Evaluation of Stem Cell Marker Expression in Canine B-Cell Lymphoma Cell Lines, B-Cell Lymphoma-generated Spheres and Primary Samples

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Abstract. *Background: Canine lymphoma has lately drawn focus as a model of human non-Hodgkin's lymphoma due to its spontaneous occurrence and similar biological behavior. Cells with stem cell-like characteristics are believed to play a key role in therapeutic failure. Thus, an initial characterization and the possibility of specific detection of such cells could bear significant value. Materials and Methods: Expression of 12 stem cell markers were analyzed in two canine B-cell lymphoma cell lines, their generated spheres, and in primary lymphoma samples by quantitative real-time polymerase chain reaction and partially by flow cytometry and immunocytochemistry. Results: Expression of maternal embryonic leucine zipper kinase (Melk) was significantly higher in CLBL-1, CLBL-1M and in primary B-cell lymphoma samples compared to non-neoplastic lymph nodes. Spheres displayed a higher expression of v-myc myelocytomatosis viral oncogene homolog (Myc) and lower expression of Cd44 compared to original cell lines and primary B-cell lymphoma samples. Conclusion: The results suggest a potential interesting role of Melk in canine B-cell lymphoma. Furthermore, the up-regulation of Myc in serum-free-generated spheres offers interesting possibilities for functional assays characterizing the specific generated sub-population.*

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Lymphoma is one of the most common hematopoietic malignancies seen in humans and dogs. In man, approximately 88.5% of these tumors are non-Hodgkin's lymphomas, representing about 5.1% of all newly-diagnosed cancer cases per year in the world (1). Akin to men, lymphoma in dogs represents 7% to 24% of all canine neoplasias, and 83% of all canine hematopoietic malignancies (2). B-Cell lymphomas of humans and dogs share several common characteristics, including clinical presentation, tumor biology, and response to therapeutic agents such as conventional chemotherapy (3, 4). The malignancy occurs spontaneously in dogs, and thus canine lymphoma has lately been considered to be of significant value for human lymphoma research, providing a naturally-occurring model for its human counterpart (5, 6).

Identifying and characterizing the cell population capable of generating a tumor, and causing metastasis and tumor relapse is currently a critical issue in cancer research. In this context, cell populations with a stem cell-like character, also referred to commonly as cancer stem cells (CSCs), have been identified in different cancer types. These cells are considered to play a major role in tumor development and relapse, representing a rather small cell sub-population of tumor-forming cells (7-9). Since Bonnet and Dick isolated a small subset of cells with these stem cell characteristics from acute myeloid leukemia in 1997 (10), CSCs from several types of human solid tumors have been identified, including breast, prostate, lung and pancreatic cancer, melanoma, glioblastoma and hepatocellular carcinoma (10-19). These cells exhibited a strong tumorigenic character in the fact that as few as 100 cells with this phenotype were able to form tumors in mice. In dogs, potential CSCs, or cancer-initiating

cells, have been identified akin to their human counterparts in osteosarcoma, mammary carcinoma, prostate cancer, lung cancer and glioblastoma (20-24).

The expression of stem cell markers is frequently used as a tool to facilitate the identification and isolation of CSCs from multiple human tumor entities (25-28). However, CSCs from different tumors have different patterns of expression of various stem cell markers, sometimes even varying within a tumor. Furthermore, the frequency of CSCs-expressing stem cell markers can vary dramatically in different tumors, from 0.01% to 41%. However, in most cases, the respective subpopulation is rather small, leading to difficulties in cultivation and isolation, especially from primary tumor samples (18, 29, 30). Despite such marker expression differences, some stem cell surface markers appear to be frequently present and are most commonly used to identify potential CSCs, such as CD44 and CD133 (31-33). As these cell populations are considered to play a major role in tumor development, resistance to chemotherapy and relapse, the isolation of these cells is of major interest for the development of therapeutic approaches aiming at directed targeting of these cells. In this context, cell lines are key, providing a stable tool for the isolation and detailed characterization of such cells.

However, a pre-condition for further isolation and detailed characterization of these cells is the identification and evaluation of a marker panel allowing for detection of these sub-populations. As the respective cell populations are usually present at low percentages, their isolation and marker evaluation from primary material by surface markers can be challenging (34). This challenge is of major impact when visualizing a respective marker screening for diagnostic purposes or directed targeting. Thus, despite methodical disadvantages, nucleic acid-based detection is able to offer an alternative for initial screening processes.

In order to evaluate if a characteristic stem cell marker expression panel can be characterized in canine B-cell lymphoma, we analyzed two cell lines and primary samples. CLBL-1 was derived from a fine-needle aspirate from a lymph node in stage IV diffuse large B-cell lymphoma, and CLBL-1M was established from CLBL-1-generated tumor material in recombination activating gene 2 deficient (*Rag2*$^{-/-}$) γc mice (35, 36). We evaluated 12 stem cell-associated markers which have been widely used in different combinations and tumor types to identify potential CSC populations: CD34, CD133, c-KIT (CD117), CD44, integrin-α6 (ITGA6, aka CD49f), octamer-binding transcription factor 4 (OCT4), NANOG, Kruppel-like factor 4 (KLF4), SOX2, MYC, maternal embryonic leucine zipper kinase (MELK) and [DEAD (Asp-Glu-Ala-Asp) box helicase 5](DDX5) in the two B-cell lymphoma cell lines and additionally in 14 primary lymphomas and seven non-neoplastic lymph nodes by real-time polymerase chain reaction (PCR), partially by flow cytometry and immunocytochemistry. Spheres from CLBL-1 and CLBL-1M cells were also generated under serum-free conditions, subsequently analyzed by flow cytometry, and quantitative relative real-time PCR and comparatively analyzed with the non-enriched CLBL-1 and CLBL-1M cells and the primary B-cell lymphoma samples.

Materials and Methods

Cell lines and tissue samples. The canine B-cell lymphoma cell line CLBL-1 (35) and the derived daughter cell line CLBL-1M (36) were cultured routinely in RPMI-1640 with 20% fetal bovine serum (FBS) and 2% penicillin-streptomycin (all from Biochrom AG, Berlin, Germany) medium. All cells were maintained in a humidified incubator at 37˚C with 5% CO_2.

Samples from dogs with naturally-occurring lymphoma (N=14) were collected at the Small Animal Clinic, University of Veterinary Medicine Hannover, Germany according to the legislation of the state of Lower Saxony, Germany. Fine-needle aspirates (FNA) of enlarged lymph nodes from these 14 patients with lymphoma (12 multicentric B-cell lymphomas, one relapsed B-cell lymphoma and one T-cell lymphoma) were used for analysis. Details of the sample set were previously described by us (37). Seven non-neoplastic lymph nodes were used as controls.

RNA isolation and cDNA synthesis. Total RNA was extracted from CLBL-1, CLBL-1M and the primary samples using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. On-column DNase digestion was performed with the RNase-Free DNase Set (Qiagen) to avoid genomic DNA contamination. Subsequently, cDNA synthesis was performed using the QuantiTect Reverse Transcription Kit (Qiagen), using 500 ng total RNA of each sample for each reaction in 20 μl total volume.

Quantitative real-time PCR for quantitative stem cell marker expression analyses. For quantitative marker analysis, two cell lines, the primary lymphoma and reference samples were analyzed by absolute and relative quantitative real-time PCR. Absolute quantitative real-time PCR was performed to quantify the expression levels of stem cell marker genes in CLBL-1 and CLBL-1M cell lines. As the total amount of RNA isolated from primary samples and spheres was not predictable, relative quantitative real-time PCR was additionally performed to evaluate the relative expression level of the stem cell marker genes in the two cell lines, primary lymphoma samples and non-neoplastic lymph nodes. Relative quantitative real-time PCR was also applied to the cell line-generated spheres and the non-enriched cell lines comparatively.

Absolute quantification: Copy number quantification in CLBL-1 and CLBL-1M cells was performed using a one-step QuantiTect SYBR Green PCR Kit (Qiagen). Fifty nanograms of total RNA was used in each reaction and all assays were performed in triplicates. For each gene, a standard curve was generated using serial 10-fold dilution of a standard DNA oligonucleotide sequence from 10^3 to 10^{10} copies. Melting-curve analysis was performed after amplification as control for specificity of the reaction and to exclude false positives. No template controls (NTC) and no reverse

Table I. *Primer pairs used in conventional PCR and quantitative real-time PCR.*

Gene	Gene name	Forward primer (5'-3')	Reverse primer (5'-3')	Amplicon (bp)
Cd34	CD34 molecule	ACCAGAGCTATTCCCGCAAG	TTTCTCCTGTAGGGCTCCAA	120
Cd133	prominin 1	CTTTCTCATGGTCGGAGTTGG	TGGAATAGTTTCCTGTTCTGGTAAG	135
c-KIT	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog	AGAAACGTGAAGCGCGAGTA	ACACAACTGGTACAGCTCGATGG	129
Itga6	integrin, alpha 6	TCAGACCCTTAACTGCAGCA	CATAACCTCGAGCGCAGAA	132
Cd44	CD44 molecule	AATGCTTCAGCTCCACCTG	CGGTTAACGATGGTTATGGTAATT	92
Oct4	POU class 5 homeobox 1	CGAGGAGTCCCAAGACATCA	AACACCTTCCCAAAGAGAACC	138
Nanog[#]	Nanog homeobox	CTATAGAGGAGAGCACAGTGAAG	GTTCGGATCTACTTTAGAGTGAGG	141
Klf4	Kruppel-like factor 4	CCACATTAATGAGGCAGCCA	CTCCCGCCAGCGGTTATT	146
Sox2	(sex determining region Y)-box 2	GGAAACTTTTGTCGGAGACG	CGGGGCCGGTATTTATAATC	103
Myc	v-myc myelocytomatosis viral oncogene homolog	TCGGACTCTCTGCTCTCCTC	TTCTTCCTCCGAGTCGCT	108
Melk	maternal embryonic leucine zipper kinase	CCAAGGGTAACAAGGACTAC	CTCCAAACATCTGCCTCTGA	112
Ddx5	DEAD (Asp-Glu-Ala-Asp) box helicase 5	AACTTCCCTGCAAATGTAATGGA	AGTCTGTGCTACTCCAACCAT	123
Actb	β-actin	TCGCTGACAGGATGCAGAAG	GTGGACAGTGAGGCCAGGAT	127

Nanog[#]: Described previously (20).

transcriptase controls were included in each run. The sequences of the primer pairs used for amplification of the stem cell markers are listed in Table I.

Relative quantification: Expression of the stem cell marker genes *Cd44, Myc, Melk, Itga6, Ddx5* and *c-Kit* were quantified relatively in the cell lines CLBL-1, CLBL-1M, the primary lymphoma samples and the non-neoplastic lymph node samples. As biological replicates, three different passages of CLBL-1 and CLBL-1M cells were analyzed. The assays correspond to those described for absolute quantitative real-time PCR. As reference gene canine β-actin (*Actb*) was chosen. The respective primer pair is listed in Table I. The analyses of each sample were performed in triplicates in a 96-well plate using a two-step QuantiTect SYBR Green PCR Kit (Qiagen) in an Eppendorf Mastercycler®. For each real-time PCR reaction, 1 μl cDNA generated by reverse transcription of mRNA was used. Data were analyzed by Rest 2009 software (Qiagen). The mean expression level of seven non-neoplastic lymph nodes was set as 1. The relative quantitative ratio (RQ) of *Cd44, Myc, Melk, Itga6, Ddx5* and *c-Kit* were normalized to the reference gene *β-actin* (*Actb*).

Flow cytometric analyses of CLBL-1 and CLBL-1M cells. The four cell surface marker proteins CD44, CD133, CD34 and CD49f were analyzed by flow cytometry on the cultured cell lines CLBL-1 and CLBL-1M. The examinations were performed in triplicates in different passages. The cells were harvested from culture flasks and washed with PBS. The cells were resuspended in PBS and gently pipetted in order to obtain a single-cell suspension. The viability was determined by Trypan blue (Invitrogen, Darmstadt, Germany) staining. For each reaction, 1×10^6 cells were placed in 100 μl fluorescence-activated cell sorting (FACS) buffer (1% BSA/PBS). Subsequently, cells were incubated for 30 min with a primary antibody or a matching isotype control at 4˚C. The primary antibodies and their corresponding isotypes are described in Table II. After washing three times with FACS buffer, the CD44-, CD133- and CD34-labeled cells were analyzed with a FACSCalibur (BD Bioscience, Heidelberg, Germany). The CD49f labeled cells were incubated for 30 min at 4˚C with a secondary rabbit anti-rat fluorescein isothiocyanate (FITC) (STAR17B; AbD Serotec, Puchheim, Germany) antibody and washed three times with FACS buffer. After staining, the data were analyzed using FlowJoV10 (TreeStar, Ashland, OR, USA). Each measurement was performed three times independently.

Immunocytochemistry. Further verification of target protein expression was performed on CLBL-1 and CLBL-1M cells to characterize the expression of the stem cell-associated cell surface markers CD44, CD49f and c-KIT by immunocytochemistry. Cell pellets of the described cell lines (approximately 1×10^7 cells) were fixed in 4% paraformaldehyde/PBS, embedded in paraffin and cut into 3-4 μm sections. Immunodetections were performed using purified primary antibodies directed against CD44 (ab119863; Abcam, Cambridge, UK), CD49f (555734; BD Bioscience), and c-KIT (CD117) (A450229-2; DAKO, Hamburg, Germany). As secondary antibodies, biotinylated goat anti-rat IgG and goat anti-rabbit IgG (both Vector Laboratories, Peterborough,UK) were used, respectively. Avidin-biotin-peroxidase reagent (Vector Laboratories) was applied according to the manufacturer's instructions. The chromogen used was 3'3-diaminobenzidine-tetrahydrochloride (Sigma-Aldrich, Seelze, Germany). Negative controls were performed by replacing the primary antibodies with normal rat serum (for the anti-CD44 and anti-CD49f antibodies) and normal rabbit serum (for the anti c-KIT antibody). All reactions were carried-out twice.

Generation of CLBL-1 and CLBL-1M spheres in serum-free medium and marker analyses. Serum-free culture: CLBL-1 and CLBL-1M cells were collected and washed by PBS to remove serum. Subsequently, the cells were seeded at a density of 5×10^4 cells/ml in a 6-well plate and cultured in DMEM/F12 (Biochrom) serum-free medium containing 2 mM L-glutamine (Sigma-Aldrich), 5 μg/ml Insulin (Sigma-Aldrich), 20 ng/ml human epidermal growth factor

Table II. *Antibodies and corresponding isotypes used in this study.*

Antibody	Clone	Marker identified	Isotype
Mouse anti-dog CD34: RPE AbD Serotec	1H6	CD34	Mouse IgG1 RPE AbD Serotec
Anti-Canine CD44 FITC eBioscience	YKIX337.8	CD44	Rat IgG2aκ FITC BD Bioscience
PE anti-mouse CD133[a] eBioscience	13A4	CD133	Rat IgG1κ PE eBioscience
Purified Rat Anti-Human CD49f[b] BD Bioscience	GoH3	CD49f	Rat IgG2aκ purified BD Bioscience

PE/RPE: R-Phycoerythrin; FITC: fluorescein isothiocyanate. [a]Reported canine CD133 cross-reactivity (41, 65). [b]Reported canine CD49f cross-reactivity (22).

Table III. *Absolute quantitative real-time PCR analyses of CLBL-1 and CLBL-1M cell lines.*

	Gene										
Cells	*Cd34*	*Cd133*	*c-Kit*	*Cd44*	*Itga6*	*Oct4*	*Nanog*	*Klf4*	*Sox2*	*Myc*	*Melk*	*Ddx5*
CLBL-1	139	101	2266	1.32×10^6	5.69×10^4	103	1091	1607	–	1.18×10^6	3.98×10^5	2.08×10^6
CLBL-1M	31.6	75.2	416	1.01×10^6	9.43×10^4	–	1691	1143	425	1.62×10^6	6.08×10^5	3.66×10^6

–: No signal could be detected. Data shown are mean copy numbers in 50 ng total RNA of triplicates.

(EGF) (Biochrom), 20 ng/ml human basic fibroblast growth factor (bFGF) (Life Technologies, Darmstadt, Germany) and 2% B27 supplement without vitamin A (Life Technologies). New medium was added every two or three days depending on cell condition. The serum-free cultured cells were passaged six times. Images were captured on the first, fifth and ninth day of cultivation using a Leica DMI6000B microscope (Leica Microsystem Vertrieb GmbH, Wetzlar, Germany). The serum-free cultured cells were named CLBL-1S and CLBL-1MS.

Marker analyses by conventional PCR, relative real-time PCR and flow cytometry: On the ninth day, the cells were harvested for relative quantitative real-time PCR and flow cytometric analysis. We screened all 12 marker genes previously in the serum-free cultured cells by conventional PCR (data not shown). Additionally, *Cd44, Itga6, Myc, Ddx5* and *Melk* mRNA expression levels were compared among the serum-free cultured CLBL-1S and CLBL-1MS, the corresponding native cells and primary B-cell lymphoma samples by relative real-time PCR. The analyses of the marker gene expression were performed as described above. The respective expression level was normalized to the reference gene *Actb*. To characterize the cell surface marker expressions of CD44 and CD133, flow cytometric analyses were performed for CLBL-1S and CLBL-1MS cells as described above.

Statistical analysis. The relative real-time PCR data were analyzed by Rest 2009 software (Qiagen) to discriminate the significant differences between the non-neoplastic lymph nodes samples and other groups ($p<0.05$ was considered as statistically significant).

Results

Marker expression analyses by absolute quantitative real-time PCR in CLBL-1 and CLBL-1M cells. The expression of the stem cell marker genes in CLBL-1 and CLBL-1M cells are summarized in Table III. The results were consistent with previous results generated by conventional PCR results showing expression of *Cd44, Itga6, Myc, Melk* and *Ddx5* (data not shown in detail). Furthermore, *Itga6* and *Melk* displayed very low expression levels in CLBL-1 and CLBL-1M cells. CLBL-1 had 5.69×10^4 copies and CLBL-1M 9.43×10^4 copies of *Itga6* in 50 ng total RNA. *Melk* had 3.98×10^5 copies in CLBL-1 and 6.08×10^5 copies in CLBL-1M in 50 ng total RNA. Marker expression analyses requiring Ct values of more than 30 cycles were considered to be not or only weakly-expressed. Markers with Ct values beyond 30 were: *Cd34, Cd133, c-Kit, Oct4, Nanog, Klf4* and *Sox2*.

Marker expression analyses by relative quantitative real-time PCR in CLBL1, CLBL-1M cells and primary B-cell lymphoma samples. According to conventional PCR and absolute real-time PCR results, *Cd44, Itga6, Myc, Melk* and *Ddx5* were detected in all cell lines and primary samples, thus we focused on these five genes and *c-Kit* which is a hematopoietic stem cell marker in relative real-time PCR.

Table IV. *Relative expression of stem cell marker genes in CLBL-1, CLBL-1M and primary canine lymphoma samples.*

Markers		Cell lines and lymphoma samples					
		CLBL-1 (n=3)	CLBL-1M (n=3)	B-Cell lymphoma (n=12) (relapse; n=1)	B-Cell lymphoma (n=1)	T-Cell lymphoma	Lymph node (n=7)
Cd44	Expression	0.440	0.456	0.305	0.252	1.000	1.000
	Std. error	0.287-0.682	0.304-0.713	0.162-0.603			
	p-Value	0.005	0.009	<0.0001			
Myc	Expression	0.672	0.522	1.095	1.464	0.783	1.000
	Std. error	0.379-1.364	0.299-0.907	0.552-2.219			
	p-Value	0.289	0.061	0.721			
Melk	Expression	3.257	2.895	2.192	2.738	1.128	1.000
	Std. error	1.333-5.780	1.846-4.077	1.450-3.342			
	p-Value	0.004	0.008	<0.0001			
Itga6[a]	Expression	0.278	0.268	0.115	0.088	0.415	1.000
	Std. error	0.144-0.522	0.150-0.553	0.054-0.245			
	p-Value	0.019	0.013	<0.0001			
Ddx5[b]	Expression	0.306	0.335	0.457	0.611	0.825	1.000
	Std. error	0.229-0.407	0.240-0.420	0.310-0.645			
	p-Value	<0.0001	0.003	<0.0001			
c-Kit	Expression	0.001	0.001	0.034	0.045	0.118	1.000
	Std. error			0.016-0.079			
	p-Value	<0.0001	<0.0001	<0.0001			

[a]CLBL-1 and CLBL-1M cells showed significantly higher expression of ITGA6 compared to the primary B-cell lymphoma samples; [b]CLBL-1 cells showed significantly lower expression of DDX5 compared to the primary B-cell lymphoma samples. Std. error: standard error.

The expression levels of *Cd44, Itga6, Ddx5* and *c-Kit* in CLBL-1, CLBL-1M and the primary B-cell lymphoma samples were significantly lower compared to the non-neoplastic lymph nodes. Compared to the mean value for seven non-neoplastic lymph nodes, significantly higher expressions of *Melk* were observed in CLBL-1, CLBL-1M and the primary B-cell lymphoma samples. The B-cell lymphoma relapse sample had an expression profile similar to that of B-cell lymphoma samples. In contrast, the T-cell lymphoma sample had a higher expression of *Cd44* and a lower expression of *Melk* when compared to the B-cell lymphoma samples. In addition, the CLBL-1 and CLBL-1M cell lines had a significantly higher expression of *Itga6* (p=0.012; p=0.009) with respect to the primary B-cell lymphoma samples, while *Ddx5* expression (p=0.04) was significantly lower in CLBL-1 compared to the primary B-cell lymphoma samples. All data are summarized in Table IV.

Flow cytometric analyses of cell surface stem cell markers. Flow cytometric expression analyses of the cell surface markers CD34, CD44, CD133 and CD49f in CLBL-1 and CLBL-1M cell lines is shown in Figure 1. All markers were measured in triplicates. In Figure 1, we show one of the three histograms. The mean fluorescence intensity (MFI) of each marker was the mean level of three measurements.

In the two cell lines, almost all of cells expressed CD44, with a positivity above 99%. Both cell lines were negative for CD34 and CD133 expression when compared to the isotype control. CD49f was weakly expressed in CLBL-1 and CLBL-1M cells, but the frequency of positive cells varied slightly in each measurement. The MFI was measured as a geometric mean and normalized to that of the isotype control. The data are expressed as normalized MFI to evaluate the expression levels of the indicated cell surface markers (Figure 1B). The normalized MFI showed high expression of CD44, low expression of CD49f and no expression of CD34 and CD133 in CLBL-1 and CLBL-1M cell lines. This result was consistent with the absolute quantitative real-time PCR.

Immunocytochemical analyses of CD44, CD49f and c-KIT. CLBL-1 and CLBL-1M were both positive for CD44 and negative for CD49f and c-KIT (Figure 2). All CLBL-1 and CLBL-1M cells (100%) showed intense membranous immunolabeling for CD44. With the CD49f and the c-KIT antibodies, no immunolabeling was detected.

Generation of CLBL-1 and CLBL-1M spheres in serum-free medium and marker analyses.

Serum-free culture: CLBL-1 and CLBL-1M cells were cultured and monitored under serum-free conditions for days.

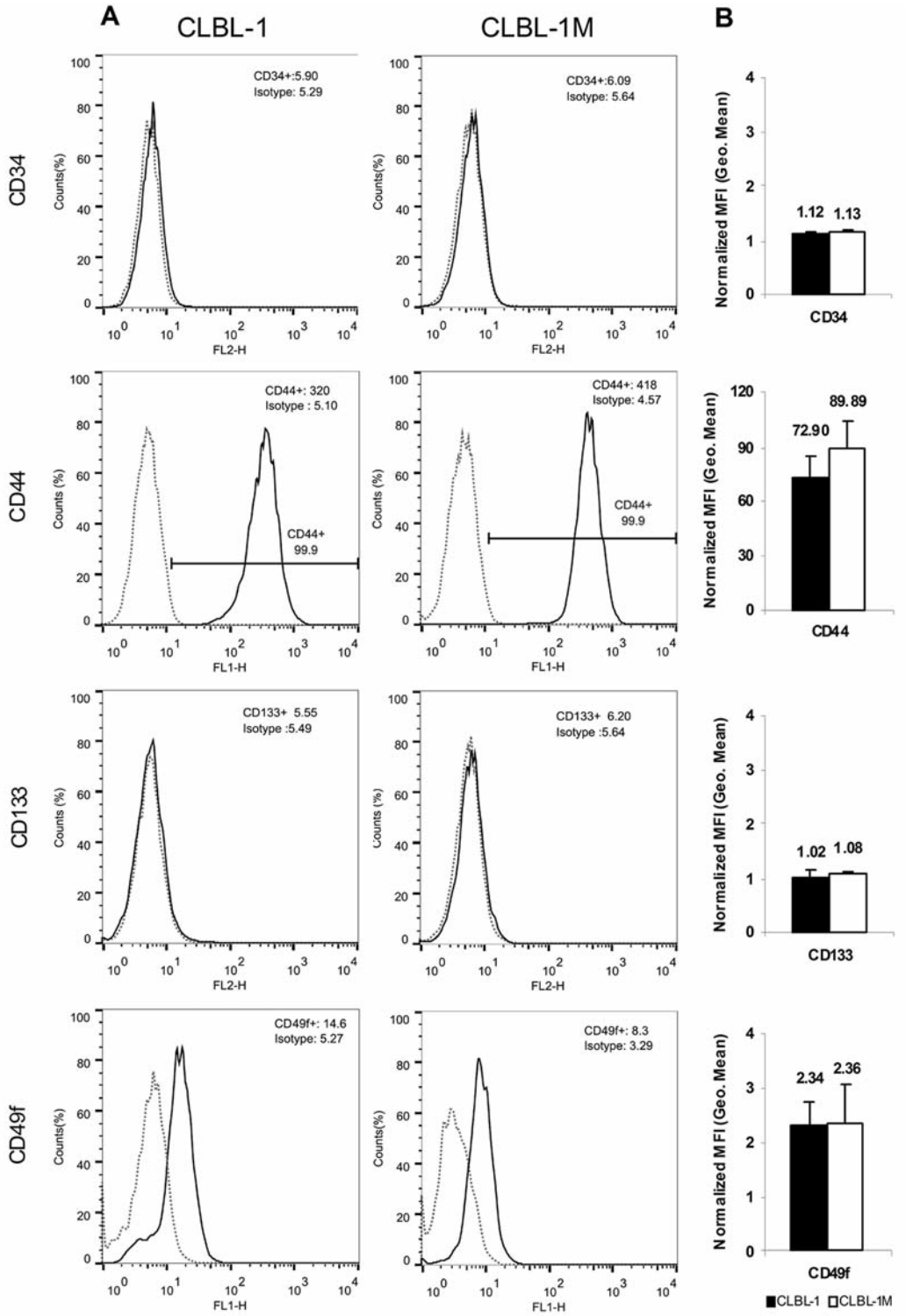

Figure 1. *Flow cytometric analyses assessing the expression of the cell surface markers. A: Representative histograms of CD34, CD44, CD133 and CD49f expressions on CLBL-1 and CLBL-1M cell lines. Gray-dashed line, isotype control; black solid line, specific antibody. Both cell lines were negative for CD34 antigen (CD34) and CD133, with staining of antibody equivalent to isotype control. Both cell lines were strongly positive for CD44 and weakly positive for CD49f. Almost all cells expressed CD44 (>99%). B: The normalized mean florescence intensity (MFI) of flow cytometric analysis is shown. The geometric mean (Geo. Mean) was used to analyze the flow cytometric data. The geometric mean of the MFI of cells stained with specific CD34, CD44, CD133 or CD49f antibodies was divided by that of the isotype staining to determine the expression ratio. Error bars represent the standard deviation. Each marker was measured three times.*

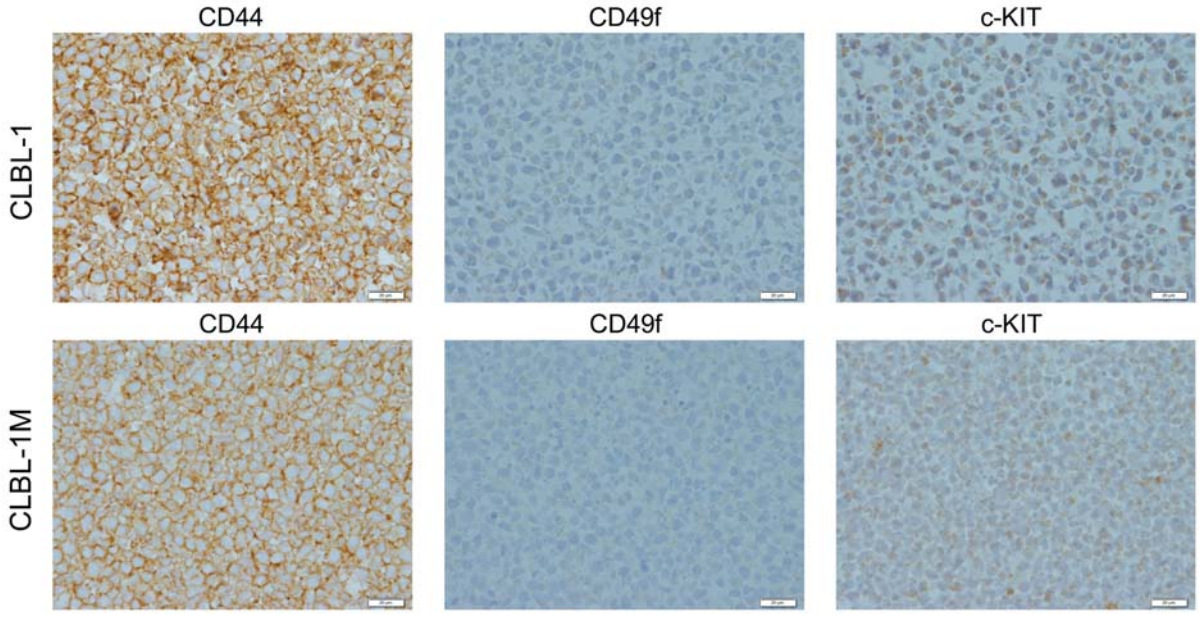

Figure 2. *Immunocytochemistry for CD44 antigen (CD44), CD49f and tyrosine-protein kinase Kit (c-KIT) in CLBL-1 and CLBL-1M cells. All CLBL-1 and CLBL-1M cells expressed CD44. Immunocytochemistry was negative for CD49f and c-KIT in both cell lines (×400).*

The results showed that CLBL-1 and CLBL-1M cells were able to grow in serum-free medium and form spheres, although a large number of dead cells were observed in the first three days (Figure 3A).

Marker analyses by conventional PCR, relative real-time PCR and flow cytometry: The expression of *Cd34, Cd133, c-Kit, Cd44, Itga6, Oct4, Nanog, Klf4, Sox2, Myc, Melk* and *Ddx5* were analyzed by conventional PCR (data not shown). *Cd44, Itga6, Myc, Ddx5* and *Melk* were detected in both CLBL-1S and CLBL-1MS cells, while expression of the other marker genes was undetectable, therefore we focused on *Cd44, Itga6, Myc, Ddx5* and *Melk* in relative real-time PCR.

We analyzed the expression levels of *Cd44, Itga6, Myc, Ddx5* and *Melk* in non-enriched cell lines, primary B-cell lymphoma samples and serum-free cultured spheres by relative real-time PCR comparatively (Figure 3C). The expression level of Cd44 in CLBL-1S was significant lower compared to CLBL-1 (p=0.045), and the expression of *Myc* was significant higher in CLBL-1S in comparison to CLBL-1 (p=0.001) and B-cell lymphoma samples (p=0.004). B-Cell lymphoma samples showed significantly higher expression of *Ddx5* when compared to CLBL-1 (p=0.013). Furthermore, *Itga6* expression was significant lower in B-cell lymphoma samples compared to CLBL-1 (p=0.002) and CLBL-1S (p=0.008). No significant difference was observed in the expression of *Melk*. Moreover, CLBL-1MS also had a significantly lower expression of Cd44 (p=0.027), but a significantly higher expression of *Myc* when compared to CLBL-1M (p=0.025). Compared to B-cell lymphoma samples, a high expression of *Myc* was also observed in CLBL-1MS but was not significant. Additionally, B-cell lymphoma samples (p=0.007) and CLBL-1MS (p=0.019) showed significant lower expression of *Itga6* when compared to CLBL-1M. Similarly, no significant differences were found in *Ddx5* and *Melk* expression.

Cell surface marker analyses of CD44 and CD133 of CLBL-1S and CLBL-1MS cells are shown in Figure 3B. The CLBL-1S and CLBL-1MS cells displayed strong positivity for CD44 (>99%) and negativity for CD133, nonetheless there were no distinct subpopulations. In addition, we examined CD44 and CD133 in CLBL-1S and CLBL-1MS cells by flow cytometry on six consecutive days from the fourth day to the ninth day, passage 1, passage 3 and passage 6. No distinct double-positive subpopulations were detected.

Discussion

Despite their discovery in leukemia and a number of solid tumors (10, 17, 18, 24, 38), the potential existence of CSCs in lymphoma is still controversially discussed in humans and dogs (39). Kim *et al*. evaluated a Hoechst 33342-positive side-population of canine lymphoma cells from cell lines and clinical samples by flow cytometry in 2013. The three canine

lymphoma cell lines GL-1, 17-71 and CL-1 used within their study revealed a content of 0.13%, 4.07% and 0.73% side-population cells respectively and canine B-cell lymphoma samples contained 68-78% side-population cells. Nevertheless, as stated by the authors, due to methodical aspects, an appropriate CSC subpopulation was finally not detected (40). The difference in the described percentages is particularly interesting, as conventionally potential CSCs are considered to be strongly resistant to chemotherapeutical drugs. Instead canine lymphoma can be targeted highly efficient by chemotherapeutic protocols, leading to nearly complete reduction of the neoplastic load.

An expanded population of lymphoid progenitor cells, co-expressing the hematopoietic progenitor markers CD34, c-KIT and/or CD133, leukocyte antigen CD45, CD21 and CD22, was characterized by Endicott *et al*. in lymph nodes of dogs with malignant B-cell lymphoma and related to malignant cells (41). These results were used to evaluate and describe the existence of a hierarchy in canine malignant B-cell lymphoma. Thus, the identification of potential CSCs of lymphomas is still a challenge and alternative detection approaches are able to complete previous work and help elucidate if cells with a stem cell-like character are stably detectable in canine lymphoma. The possibility of identifying cells with a stem cell-like character will be a major advance in understanding the origin of lymphoma, and may also provide possibilities for diagnostic approaches or for developing effective novel therapeutic approaches by specifically targeting these cells.

In general, the most important characteristics of stem cells are self-renewal, differentiation and proliferation. Candidate CSCs are considered to arise from mutated normal stem cells or progenitors (42). Therefore, they are considered to retain these stem-like properties and show 'stemness' (8, 9, 30). In humans, CD44 has been used as a single marker, or in conjunction with others such as CD133, CD24, epithelial specific antigen and CD49f (integrin-α6) to identify cells with stem cell-like character in primary tumors and cancer cell lines (11, 43). CD133, in its function as a hematopoietic and neural stem cell marker, has been widely used as a cancer stem cell marker in numerous types of tumors of humans and dogs. In part, CD133 is also discussed as a unique signature for CSCs, with effects on self-renewal, tumorigenesis and metastasis (44-46). Furthermore, *Oct4, Sox2, Klf4* and *Myc* are four genes described as being involved in re-programming stem cells and being used to produce induced pluripotent stem cells. These cells have also been shown to express the pluripotency marker NANOG (27, 47, 48).

In the present study, we analyzed a panel of molecular markers which have been described as associated in general with stem cells in different functions. Our results indicate *Cd44, Myc, Melk, Ddx5* and *Itga6* expression in the B-cell lymphoma cell lines CLBL-1 and CLBL-1M. Nearly all cells in both analyzed cell lines were CD44-positive flow cytometrically and very similar results were also demonstrated by immunocytochemistry. However, the gene expression level of *Cd44* in canine B-cell lymphoma cell lines and primary samples was lower than those in the reference lymph nodes. Interestingly, the same situation was also found concerning *Ddx5* and *Itga6* expression. *c-Kit* was stably expressed in the reference lymph nodes, in contrast it was undetectable in CLBL-1 and CLBL-1M cells, and expressed in only two primary B-cell lymphoma samples. MELK as an embryonic stem cell marker is involved in cell cycle, cell proliferation as well as apoptosis. It was demonstrated that *Melk* is overexpressed in various human cancer types, such as breast, colorectal, lung and ovarian cancer (49-51). Our result of *Melk* expression analysis demonstrated significantly high expression in the cell lines and primary samples. This suggests that *Melk* may play an interesting role in B-cell lymphoma development and can be used for further functional in vitro assays analyzing its role in canine B-cell lymphoma cell lines.

Consistent with previous research in dogs (40), Nanog expression was not detected in our B-cell lymphoma cell lines and all primary lymphoma samples. The remaining markers showed an alternating expression pattern. Only in a few number of the analyzed primary lymphoma samples did we observe expression of *Cd34, Cd133, c-Kit, Oct4, Klf4* and *Sox2*. This suggests that if these genes play a role, their expression is limited to a small amount. In this case, it remains questionable if the potential expression is sufficient to cause a biological effect. However, as some samples were found to be positive for their expression, it might also be explained by higher heterogeneity in primary tumor samples compared to the established cell lines. According to this, the primary samples could contain different stem cell types or progenitors such as lymphoid precursor cells. However, as our results showed, the expression patterns are different in the individual patients indicating that no general characteristic expression pattern exists. Apparently it remains a challenge to establish a standard procedure for the isolation of cells with stem cell-like character (potential CSCs) which would allow the development of a strategy specifically to target these cell populations. However, the idea of developing a combined therapeutic approach adding directed CSC targeting depending on the marker expression remains desirable.

The present study attempted to evaluate if spheres from B-cell lymphoma can be enriched using a formation assay derived from the neurosphere assay (17, 52, 53). This method has been widely used to identify CSCs in various cancer types. Previous studies have demonstrated that spheroid cells had higher drug resistance, more stem cell-like capabilities and a higher tumorigenicity in animal models (20, 21, 54, 55). Our study showed that CLBL-1 and CLBL-1M cells were able to grow under serum-free conditions. The

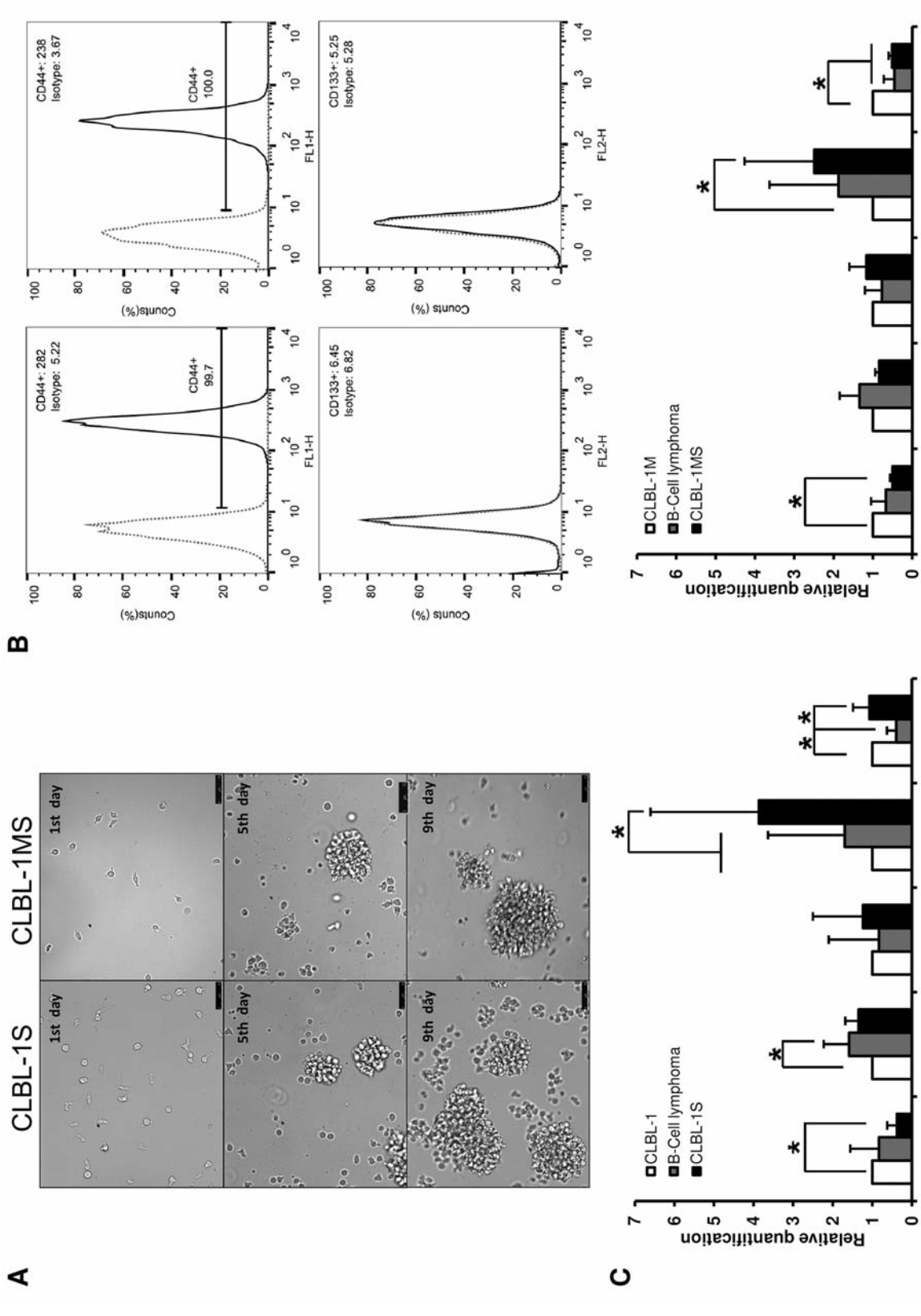

Figure 3. *Evaluation of stem cell marker genes in serum-free-cultured CLBL-1S and CLBL-1MS cells. A: Morphology of CLBL-1 and CLBL-1M cells cultured in serum-free medium. Images were captured on the indicated day of culture (scale bar: 50 μm). B: Analyses of cell surface markers CD44 and CD133 by flow cytometry. All cells were positive for CD44 and negative for CD133. No discriminate subpopulation was demonstrated. C: The expression levels of stem cell markers in CLBL-1S, CLBL-1MS and primary B-cell lymphoma samples relative to CLBL-1 and CLBL-1M cells. Statistical analyses were performed by the Rest 2009 software. Significantly different at *p<0.05. Error bars represent the standard deviation.*

observations revealed that the spheres formed by CLBL-1 and CLBL-1M cells appear not to be as regular and compact in shape as seen in solid tumors such as glioblastoma, prostate cancer and mammary tumor (17, 22, 24, 56). The PCR results showed that *Cd34, Cd133, c-Kit, Oct4, Klf4* and *Sox2* were not expressed in CLBL-1 and CLBL-1M cells nor in serum-free cultured cells. Therefore, these markers appear not be suitable for serving as CSC markers for the canine B-cell lymphoma cell lines analyzed here.

In contrast to this, *Myc* expression revealed a more interesting pattern. In general, *Myc* is a regulator gene which causes unregulated expression of many genes involved in cell proliferation, cell growth, apoptosis, differentiation and stem cell self-renewal (39, 57). Mutations and aberrant expression of *Myc* in B-cell lymphomas have been already reported. In humans, these mutations and deregulations are usually caused by chromosomal translocations (58-60). Our study showed that compared to non-neoplastic lymph nodes, the tested B-cell lymphoma cell lines and primary lymphoma samples did not exhibit significant up-regulation of *Myc* expression. CLBL-1 and CLBL-1M revealed slightly lower *Myc* expression compared to non-neoplastic lymph nodes. Interestingly, our results indicate that *Myc* expression was significantly up-regulated in serum-free-cultured cells, suggesting that *Myc* expression characterizes the serum-free culture-generated subpopulation. Consequently, akin to our results seen for *Melk*, it remains interesting to analyze the role *Myc* in further functional sphere assays.

Flow cytometric analyses comparing non-enriched CLBL-1 and CLBL-1M cells with serum-free cultured CLBL-1S and CLBL-1MS cells showed similar expression patterns for CD44 and CD133, even though the mRNA level of *Cd44* in serum-free cultured cells was lower when compared to the 'native' non-enriched cell lines. Matching our PCR results, a distinct $CD44^-$ or $CD133^+$ sub-population was not detected. This suggests that when CSC-like cells are present, the cell surface markers CD44 and CD133 are not suitable for the isolation of a potential CSC sub-population from the B-cell lymphoma cell lines CLBL-1 and CLBL-1M. Consistent with our results, previous studies reported that no CD34, CD133 and CD117 sub-populations were detectable in several analyzed canine cancer cell lines including a canine 3132 B-cell lymphoma cell line. However, CD44 was expressed by almost all cells and thus it appears that in cultured canine cells CD44 is not a specific marker for potential CSCs (61, 62). A further discussed possibility is that canine B-cell lymphoma CSCs might be isolated by alternative markers based on the hypothesis that lymphomas derive from the normal B-cell counterpart (63, 64). However, interestingly the significant down-regulation of *Cd44* expression in serum-free cultured cells indicates that serum-free culture enriched the slowly proliferating cells.

In conclusion, our results indicate that a specific pattern of stem cell marker expression indicating the presence of stem cell-like cells is not present in the canine B-cell lymphoma sources we analyzed herein. In general, stem cell marker expression appeared to be down-regulated in B-cell lymphoma primary samples and cell lines. However, the finding of up-regulation *Melk* in the B-cell lymphoma primary samples and cell lines indicates a potential interesting role for *Melk* in canine B-cell lymphoma. Furthermore, the up-regulation of *Myc* in serum-free-generated spheres offers interesting possibilities for functional assays characterizing the generated specific sub-population.

Competing Interests

The Authors declare that they have no competing interests.

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