Comparative Characterization of Stem Cell Marker Expression, Metabolic Activity and Resistance to Doxorubicin in Adherent and Spheroid Cells Derived from the Canine Prostate Adenocarcinoma Cell Line CT1258

WEN LIU[1,2*], MOHAMMED MOULAY[1*], SASKIA WILLENBROCK[1], CATRIN ROOLF[2],
CHRISTIAN JUNGHANSS[2], ANACLET NGENAZAHAYO[3], INGO NOLTE[1] and HUGO MURUA ESCOBAR[1,2]

[1]Small Animal Clinic, University of Veterinary Medicine Hannover, Hannover, Germany;
[2]Division of Medicine, Dept. of Haematology, Oncology and Palliative Medicine,
University of Rostock, Rostock, Germany;
[3]Institute of Biophysics, Leibniz University Hannover, Hannover, Germany

Abstract. *Background: Canine prostate cancer represents a spontaneous animal model for the human counterpart. Cells with stem cell-like character are considered to play a major role in therapeutic resistance and tumor relapse. Thus, the identification of markers allowing for recognition and characterization of these cells is essential. Materials and Methods: Expression of 12 stem cell marker genes in the canine prostate cancer cell line CT1258 and spheroid cells generated from these was analyzed by quantitative real-time PCR. In CT1258 and the generated spheroid cells, CD44 and CD133 expression was analyzed by flow cytometry, as well as proliferation and doxorubicin resistance. Results: Integrin alpha-6 (ITGA6) expression and metabolic activity were significantly up-regulated in CT1258-derived spheroid cells, while doxorubicin resistance remained comparable. Conclusion: ITGA6 de-regulation and metabolic activity appear to be characteristic of the generated spheres, indicating potential intervention targets.*

Prostate cancer is, following lung cancer, considered to be the second leading cause of cancer-related death in men in the Western societies (1). Besides man, the dog is the only domesticated mammalian species in which this malignancy also occurs spontaneously (2, 3). In both species, a comparable progression with local invasive growth, metastatic behaviour and histopathological phenotype can be observed (2, 4-6). Unlike the condition in men, the risk of prostate cancer development in dogs is considered to be significantly lower, with an estimated incidence of 0.2-0.6% (7, 8). Regardless of this, the prognosis for canine prostate cancer is poor as the disease is highly aggressive and mostly diagnosed at a very late stage (9, 10). Akin to men, elderly canine individuals are predominantly affected at an average age of 10 years (3, 5, 11). However, some differences in prostate cancer of both species remain, such as different incidence, response to androgen deprivation therapy and different expression of diagnostic markers such as prostate-specific antigen (PSA) (12, 13). Despite these differences, the dog is considered to represent an appropriate natural complementary animal model for human prostate cancer, especially for late-stage human prostatic carcinoma (2, 14-19). Consequently, the evaluation of novel diagnostic and therapeutic regimens provides benefit for both humans and dogs (15, 20).

Cancer-related therapeutic failure after chemotherapeutic regimens was lately suspected to be caused by a minor, highly drug-resistant cell population with stem cell-like character commonly referred to as cancer stem cells (CSCs). These cells are assumed to play a major role in tumor relapse and metastasis in advanced stages of the disease (21-24). However, these sub-populations usually represent a rather small percentage of the total tumor burden, constituting a minor fraction of the cancer cells (25).

Several stem cell markers have been described as being expressed in these cells, allowing screening and isolation of these sub-populations (26, 27). Despite the fact that some of the genes for these markers are also expressed in non-neoplastic cells, the expression of *CD44* (28), prominin 1

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*These Authors contributed equally to this study.

Correspondence to: Professor Ingo Nolte, Small Animal Clinic, University of Veterinary Medicine Hannover, Bünteweg 9, 30559 Hannover, Germany. Tel: +49 5119536202, Fax: +49 511953-6204, e-mail: ingo.nolte@tiho-hannover.de

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

Gene	Full 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
CD44	CD44 molecule	AATGCTTCAGCTCCACCTG	CGGTTAACGATGGTTATGGTAATT	92
ITGA6	integrin, alpha 6	TCAGACCCTTAACTGCAGCA	CATAACCTCGAGCGCAGAA	132
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

Table II. *Antibodies used for flow cytometric analyses.*

Monoclonal antibody	Specificity	Clone	Marker identified	Monoclonal isotype
Anti-canine CD44 FITC	Dog	YKIX337.8	CD44	Rat IgG2aϰ FITC
PE anti-mouse CD133	Mouse/Dog	13A4	CD133	Rat IgG1ϰ PE

FITC: Fluorescein isothiocyanate; PE: phycoerythrin.

(*CD133*) (29), v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog (*c-KIT*) (30), *CD34* (31), integrin alpha 6 (*ITGA6*) (32-34), v-myc myelocytomatosis viral oncogene homolog (*MYC*) (35), Nanog homeobox (*NANOG*) (36, 37), DEAD (Asp-Glu-Ala-Asp) box helicase 5 (*DDX5*) (38), kruppel-like factor 4 (*KLF4*) (39), (sex determining region Y)-box 2 (*SOX2*) (28), maternal embryonic leucine zipper kinase (*MELK*) (40) and POU class 5 homeobox 1 (*OCT4*) (41) has been widely considered as a potent tool to affirm the presence of these cell sub-populations in different human cancer types such as breast, haematopoietic, lung, brain and prostate cancer (42). In human prostate cancer, selection of these cells was achieved by sorting CD44 and CD133 double-positive prostate cancer cells (43). As the proportion of these cells in the total tumour cell population is low, their sorting and detection by fluorescence-activated cell sorting can be challenging (43), and thus cultivation-based enrichment strategies are employed (21, 43-45). The possibility of isolating and handling these cells allows for detailed characterization of the respective sub-populations and consequently the development of strategies aiming at a directed targeting.

Considering the lack of therapeutic options in dogs, the characterization of potential cancer cell populations with a stem cell-like character in canine prostate cancer could be of significant value for the development of therapeutic approaches. In previous studies, we showed that the phenotype of the canine prostate adenocarcinoma cell line CT1258 provides a constantly stable cell line model (46). In the present study, we attempted to enrich potential cancer stem-like cells by serum-free cultivation, investigate the expression levels of 12 stem cell marker genes and characterise the doxorubicin resistance.

Materials and Methods

Adherent and suspension cell culture. The adherent canine prostate adenocarcinoma cell line CT1258 (47) was cultivated in medium 199 (Gibco, Karlsruhe, Germany), supplemented with 10% Fetal Bovine Serum (FBS) Superior (Biochrom AG, Berlin, Germany) and 2% penicillin/streptomycin (Biochrom AG). The cells were incubated at 37°C with 5% CO_2. For the suspension culture, adherent cultivated cells were isolated by trypsinization and washed with phosphate-buffered saline (PBS). The cells were re-cultivated at a density of 10000 cells/ml in 100 cm2 dishes (TPP Techno Plastic Products AG, Trasadingen, Switzerland) coated with 1% agarose. Serum-free DMEM/F12 medium (Biochrom AG) was supplemented with 5 µg/ml insulin (Sigma-Aldrich, Seelze, Germany), 2 mM L-glutamine (Sigma-Aldrich), 2% B27 supplement minus vitamin A (Life Technologies
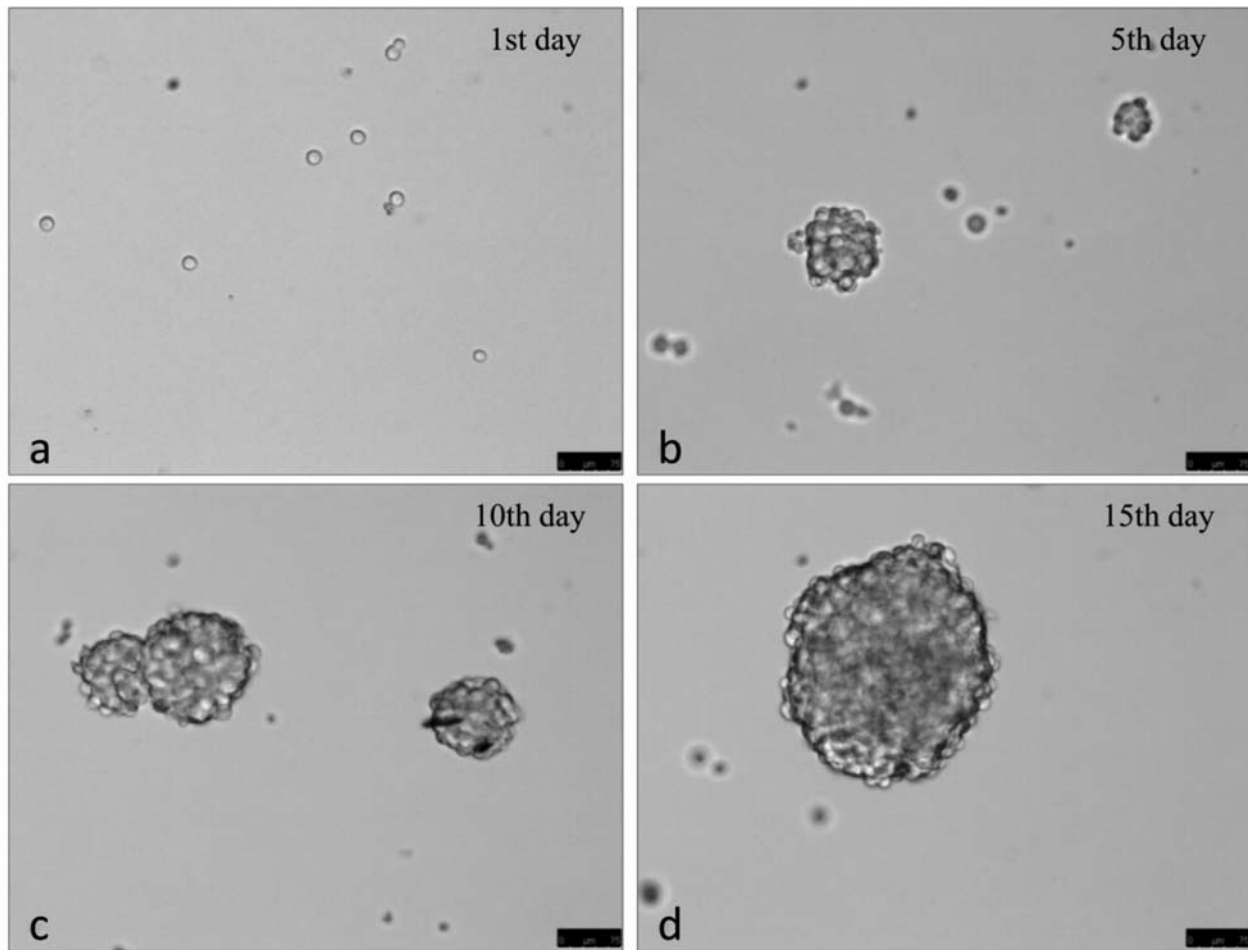
Figure 1. *Sphere-formation of CT1258 cells in suspension in serum-free culture medium. CT1258 sphere-forming cells were imaged on the indicated day of culture. Day 1: After trypsinization, a defined number of 10^5 CT1258 cells/ml was transferred in suspension medium. Day 5: Few floating cells started to construct spheroids, the majority of cells were dead. Day 10: The dead cells were reduced compared to day 5 and the number of spheres increased. Day 15: The volume of the formed spheres increased, while a low number of dead cells was recorded.*

GmbH, Darmstadt, Germany), 20 ng/ml EGF (Biochrom AG) and 20 ng/ml bFGF (Invitrogen, Darmstadt, Germany).

Two different serum-free cultivation periods were chosen. Both cultivation periods utilised two dishes, and cells were cultivated for 10 days (CT1258 S10d) and 15 days (CT1258 S15d). The medium was changed under both incubation periods every three days. The cells were incubated at 37°C with 5% CO_2 with manual shaking of the dishes at least once every two days during the whole serum-free cultivation period. The cultivations were performed three times independently, providing three biological replicates for analyses.

To monitor the formation of cell spheres, images were taken at 1, 5, 10 and 15 days of culture in suspension medium at 100-fold magnification using a Leica DMI6000B microscope (Leica Mikrosysteme Vertrieb GmbH, Wetzlar, Germany).

At the end of the cultivation period, spheres in each dish from both incubation periods were collected in a single sterile 15 ml tube (Greiner bio-one GmbH, Frickenhausen, Germany) and centrifuged for 5 min at 118 ×g. Subsequently, the spheres were washed with 1 ml of PBS and used for further analyses.

RNA isolation and cDNA synthesis for quantitative real-time PCR. The isolation of total RNA from adherent CT1258 cells and cell spheroids S10d and S15d was carried out using RNeasy mini Kit (Qiagen, Hilden, Germany). On-column DNase digestion was performed for eliminating genomic DNA during this experiment. cDNA synthesis was carried out using 500 ng of total RNA in 20 μl according to the manufacturer's protocol for the QuantiTect Reverse Transcription Kit (Qiagen).

Analysis of stem cell marker gene expression by qPCR. The specific gene expression assays of selected markers described in Table I were used to perform relative qPCR. Beta-actin (*ACTB*) was utilised as endogenous control. The qPCR results were analysed using the delta delta CT (ΔΔCT) method.

The qPCR reactions were carried out using the Eppendorf Mastercycler® ep *realplex* real-time PCR system (Eppendorf, Hamburg, Germany) and QuantiTect SYBR green qPCR Kit (Qiagen). In each reaction, 1 μl cDNA equivalent to 25 ng total RNA was used in a final volume of 20 μl. The program of qPCR reactions was
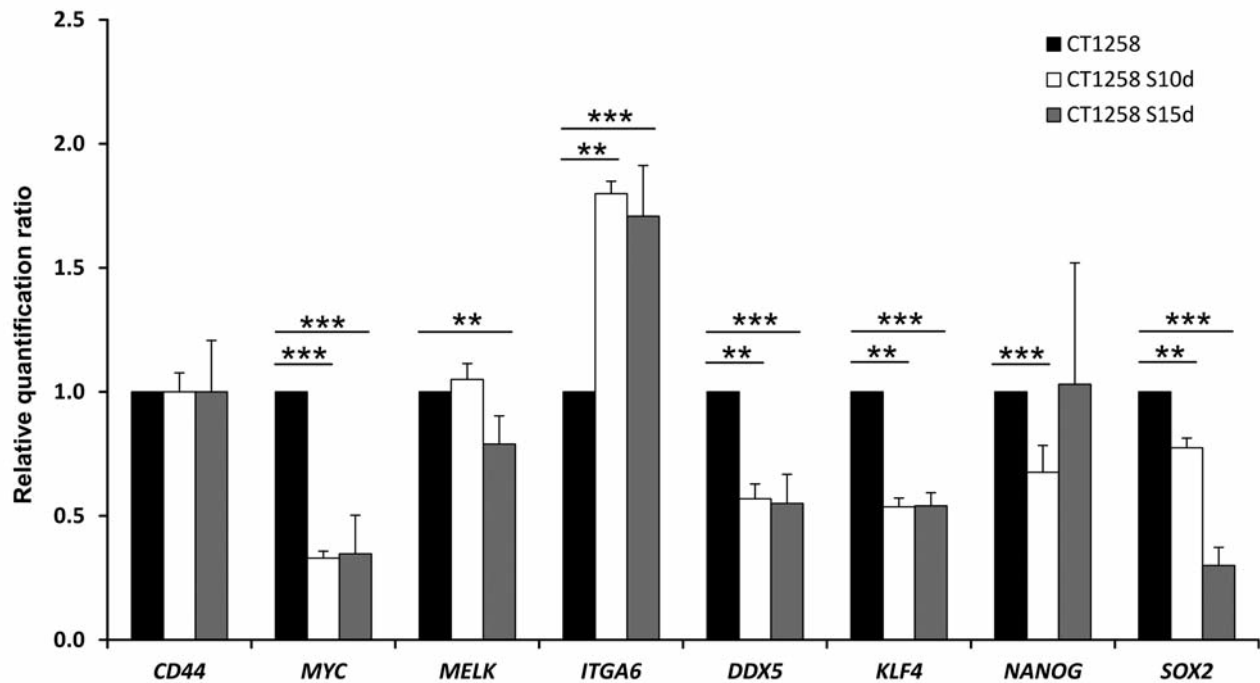
Figure 2. *Relative expression of selected markers in CT1258 S10d and S15d spheroid cells compared to adherent CT1258 cells. Quantitative real-time PCR analyses of the expression of 12 selected stem cell markers in spheroid compared with adherent CT1258 cells. CT1258: Adherent CT1258 cells were used as the calibrator for the spheroid cells. CT1258 S10d: Spheroid cells cultivated for 10 days. CT1258 S15d: Spheroid cells cultivated for 15 days. *p<0.05; **p<0.01; ***p<0.001.*

performed according to the manufacturer's protocol for the Quanti-Tect SYBR green qPCR Kit (Qiagen). All samples were analysed in triplicates including non-template and non-reverse transcriptase controls for each reaction.

The efficiency test for all analysed genes was used by applying serial dilutions of standard DNA for 1:2 and 1:10, and all assay efficiency were between 0.91 and 1.10.

As the expression of the majority of the analysed markers was evaluated by us previously in adherent CT1258 cells, the non-enriched CT1258 cell was selected as calibrator and was set a value of '1' for $\Delta\Delta$CT analysis for all target genes referenced to the expression level.

Flow cytometric analyses of CD44 and CD133. For flow cytometric analyses, the spheroid CT1258 cells were centrifuged at 118 ×g for 5 min. Subsequently, the pellet was dissociated by treatment with Accutase Cell Dissociation Reagent (Life Technologies GmbH, Frankfurt, Germany) for 5 min in order to obtain single-cell suspensions for flow cytometric analyses. Adherent CT1258 cells cultivated under regular conditions (47) were used as reference for later comparison with the generated CT1258 spheroids. After a washing step with PBS, the adherent CT1258 cells were also treated with Accutase Cell Dissociation Reagent as described above.

All different CT1258 cell suspensions were adjusted to a total number of 10^5 cells in 100 µl using an Auto T4 automated cell counter (Nexcelom Bioscience, Washington, DC, USA). The adherent CT1258 cells, as well as the spheroids, were incubated for 30 min at 4°C with either monoclonal anti-CD44-FITC (eBioscience, Frankfurt, Germany), or with anti-CD133-PE (eBioscience)

(Table II) antibodies. The samples were analysed via flow cytometry as described previously (46).

Characterization of metabolic activity and doxorubicin resistance. Adherent CT1258 cells and spheroids were harvested and dissociated to single-cell suspensions as described above. Cell viability was determined by conventional trypan blue staining.

For metabolic studies, 22500 cells (0.5 ml medium/well) were plated in triplicate in a 24-well plate. Cell counts were determined after 72 h with a haemocytometer. At the same time, 5000 cells per well were plated in triplicate in 100 µl in 96-well plates. Metabolic activity was analysed after 72 h using tetrazolium compound WST-1 reagent (Roche, Mannheim, Germany).

The doxorubicin resistance of CT1258 spheroid cells was analysed by cell count and WST-1 cell proliferation assay. Cells were plated as described above, 22500 cells/well in a 24-well plate in 0.5 ml medium, and treated with 50 nM and 100 nM doxorubicin. Cell counts were determined after 72 h by trypan blue staining with a haemocytometer. In 96-well plates, 5000 cells/well were plated in 100 µl medium with 0 nM, 10 nM, 50 nM, 100 nM, 150 nM and 200 nM doxorubicin. Each concentration was performed in triplicate. Cell metabolic activity was analysed after 72 h using tetrazolium compound WST-1 reagent. A complete culture medium 199 was used in the experiments.

Statistical analysis. Results of each experiment were expressed as the mean±standard deviation. Significant differences were calculated using Student's *t*-test, where a *p*-value of less than 0.05 was considered to be statistically significant.
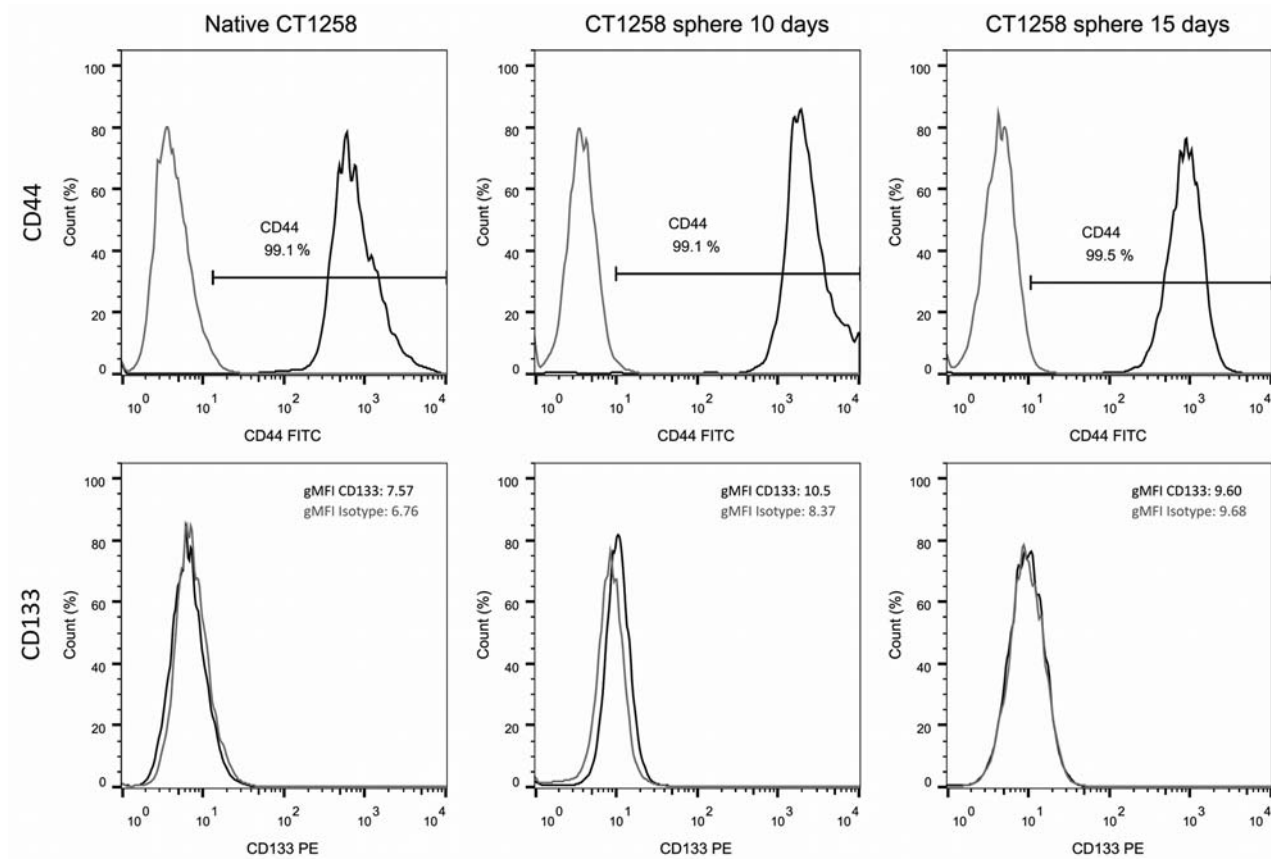
Figure 3. *Flow cytometric analyses of adherent and spheroid CT1258 cells. Monoclonal antibodies against mouse/dog CD44 and CD133 labeled with Fluorescein isothiocyanate (FITC) and Phycoerythrin (PE) fluorophore substances were used. The representative histograms show the analyzed cell lines stained with CD44 and CD133 antibodies (black lines) compared to the corresponding isotype controls (gray lines). The geometric fluorescence intensities (gMFI) are shown for CD133 PE staining.*

Results

CT1258 cell sphere cultivation. At the beginning of culti-vation, a significant number of non-vital cells was observed. Viable cells growing in floating spheres began to arise after a cultivation period of five days. The formation of new floating spheres increased until day 10. Thereafter, the num-ber of formed spheres stayed stable until day 15 (Figure 1). The formed spheres showed tight cell-cell adherence and were difficult to separate into a single-cell suspension. The images of forming spheres were taken consecutively at 100-fold magnification at days 1, 5, 10 and 15 (Figure 1).

Expression of stem cell marker genes in CT1258 spheroid cells. The qPCR of spheroid and adherent CT1258 cells showed in general a comparable stem cell marker expression pattern to our previous analyses of adherent CT1258 cells (46) (Figure 2).

The expression of *CD133*, *c-KIT*, *CD34* and *OCT4* was undetectable in analyzed CT1258 spheroid cells after 40 cycles. *CD44* was shown to be highly expressed in S10d (p=0.9684) and S15d (p=0.9668) cells. *MYC* was shown to be down-regulated in S10d (p=0.0001) and S15d (p=0.0006) cells, when compared to the calibrator CT1258 cell line. *MELK* was shown to be slightly increased in S10d (p=0.3024) and down-regulated in S15d (p=0.0061) compa-red to the calibrator. *ITGA6* was found to be strongly up-regulated in S10d (p=0.0013) and S15d (p=0.0004) compa-red to the calibrator. *DDX5* showed a decreased expression in S10d (p=0.0064) and S15d (p=0.0002) compared to the calibrator. *KLF4* expression was decreased in S10d (p=0.0019) and S15d (p=0.0001) compared to the calibrator. *NANOG* expression was also shown to be decreased in S10d (p=0.0001) while being similar in S15d (p=0.8760), compa-red to the calibrator. *SOX2* was down-regulated in S10d (p=0.0099) and S15d (p=0.0000) compared to the calibrator.
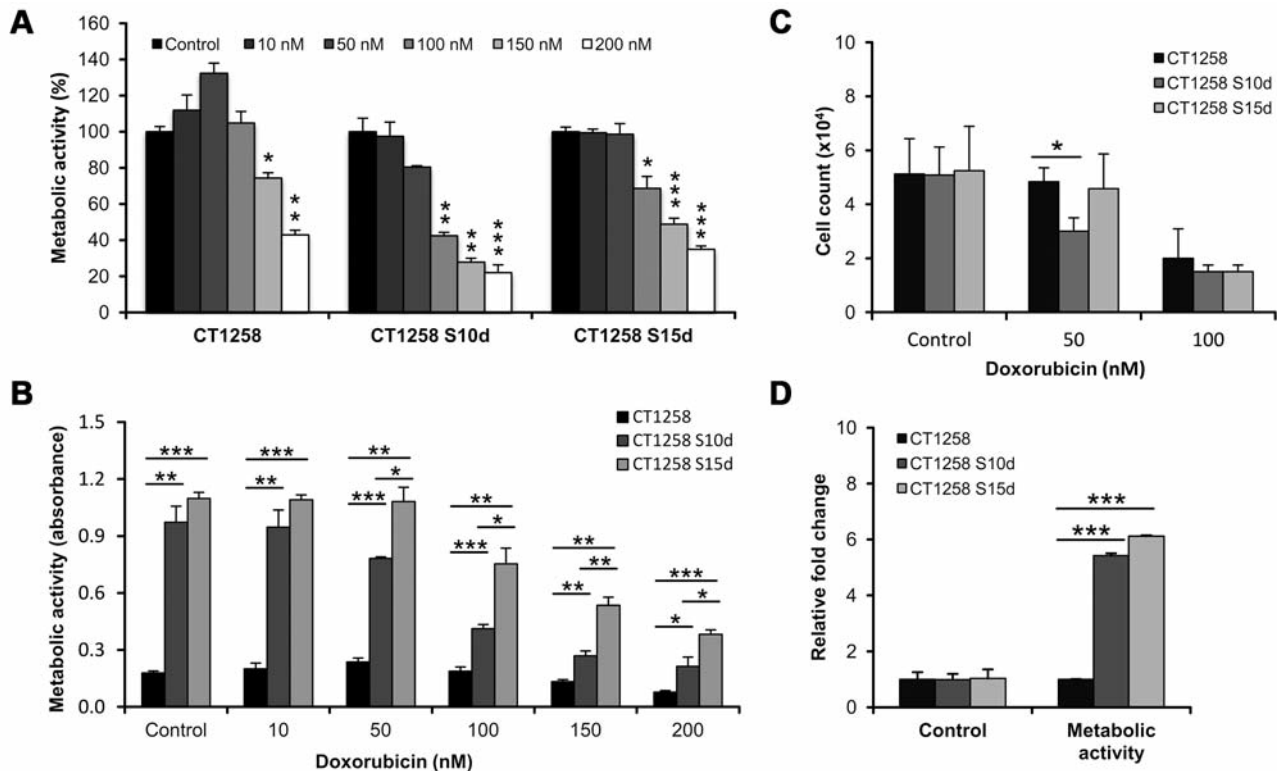
Figure 4. *Cell proliferation and doxorubicin resistance analyses of adherent and spheroid CT1258 cells. a: Doxorubicin resistance analyses of adherent and spheroid CT1258 cells. Results were calculated by dividing the absorbance value of doxorubicin-treated cells by that of untreated adherent cells and spheroid cells respectively. Data shown are the relative mean±SD of metabolically active cells (%) (n=3). b: The metabolic activity of adherent and spheroid CT1258 cells. Data shown are the mean absorbance value±SD (n=3). c: The data are the cell counts of adherent and spheroid cells untreated as well as treated with 50 nM and 100 nM doxorubicin (n=3). d: Comparison of cell proliferation and metabolic activity in untreated cells. Cell proliferation is shown as the relative fold change of cell counts (spheroid vs. adherent cells); metabolic activity was determined by the WST-1 assay. Data shown are the relative fold change of absorbance value (spheroid vs. adherent cells)±SD (n=3). *p<0.05; **p<0.01; ***p<0.001. Cells were incubated for 72 h in all experiments.*

Flow cytometric analyses of CD44 and CD133. In the adherent CT1258 and the generated spheroid cells, the flow cytometric analyses showed strong expression of CD44, with positivity above 99% cells for CD44. The analyses of CD133 revealed a weak signal in adherent and generated spheroid CT1258 cells compared to the corresponding isotype controls (Figure 3).

For better comparability of the flow cytometric data, normalized geometric mean fluorescent intensities (gMFIs) of the specific CD133 staining were calculated by division of the gMFI of CD133-labled cells by the gMFI of the respective isotype control. The normalisation of the CD133 staining led to low gMFIs for adherent CT1258 cells (1.08±0.07), while the normalized gMFI slightly increased for S10d cells (1.09±0.11) and slightly decreased in S15d cells (0.95±0.07).

Nonetheless, the flow cytometric data for CD133 and CD44 showed no distinct double-positive subpopulations in spheroid cells.

Characterization of metabolic activity and doxorubicin resistance. The doxorubicin resistance of adherent and sphe-roid CT1258 cells was analyzed by incubation with different concentrations of doxorubicin for 72 h (Figure 4a-c).

To interpret the doxorubicin resistance data of the WST-1 assay, values are shown as the percentage of metabolic acti-vity, normalized to that of untreated cells. Compared to untreated adherent CT1258 cells, the metabolic activity of doxorubicin-treated CT1258 cells decreased, starting at 150 nM (Figure 4a). In CT1258 S10d cells, compared to untreated S10d cells, the metabolic activity also apparently decreased to a similar level but at 50 nM (Figure 4a). Like-wise, cell count analyses also showed a significant decrease at 50 nM in S10d cells (Figure 4c). In CT1258 S15d cells, metabolic activity significantly decreased starting at 100 nM (Figure 4a). Furthermore, cell count analyses showed that CT1258 S15d cells display a similar value as adherent CT1258 cells at 50 nM and 100 nM (Figure 4c).

Interestingly, according to the raw absorbance value, the metabolic activities of CT1258 spheroid cells were remarkably higher when compared to their adherent counterparts, untreated or doxorubicin-treated cells (Figure 4b and d). In untreated cells, cell counts of adherent cell and spheroid cells did not show any major differences. However, the metabolic activities of CT1258 S10d and S15d cells were more than 5-fold higher than that of adherent CT1258 cells (Figure 4d). Doxorubicin-treated CT1258 spheroid cells also showed significantly higher metabolic activities than the CT1258 adherent cells. Furthermore, starting at 50 nM, CT1258 S15d cells displayed a significant higher metabolic activity compared to CT1258 S10d cells (Figure 4b).

Discussion

CSCs are increasingly being the center of attention in cancer research due to their suspected key role in tumor progression, metastasis, resistance to therapeutics and recurrence of cancer (21, 22). A development of therapeutic strategies allowing for selective targeting of these cells would be of considerable value, especially in cases where conventional therapeutic options are limited.

In recent years, several research groups have reported isolation of CSCs from prostate cancer (43, 48-50). Commonly, CSCs were identified and isolated using three methods: sorting of specific cell side populations, activated cell sorting based on specific surface markers, and sphere-forming culture (48). These initial sphere generation steps are often followed by verification of tumor-forming capacity *in vivo*. Yamamoto *et al*. generated spheres from human benign prostatic hyperplasia cells (BPH) in serum-free medium. In their evaluation of the generated sphere-forming cells, flow cytometric analyses showed that CD49f (ITGA6) exhibited a stronger marker character in comparison to CD44 and CD133 (34). In contrast, Fan *et al*. were not able to generate spheroids from the human prostate cancer cell line LNCaP (50). However, CD44 and CD133 are described to characterise putative CSCs in different canine tumour models (51), as well as human prostate cancer (43, 52). Additionally, further markers such as C-KIT, CD34, DDX5 and MELK were used as single markers or in a marker panel in combination with CD44 and CD133 to identify putative CSC populations (53-60). However, due to the different materials and methods used in these studies, as well as the heterogeneous results, it was still not possible to unify and define a specific biomarker set for prostate CSCs. Consequently the question can be posed if a uniform marker panel allowing for reliable detection of these cells even exists.

In the present study, a conventional suspension culture method was used to stimulate the formation of spheres from the canine prostate adenocarcinoma cell line CT1258. The results showed that CT1258 cells form spheres when grown in serum-free media supplemented with growth factors. During the first five days, high numbers of non-vital cells were observed, suggesting that serum-free conditions could have advantages for specific CT1258 cell clone selection and enrichment. The number of generated spheres increased during the first 10 days of cultivation. Between the 10th and 15th day, the diameter of the spheroids increased. As mentioned, human prostate cancer cell lines have different abilities for generating spheres independent of their cell line-specific nature and metastatic potential (48, 50, 61). Consequently, sphere-forming ability per se is likely not to be sufficient to fully characterize and identify potential cancer cells with stem cell-like character. Thus, the combination of sphere-forming capacity and evaluation of stem cell marker expression at the gene and protein levels might help to identify and selectively enrich potential subpopulations with stem cell-like character in culture.

In general, several stem cell markers in different panel combinations are currently used to isolate potential CSCs. As previously described, the dual staining of CD44 and CD133 is commonly used to characterize potential CSCs in different types of cancer (43, 51, 62). In human prostatic lesions, small numbers of CD133$^+$ cells of up to 1% were identified (43, 62). In the present study, flow cytometric analyses showed that a high number of CD44$^+$ cells were detected in the spheres formed after 10 and 15 days of cultivation in serum-free medium. While CD44 was strongly expressed, no distinct expression of CD133 was detected within the spheres formed after both these cultivation periods. These results indicate that the combination of the surface markers CD44 and CD133 for CT1258 cells is not suitable for the definition of subpopulations with stem cell-like characteristics.

The qPCR analyses showed that *c-KIT, CD133, CD34* and *OCT4* were not detectable in CT1258 S10d and S15d cells, nor in the adherent CT1258 cells cultivated under regular conditions as previously described by us (46). In Man, these markers were reported to be significantly expressed in prostate cancer and partially in the prostate cancer-derived bone metastatic cell line PC-3 (63, 64).

DDX5 and *MELK* are considered stem cell marker genes and also prostate cancer marker candidate genes, as both were reported to be overexpressed in the prostate cancer cell lines PC-3 and LNCaP (56, 57, 65, 66). In the current study, expression of *DDX5* and *MELK* did not differ between the spheroid and adherent CT1258 cells.

The transcription factor genes *SOX2, KLF4, OCT4* and *NANOG* were described to be involved in the induction of pluripotency and the maintenance of stem cells in their undifferentiated state (36, 56, 67). Furthermore, the expression of these genes was suggested to correlate with the degree of malignancy in human prostate cancer (68, 69). The results of our study showed that these transcription factor genes were weakly expressed in serum-free cultured spheroid cells, leading

to the conclusion that these genes are not predominantly involved in the formation of spheres from CT1258 cells.

MYC is known to be overexpressed in human prostate cancer (70, 71). In contrast to these findings, our qPCR analyses showed down-regulation of MYC in both CT1258 spheroid cultures when compared to adherent CT1258 cells. Concerning the aggressive character of the cell line CT1258 *in vivo*, this result was unexpected, indicating that alternative mechanisms leading to cell proliferation appear to be active in CT1258 (72).

The influence of ITGA6 on the functions of potential CSCs was clearly shown by enhancing their efficiency in tumourigenesis (69, 73, 74). In human prostate cancer, ITGA6 was described as keeping CSCs undifferentiated and increasing the ability of the potential CSCs to migrate into other organs such as the neural system or bones (73-76). ITGA6 was found to be highly increased in sphere-forming BPH cells to up to 98% in comparison to freshly isolated BPH cells in which 5.6±3.1% ITGA6-positive cells were detected (34). Similarly, our qPCR analyses demonstrated that *ITGA6* expression was significantly increased in CT1258 S10d and S15d spheroid cells compared to adherent CT1258 cells. As mentioned before, flow cytometric analyses of human prostate cancer generated sphere-forming cells showed that ITGA6 (CD49f) revealed a stronger marker character in comparison to CD44 and CD133 (34) for potential CSC selection. These results in human prostate cancer and the spheroid qPCR expression result of our study suggest that ITGA6 could be a potential indicator for the existence of a specific cell subpopulation in selectively cultivated CT1258 sphere subclones.

As described, the comparative expression analyses revealed that no up-regulation of CD44 and CD133 was observed at the gene expression level in the CT1258 spheres. Thus, as flow cytometric data verified these findings, it can be stated that 'conventional' CSC enrichment did not take place in CT1258 cells. This fact does not imply that in general CT1258 lacks a potential subpopulation of cells with stem cell-like character. These findings indicate that apparently the 'typical' CD44/CD133 double-positive pattern is not characteristic of CT1258 cells, as has been also reported in human prostate cancer stem cell studies (34, 49). However, potential specific cell populations with stem cell-like character in CT1258 might be characterized by an individual characteristic stem cell marker panel with ITGA6 as the predominant marker. In summary, the results and the comparison to the human counterpart again raises the question if in general a 'stable' marker set actually exists for CSCs, or if stem cell marker expression varies individually as the presentation of tumors does. As mentioned above, in our study ITGA6 appears to characterize an enriched specific sub-population of CT1258 cells. Consequently, besides the evaluation of the tumor-forming potential of the generated spheres *in vivo*, it is tempting to further characterize the role of ITGA6 in CT1258 cells and the enriched CT1258 subclones.

In general, cells with stem-like properties are discussed as being more resistant to conventional chemotherapy than the bulk of overall cancer cell populations (21, 61, 77). Accordingly, this may also be the main reason for cancer recurrence. Metabolic alteration is considered a hallmark of cancer cells and an important factor contributing to cancer cell survival (78). CSCs were reported to have special metabolic features that can distinguish them from other cancer cells (79). Cancer cells generally increase their glycolysis and CSCs are discussed as being characterized by an even higher glycolytic activity (78, 80). In the present study, we analyzed the metabolic activity and doxorubicin-resistant capability of the generated spheres. However, the spheroid cells did not show a significantly higher resistance to doxorubicin. Interestingly, the metabolic activities of spheroid cells were remarkably higher when compared to adherent CT1258 cells. In addition, CT1258 S15d cells displayed higher metabolic activities compared to S10d cells. These observations suggest that during cultivation, certain populations are enriched.

In summary, the present study demonstrated that CT1258 cells can form spheres in suspension in a serum-free culture medium. Furthermore, ITGA6 expression and elevated metabolic activity appear to characterize a specific cell subpopulation which potentially bears the characteristics described for cancer cells with a stem cell-like character. However, these results must be complemented *in vivo* by evaluating the potential to generate tumours by *s.c.* injection of the spheres. If successful, analysis comparative to our previously established CT1258 *in vivo* model can potentially provide a stable model for further studies characterizing the sub-populations. Furthermore, our results indicate that as *MYC* and *ITGA6* are significantly de-regulated in the generated spheres, these genes could consequently play a major role in CT1258 cell line spheroid formation and biology. Taking into account the lack of therapeutic options for dogs and the unique model character for human neoplasia, further characterization of the *in vitro* model herein described using enriched cells could be of major value for both prostate cancer-affected species.

Competing Interests

The Authors declare that they have no competing interests with regard to this study.

Authors' contributions

WL performed all the experiments, data analysis and partially drafted the manuscript. MM partially performed the sphere cultivation, real-time PCR, and flow cytometric analysis. SW partially participated in data analyses and manuscript drafting, AN participated in drafting and finalization of the manuscript. CR and CJ participated in chemo-resistance assays. IN is the head of the research group, participated in study designing and edited and approved the final manuscript. HME was

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