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Modulation of fatty acid metabolism and immune suppression are features of *in vitro* tumor sphere formation in ontogenetically distinct dog cancers

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Abstract

Non-adherent, three-dimensional sphere formation is used as an *in vitro* surrogate to evaluate cellular potential for tumor initiation and self-renewal. To determine if a shared molecular program underlies the capacity for sphere formation by cells originating from diverse tumor types, we characterized molecular and functional properties of ten independent cell lines derived from three ontogenetically distinct dog cancers: hemangiosarcoma, osteosarcoma, and glial brain tumors. Genome-wide gene expression profiling identified tumor-of-origin-dependent patterns of adjustment to sphere formation in a uniform culture condition. However, expression of the stem/progenitor markers CD34 and CD117, resistance to cytotoxic drugs, and dye efflux (side population assays) showed no association with these gene expression profiles. Instead, primary sphere-forming capacity was inversely correlated with the ability to reform secondary spheres, regardless of tumor ontogeny. Primary sphere formation seemed to be proportional to the number

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Conflict of interest

The authors declare that they have no competing interests.

Authors' contributions

AMF, TDO, EBD, and JFM conceived and designed the experiments. AMF and BHGK performed the experiments. AMF prepared the samples for expression studies. AMF and ALS generated the expression data. JHK, AMF, ALS, and JFM analyzed and interpreted the data. JHK, AMF, ML, EBD, and JFM wrote the first draft of the manuscript. JHK prepared the graphical illustrations. ML coordinated, collected, and prepared the animal tissue samples for the study. JHK, EBD, and JFM were responsible for formulating the conclusions from the data and for assembling the final version of the manuscript. All authors read and approved the manuscript.

of pre-existing cells with sphere-forming capacity in the cell lines. Cell lines where secondary sphere formation was more proficient than primary sphere formation showed enrichment of genes involved in fatty acid synthesis and immunosuppressive cytokines. In contrast, cell lines where secondary sphere formation was approximately equivalent to or less proficient than primary sphere formation showed upregulation of CD40 and enrichment of genes involved in fatty acid oxidation. Our data suggest that *in vitro* sphere formation is associated with upregulation of gene clusters involved in metabolic and immunosuppressive functions, which might be necessary for self-renewal and for tumor initiation and/or tumor propagation *in vivo*.

Keywords

cancer; dog; genome-wide gene expression; sphere; stem cell; tumor-initiating cell

Introduction

The properties that endow cells with tumor-initiating and self-renewal capacity have been the subject of extensive investigation.¹ Tumor-initiating cells (TICs, also known as cancer stem cells) promote an organizational hierarchy that is critical for self-renewal and, consequently, tumor progression. Several cell surface markers and functional assays have been proposed to identify TICs *in vitro* in hematopoietic and solid tumors.^{2–5} However, TICs show extensive intra- and inter-tumor heterogeneity, and a universal set of markers has not been identified to broadly define these cells.⁴ TICs can be defined functionally, and since the original reports showing that adult neural stem cells were enriched by formation of non-adherent, three-dimensional spheres under serum-free conditions,⁶ sphere-forming assays have been used as an *in vitro* tool to enrich putative TICs.^{5, 7, 8} Still, sphere cells also are variable among different tumors, and common features have not been standardized.

Here we investigated if sphere-forming cells from three distinct cancer types had shared molecular programs using a genome-wide gene expression microarray platform to enable hypothesis generation. We included dog cell lines derived from hemangiosarcoma (HSA), osteosarcoma (OS), and glial brain tumors to study functional, biochemical, molecular, and morphological events associated with formation and maintenance of tumor cell-derived spheres in culture. Our data suggest that modulation of gene clusters that regulate metabolic and immunosuppressive functions is a consistent feature of sphere-forming cells from these tumors.

Materials and methods

Samples

Samples were collected from client-owned dogs with naturally occurring HSA, OS, and glial brain tumors. Informed consent was obtained from all dog owners prior to the sample collection. The process of collection and generation for some of the cell lines used has been described previously (see below). For all new samples used in this study, collection protocols were approved and reviewed by the Institutional Animal Care and Use Committee (IACUC) at the University of Minnesota Veterinary Medical Center. Animal care and

experiments were carried out in accordance with all applicable institutional, local, and national guidelines; dogs were under the care of licensed veterinarians and tumor tissue donation did not influence decisions of care. Cancer cell lines from four HSA (SB, Frog, Emma, and JHE), three OS (OSCA-2, OSCA-8, OSCA-32), and three glial brain tumors (GRCR, CADE, and MARO) were used for this study.^{9–14} HSA cell lines Frog, Emma, and JHE were derived by the Modiano laboratory in 2005, 2008, and 2010, respectively, and authenticated in 2015 using STR testing (DNA Diagnostic Center, Fairfield, OH, USA). HSA cell line SB was derived by the Helfand laboratory in 2002. It was authenticated in 2015 using STR testing (DNA Diagnostic Center). OS lines OSCA-2, OSCA-8, and OSCA-32 were derived in the Modiano laboratory in 2000, 2001, and 2004, respectively. OSCA-8 and OSCA-32 cells were authenticated in 2015 using STR testing (DNA Diagnostic Center). Glial tumor cell lines, GRCR, CADE, and MARO, were generously provided by Drs. John Ohlfest and Liz Pluhar (University of Minnesota, Minneapolis, MN, USA) in 2009. OSCA-2 cells and the glial tumor cell lines used for these experiments were passaged fewer than 20 times in our laboratory.

Cell culture and sphere formation

HSA and OS cell lines were cultured as adherent monolayers as described.^{15, 16} Glial brain tumor cell lines were cultured in a similar fashion, using Neurobasal medium supplemented with N2 and B27 (ThermoFisher, Carlsbad, CA, USA), 10% FCS (Atlas, Fort Collins CO, USA), L-glutamine, and Primocin (ThermoFisher). Sphere formation assays were done under conditions that favor non-adherent cell growth.¹⁷ For primary sphere formation, cells were plated at a concentration of 3×10^5 cells/mL in ultra-low binding dishes (Corning, Lowell, MA, USA) in DMEM/F-12 medium containing L-glutamine and sodium pyruvate (ThermoFisher) and supplemented with 10 ng/mL basic fibroblast growth factor (bFGF, Peprotech, Rocky Hill, NJ, USA), 20 ng/mL epidermal growth factor (EGF, Peprotech), 5 µg/mL insulin (Sigma, St. Louis, MO, USA), and 0.4% bovine serum albumin (MP Biomedicals, Solon, OH, Peprotech). Cells were maintained in a 37°C incubator with a 5% CO₂ atmosphere, and medium with fresh growth factors was replaced every two to three 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 the spheres were allowed to settle to the tube bottom at room temperature over a period of 10 minutes. The spheres were washed once with 1X PBS without Ca²⁺ or Mg²⁺ and centrifuged at 240 x *g* for 5 minutes. The cell pellet was resuspended in 1 mL of Accutase (Sigma) and incubated for 10 minutes at room temperature with gentle agitation every two to three minutes. The cells were passed through a 200 µL gel loading tip (Laboratory Sales Limited, Rochdale, UK) 50 to 100 times to break up the spheres. Cells were examined microscopically in a hemocytometer at 10X magnification to ensure dissociation of the spheres. For the secondary sphere formation assays, we used primary sphere cells that had been maintained in sphere culture for 2 weeks as the starting material and essentially repeated the assays as described above.

Images of the cells were obtained using an Olympus IX71 microscope with an Olympus DP70 cooled digital camera (Leeds Precision Instruments, Golden Valley, MN, USA) and Olympus DP Controller software (Olympus America Inc., Center Valley, PA, USA). Images were converted from RGB to Grayscale (Adobe Systems, Inc., San Jose, CA, USA). No other image processing was performed.

RNA isolation, array hybridization, and array data analysis—RNA was extracted from the sphere and monolayer cultures of each cell line. RNA was quantified and assessed for quality as described.¹⁰ Bioanalyzer quality control, RNA labeling, and hybridization to dog 4 × 44,000 microarray chips (Agilent Technologies, Santa Clara, CA, USA) were done at the University of Minnesota Genomics Center.¹⁰

Agilent algorithms in Expressionist Refiner Module (Genedata, Basel, Switzerland, v. 7.1) were used to confirm that data from each chip met the manufacturer's standards for quality control and quality assurance. Out of 45,220 features on each array, 35,676 with annotation to known genes were used for analysis. Annotated data were quantile normalized using the Expressionist Analyst Module (v. 7.1), and these normalized data were then mean centered and log2 transformed. To show the transcriptional state of the “sphere” samples relative to the transcriptional state of its corresponding “monolayer” sample the value of the sphere sample for each annotated gene was divided by the value of the basal monolayer state. For each tumor type, a two group T-tests was performed to determine genes that were differentially expressed between the sphere state and the monolayer state. Unsupervised hierarchical clustering was based on average linkage using Gene Cluster 3.0 for Mac OS X. Multiple testing correction was done using the Benjamini-Hochberg (BH) correction. Sample gene lists were randomly permuted, and each result was reanalyzed using the permuted data to verify that outcomes were not the result of statistical chance or data artifacts. Biological functions and canonical pathways of significantly differently expressed genes were defined by Ingenuity Pathway Analysis (Ingenuity Systems, Redwood City, CA, USA).

Sphere-forming efficiency assays

For primary sphere formation, single-cell suspensions made from cells in monolayer cultures were counted using a Countess[®] automated cell counter (ThermoFisher) and plated in parallel in 96-well ultra-low binding plates (Corning, Lowell, MA, USA) in serial dilutions ranging from one cell to 300 cells/well with at least 10 wells plated for each cell concentration. For secondary sphere formation assays, we made single-cell suspensions from primary spheres cultured in non-adherent, serum-free conditions for 2 weeks. The cells were plated in serial dilutions using the same method described for primary sphere formation. Wells were visualized under light microscopy and scored for formation of spheres seven days after plating. For this study, we use the term “sphere-forming efficiency” to define the number of spheres formed in secondary sphere cultures divided over the number of spheres formed in primary sphere cultures from the same cell line.

Surface marker staining and DyeCycle® Violet dye-efflux assays

Surface marker expression and DyeCycle® Violet dye-efflux assays were performed by flow cytometry as described.^{18, 19} The primary antibodies used for surface markers staining were: anti-CD117-PE (c-Kit) and anti-CD34-PE (eBioscience, San Diego, CA, USA). Fifty thousand events per sample were collected on a BD FACSCalibur (BD Biosciences, San Jose, CA, USA). DCV emission was detected using 450/50 nm band-pass (blue) and 650 nm long-pass (red) filters in response to excitation by a violet diode laser (405 nm) using a BD™ LSR II flow cytometer (BD Biosciences). Propidium iodide was added to each sample immediately before collection to exclude dead cells from analysis. Data were analyzed using FlowJo software (Tree Star Inc., Ashland, OR, USA).

Chemosensitivity assays

Chemosensitivity of monolayer and sphere cells was determined using the colorimetric Cell Titer 96® Aqueous Non-Radioactive Cell Proliferation Assay (MTS assay, Promega, Madison, WI). The optimal conditions for each assay were determined from time course and dose response experiments in cells cultured with paclitaxel (TEVA Pharmaceuticals Inc., North Wales, PA, USA) and in cells cultured with doxorubicin (Bedford Laboratories, Bedford, OH, USA).^{19–21} Conditions were performed in triplicate using 5,000 cells per well in 100 µL of culture medium. Absorbance (A₄₉₀) was measured after 72 hours in culture using a Wallac Victor² 1420 Multilabel Counter (Perkin Elmer, Waltham, MA, USA). Viability was expressed as the percentage of the A₄₉₀ of drug-treated cells relative to untreated cells according to the following equation: % Viability = 100 x (A₄₉₀ drug-treated/A₄₉₀ untreated).

Statistics and data visualization

GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA) was used for statistical analysis. Where appropriate, descriptive statistics were done for each experimental condition (mean, SD, range), and the Mann-Whitney test was used to evaluate differences between sphere and monolayer cultures for the same cell line. For viability assays, the half maximal effective dose (EC₅₀) for cytotoxicity of each drug was calculated using linear regression and the line of best fit. Nonparametric Spearman correlation was used to determine the r-values between variables. Unsupervised hierarchical clustering and heat maps were visualized using Java TreeView version 1.1.6. Data were organized using Microsoft Excel (Microsoft Corporation, Redmond, WA, USA), GraphPad Prism 6, and Gene-E (the Broad Institute, Cambridge, MA, USA) (<http://www.broadinstitute.org/cancer/software/GENE-E/>).

Results

Ontogenetically distinct dog cancer cell lines retain lineage-specific gene signatures during sphere formation

To determine whether sphere formation creates a shared molecular program in cells from distinct cancers, we first analyzed genome-wide gene expression profiles for spheres generated from 10 dog cancer cell lines. Figure 1A shows an overview of the process used to generate the cell lines, the monolayer and sphere cell samples, and the genome-wide

analysis for the gene expression on spotted microarrays. The results revealed 799 differentially expressed genes (fold change > 3; $P < 0.05$) in spheres compared to monolayer cells. A tumor of origin-dependent pattern of adjustment to sphere culture is present (Figure 1B). Ingenuity pathway analysis of differentially expressed genes revealed that cells from each tumor type were associated with distinct canonical pathways and biological functions (Tables S1–3). These data suggest that cells from these three tumor types preserved unique ontogenetic and biological features through the process of sphere formation.

The sphere-forming efficiency of dog cancer cell lines is inversely proportional to primary sphere formation

All cancer cell lines used in this study successfully formed spheres and re-formed monolayers, indicating that sphere formation and sphere cell maintenance were reversible processes (Figure S1). Both the total number of spheres formed and the kinetics of sphere formation were variable among the cell lines. We therefore examined primary sphere formation and secondary sphere formation for each cell line using *in vitro* limiting dilution assays as a way to reduce the influence of these variables. The percentage of primary sphere-forming cells ranged from 0.13% to 4.34%, and the percentage of secondary sphere-forming cells ranged from 0.32% to 2.77% (Figure 2A). The HSA-derived cell lines had significantly fewer sphere-forming cells than either the OS or the glial brain tumor-derived cell lines ($P = 0.012$ and $P < 0.0001$, respectively), and the glial brain tumor-derived cell lines formed more spheres than the OS-derived cell lines ($P = 0.008$) (Figure 2B). In addition, sphere-forming efficiency (*i.e.*, the ratio of secondary sphere formation relative to primary sphere formation) was inversely correlated with primary sphere formation (Figure 2C; Spearman $r = -0.78$; $P = 0.009$). These findings suggest that cells from distinct tumor types have different capacities to form spheres and that cell lines where primary sphere-forming cells are infrequent show significantly increased secondary sphere-forming capacity, regardless of ontogeny.

The relationship among different traits associated with functions of TICs is conserved in spheres from different dog cancer cell lines

The phenotypic markers CD34 and CD117 have been used to identify putative TICs in hematopoietic and also in some mesenchymal cancers.^{17, 22–24} Our flow cytometry results showed increased CD34 or CD117 expression in spheres compared to monolayer cells for some of the cell lines; however, this was not observed in all of the cell lines we tested (Table S4 and Figure S2). Drug resistance is another feature that has been used to identify and enrich TICs from both human and dog cancers.^{19, 25, 26} As was true for expression of CD34 and CD117, sphere cells from some of the tumor cell lines were more resistant to doxorubicin and paclitaxel than their corresponding monolayer cells, but others were not (Table S5). To determine if this variability was caused by activity of ABC transporters, we analyzed the efflux of DyeCycle Violet by cells cultured as spheres and as monolayers (Figure S3). Sphere cells from seven of eight cell lines tested showed increased verapamil-resistant dye efflux as compared to monolayer cells (Table S6), suggesting that spheres express different subsets or greater levels of ABC transporters.

Properties such as number of sphere-forming cells, gene expression profiles, presence of early progenitor markers on the cell surface, chemoresistance, and ABC transporter-

mediated efflux appeared quite heterogeneous across the different cell lines. Therefore, we examined correlations among these different features to uncover potential shared properties among the sphere cells. The results of this analysis are shown as a heatmap in Figure 3. The data indicate statistically significant negative correlations ($P < 0.01$) between primary sphere formation and sphere-forming efficiency and between primary sphere formation and verapamil-resistant dye efflux. In addition, secondary sphere formation was negatively correlated ($P < 0.05$) with *in vitro* drug resistance. Conversely, CD34 expression was positively correlated ($P < 0.05$) with sphere-forming efficiency, and resistance to doxorubicin was positively correlated ($P < 0.05$) with resistance to paclitaxel, suggesting that sphere cells engage common mechanisms of drug resistance. However, neither total dye efflux nor verapamil-resistant dye efflux showed consistent correlations with drug resistance in the sphere cells, suggesting that these properties are not mechanistically linked.

Cancer cell metabolism and immune programs are associated with sphere formation

Finally, we sought to identify molecular mechanisms that contribute to sphere-forming capacity. For this purpose, we compared cell lines with the highest (SB, Frog, and GRRC) and the lowest (Emma, CADE, and MARO) sphere-forming efficiency (Figure 4A). Primary spheres generated from these cell lines were cultured under identical conditions to minimize potential contributions from the cell culture medium or the environment. This comparison generated a distinct profile comprising 242 differentially expressed genes ($P < 0.05$; fold change > 2) between the two groups (Figure 4A), which offers insight into gene expression patterns associated with sphere-forming capacity (Figure 4B). Ingenuity pathway analysis showed that these differential gene signatures were associated with biological functions including inflammatory and immune responses, lipid and drug metabolism, cell cycle, and cancer (Figure 4B, Figure S4, and Table S7). Upstream regulators of these genes were predicted to activate *IL10RA* and *RARA* and also to inhibit *RIPK2*, *STAT6*, *SMARCA4* and several cytokines such as *IFNG*, *IL-6*, *OSM*, and *IL1B* (Figure 4B and Table S8). The profile analysis also identified the top significant canonical pathways associated with fatty acid metabolism and immune signaling, including *ACSL1* and *ACSL5*, *FASN*, and *CD40* genes. Figure 5 displays a summary of the results, including a hypothetical model proposing that the core molecular systems, which control sphere-forming capacity, are associated with fatty acid metabolism and immune suppression in cancer cells.

Discussion

Sphere-forming assays have been used as *in vitro* surrogates to identify and enrich TICs. Indeed, *in vitro* formation of non-adherent three-dimensional tumor spheres has been previously reported to maintain and enrich cells with tumor-propagating potential in human and dog cancers.^{5, 7, 8, 27–30} However, the functional mechanisms utilized by cells in non-adherent sphere culture to maintain and regulate self-renewal are not well understood. For this study, we examined phenotypic, functional, and molecular features of sphere cells from three different dog cancers under uniform culture conditions to identify a set of conserved traits that define TICs. Our data show that sphere cells derived from dog cancer cell lines retain their distinct ontogenetic features, suggesting that spheres consist of lineage-committed subpopulations for each cancer type. Sphere-forming efficiency, that is, the ratio

of secondary relative to primary sphere formation, was negatively correlated with primary sphere-formation in these cell lines. In other words, the lines where primary sphere-forming cells were infrequent showed the most substantial gains under conditions of secondary sphere formation. This could be due to greater enrichment of TICs in sphere cultures from cell lines where these cells were rare, and it could explain the heterogeneity of surface marker expression, drug efflux, and chemoresistance observed among the cell lines.

Our gene expression profiling data also highlight another non-mutually exclusive possibility. Spheres from cell lines with high sphere-forming efficiency showed consistent downregulation of *ACSL1* and *ACSL5* genes, encoding long-chain-fatty-acid-CoA ligase (ACSL) 1 and 5, with concomitant upregulation of the *FASN* gene, encoding fatty acid synthase. Proteins of the ACSL family are mainly expressed in mitochondria, where they play a role in modulating lipid biosynthesis and fatty acid degradation.³¹ Fatty acid synthase, encoded by *FASN*, is a multifunctional enzyme that catalyzes the synthesis of fatty acids (e.g., palmitic acid production from acetyl-CoA and malonyl-CoA).^{32, 33} Recent data suggest that FASN-dependent lipogenesis supports proliferation and differentiation of stem cells into lineage-committed cells,^{34, 35} and that fatty acid oxidation maintains quiescence of hematopoietic stem cells to prevent them from exhaustion.^{36, 37} Thus, a model has been proposed where a potential shift from a fatty acid oxidation-dominant phenotype to a fatty acid synthesis-dominant phenotype determines the transition from quiescent, normal stem cells to proliferating, normal progenitor cells.^{35, 36}

A similar process might be operative in cancer cells that require metabolic reprogramming to regulate stemness and self-renewal,^{38, 39} since emerging data suggest that metabolic reprogramming can be used to discriminate features of heterogeneous cancer cell populations. Specifically, TICs have been shown to have metabolic properties distinct from bulk tumor cells that consist of proliferative and differentiated cell populations.^{40, 41} Furthermore, it has been suggested that TICs rely on fatty acid metabolism for their cell plasticity to maintain quiescence and self-renewal.^{39, 42} In this context, and based on our data, we advance the hypothesis that cell lines with greater sphere-forming efficiency contain proliferating progenitor cells that use fatty acid synthesis for growth, whereas the cell lines that do not show a significant increment in sphere-forming efficiency keep a steady state of sphere formation by promoting fatty acid oxidation that maintains quiescent stem-like cells. Additional work will be necessary to test this hypothesis, and to determine if sphere-forming assays are suitable models to test the roles of fatty acid metabolism in maintaining quiescence or in promoting asymmetric or symmetric division of TICs.

Reprogramming of fatty acid metabolism in immunity and inflammation has recently become an area of increasing interest.^{43, 44} For instance, fatty acid oxidation regulates T-cell activation and cytokine production; furthermore, pro-inflammatory signals inhibit proliferation through altered macrophage metabolism.^{45, 46} An intriguing finding from this study was the apparent induction of immunosuppressive pathways in sphere cells. Specifically, gene expression profiles in spheres from cell lines with high sphere-forming efficiency (*i.e.*, those with elevated fatty acid synthesis) showed upregulation of the *IL-10* and *TGF- β* genes and inhibition of the *IFNG*, *IL1*, and *IL6* genes; furthermore, *CD40* gene expression was downregulated in these spheres. In contrast, cell lines with low sphere-

forming efficiency (*i.e.*, those with elevated fatty acid oxidation) showed upregulation of the *CD40* gene. These findings are consistent with a role for TICs in immunomodulation of the tumor niche.^{47, 48}

In our hypothetical model, the progenitor pool would create a milieu where regulatory T cell activity is enhanced and conventional helper and cytotoxic T cells are suppressed. Previous studies suggest that TICs can potentiate secretion of IL-10 and TGF- β produced by tumor-infiltrating macrophages, further amplifying functional immunosuppression.⁴⁹ CD40, which is a costimulatory protein receptor detected on antigen presenting cells such as B cells and macrophages, is essential for the activation of immune response by binding of CD40 ligand on T helper cells.^{50, 51} Thus, in our model, downregulation of CD40 in tumor cells could help TICs and/or tumor progenitor cells to evade immune cells in the tumor niche, enabling them to undergo expansion.

Conversely, our findings revealed upregulation of the *CD40* gene in the putative self-renewing or quiescent stem-like cells where pro-inflammatory molecules were activated. CD40 expression is a prototypical feature during lineage commitment of hematopoietic stem cells, epithelial stem cells, and embryonic stem cells,^{52, 53} indicating that it is important for cell development and differentiation. An increase of CD40 on tumor cells with upregulation of pro-inflammatory cytokines could compete with its expression on other antigen presenting cells, potentially creating an immunologically permissive tumor niche.⁵⁴ Interestingly, previous studies have shown that signaling through CD40 increases PD-L1 expression on tumor-infiltrating antigen presenting cells, thus increasing PD-1 blockade and T-cell exhaustion.^{55, 56} Our model thus would suggest that the pool of proliferating, progenitor-like TICs and the pool of quiescent, stem-like TICs might regulate the tumor immunosuppressive barrier using different mechanisms.

In conclusion, we demonstrate that ten cancer cell lines derived from three distinct dog tumor types preserve their ontogenetic transcriptional profiles in uniform *in vitro* culture conditions. By utilizing primary and secondary sphere-forming assays combined with genome-wide gene expression profiling, we found that sphere-forming efficiency is inversely proportional to primary sphere formation and is associated with the presence of distinct gene signatures in these cell lines. We propose a model where primary sphere-forming cells are dependent on fatty acid metabolism. The gene expression signatures in these sphere cells also highlight their potential to mold the tumor immunosuppressive barrier. Additional work will be needed to determine the interconnections between fatty acid metabolism and the creation of immunologically permissive tumor niches.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Tysnes BB. Tumor-initiating and -propagating cells: cells that we would like to identify and control. *Neoplasia*. 2010; 12(7):506–15. [PubMed: 20651980]
2. Schatton T, Frank NY, Frank MH. Identification and targeting of cancer stem cells. *Bioessays*. 2009; 31(10):1038–49. [PubMed: 19708024]
3. Akbari-Birgani S, Paranjothy T, Zuse A, et al. Cancer stem cells, cancer-initiating cells and methods for their detection. *Drug Discov Today*. 2016; 21(5):836–42. [PubMed: 26976692]
4. Medema JP. Cancer stem cells: the challenges ahead. *Nat Cell Biol*. 2013; 15(4):338–44. [PubMed: 23548926]
5. Duan JJ, Qiu W, Xu SL, et al. Strategies for isolating and enriching cancer stem cells: well begun is half done. *Stem Cells Dev*. 2013; 22(16):2221–39. [PubMed: 23540661]
6. Reynolds BA, Weiss S. Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. *Science*. 1992; 255(5052):1707–10. [PubMed: 1553558]
7. Ishiguro T, Ohata H, Sato A, et al. Tumor-derived spheroids: Relevance to cancer stem cells and clinical applications. *Cancer Sci*. 2017
8. Weiswald LB, Bellet D, Dangles-Marie V. Spherical cancer models in tumor biology. *Neoplasia*. 2015; 17(1):1–15. [PubMed: 25622895]
9. Akhtar N, Padilla ML, Dickerson EB, et al. Interleukin-12 inhibits tumor growth in a novel angiogenesis canine hemangiosarcoma xenograft model. *Neoplasia*. 2004; 6(2):106–16. [PubMed: 15140399]
10. Kim JH, Frantz AM, Anderson KL, et al. Interleukin-8 promotes canine hemangiosarcoma growth by regulating the tumor microenvironment. *Exp Cell Res*. 2014; 323(1):155–64. [PubMed: 24582862]
11. Scott MC, Sarver AL, Gavin KJ, et al. Molecular subtypes of osteosarcoma identified by reducing tumor heterogeneity through an interspecies comparative approach. *Bone*. 2011; 49(3):356–67. [PubMed: 21621658]
12. Lamerato-Kozicki AR, Helm KM, Jubala CM, et al. Canine hemangiosarcoma originates from hematopoietic precursors with potential for endothelial differentiation. *Exp Hematol*. 2006; 34(7):870–8. [PubMed: 16797414]
13. Tamburini BA, Trapp S, Phang TL, et al. Gene expression profiles of sporadic canine hemangiosarcoma are uniquely associated with breed. *PLoS One*. 2009; 4(5):e5549. [PubMed: 19461996]
14. Thomas R, Scott A, Langford CF, et al. Construction of a 2-Mb resolution BAC microarray for CGH analysis of canine tumors. *Genome Res*. 2005; 15(12):1831–7. [PubMed: 16339382]
15. Fosmire SP, Dickerson EB, Scott AM, et al. Canine malignant hemangiosarcoma as a model of primitive angiogenic endothelium. *Lab Invest*. 2004; 84(5):562–72. [PubMed: 15064773]
16. Cannon CM, Pozniak J, Scott MC, et al. Canine osteosarcoma cells exhibit resistance to aurora kinase inhibitors. *Vet Comp Oncol*. 2015; 13(1):48–59. [PubMed: 23410058]
17. Gorden BH, Kim JH, Sarver AL, et al. Identification of three molecular and functional subtypes in canine hemangiosarcoma through gene expression profiling and progenitor cell characterization. *Am J Pathol*. 2014; 184(4):985–95. [PubMed: 24525151]

18. Gorden BH, Saha J, Khammanivong A, et al. Lysosomal drug sequestration as a mechanism of drug resistance in vascular sarcoma cells marked by high CSF-1R expression. *Vasc Cell*. 2014; 6:20. [PubMed: 25295160]
19. Khammanivong A, Gorden BH, Frantz AM, et al. Identification of drug-resistant subpopulations in canine hemangiosarcoma. *Vet Comp Oncol*. 2016; 14(3):e113–25. [PubMed: 25112808]
20. Schappa JT, Frantz AM, Gorden BH, et al. Hemangiosarcoma and its cancer stem cell subpopulation are effectively killed by a toxin targeted through epidermal growth factor and urokinase receptors. *Int J Cancer*. 2013; 133(8):1936–44. [PubMed: 23553371]
21. Rodriguez AM, Graef AJ, LeVine DN, et al. Association of Sphingosine-1-phosphate (S1P)/S1P Receptor-1 Pathway with Cell Proliferation and Survival in Canine Hemangiosarcoma. *J Vet Intern Med*. 2015; 29(4):1088–97. [PubMed: 26118793]
22. Adhikari AS, Agarwal N, Wood BM, et al. CD117 and Stro-1 identify osteosarcoma tumor-initiating cells associated with metastasis and drug resistance. *Cancer Res*. 2010; 70(11):4602–12. [PubMed: 20460510]
23. Guth AM, Deogracias M, Dow SW. Comparison of cancer stem cell antigen expression by tumor cell lines and by tumor biopsies from dogs with melanoma and osteosarcoma. *Vet Immunol Immunopathol*. 2014; 161(3–4):132–40. [PubMed: 25146881]
24. Zhao Y, Dong J, Huang Q, et al. Endothelial cell transdifferentiation of human glioma stem progenitor cells in vitro. *Brain Res Bull*. 2010; 82(5–6):308–12. [PubMed: 20599593]
25. Dean M, Fojo T, Bates S. Tumour stem cells and drug resistance. *Nat Rev Cancer*. 2005; 5(4):275–84. [PubMed: 15803154]
26. Reya T, Morrison SJ, Clarke MF, et al. Stem cells, cancer, and cancer stem cells. *Nature*. 2001; 414(6859):105–11. [PubMed: 11689955]
27. Michishita M, Ezaki S, Ogihara K, et al. Identification of tumor-initiating cells in a canine hepatocellular carcinoma cell line. *Res Vet Sci*. 2014; 96(2):315–22. [PubMed: 24534130]
28. Pang LY, Gatenby EL, Kamida A, et al. Global gene expression analysis of canine osteosarcoma stem cells reveals a novel role for COX-2 in tumour initiation. *PLoS One*. 2014; 9(1):e83144. [PubMed: 24416158]
29. Michishita M, Akiyoshi R, Yoshimura H, et al. Characterization of spheres derived from canine mammary gland adenocarcinoma cell lines. *Res Vet Sci*. 2011; 91(2):254–60. [PubMed: 21190702]
30. Stoica G, Lungu G, Martini-Stoica H, et al. Identification of cancer stem cells in dog glioblastoma. *Vet Pathol*. 2009; 46(3):391–406. [PubMed: 19176492]
31. Singh I, Lazo O, Kremser K. Purification of peroxisomes and subcellular distribution of enzyme activities for activation and oxidation of very-long-chain fatty acids in rat brain. *Biochim Biophys Acta*. 1993; 1170(1):44–52. [PubMed: 8399326]
32. Jayakumar A, Tai MH, Huang WY, et al. Human fatty acid synthase: properties and molecular cloning. *Proc Natl Acad Sci U S A*. 1995; 92(19):8695–9. [PubMed: 7567999]
33. Wakil SJ. Fatty acid synthase, a proficient multifunctional enzyme. *Biochemistry*. 1989; 28(11):4523–30. [PubMed: 2669958]
34. Knobloch M, Braun SM, Zurkirchen L, et al. Metabolic control of adult neural stem cell activity by Fasn-dependent lipogenesis. *Nature*. 2013; 493(7431):226–30. [PubMed: 23201681]
35. Folmes CD, Dzeja PP, Nelson TJ, et al. Metabolic plasticity in stem cell homeostasis and differentiation. *Cell Stem Cell*. 2012; 11(5):596–606. [PubMed: 23122287]
36. Ito K, Suda T. Metabolic requirements for the maintenance of self-renewing stem cells. *Nat Rev Mol Cell Biol*. 2014; 15(4):243–56. [PubMed: 24651542]
37. Ito K, Carracedo A, Weiss D, et al. A PML-PPAR-delta pathway for fatty acid oxidation regulates hematopoietic stem cell maintenance. *Nat Med*. 2012; 18(9):1350–8. [PubMed: 22902876]
38. Ito K, Bernardi R, Morotti A, et al. PML targeting eradicates quiescent leukaemia-initiating cells. *Nature*. 2008; 453(7198):1072–8. [PubMed: 18469801]
39. Peiris-Pages M, Martinez-Outschoorn UE, Pestell RG, et al. Cancer stem cell metabolism. *Breast Cancer Res*. 2016; 18(1):55. [PubMed: 27220421]

40. Wu M, Neilson A, Swift AL, et al. Multiparameter metabolic analysis reveals a close link between attenuated mitochondrial bioenergetic function and enhanced glycolysis dependency in human tumor cells. *Am J Physiol Cell Physiol*. 2007; 292(1):C125–36. [PubMed: 16971499]
41. Liu PP, Liao J, Tang ZJ, et al. Metabolic regulation of cancer cell side population by glucose through activation of the Akt pathway. *Cell Death Differ*. 2014; 21(1):124–35. [PubMed: 24096870]
42. Sancho P, Barneda D, Heeschen C. Hallmarks of cancer stem cell metabolism. *Br J Cancer*. 2016; 114(12):1305–12. [PubMed: 27219018]
43. Byersdorfer CA. The role of Fatty Acid oxidation in the metabolic reprogramming of activated t-cells. *Front Immunol*. 2014; 5:641. [PubMed: 25566254]
44. Kominsky DJ, Campbell EL, Colgan SP. Metabolic shifts in immunity and inflammation. *J Immunol*. 2010; 184(8):4062–8. [PubMed: 20368286]
45. Liu L, Lu Y, Martinez J, et al. Proinflammatory signal suppresses proliferation and shifts macrophage metabolism from Myc-dependent to HIF1alpha-dependent. *Proc Natl Acad Sci U S A*. 2016; 113(6):1564–9. [PubMed: 26811453]
46. Shriver LP, Manchester M. Inhibition of fatty acid metabolism ameliorates disease activity in an animal model of multiple sclerosis. *Sci Rep*. 2011; 1:79. [PubMed: 22355598]
47. Kleffel S, Schatton T. Tumor dormancy and cancer stem cells: two sides of the same coin? *Adv Exp Med Biol*. 2013; 734:145–79. [PubMed: 23143979]
48. Park TS, Donnenberg VS, Donnenberg AD, et al. Dynamic Interactions Between Cancer Stem Cells And Their Stromal Partners. *Curr Pathobiol Rep*. 2014; 2(1):41–52. [PubMed: 24660130]
49. Raggi C, Mousa HS, Correnti M, et al. Cancer stem cells and tumor-associated macrophages: a roadmap for multitargeting strategies. *Oncogene*. 2016; 35(6):671–82. [PubMed: 25961921]
50. Grewal IS, Flavell RA. CD40 and CD154 in cell-mediated immunity. *Annu Rev Immunol*. 1998; 16:111–35. [PubMed: 9597126]
51. van Kooten C, Banchereau J. CD40-CD40 ligand. *J Leukoc Biol*. 2000; 67(1):2–17. [PubMed: 10647992]
52. Pearson S, Lancrin C, Lacaud G, et al. The sequential expression of CD40 and Icam2 defines progressive steps in the formation of blood precursors from the mesoderm germ layer. *Stem Cells*. 2010; 28(6):1089–98. [PubMed: 20506544]
53. Tong AW, Stone MJ. Prospects for CD40-directed experimental therapy of human cancer. *Cancer Gene Ther*. 2003; 10(1):1–13. [PubMed: 12489023]
54. Quail DF, Joyce JA. Microenvironmental regulation of tumor progression and metastasis. *Nat Med*. 2013; 19(11):1423–37. [PubMed: 24202395]
55. Zippelius A, Schreiner J, Herzig P, et al. Induced PD-L1 expression mediates acquired resistance to agonistic anti-CD40 treatment. *Cancer Immunol Res*. 2015; 3(3):236–44. [PubMed: 25623164]
56. Luheshi NM, Coates-Ulrichsen J, Harper J, et al. Transformation of the tumour microenvironment by a CD40 agonist antibody correlates with improved responses to PD-L1 blockade in a mouse orthotopic pancreatic tumour model. *Oncotarget*. 2016; 7(14):18508–20. [PubMed: 26918344]

Figure 1. Overview of methods used to analyze gene expression profiles between sphere and monolayer cells in ontogenetically distinct dog cancers

(A) Three types of spontaneous dog cancers were harvested and cells were cultured to establish primary cancer cell lines *in vitro*. Cells were cultured either in conventional monolayer cell culture condition in adherent flasks or in three-dimensional sphere conditions in non-adherent flasks. After isolation of RNA and synthesis of cDNA, genome-wide gene expression was compared between sphere and monolayer samples. (B) Heatmap shows transcriptional changes of spheres relative to monolayer cells in ten tumor cell lines from three types of dog cancers. Sphere samples are shown relative to the corresponding monolayer samples. 799 genes were observed to be statistically differentially expressed in at least one of the three types of tumors examined ($P < 0.05$; $> \text{three-fold change}$). A tumor of origin-dependent pattern of adjustment to sphere culture is present. Red bar = hemangiosarcoma; green = osteosarcoma; orange = glial brain tumor.

Figure 2. Sphere-forming efficiency in dog cancer cell lines

In vitro limiting dilution assays of monolayer cells and their respective cells cultured as spheres for 14 days were plated at densities ranging from one to three-hundred cells per well in 96-well non-adherent conditions favoring sphere formation. (A) Bar graph displays percentage of primary (from monolayer cells) and secondary (from primary sphere) sphere-forming cells in HSA (SB, Frog, and Emma), OS (OSCA-2, OSCA-8, and OSCA-32), and glial brain tumor (GRCR, CADE, and MARO) cell lines. (B) Mean percentage of primary sphere-forming cells are presented for each tumor type. Mann-Whitney test (* = $P < 0.05$; ** = $P < 0.01$; **** = $P < 0.0001$). (C) Relative sphere-forming efficiencies (the ratio of

secondary sphere formation relative to primary sphere formation) (Spearman $r = -0.78$; $P = 0.009$).

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Figure 3. Relationship among traits associated with TICs in spheres from dog cancer cell lines Heatmap representation of the correlation (Spearman r values) among primary and secondary sphere formation, sphere-forming efficiency (defined as the ratio of secondary sphere formation relative to primary sphere formation), CD34 and CD117 expression, total and verapamil-resistant dye efflux, and resistance to doxorubicin and paclitaxel. Positive correlations are shown in blue and negative correlations are shown in red (* = $P < 0.05$; ** = $P < 0.01$).

Figure 4. Identification of gene signature and canonical pathways between different sphere-forming efficiency (SFE) groups

(A) Scheme illustrates cancer cells showing distinct capacity to form and maintain spheres, and how to define SFE groups. (B) Heatmap clustering displays 242 differentially expressed genes ($P < 0.05$; $> \text{two-fold change}$; yellow = upregulated; blue = downregulated) between different high and low SFE groups. Ingenuity pathway analysis identifies biological functions and upstream regulators associated with metabolism and immune suppression. Prediction of activated (red arrow) or inhibited (blue arrow) upstream regulators is analyzed in high SFE group.

Figure 5.

Illustration of a proposed model of cancer cells showing their distinct capacity to form and maintain spheres.