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Characterization of spheres derived from canine mammary gland adenocarcinoma cell lines

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ABSTRACT

There is increasing evidence for the presence of cancer stem cells in several solid tumors, and these cancer stem cells have a potential role in tumor initiation, aggression, and recurrence. The stem cell-like properties of spheres derived from canine mammary tumors remain largely elusive. We attempted to induce sphere formation using four cell lines of canine mammary adenocarcinoma, and characterized the spheres derived from a CHMp line *in vitro* and *in vivo*. The CHMp-derived spheres showed predominantly $CD44^{+}CD24^{-}$ population, higher expression of stem cell-related genes, such as CD133, Notch3 and MDR, and higher resistance to doxorubicin compared with the CHMp-derived adherent cells. Xenograft transplantations in nude mice demonstrated that only 1×10^{4} sphere cells were sufficient for tumor formation. Use of the sphere assay on these sphere-derived tumors showed that sphere-forming cells were present in the tumors, and were maintained in serial transplantation. We propose that spheres derived from canine mammary adenocarcinoma cell lines possess a potential characteristic of cancer stem cells. Spheres derived from canine mammary tumors could be a powerful tool with which to investigate novel therapeutic drugs and to elucidate the molecular and cellular mechanisms that underlie tumorigenesis.

1. Introduction

A small subpopulation of cells, referred to as cancer stem cells or tumor-initiating cells, which have the capacity to self-renew, differentiate and initiate tumors at high frequency, are the source of cancers including leukemia (Bonnet and Dick, 1997) and solid tumors, such as brain, colon and breast tumors (Al-Hajj et al., 2003; O'Brien et al., 2007; Ponti et al., 2005; Ricci-Vitiani et al., 2007). Cancer stem cells have been characterized in several tumors using various stem cell markers, including CD133, CD90, and CD44 (Al-Hajj et al., 2003; O'Brien et al., 2007; Ricci-Vitiani et al., 2007; Yang et al., 2008). The sphere assay is one of the strategies used to identify cancer stem cells (Dontu et al., 2003). Free-floating spheres are a characteristic of the proliferation of solid tumors *in vitro*, they are rich in stem cells and progenitors, and they exhibit stem-like properties when cultured under conditions of anchorage independence and serum starvation (Dontu et al., 2003). Many researchers have investigated the presence of cancer stem cells in glioblastoma (Stoica et al., 2009; Yuan et al., 2004), mammary tumor (Cocola et al., 2009; Grange et al., 2008; Penzo et al., 2009), and sarcoma cell lines (Fujii et al., 2009; Wilson et al., 2008) by use of the sphere assay. In addition to the identification of cancer stem cells, the sphere assay has also been used to evaluate the effect of conventional chemotherapeutic drugs on spheres or parental cells (Fujii et al., 2009; Grange et al., 2008; Penzo et al., 2009).

Mammary gland tumors are the most common tumors in dogs, and approximately 50% are considered malignant (Misdorp et al., 1999; Rutteman et al., 2001). Therefore, mammary cancer has become a clinically important disease in veterinary medicine as well as in human medicine. Most cancer treatments, such as chemotherapy, radiation, immunotherapy, or their combinations, are not very effective in preventing recurrence (MacEwen, 1990). Therefore, it is essential to develop a novel strategy targeting cancer stem cells for the eradication of mammary tumors in dogs as well as in humans. In humans, breast cancer stem cells are characterized mainly by three properties: (i) $CD44^{+}CD24^{-/low}Lin^{-}$ cells (Al-Hajj et al., 2003), (ii) sphere formation (Ponti et al., 2005), and (iii) aldehyde dehydrogenase (ALDH) activity (Ginestier et al., 2007). In dogs, however, very little research on mammary cancer stem cells has been done. Recently, Cocola et al. reported that spheres were generated from normal and tumorous mammary

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Table 1
Primer pairs used in RT-PCR.

Gene	Forward primer 5′–3′	Reverse primer 5′–3′
CD133	GCTGCTCTTTGTGATTCTGATG	ATCACCAGGAGGGAGACTGTAA
CD34	TGAGACCTCCAGCTGTGA	CAGGTGTTGTCTTGCTGAATGG
Bmi-1	CACTGTGAATAATGACTTCTTGCAT	AAGTTTACTTTCCTTTGATCGGTTT
MDR	GAGGACTTGAATGAGAATGTTCCT	CGGGTAAAGATCCCTATAATCCTT
Oct-4	CTCTGCAGCCAATCAACCACAA	GGAGAGGGGGATGAGAAGTACAAT
Nanog	CTATAGAGGAGAGCACAGTGAAG	GTTCGGATCTACTTTAGAGTGAGG
Sox-2	AACCCCAAGATGCACAACTC	CGGGGCCGGTATTTATAATC
c-myc	AATAGGAACTATGACCTCGAC	AGCAGCTCGAATTTCTTCCAG
Stat3	GTGGAGAAGGACATCAGCGGTAA	AACTTGGTCTTCAGGTATGGGGC
Gli-1	ACCTCCATGATAGGCAGTGG	ACTCACCCCATGGTTCAGAG
Notch1	AGGACCGTGACAATGCCTAC	ACACTCGTAACCGTCGATCC
Notch2	ATTTCATGCAGGTTAGAGAAGGAC	CTGTCTGAGAGCTCAGTGACCTTA
Notch3	GCGTCCTTCTACTGCCTTTG	CCCATGTAGCCTTGACAGGT
β-Actin	CATGTTTGAGACCTTCAACACCCC	GCCATCTCTTGCTCGAAGTCCAG

tissues (Cocola et al., 2009). Another study has shown that the sphere assay is useful for evaluation of the effect of interferon-ω against canine mammary carcinoma (Penzo et al., 2009). However, the stem cell-like property of such spheres remains unknown. Further characterization of spheres derived from canine mammary tumors is needed to define novel therapies that target cancer stem cells, and to elucidate the biological and molecular mechanisms that underlie oncogenesis. In this study, we attempted to induce the formation of spheres from established canine mammary tumor cell lines. We examined whether spheres derived from these cell lines were enriched with breast cancer stem cells, namely a $CD44^{+}CD24^{-}$ cell population, and whether spheres from CHMp, a canine mammary tumor cell line, showed up-regulation of stem cell-related genes, such as CD133, CD34, MDR and Sox-2. Furthermore, the capacity to generate tumor nodules was analyzed in immunodeficient mice transplanted with sphere cells.

Table 2
The cell surface phenotypes of canine mammary adenocarcinoma cell lines.

	$CD44^{-}$ $CD24^{+}$	$CD44^{+}$ $CD24^{+}$	$CD44^{+}$ $CD24^{-}$	$CD44^{-}$ $CD24^{-}$
CHMp	3.9 ± 0.10	1.5 ± 0.15	91.9 ± 0.40	2.7 ± 0.40
CIPp	1.8 ± 0.20	1.5 ± 0.15	93.0 ± 0.72	3.7 ± 0.61
CNMp	0.8 ± 0.35	0.5 ± 0.06	76.2 ± 5.57	22.6 ± 5.18
CTBp	0.5 ± 0.00	0.9 ± 0.23	44.3 ± 2.40	54.3 ± 2.67

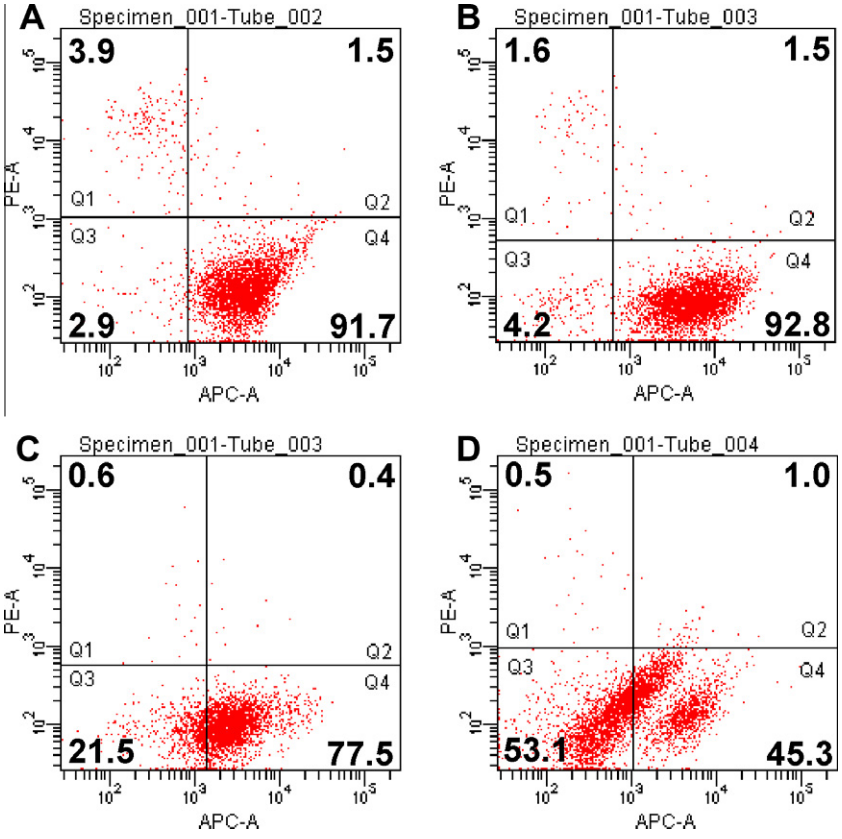

Fig. 1. Flow cytometry analysis of the expression of CD44 and CD24 in canine mammary adenocarcinoma cell lines. The $CD44^{+}CD24^{-}$ cells were detected in all cell lines, CHMp (A), CIPp (B), CNMp (C), and CTBp (D). The average percentage of cells is shown in each quadrant drawn according to staining of the isotype-control. The cells in Q4 correspond to $CD44^{+}CD24^{-}$ cells. The results shown are representative of at least three independent experiments.

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2. Materials and methods

2.1. Cell lines

Four canine mammary gland adenocarcinoma cell lines, CHMp, CNMp, CIPp and CTBp, were used as described previously (Uyama et al., 2006). All the cells were maintained in RPMI1640 (Wako) supplemented with 10% fetal bovine serum (FBS, Invitrogen), streptomycin and amphotericin B (Invitrogen), and incubated at 37 °C in an atmosphere containing 5% CO_2.

2.2. Flow cytometry

The cells were washed with phosphate-buffered saline (PBS), and then enzymatically dissociated with 0.25% trypsin–EDTA (Invitrogen). The cells were washed with PBS containing 2% FBS (FACS buffer), resuspended with FACS buffer, and then incubated with APC-conjugated anti-CD44 (clone IM7, BD Bioscience) and PE-conjugated anti-CD24 (clone M1/69, BD Bioscience) at 4 °C for 45 min. After washing, the labeled cells were analyzed on a FACSAria (BD Bioscience), and the data were analyzed using FACSDiVa software (BD Bioscience).

2.3. Sphere assay

The sphere assay was performed as described previously (Dontu et al., 2003), with minor modification. In brief, singly suspended cells were plated at a density of 1×10^4 or 1×10^5 viable cells per ultralow attachment 6-well plate or 10 cm dish (Corning), respectively. The cells were grown in serum-free DMEM/F12 (Invitrogen) supplemented with 10 ng/ml bFGF (Invitrogen), 10 ng/ml EGF (Invitrogen), 4 μg/ml heparin (Sigma) and B27 (Gibco) for 7–10 days. Spheres were counted under low magnification, collected, and used for flow cytometry and the following analyses.

2.4. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from adherent cells or spheres derived from CHMp using ISOGEN reagent according to the manufacturer's protocol (Nippon Gene, Japan). The quality and quantity of the RNA were confirmed using RNA LabChip and a 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). First strand cDNA synthesis was performed with 500 ng of total RNA using Superscript III (Invitrogen). Thermal cycling was performed using 30 cycles of 96 °C for 10 s, 64 °C for 20 s, and 72 °C for 1 min. The primer sequences for CD133, c-myc, MDR, Sox2, CD34, Bmi-1, Notch1, Notch2, Notch3, Gli-1, Oct-4, STAT3, Nanog and β-actin are summarized in Table 1. Primer pairs for Oct-4, Stat3, Nanog and β-actin were used as described previously (Wilson et al., 2008). The PCR products were electrophoresed on 2% agarose gels containing ethidium bromide. Detectable bands were photographed by ultraviolet transilluminator (ATTO) and measured by densitometer using Image J (NIH) software.

2.5. Drug resistance assay

Sensitivity to doxorubicin was examined for the adherent cells and spheres derived from CHMp. Singly suspended cells were seeded on cell culture or ultra low attachment dishes for adherent culture or sphere assay, respectively. The adherent cells were cultured for 24 h, and stimulated with culture medium containing three different doses (final conc. 250, 500 or 1000 ng/ml) of doxorubicin. The number of surviving cells was counted by using a trypan blue stain after 48 h of treatment. For the sphere assay, singly suspended cells were cultured in the presence of doxorubicin from the beginning, and the total number of spheres was counted after 7 days.

2.6. Xenografts in BALB/c nude mice

Female BALB/c nude mice, aged 5–7 weeks, were purchased from CLEA Inc. (Tokyo, Japan). Various numbers of sphere cells or adherent cells (1×10^4, 1×10^5 and 1×10^6 cells each) were injected subcutaneously into the ventro-lateral area under anesthesia. Tumor formation was monitored weekly for 6 weeks. The tumor volume (V) was estimated using the equation $V = (\text{length}) \times (\text{width})^2/2$. For serial transplantation, a part of tumors induced by sphere cells were minced in sterile PBS. Small pieces of tumor were excised and digested with 0.4% collagenase/DMEM. After filtration with a 70 μm cell-strainer (BD Falcon), singly suspended cells were submitted to the sphere assay and transplanted into nude mice again, as detailed above. The procedure described above was repeated three times. The experiments were approved by the Animal Experiments Committee of the Nippon Veterinary and Life Science University.

2.7. Histopathology

The tumors formed in nude mice were removed 6 weeks after transplantation, fixed with 10% neutral buffered formalin, and routinely embedded in paraffin wax for histopathological examination. Sections were stained with hematoxylin and eosin (HE).

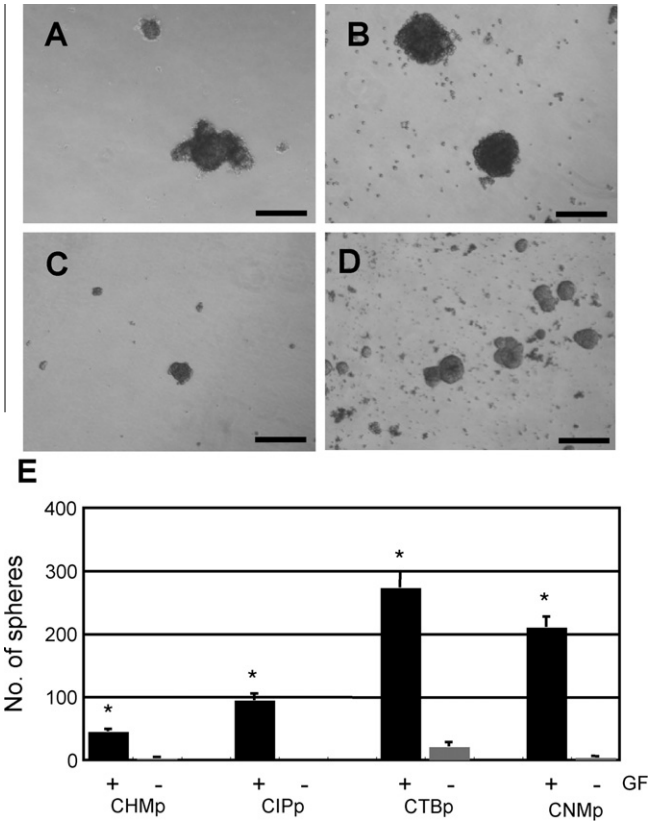

Fig. 2. Phase-contrast photographs of spheres formed from self-renewing cells. Spheres formed from CHMp (A), CIPp (B), CNMp (C) and CTBp (D) plated at a density of 1×10^4 cells/well in six-well ultralow attachment plates for 7 days. All experiments were repeated at least three times with similar results. Bar = 400 μm. (E) Evaluation of the number of spheres from four cell lines. Singly suspended cells were grown in serum-free DMEM/F12 supplement with (+) or without (−) growth factors for 7 days. Statistical analysis was performed using Student's t-test. $^*P < 0.001$. Data are the means ± SD.

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2.8. Statistical analysis

The results are presented as means ± SD. Statistical differences were determined using Student's *t*-test or a one-way analysis of variance (ANOVA). Probabilities of less than 5% ($P < 0.05$) were considered significant.

3. Results

3.1. Identification of $CD44^+CD24^-$ subpopulations in canine mammary adenocarcinoma cell lines

The canine mammary adenocarcinoma cell lines CHMp, CIPp, CNMp and CTBp were cultured under the adherent condition, reacted with fluorescence-conjugated CD44 and CD24 antibodies, and subjected to flow cytometry. The results of the analysis are summarized with respect to three fractions: $CD44^+CD24^-$, $CD44^-CD24^+$, and $CD44^+CD24^+$ (Fig. 1 and Table 2). The $CD44^+CD24^-$ cells, which are similar to human breast cancer stem cells, were identified in all four cell lines, and constituted 91.7%, 92.8%, 77.5%, and 45.3% of the cells in the CHMp, CIPp, CNMp, and CTBp lines, respectively. In contrast, only a small population of $CD44^-CD24^+$ and $CD44^+CD24^+$ cells was detected in the four cell lines (Table 2).

3.2. Identification of spheres derived from canine mammary adenocarcinoma cell lines

To confirm the existence of cancer stem cells in canine mammary gland tumors, we examined the capability of four cell lines to form spheres using the sphere assay. All the cell lines formed free-floating spheres when cultured in serum-free culture medium supplemented with growth factors for 7 days. The spheres exhibited round, sharp, uneven edges (Fig. 2A–D). The number of spheres was 45.33 ± 4.04, 95.00 ± 10.54, 273.00 ± 26.15, and 210.67 ± 16.62 (mean ± SD/well) in the cultured CHMp, CIPp, CTBp, and CNMp cells, respectively (Fig. 2E). Overall, the spheres in the CHMp and CIPp cell lines were larger than those of the CTBp and CNMp lines. In contrast, spheres were completely absent from or only a few were detected in any cell line in the absence of growth factors. Serial passages of CHMp cells formed spheres at a similar rate to the first plating (data not shown), which suggests the existence of cancer stem cells in canine mammary tumors. The CHMp

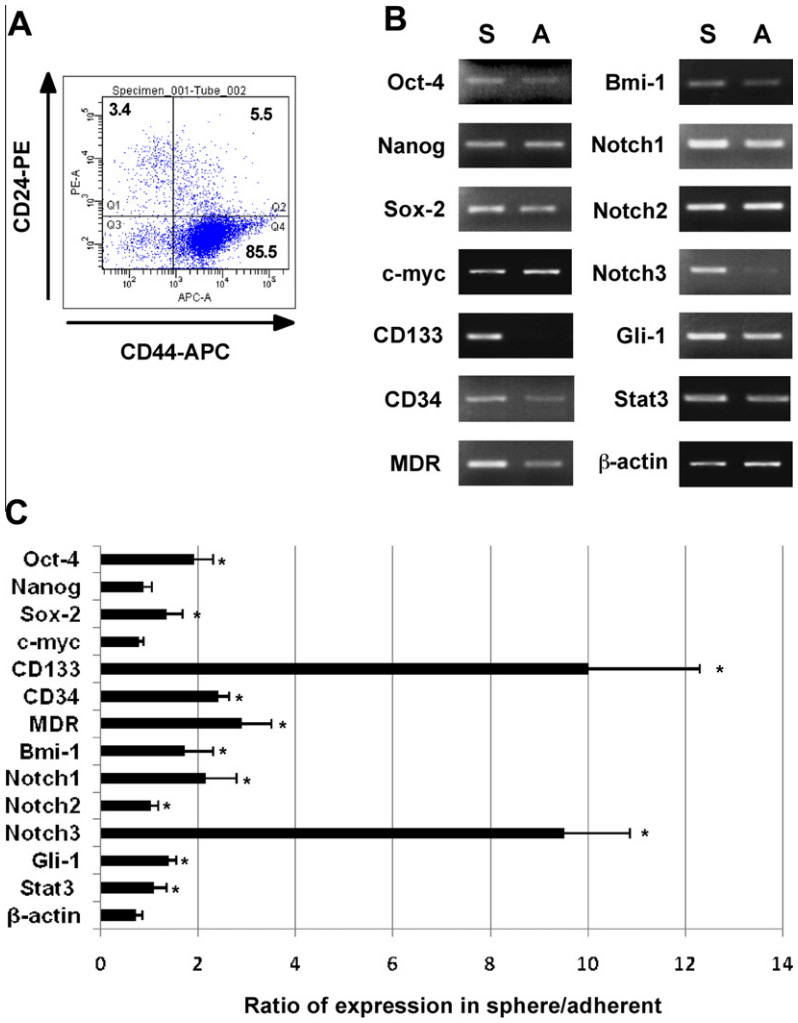

Fig. 3. Characterization of spheres derived from CHMp cells. (A) Flow cytometry analysis of the human breast cancer stem cell marker, $CD44^+CD24^-$, subpopulation in the sphere cells derived from CHMp. Quadrant lines were drawn according to staining of the isotype-control. (B) RT-PCR analysis of expression of the stemness-related genes in sphere cells (S) compared with adherent cells (A). β-Actin was used as an internal control. One of three experiments with similar results is represented, respectively. (C) Semi-quantitative analysis of the mRNA expression. The index of expression was defined as the ratio of sphere cells to adherent cells. Results are indicated as the means ± SD of at least three experiments. Statistical analysis was performed using Student's *t*-test. $^*P < 0.05$ vs. β-actin.

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line was used for the subsequent study, which was involved in characterization of the spheres.

3.3. Characterization of spheres derived from CHMp cells

Detailed analyses of spheres derived from CHMp cells were performed by flow cytometry and RT-PCR. Similar to the adherent cell phenotype, the spheres were also enriched with the $CD44^{+}CD24^{-}$ subpopulation, like human breast cancer stem cells (Fig. 3A). To evaluate the expression of the mRNAs of several marker genes, including stem cell-related genes, spheres were analyzed by RT-PCR in comparison with the corresponding adherent cells. As shown in Fig. 3B and C, the expressions of Oct-4, Sox-2, Bmi-1, Stat3, CD34, Notch1, Notch2, Notch3, Gli-1, MDR, and CD133 were higher in sphere cells than adherent cells. CD133 was expressed at a markedly higher rate in sphere cells, but was almost undetectable in adherent cells. The expression of Nanog and c-myc in sphere cells was comparable to that in adherent cells.

3.4. Effect of doxorubicin on adherent and sphere cells from CHMp

Next, we examined the effect of the cytotoxic drug doxorubicin on adherent cultures and sphere formation. The total number of surviving adherent cells decreased significantly after 48 h of doxorubicin treatment (Fig. 4A). On the other hand, the number of spheres formed at 7 days did not change significantly, despite the presence of doxorubicin (Fig. 4B). Morphologically, the spheres exhibited irregular aggregates in the presence of doxorubicin, although they were able to produce round bodies in the absence of doxorubicin (Fig. 4C–F). These results suggest that the sphere cells derived from CHMp may include cancer stem cells that have chemoresistant characteristics.

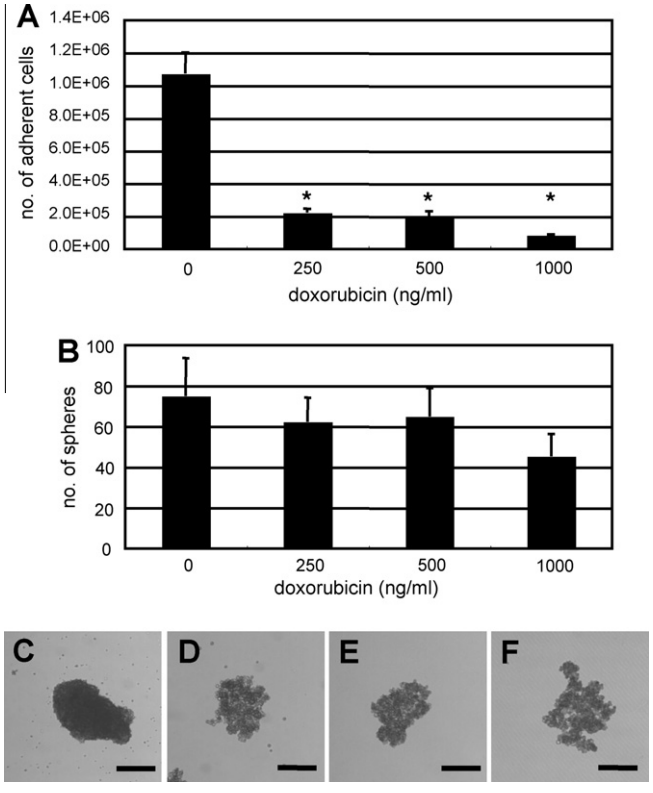

Fig. 4. Doxorubicin resistance of sphere-forming cells. (A) Adherent cells from CHMp were treated with three different doses of doxorubicin for 48 h. The total number of surviving cells was counted. Statistical analysis was performed using one-way ANOVA. $^{*}P < 0.05$. Data are the means ± SD. (B) Singly suspended cells were plated at a density of 1000 cells/well, and treated with doxorubicin for 7 days. The spheres were observed and counted under low magnification. Data indicate the means ± SD. (C–F) Representative micrographs showing the morphology of spheres from cells treated with doxorubicin at 0 ng/ml (C), 250 ng/ml (D), 500 ng/ml (E) and 1000 ng/ml (F). Bar = 100 μm.

3.5. Xenografts in BALB/c nude mice

To examine whether the tumorigenic capacity differs between adherent cells and sphere cells derived from CHMp, various numbers of these cells were transplanted into BALB/c nude mice (Fig. 5A). As few as 1×10^4 sphere cells could initiate tumors in all mice injected. An injection of 1×10^6 adherent cells could induce tumors in all mice. However, 1×10^5 adherent cells induced tumors in only two of three mice injected, and 1×10^4 adherent cells failed to induce tumor formation in all three mice tested. We also compared the size of the tumors 6 weeks after injection of 1×10^5 sphere cells or adherent cells. Sphere cells produced larger tumor xenografts than adherent cells (Fig. 5B–D). To elucidate whether sphere-forming cells existed in the tumors, we repeated the sphere assay. The number of spheres per well was 17.11 ± 5.37 (mean ± SD) in the presence of growth factors, whereas it was 1.44 ± 1.01/well in their absence (Fig. 5E). The histology of xenograft tumors of the sphere cells was similar to that of adherent cells (Fig. 5F and G). The tumors were composed of undifferentiated carcinoma cells, which were arranged haphazardly but not in an epithelial pattern. No metastasis of the tumor cells to other organs, including the neighboring lymph nodes, lung or bone, was observed (data not shown). The spheres could form tumors in at least three generations of mice transplanted with serial xenografts, and these tumors also showed similar histology to that described above (data not shown). These results demonstrate that sphere cells have higher tumorigenicity than adherent cells.

4. Discussion

Cancers are composed of heterogeneous cell populations with a small subset of cancer stem cells that sustain tumor formation, metastasis, recurrence, and aggression (Pardal et al., 2003; Reya et al., 2001). In breast tumors, cancer stem cells can be identified as $CD44^{+}CD24^{-}Lin^{-}$ cells, $ALDH^{+}$ cells, side population cells, or sphere-forming cells by various techniques (Al-Hajj et al., 2003; Charafe-Jauffret et al., 2008; Ginestier et al., 2007). $CD44^{+}CD24^{-}$ cells are regarded as not only cancer stem cells but also basal/mesenchymal cells in human breast cancers (Al-Hajj et al. 2003; Honeth et al. 2008; Sheridan et al. 2006). Spheres have been claimed to be enriched with stem cells in normal breast tissues and in cancer in humans and dogs, and the sphere assay may be a valuable way to identify cells with the characteristics of cancer stem cells (Cocola et al., 2009; Dontu et al., 2003; Penzo et al., 2009; Ponti et al., 2005). Spheres have been identified in canine mammary tumors, and were used to assess the sensitivity to several antitumor drugs, including interferon-ω and doxorubicin (Grange et al., 2008; Penzo et al. 2009). However, there remained some doubt about whether the spheres had stem cell-like properties. In this study, we characterized spheres derived from a canine mammary adenocarcinoma line, CHMp. We demonstrated that the spheres: (i) contained $CD44^{+}CD24^{-}$ cells, (ii) showed increased expression of stem cell-related genes, such as CD133, CD34 and MDR, (iii) had higher tumorigenicity in immunodeficient mice than adherent cells, (iv) exhibited drug resistance against doxorubicin, and (v) induced tumors containing several sphere-forming cells. Based on the above mentioned evidence, the existence of cancer stem cells in canine mammary tumors has been demonstrated.

A previous study has demonstrated the tumorigenic potential of and expression of stem cell markers in sphere cells derived from

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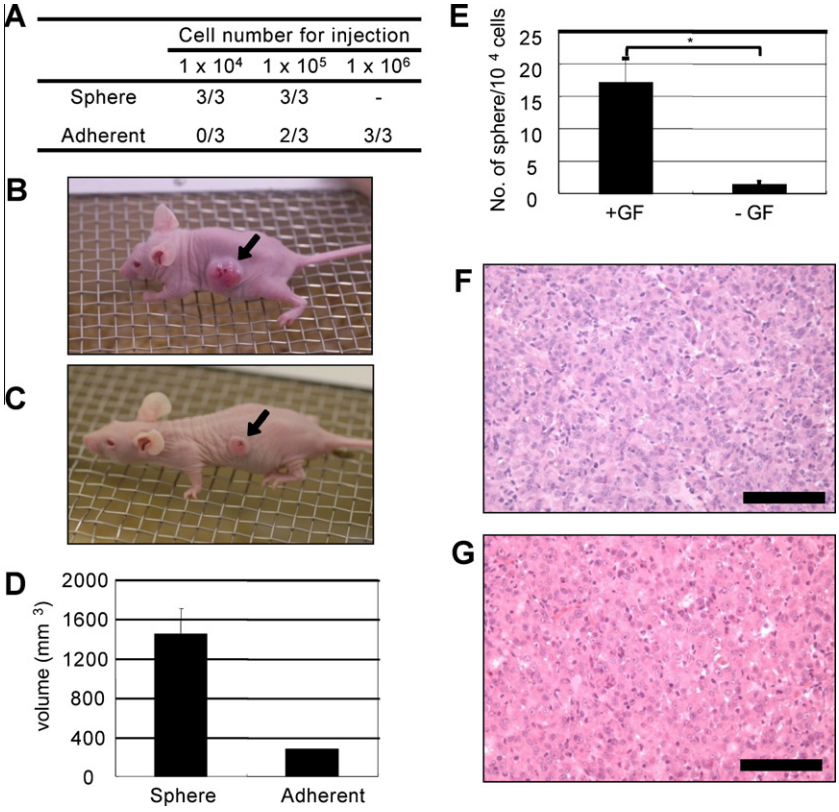

Fig. 5. Tumorigenic capacity of the spheres derived from CHMp. (A) Various numbers of singly suspended CHMp adherent or sphere cells were injected into nude mice. Tumor formation was observed for 6 weeks after injection. Subcutaneous tumors (arrows) formed by injection of 1×10^5 sphere-forming cells (B) and adherent cells (C). (D) Tumor volume (mm^3), calculated as length $\times$ $(width)^2 \times ½$, was compared between sphere and parental cells at 6 weeks after transplantation. The data from sphere-induced tumors indicate the means ± SD of three mice. The data from adherent cell-induced tumors indicate the means from two mice. (E) Sphere assay on sphere-induced tumor. Removed tumors were dissociated enzymatically, and single cells were grown in serum-free DMEM/F12 supplemented with (+) or without (−) growth factors. Statistical analysis was performed using Student's *t*-test. $^*P < 0.001$. Data represent the means ± SD ($n = 9$) derived from three tumors. HE staining of the tumors induced by sphere (F) and adherent cells (G). Both tumors show similar haphazard growth of differentiated cells. Bar = 100 μm.

primary mammary tumors in dogs (Cocola et al., 2009). However, evidence of a cellular hierarchy has not been established. Our data demonstrate that the tumors induced by xenografts are organized in a heterogeneous population containing a few sphere-forming cells that possess the ability to self-renew and initiate tumors. This indicates the further possibility that mammary tumors in dogs can arise from their cancer stem cells. Nevertheless, our expression profile of stem cell markers differed from the data described by Cocola et al. (Cocola et al., 2009). Spheres from the primary mammary tumors identified by Cocola et al. demonstrated the expression of mouse mammary stem cell markers, such as CD49f and CD29, but not the human stem cell markers CD133 and CD44 (Cocola et al., 2009). Contrary to the previous report, our study demonstrated a higher level of expression of CD44 and CD133 in spheres derived from CHMp by flow cytometry and RT-PCR, which strongly supports the stem cell-like properties of the spheres in canine mammary tumors. Moreover, other genes, such as Sox-2, Oct-4, Nanog and c-myc, which are associated with the maintenance of stem cells (Takahashi and Yamanaka, 2006), were expressed in adherent cells as well as in sphere cells. These results suggest that these molecules may be involved in the growth of cancer cells as well as in the maintenance of stemness in cancer stem cells.

The cell surface analysis for CD44 and CD24 revealed the constituents of each cell line. All cell lines except for CTBp contain the majority of $CD44^+CD24^-$ cells as a majority of cells and few others. In human breast cancers, $CD44^+CD24^-$ cells have been reported to show stem/progenitor cell properties (Al-Hajj et al. 2003). Besides, $CD44^+CD24^-$ cells are involved in more enhanced invasiveness as well as higher tumorigenicity compared with $CD44^+CD24^+$ cells (Al-Hajj et al. 2003; Sheridan et al. 2006). On the other hand, $CD44^+CD24^+$ cells may be more differentiated cancer cells rather than cancer stem cells, although the precise properties of $CD44^+CD24^+$ cells remain unclear in the previous or present studies. The percentage of the $CD44^+CD24^-$ cells has varied markedly among human breast cancer cell lines (Sheridan et al. 2006). CTBp may differ in biological behavior from CHMp because the former contains less $CD44^+CD24^-$ cells compared with the latter. Further studies will be needed to elucidate the properties of other three cell lines.

Sphere generation is a useful tool for the identification of cancer stem cells, and it can be used to estimate the sensitivity to the antitumor effect of chemotherapeutic drugs. In canine mammary tumors, spheres derived from the REM134 cell line are significantly more resistant to recombinant interferon-ω than adherent cells, whereas the effect of chemotherapeutic drugs, including doxorubicin, on sphere generation has not been investigated (Penzo et al., 2009). A previous study has demonstrated that tumor spheres from mammary tumors of BALB-neu transgenic mice are chemoresistant to doxorubicin (Grange et al., 2008). Similarly, our findings demonstrate that sphere cells are resistant to doxorubicin, although the morphology of the spheres showed an irregular appearance in the presence of doxorubicin. Goodell et al. have demonstrated that ATP-binding transporters such as MDR may be involved in the efflux capacity of the side population that is enriched with stem cells (Goodell et al., 1996). Therefore, the resistance machinery against doxorubicin may be associated with the efflux capacity of sphere

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cells via MDR, which is highly expressed in sphere-forming cells derived from CHMp.

In conclusion, by the detailed analyses of spheres derived from canine mammary tumors, we have confirmed the existence of cancer stem cells. The further characterization of spheres from mammary tumors in dogs is needed to define novel therapies targeted against cancer stem cells, and this will contribute to the elucidation of the molecular and cellular mechanisms that underlie tumorigenesis.

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References

Al-Hajj, M., Wicha, M.S., Benito-Hernandez, A., Morrison, S.J., Clarke, M.F., 2003. Prospective identification of tumorigenic breast cancer cells. Proceeding of the National Academy of Sciences of the United States of America. 100 (7), 3983–3988.

Bonnet, D., Dick, J.E., 1997. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Nature Medicine 3 (7), 730–737.

Charafe-Jauffret, E., Monville, F., Ginestier, C., Dontu, G., Birnbaum, D., Wicha, M.S., 2008. Cancer stem cells in breast: current opinion and future challenges. Pathobiology 75 (2), 75–84.

Cocola, C., Anastasi, P., Astigiano, S., Piscitelli, E., Pelucchi, P., Vilardo, L., Bertoli, G., Beccaglia, M., Veronesi, M.C., Sanzone, S., Barbieri, O., Reinbold, R.A., Luvoni, G.C., Zucchi, I., 2009. Isolation of canine mammary cells with stem cell properties and tumor-initiating potential. Reproduction in Domestic Animals 44 (Suppl. 2), 214–217.

Dontu, G., Abdallah, W.M., Foley, J.M., Jackson, K.W., Clarke, M.F., Kawamura, M.J., Wicha, M.S., 2003. In vitro propagation and transcriptional profilining of human mammary stem/progenitor cells. Genes and Development 17 (10), 1253–1270.

Fujii, H., Honoki, K., Tsujiuchi, T., Kido, A., Yoshitani, K., Takakura, Y., 2009. Sphere-forming stem-like cell populations with drug resistance in human sarcoma cell lines. International of Journal Oncology 34 (59), 1381–1386.

Ginestier, C., Hur, M.H., Charafe-Jauffret, E., Monville, F., Dutcher, J., Brown, M., Jacquemier, J., Viens, P., Kleer, C.G., Liu, S., Schott, A., Hayes, D., Birnbaum, D., Wicha, M.S., Dontu, G., 2007. ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome. Cell Stem Cell 1 (5), 555–567.

Goodell, M.A., Brose, K., Paradis, G., Conner, A.S., Mulligan, R.C., 1996. Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. The Journal of Experimental Medicine 183 (4), 1797–1806.

Grange, C., Lanzardo, S., Cavallo, F., Camussi, G., Bussolati, B., 2008. Sca-1 identifies the tumor-initiating cells in mammary tumors of BALB-neuT transgenic mice. Neoplasia 10 (12), 1433–1443.

Honeth, G., Bendahl, P.O., Ringner, M., Saal, L.H., Gruvberger-Saal, S.K., Lovgren, K., Grabau, D., Ferno, M., Borg, A., Hegardt, C., 2008. The $CD44^+/CD24^-$ phenotype is enriched in basal-like breast tumors. Breast Cancer Research 10, R53.

MacEwen, E.G., 1990. Spontaneous tumors in dogs and cats: models for the study of cancer biology and treatment. Cancer Metastasis Reviews 9, 125–136.

Misdorp, W., Else, R.W., Hellmén, E.,Lipscomb, T.P., 1999. Histological Classification of Mammary Tumors of the Dog and the Cat. second ed., Armed Forces Institute of Pathology in Cooperation with the American Registry of Pathology and the World Health Organization Collaborating Centre for Worldwide Reference on Comparative Oncology, Washington, DC.

O'Brien, C.A., Pollett, A., Gallinger, S., Dick, J.E., 2007. A human colon cancer stem cell capable of initiating tumour growth in immunodeficient mice. Nature 445 (7123), 106–110.

Pardal, R., Clarke, M.F., Morrison, S.J., 2003. Applying the principles of stem-cell biology to cancer. Nature Reviews. Cancer 3 (12), 895–902.

Penzo, C., Ross, M., Muirhead, R., Else, R., Argyle, D.J., 2009. Effect of recombinant feline interferon-w alone and in combination with chemotherapeutic agents on putative tumour-initiating cells and daughter cells derived from canine and feline mammary tumours. Veterinary and Comparative Oncology 7 (4), 222–229.

Ponti, D., Costa, A., Zaffaroni, N., Pratesi, G., Petrangolini, G., Coradini, D., Pilotti, S., Pierotti, M.A., Daidone, M.G., 2005. Isolation and in vitro propagation of tumorigenic breast cancer stem cells with stem/progenitor cell properties. Cancer Research 65 (13), 5506–5511.

Reya, T., Morrison, S.J., Clarke, M.F., Weissman, I.L., 2001. Stem cells, and cancer stem cells. Nature 414 (6859), 105–111.

Ricci-Vitiani, L., Lombardi, D.G., Pilozzi, E., Biffoni, M., Todaro, M., Peschle, C., De Maria, R., 2007. Identification and expansion of human colon-cancer-initiating cells. Nature 445 (7123), 111–115.

Rutteman, G.R., Withrow, S.J., MacEwn, E.G., 2001. Tumors of the mammary gland. In: Withrow, S.J., MacEwen, E.G. (Eds.), Small Animal Clinical Oncology, third ed. W.B. Saunders, Philadelphia, pp. 455–477.

Sheridan, C., Lishimoto, H., Fuchs, R.K., Mehrotra, S., Bhat-Nakshatri, P., Turner, C.H., Goulet Jr., R., Badve, S., Nakshatri, H., 2006. $CD44^+/CD24^-$ breast cancer cells exhibit enhanced invasive properties: an early step necessary for metastasis. Breast Cancer Research 8, R59.

Stoica, G., Lungu, G., Martini-Stoica, H., Waghela, S., Levine, J., Smith, R., 2009. Identification of cancer stem cells in dog glioblastoma. Veterinary Pathology 46 (3), 391–406.

Takahashi, K., Yamanaka, S., 2006. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126 (4), 663–676.

Uyama, R., Nakagawa, T., Hong, S.H., Mochizuki, M., Nishimura, R., Sasaki, N., 2006. Establishment of four pairs of canine mammary tumour cell lines derived from primary and metastatic origin and their E-cadherin expression. Veterinary and Comparative Oncology 4 (2), 104–113.

Wilson, H., Huelsmeyer, M., Chun, R., Young, K.M., Friedrichs, K., Argyle, D.J., 2008. Isolation and characterization of cancer stem cells from canine osteosarcoma. The Veterinary Journal 178 (1), 69–75.

Yang, Z.F., Ho, D.W., Ng, M.N., Lau, C.K., Yu, W.C., Ngai, P., Chu, P.W., Lam, C.T., Poon, R.T., Fan, S.T., 2008. Significance of CD90+ cancer stem cells in human liver cancer. Cancer Cell 13 (2), 153–166.

Yuan, X., Curtin, J., Xiong, Y., Liu, G., Waschsmann-Hogiu, S., Farkas, D.L., Black, K.L., Yu, J.S., 2004. Isolation of cancer stem cells from adult glioblastoma multiforme. Oncogene 23 (58), 9392–9400.

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