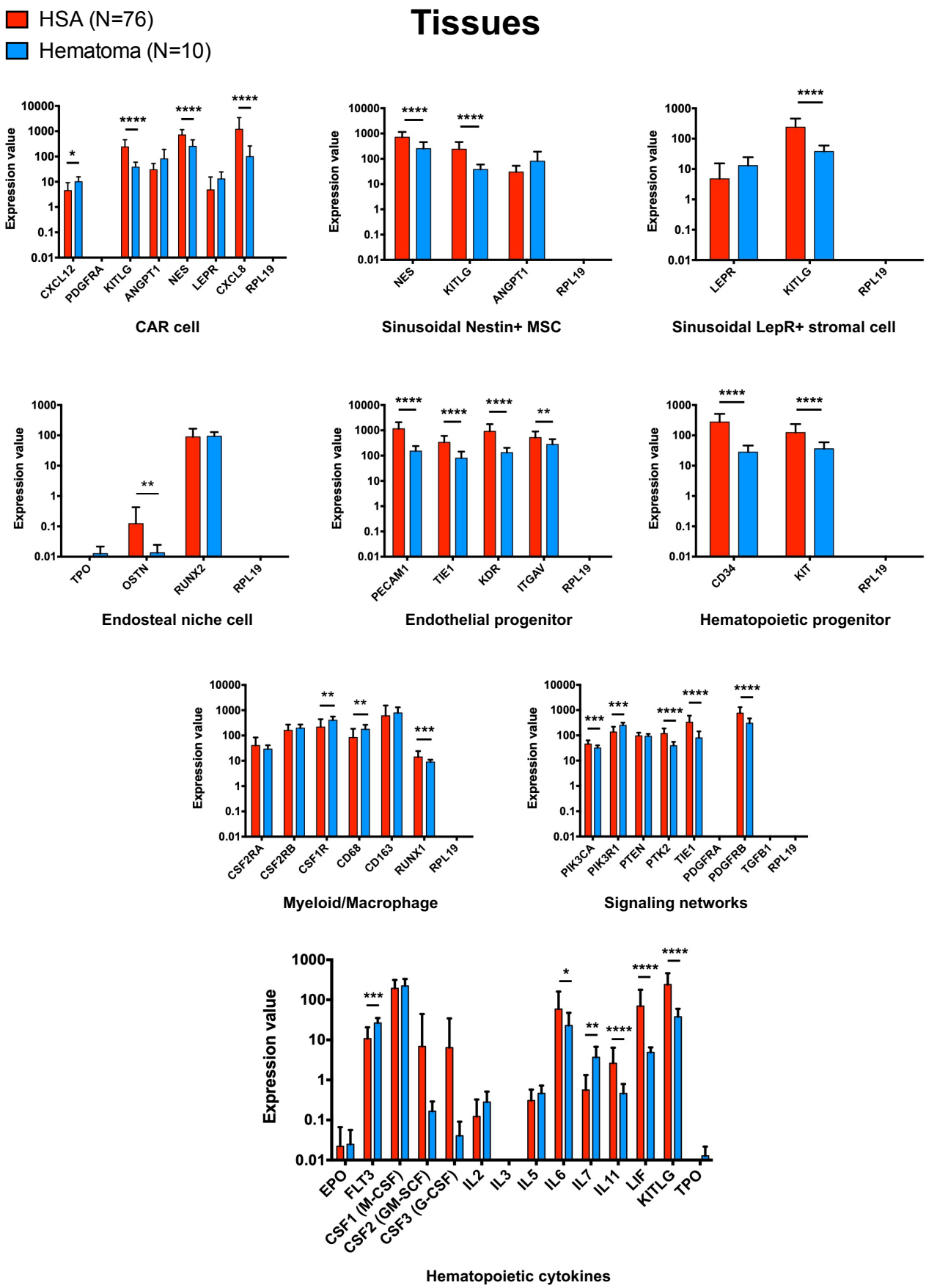

HSA (N=76)
Hematoma (N=10)
Tissues
Expression value
CXCL12
PDGFRA
KITLG
ANGPT1
NES
LEPR
CXCL8
RPL19
CAR cell
NES
KITLG
ANGPT1
RPL19
Sinusoidal Nestin+ MSC
LEPR
KITLG
RPL19
Sinusoidal LepR+ stromal cell
TPO
OSTN
RUNX2
RPL19
Endosteal niche cell
PECAM1
TIE1
KDR
ITGAV
RPL19
Endothelial progenitor
CD34
KIT
RPL19
Hematopoietic progenitor
CSF2RA
CSF2RB
CSF1R
CD68
CD163
RUNX1
RPL19
Myeloid/Macrophage
PIK3CA
PIK3R1
PTEN
PTK2
TIE1
PDGFRA
PDGFRB
TGFB1
RPL19
Signaling networks
EPO
FLT3
CSF1 (M-CSF)
CSF2 (GM-SCF)
CSF3 (G-CSF)
IL2
IL3
IL5
IL6
IL7
IL11
LIF
KITLG
TPO
Hematopoietic cytokines

Figure 6

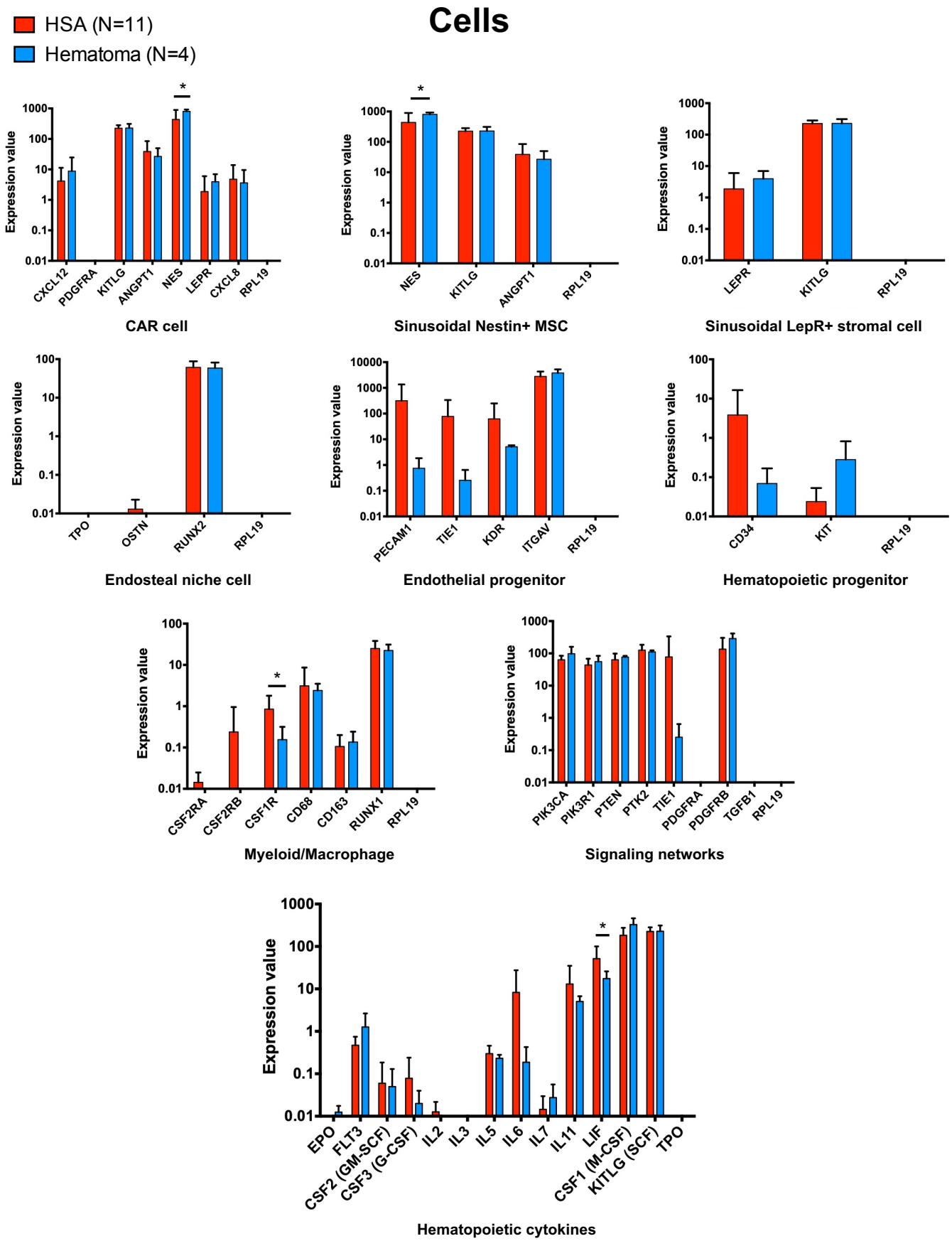
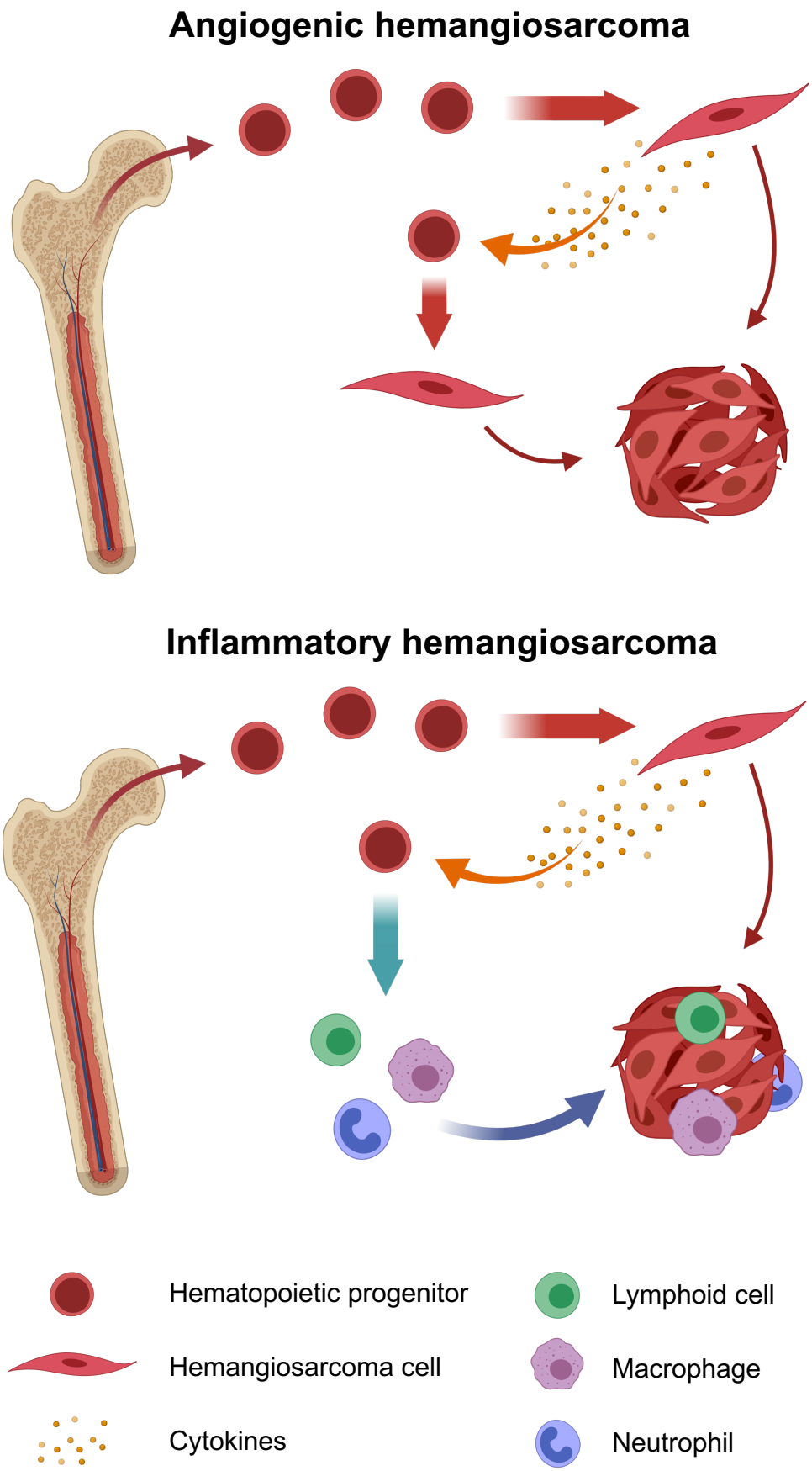

Cells
HSA (N=11)
Hematoma (N=4)
Expression value
CXCL12
PDGFRA
KITLG
ANGPT1
NES
LEPR
CXCL8
RPL19
CAR cell
Sinusoidal Nestin+ MSC
Sinusoidal LepR+ stromal cell
TPO
OSTN
RUNX2
Endosteal niche cell
PECAM1
TIE1
KDR
ITGAV
Endothelial progenitor
CD34
KIT
Hematopoietic progenitor
CSF2RA
CSF2RB
CSF1R
CD68
CD163
RUNX1
Myeloid/Macrophage
PIK3CA
PIK3R1
PTEN
PTK2
PDGFRB
TGFB1
Signaling networks
EPO
FLT3
CSF2 (GM-SCF)
CSF3 (G-CSF)
IL2
IL3
IL5
IL6
IL7
IL11
LIF
CSF1 (M-CSF)
KITLG (SCF)
Hematopoietic cytokines

Figure 7


Angiogenic hemangiosarcoma
Inflammatory hemangiosarcoma
Hematopoietic progenitor
Hemangiosarcoma cell
Cytokines
Lymphoid cell
Macrophage
Neutrophil