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# Identification of tumor-initiating cells derived from two canine rhabdomyosarcoma cell lines

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**ABSTRACT.** Cancer stem cells or tumor-initiating cells (TICs) are a small subpopulation of cells that have the capacity to self-renew, differentiate and initiate tumors. These cells may function in tumor initiation, aggression and recurrence. Whether spheres derived from canine rhabdomyosarcoma cells have stem cell-like properties is unclear. We induced sphere formation in the canine rhabdomyosarcoma cell lines, CMS-C and CMS-J, and characterized the spheres *in vitro* and *in vivo*. Sphere-forming cells were more resistant to vincristine, mitoxantrone and doxorubicin than adherent cells. Xenograft transplantation demonstrated that $1 \times 10^3$ sphere-forming cells derived from CMS-C were sufficient for tumor formation. The sphere assay showed that the sphere-forming cells were present in these tumors. These results suggest that the spheres derived from canine rhabdomyosarcoma cells may possess characteristics of TICs. This study provides the foundation for elucidating the contribution of TICs to rhabdomyosarcoma tumorigenesis.

**KEY WORDS:** canine, drug resistance, rhabdomyosarcoma, sphere, tumor-initiating cells

Rhabdomyosarcoma is the most common striated muscle tumor in domestic animals; however, it accounts for <1% of all spontaneous tumors [6, 8]. The neoplastic cells are derived from primitive mesenchymal cells and can potentially differentiate into striated muscle, invade the surrounding tissues and metastasize to other organs [6]. Rhabdomyosarcoma commonly occurs in the urinary bladder and oral cavity, including the tongue and maxillary gingiva in dogs [3, 11, 12, 17, 18]. This tumor is sub-classified as embryonal, botryoid, alveolar and pleomorphic based upon its morphological features [6]. Embryonal rhabdomyosarcoma is the most common form and is distinct in animals [6]. The alveolar type is associated with poor prognosis, rapid growth and invasion into the neighboring lymph nodes during pulmonary metastasis, resistance to radiotherapy and tumor recurrence [11, 17, 18].

Increasing evidence suggests that the progression of many tumors is controlled by a small subpopulation of cells, referred to as cancer stem cells or tumor-initiating cells (TICs). These cells are involved in tumor initiation, recurrence and metastasis [24]. TICs are defined by their self-renewal capacity, differentiation potential, tumorigenicity in immunodeficient mice, and resistance to chemotherapy and radiation [5, 19]. In humans, TICs have been characterized in several types of solid tumors, including those of the breast, brain and rhabdomyosarcoma [1, 9, 13, 21, 25]. Such characterization has been accomplished using stem cell marker analysis by flow cytometry, the aldefluor assay based on aldehyde dehydrogenase activity and the sphere assay [1, 9, 13, 21, 25]. There is increasing evidence of the presence of TICs in mammary carcinoma, osteosarcoma, hepatocellular carcinoma, pulmonary adenocarcinoma and melanoma in dogs [14–17, 20, 22, 28, 29].

The sphere assay is a useful tool for identifying TICs and evaluating their properties, including self-renewal capacity and sensitivity to chemotherapeutic drugs. In humans, spheres from rhabdomyosarcoma cell lines contain cells that exhibit an up-regulation of stem cell-associated genes, including Oct4, Nanog, Sox2 and Pax3 [25]. These spheres are also enriched in CD133-positive cells, which demonstrate higher chemoresistance and tumorigenicity in immunodeficient mice than CD133-negative cells

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[24]. A minority of fibroblast growth factor receptor 3 (FGFR3)-positive KYN-1 and RD cells have a stronger tumorigenicity than FGFR3-negative cells, indicating the existence of rhabdomyosarcoma-initiating cells [10]. Whether cells in spheres from canine rhabdomyosarcoma have stem cell-like properties remains unknown. In this study, we attempted to induce the formation of spheres in an established canine rhabdomyosarcoma cell line and examined these cells for stem cell-like properties, including a high self-renewal capacity, chemoresistance and tumorigenicity in immunodeficient mice.

## MATERIALS AND METHODS

The canine rhabdomyosarcoma cell line CMS-C [2] was maintained in Dulbecco's modified eagle medium (DMEM, Wako, Osaka, Japan) with 10% heat-inactivated fetal calf serum (Hyclone, Logan, UT, U.S.A.) and antibiotics (Nakarai tesque, Kyoto, Japan) at 37°C in an atmosphere containing 5% $CO_2$. CMS-J, bladder rhabdomyosarcoma from a 14-year-old spayed female beagle dog, was established as reported previously [2] and maintained in the same medium.

The sphere assay was performed as described previously [15]. Briefly, singly suspended cells were plated at a density of $1 \times 10^4$ or $1 \times 10^5$ viable cells per ultralow attachment 6-well plate or 10 cm dish (Corning Inc., Corning, NY, U.S.A.), respectively. The cells were grown in serum-free DMEM/F12 (Invitrogen, Carlsbad, CA, U.S.A.) supplemented with growth factors (GF); 10 *n*g/m*l* bFGF (Invitrogen), 10 *n*g/m*l* FGF (Invitrogen), 4 mg/m*l* heparin (Sigma, St. Louis, MO, U.S.A.) and NeuroBrew-21 (Miltenyi Biotech, Bergisch Gladbach, Germany) (GF+) or without GF (GF−) for 7–10 days. Spheres were counted under low magnification, collected and used for the following analyses. A colony of cells with a diameter of >50 $\mu$m is considered a sphere. For serial passages, all spheres were collected weekly, enzymatically dissociated into single cells and reseeded onto 6-well plates at a density of $1 \times 10^4$ cells/well.

Sensitivities to doxorubicin, vincristine and mitoxantrone were examined in adherent cells and spheres from CMS-C and CMS-J cells. Singly suspended cells were seeded at a density of $5 \times 10^3$ on 96-well adherent cell culture plates or $1 \times 10^3$ cells on ultralow attachment plates. The adherent cells were cultured for 24 hr and stimulated with culture medium containing seven different concentrations (1, 10 and 100 *n*g/m*l*, 1, 10 and 100 $\mu$g/m*l* and 1 mg/m*l*) for 72 hr. For the sphere assay, singly suspended cells were cultured in the presence of the chemotherapeutic drugs for 5 days. Cell viability was analyzed using CCK-8 (Dojindo laboratories, Kumamoto, Japan). The 50% inhibitory concentration ($IC_{50}$) values were calculated from triplicates, and each experiment was repeated three times in each of the cell lines.

Total RNA was isolated from CMS-C adherent cells and sphere-forming cells formed under GF+ and GF− medium using TRIzol reagent (Invitrogen), according to the manufacturer's protocol. RNA quality was determined by measurement of the absorbance at 260 nm. Total RNA (1 $\mu$g) was reverse-transcribed at 42°C for 15 min in 20 m*l* with QuantiTect (Qiagen) after inactivation of reverse transcription by heating at 95°C for 3 min. Terminal cycling was performed using 30 cycles of 96°C for 10 min, 64°C for 20 sec and 72°C for 1 min. Primer pairs for Oct-4, Nanog, Sox-2 and CD133 were used as described previously [14]. GAPDH was amplified with the following primer sets: 5′-CCCACTCTTCCACCTTCGAC-3′ and 5′-CTCCTTGGAGGAGGCCATGTG-3′ as a positive control. The PCR products were subjected to electrophoresis on 1.5% agarose gels containing ethidium bromide. Detectable bands were photographed by ultraviolet transilluminator (ATTO, Tokyo, Japan) and measured by a densitometer using ImageJ (NIH) software.

Forty-four female BALB/cAJcl-nu/nu (nude) mice, aged 8 weeks, were purchased from CLEA Inc. (Tokyo, Japan) and maintained under control laboratory conditions of 12 hr dark/light cycle, 22 ± 2°C temperature and 55 ± 5% relative humidity. Several sphere-forming cells derived from GF+ and adherent cells from the CMS-C ($1 \times 10^3$–$1 \times 10^6$ cells re-suspended in 100 $\mu l$ PBS) were injected subcutaneously into the ventrolateral area under anesthesia. Tumor formation was monitored weekly for 51 weeks. The tumor volume (V) was estimated using the following equation: $[(\text{length}) \times (\text{width})^2]/2$. For the sphere assay, parts of tumors induced by the sphere-forming cells were excised after euthanasia and digested using 0.4% collagenase/DMEM. After filtration with a 70 $\mu$m cell strainer (BD Falcon, Tokyo, Japan), singly suspended cells were analyzed using the sphere assay as described above. All experiments were approved by the Animal Experiments Committee of Nippon Veterinary and Life Science University and were performed in accordance with Guidelines for Animal Experiments by the Nippon Veterinary and Life Science University.

Parts of tumors formed in nude mice were fixed with 10% neutral-buffered formalin and routinely embedded in paraffin wax for histological examination. Sections were stained with hematoxylin and eosin (HE), periodic acid-Schiff (PAS) and phosphotungstic acid hematoxylin (PTAH). Serial sections were immunostained using the streptavidin-biotin-peroxidase method with antibodies against cytokeratin (CK, clone AE1/AE3, 1:50, Dako, Denmark A/S, Glostrup, Denmark), vimentin (clone V9, 1:200, Dako), desmin (clone 33, 1:100, Dako), MyoD1 (clone 5.8A, 1:50, Dako) and Ki67 (clone MIB-1, 1:100, Dako). Briefly, sections were treated in 0.03% $H_2O_2$ in 33% methanol at room temperature for 30 min for endogenous peroxidase blocking, following a pretreatment at 121°C for 20 min in citrate buffer (pH 6.0). Canine tissues, including fetal skeletal muscle, were used as a positive control. The Ki67 index was assessed by counting both Ki67-positive and Ki67-negative tumor cells in 10 randomly selected field at a high power (×400) field magnification and expressing the count as a percentage (Ki67-positive cells/total number of cells counted). Results are presented as mean ± standard deviation (SD). The Statcel 3 add-in (OMS Publishing, Saitama, Japan) for Microsoft Excel was used for statistical analysis. Significant differences were determined using the Tukey–Kramer, the Kruskal-Wallis test or a one-way analysis of variance (ANOVA). $P<0.05$ was considered to be significant.

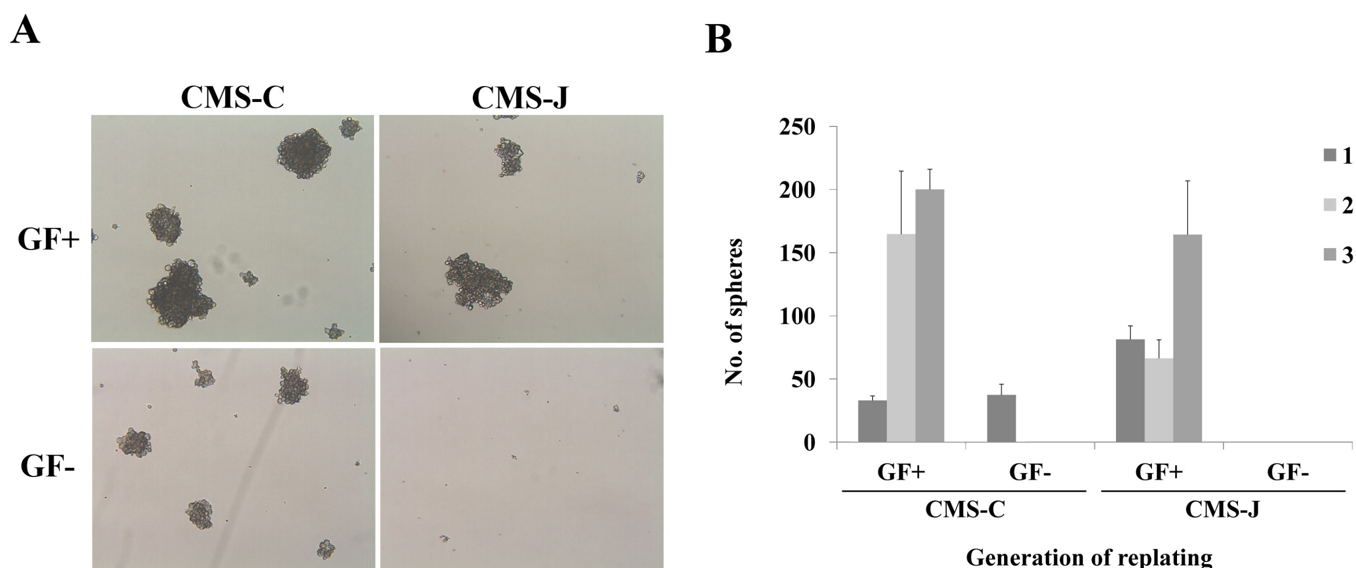


**Fig. 1.** Characterization of spheres formed from CMS-C and CMS-J cells. Representative micrographs show the morphology of spheres formed from cells grown in medium with (A, upper) or without (A, bottom) growth factors. (B) Sphere formation over serial passages. Results shown are representative of three independent experiments. Data are shown as mean ± standard deviation.

## RESULTS

To confirm the presence of TICs in canine rhabdomyosarcoma, we examined the capability of the CMS-C and CMS-J cells to form spheres. CMS-C cells formed spheres regardless of the presence or absence of GF, whereas CMS-J cells formed spheres only in the presence of GF. Both spheres cultured with GF were large, round and sharp in form (Fig. 1A). In CMS-C, cells cultured without GF were small, round, and sharp (Fig. 1A). In serial sphere assays, spheres formed in GF+ medium could be passaged at least 3 times, whereas those formed in GF−medium could not be passaged (Fig. 1B).

We compared the effect of the major chemotherapeutic drugs, vincristine, mitoxantrone and doxorubicin, which are widely used in veterinary medicine, on adherent and sphere-forming cells. Sphere-forming cells were more resistant to vincristine, mitoxantrone and doxorubicin than were adherent cells. In CMS-C, the $IC_{50}$ values of vincristine and mitoxantrone on adherent cells were 8.48 and 24.30 $\mu$g/m*l*, respectively, and 399.35 and 164.28 $\mu$g/m*l*, respectively, on sphere-forming cells were (Fig. 2A and 2C). The $IC_{50}$ value of doxorubicin was not calculated for either cell line (Fig. 2E). In CMS-J cells, the $IC_{50}$ values of vincristine, mitoxantrone and doxorubicin were 0.07, 5.46 and 32.79 $\mu$g/m*l*, respectively (Fig. 2B, 2D and 2F), while sphere-forming cells had $IC_{50}$ of 0.87, 4.09 and 69.76 $\mu$g/m*l*, respectively (Fig. 2B, 2D and 2F).

To evaluate mRNA expression of stem cell-related genes, such as Oct-4, Nanog, Sox-2 and CD133, both GF+ and GF− sphere-forming cells from CMS-C were analyzed by RT-PCR and compared with the corresponding adherent cells. The expression of stem cell-related genes was not significantly different among the cells, although the expression of Nanog and CD133 tended to increase in the GF+ sphere-forming cells compared to the others (Fig. 3).

To examine whether the tumorigenic capacity differed between sphere-forming CMS-C cells cultured in the presence of GF and adherent cells, the cells were subcutaneously transplanted into BALB/c nude mice (Table 1). As few as $1 \times 10^3$ sphere-forming cells initiated tumor formation in one of four mice injected, while $1 \times 10^4$ and $1 \times 10^5$ sphere-forming cells initiated tumor formation in three of four mice and all four mice, respectively. In contrast, injection of $5 \times 10^5$ sphere-forming cells initiated tumors in all mice. The sphere-forming cells tended to produce larger xenograft tumors than adherent cells, although these differences were not statistically significant (Fig. 4). To determine whether the sphere-forming cells were present in the induced tumors, the sphere assay was performed. The mean number of spheres in tumors induced by $1 \times 10^5$ sphere-forming cells was 52.67 ± 11.85 (mean ± SD) in the presence of GF, and no spheres were present in the absence of GF. In the xenograft of adherent cells, $1 \times 10^4$ and $1 \times 10^5$ cells initiated tumors in one of four mice, whereas $1 \times 10^3$ cells failed to induce tumors in all mice. Injection of $5 \times 10^5$ and $1 \times 10^6$ cells initiated tumors in all mice. Histologically, the tumors were composed of polygonal cells and multinuclear giant cells arranged in solid nests (Fig. 5A and 5B). The tumor cells were negative for PAS and PTAH stains. The majority of tumor cells were strongly positive for vimentin in all xenograft tumors (Fig. 5C and 5D), whereas all tumor cells were negative for CK (Fig. 5E and 5F), desmin (Fig. 5G and 5H) and MyoD1 (Fig. 5I and 5J). The Ki67 index of the sphere-forming cells and adherent cells was 59.28% and 50.95%, respectively. The Ki67 index was not significantly different among the three cells, although the Ki67 index of sphere-forming cells tended to be higher than that of adherent cells (Fig. 6). No metastasis of the tumor cells to other organs, including the neighboring lymph nodes, was observed. Xenograft tumors of the sphere-forming cells showed a pleomorphic type, similar to that of adherent cells. These results demonstrate that sphere-forming cells have higher tumorigenicity than adherent cells.

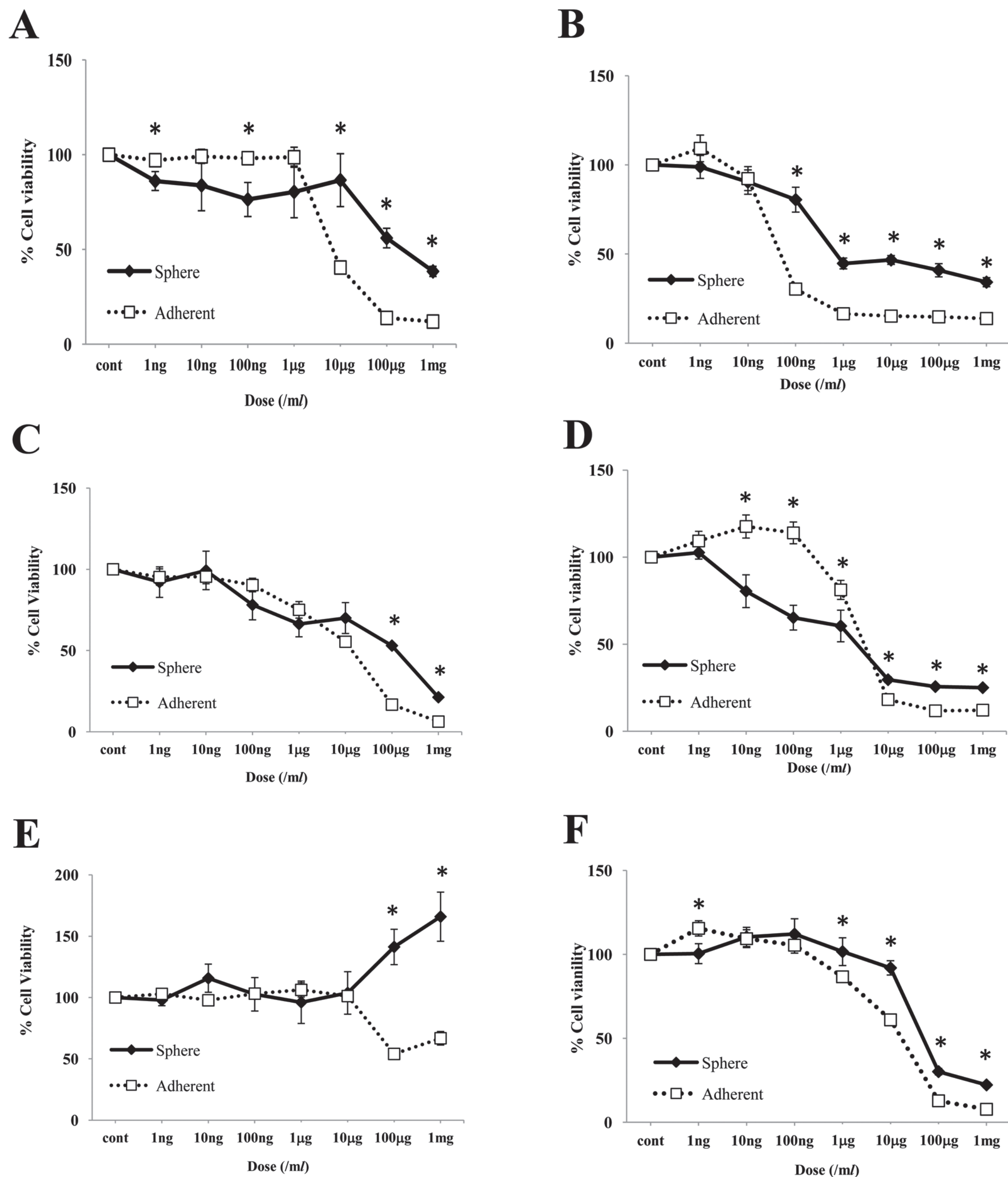


**Fig. 2.** The effect of chemotherapeutic drugs on sphere-forming and adherent cells derived from CMS-C (A, C and D) and CMS-J (B, D and F) cells. Sphere-forming cells are more resistant to vincristine (A and B), mitoxantrone (C and D) and doxorubicin (E and F) than are adherent cells. Results shown are representative of three independent experiments. Data are shown as mean ± standard deviation. **P*<0.05 (Tukey-Kramer test).

## DISCUSSION

The TIC hypothesis states that several tumors are characterized by a hierarchical structure that affects tumor progression. The sphere assay is used to enrich small populations of cells with stem cell-like properties for further analysis. In the present study, we characterized sphere formation by cells derived from canine rhabdomyosarcoma cell lines and demonstrated the following: (1) both the CMS-C and CMS-J cells grown in both the presence of GF contained a small population of sphere-forming cells with a self-renewal capacity; (2) both sphere-forming cells exhibited resistance to vincristine, mitoxantrone and doxorubicin; (3) CMS-C sphere-forming cells were more tumorigenic in nude mice than adherent cells; and (4) sphere-forming cells from CMS-C cells induced tumors, which contained a few sphere-forming cells and a majority of non-sphere-forming cells. This indicates a cellular

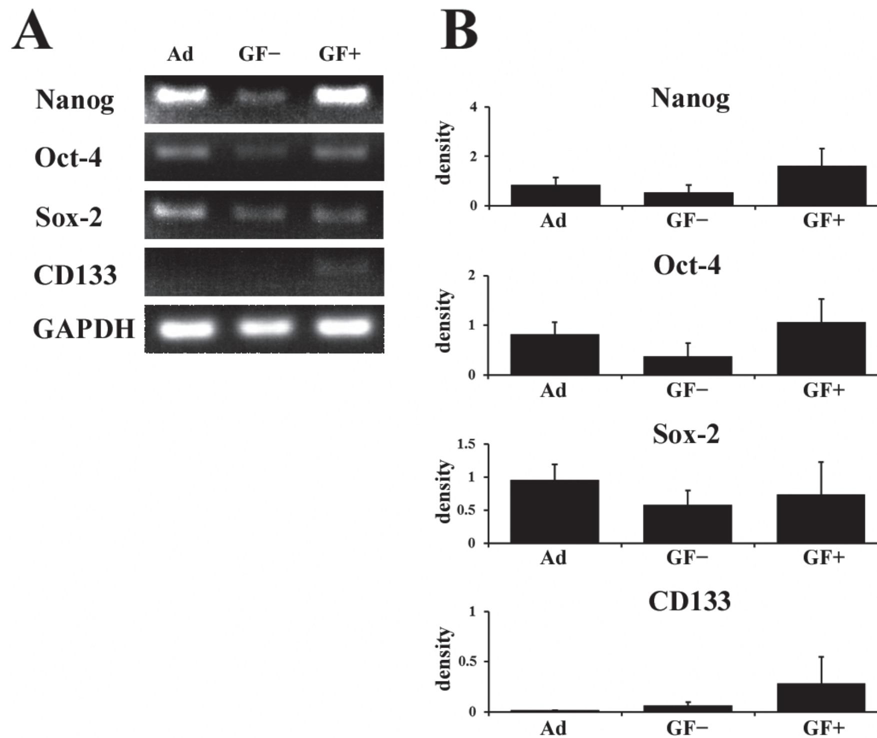


**Fig. 3.** Expression of stem cell-related genes in adherent cells and sphere-forming cells from CMS-C. (A) RT-PCR analysis of expression of Oct-4, Nanog, Sox-2 and CD133 genes in adherent cells (Ad), and sphere-forming cells formed in the GF− or GF+ medium. (B) Semi-quantitative RT-PCR results using Image J to determine the relative density of the bands compared to GAPDH. Results are indicated as the mean ± standard deviation of at least three experiments.

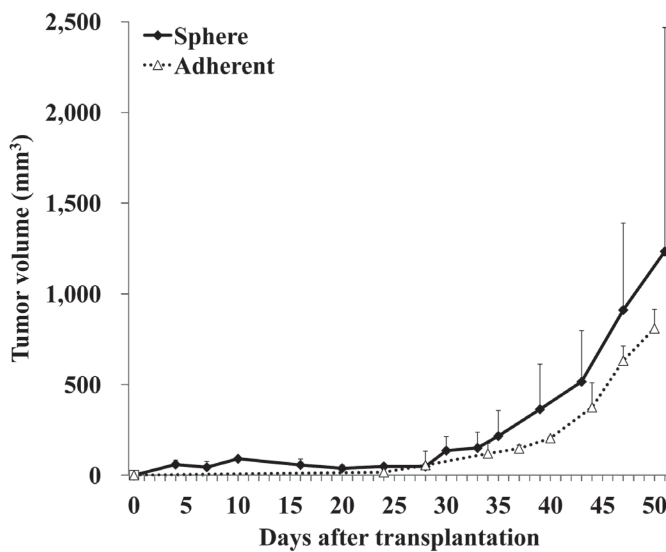


**Fig. 4.** Growth of tumors induced by 5 × $10^5$ sphere-forming and adherent cells from CMS-C. The sphere-forming cells induced larger tumors than adherent cells. The tumor volumes were not significantly different among adherent and sphere-forming cells. Data are shown as mean ± standard deviation.

**Table 1.** Tumorigenesis of sphere-forming cells from CMS-C in nude mice

| | Cell number for injection | | | | |
|---|---|---|---|---|---|
| | $1 \times 10^3$ | $1 \times 10^4$ | $1 \times 10^5$ | $5 \times 10^5$ | $1 \times 10^6$ |
| Sphere | 1/4 | 3/4 | 4/4 | 4/4 | - |
| Adherent | 0/4 | 1/4 | 1/4 | 4/4 | 4/4 |

hierarchy formed by both self-renewal and differentiation of sphere-forming cells. Therefore, we demonstrated that TICs may exist in canine rhabdomyosarcoma.

Post-operative radiation is generally considered an acceptable treatment after incomplete excision of soft tissue sarcoma in dogs.

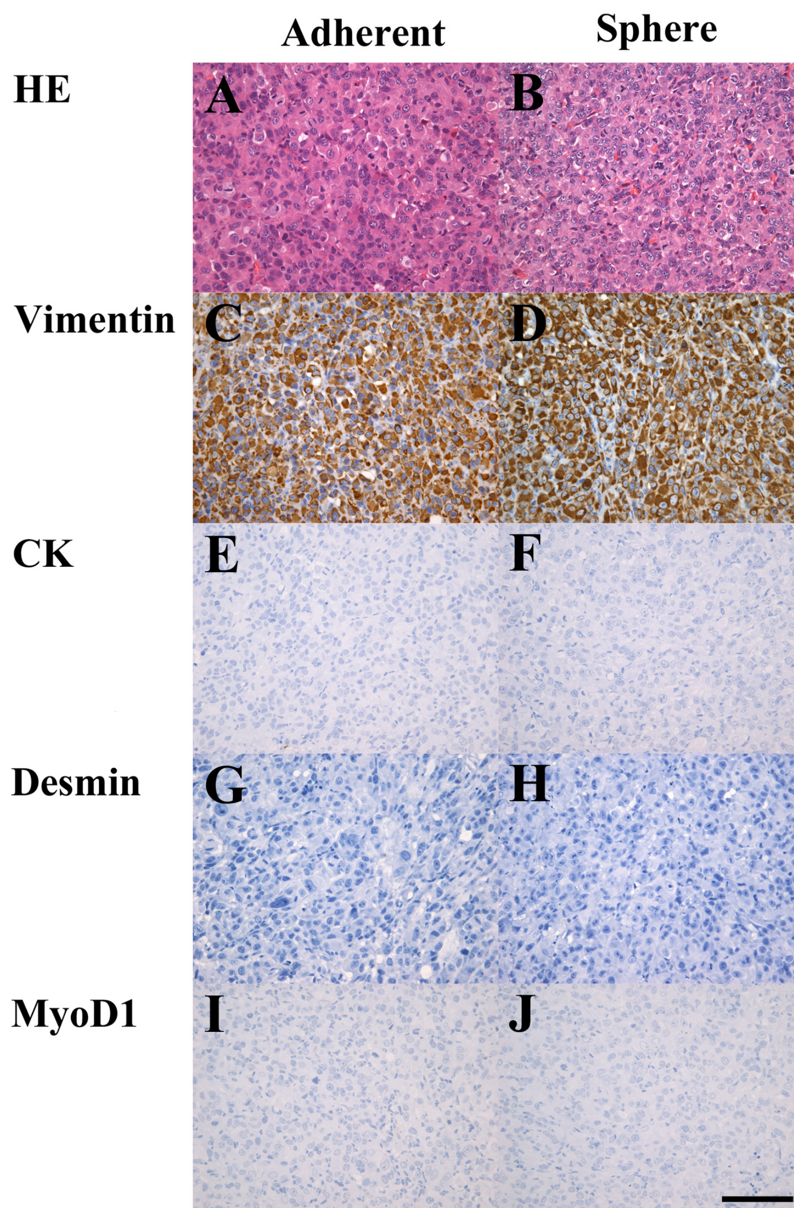


**Fig. 5.** The tumorigenic capacity of sphere-forming cells derived from CMS-C cells. Histological findings in tumors derived from 5 × $10^5$ adherent cells (A) and sphere-forming cells (B). Hematoxylin and eosin. The majority of tumor cells were strongly positive for vimentin in all xenograft tumors (C and D), whereas all tumor cells were negative for CK (E and F), desmin (G and H) and MyoD1 (I and J). Bar=100 $\mu$m.

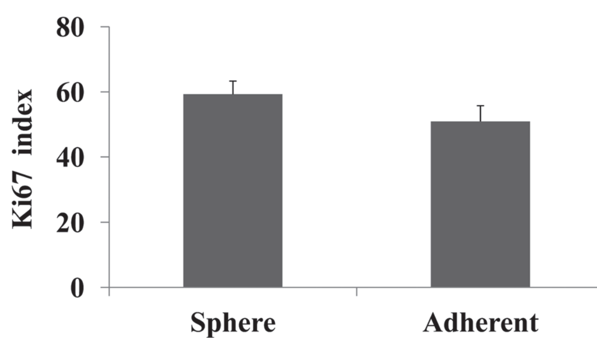


**Fig. 6.** Proliferative activity in xenograft tumors from CMS-C. The Ki67 index was not significantly different among adherent and sphere-forming cells. Data are shown as mean ± standard deviation.

However, oral rhabdomyosarcoma appears to be resistant to radiation [18]. Although adjuvant chemotherapy using doxorubicin, vincristine sulfate and cyclophosphamide is performed in dogs with rhabdomyosarcoma, effective chemotherapy regimens have not been established [23]. Therefore, a new therapeutic approach is required for the treatment of rhabdomyosarcoma in dogs. The sphere assay can be used to estimate the sensitivity of tumors to chemotherapeutic drugs. In canine mammary carcinomas, spheres are significantly more resistant to doxorubicin and interferon-ω [15, 20]. However, the *in vitro* effects of chemotherapeutic drugs on canine rhabdomyosarcoma have not been investigated. In the present study, we demonstrate the effects of chemotherapeutic drugs, such as vincristine, mitoxantrone and doxorubicin, on sphere-forming and adherent cells derived from CMS-C and CMS-J cells. Sphere-forming cells were more resistant to vincristine and mitoxantrone than were adherent cells, suggesting that the sphere-forming cells derived from CMS-C and CMS-J cells may include TICs that have chemoresistant characteristics. However, sphere-forming cells from CMS-C treated with doxorubicin showed increased viability. The mechanism of resistance in sphere-forming cells remains unclear. Further studies are needed to elucidate the properties of sphere cells to develop TIC-targeted therapies for canine rhabdomyosarcoma.

Vimentin, desmin and actin are useful immunohistochemical markers for the diagnosis of rhabdomyosarcomas [4]. MyoD1 and myogenin have been recognized as specific and sensitive markers of rhabdomyosarcoma in humans [7, 26]. Similar to the present study, previous studies have reported a double negative immunostaining for MyoD1 and myogenin of 13.6% (3 of 22 cases; 1 embryonal, 1 alveolar and 1 pleomorphic) and 3% (1 of 33 cases; 1embryonal) in human rhabdomyosarcoma [7, 26]. The significance of double negative reactivity for MyoD1 and myogenin remains unclear. Vimentin is expressed in the early phase of tumorigenesis, and desmin expression starts in the early phases and persists throughout tumor development [27]. Myogenin and MyoD1 are associated with a relatively undifferentiated tumor state [7]. Azakami *et al*. (2011) reported that MyoD1 was weakly expressed in CMS-C as indicated by RT-PCR, but not by immunohistochemistry. The expression of MyoD1 and desmin was demonstrated in the adherent cells, GF− and GF+ sphere-forming cells derived from CMS-C used in this study (data not shown) by RT-PCR, although immunoreactive cells for desmin and MyoD1 could not be demonstrated. These results suggest that the expression of MyoD1 and desmin might decrease in the tumors and that xenograft tumors could have a poorly differentiated type.

In conclusion, this study demonstrated that TICs may exist in canine rhabdomyosarcoma cell lines using detailed analyses of spheres. Understanding the characteristics of TICs may help to elucidate the mechanisms of tumorigenesis and contribute to the development of TIC-targeted therapies for rhabdomyosarcoma.

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