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FULL PAPER

Canine osteosarcoma cell lines contain stem-like cancer cells: biological and pharmacological characterization

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ABSTRACT

Cancer stem cells (CSCs) represent a small subpopulation of cells responsible for tumor formation and progression, drug resistance, tumor recurrence and metastasization. CSCs have been identified in many human tumors including osteosarcoma (OSA). CSC distinctive properties are the expression of stem cell markers, sustained growth, self-renewal and tumorigenicity. Here we report the isolation of stem-like cells from two canine OSA cultures, characterized by self-renewal, evaluated by sphere formation ability, differential marker expression, and *in vitro* proliferation when cultured in a medium containing EGF and bFGF. Current therapies for OSA increased survival time, but prognosis remains poor, due to the development of drug resistance and metastases. Chemotherapy shrinks the tumor mass but CSCs remain unaffected, leading to tumor recurrence. Metformin, a drug for type 2 diabetes, has been shown to possess antitumor properties affecting CSC survival in different human and animal cancers. Here we show that metformin has a significant antiproliferative effect on canine OSA stem-like cells, validating this *in vitro* model for further pre-clinical drug evaluations. In conclusion, our results demonstrate the feasibility of obtaining CSC-enriched cultures from primary canine OSA cells as a promising model for biological and pharmacological studies of canine and human OSAs.

Key Words: Osteosarcoma, dog, cancer stem cells, metformin, comparative oncology.

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Introduction

Canine osteosarcoma (OSA) accounts for up to 85% of primary malignant bone-originating tumors[22]. Standard treatment consists in neoadjuvant chemotherapy, followed by amputation or limb sparing, reconstructive surgery and adjuvant chemotherapy. Cisplatin or carboplatin, alone or in combination with doxorubicin, constitutes the standard chermotherapy[24]. Currently, in dogs, radiotherapy is considered a palliative treatment although, some curative protocols for primary and metastatic disease have been proposed[36].

Canine OSA is a suitable model also for the study of human tumor since it shares similar biological and clinical behavior and molecular features[40,29,30]. Accumulating evidence indicates that OSA, as several solid and hematopoietic tumors, are characterized by intratumor cell heterogeneity that could be explained by the recent cancer stem cell (CSC) hypothesis. According to this theory, a subpopulation of cancer cells with stem-like properties, therefore named CSCs, is present within the hierarchical tumor organization. CSCs are responsible for tumor development and recurrence, and drug resistance. *In vitro*, CSCs are defined by long-term self-renewal, differentiation capacity and tumorigenicity after xenotransplantation in immunocompromised mice[31].

Conventional antineoplastic agents often fail to completely eliminate CSCs, which are able to repopulate the tumor mass, causing relapse. Thus, CSCs represent a pivotal pharmacological target to obtain effective therapeutic responses in cancer[6,32,12].

Human OSA tissues and cell lines contain putative CSCs, able to grow in serum-free conditions as spherical clusters in suspension, and possess the properties of self-renewal and pluripotency[14]. These cell aggregates can be dissociated and replated to obtain secondary spheres, and have the ability to undergo osteogenic and adipogenic differentiation. OSA cells with stem-like properties express the membrane markers preferentially expressed by mesenchymal stem cells such as CD133, CD117 (c-kit) and Stro-1[1,35]. Moreover, the chemokine receptor CXCR4 has been detected in canine OSA, playing a role in cell migration[9], and Oct4 is expressed in canine OSA stem cells as indicator of cell pluripotency[39]. Currently, the definitive set of markers that can specifically identify both human and canine OSA CSCs is still undefined, and the reliability of OSA stem cell phenotype should be improved by combining stem cell markers and growth features, such as sphere-formation ability[43]. Recently, the development of specific therapies targeted to CSCs has gained relevance in preclinical oncology aimed at improving treatment efficacy in cancer patients.

Metformin is a drug used in patients with type 2 diabetes, and several epidemiological studies shown that it reduces cancer incidence and mortality compared with other treatments in diabetic patients. *In vitro* findings demonstrate that metformin inhibits the proliferation of cell lines derived from breast, colon, lung, prostate and pancreatic cancers[28,42].

We previously isolated CSCs from human glioblastomas[15], and both feline[5] and canine[4] mammary carcinomas, characterizing the intracellular pathways involved in CSC proliferation as the chemokine CXCL12 and its receptor CXCR4[11,13]. In addition, we reported that metformin selectively targets human glioblastoma[41,16] and canine mammary carcinoma CSCs[4].

In this study, cancer stem-like cells have been isolated from canine OSA primary cultures and preliminary bio-molecular characterization of these cells has been performed. In addition, the responsiveness of OSA stem-like cultures to innovative antitumor drugs such as metformin, was assayed, aimed at evaluating the sensitivity of canine OSA stem-like cells and the reliability of dog as a model for human OSA.

Materials and Methods

Cell cultures and cancer stem cell enrichment: Two canine primary OSA cultures, named OSA1 and OSA2, were isolated and characterized at the Department of Animal Pathology of the University of Torino[23]. Primary osteoblasts were obtained[8] and grown until confluence for no more than 3 passages and stored in liquid nitrogen. To confirm the osteosarcoma origin of OSA cultures, alkaline phosphatase (ALP) staining was performed. Cells were incubated with 5-bromo, 4-chloro, 3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) as a substrate for ALP. Cells were cultured in Iscove's medium supplemented with 10% fetal bovine serum (FBS), 1% glutamine, penicillin/streptomycin (all from Lonza) and maintained at 37°C in a humidified atmosphere with 5% CO_2.

To enrich the cultures in stem-like cell subpopulation, cells were harvested using trypsin-EDTA (Euroclone) and re-plated in a stem permissive medium consisting of Iscove's medium with 1% FBS, N2 supplement (Invitrogen), epidermal growth factor (EGF, 10 ng/ml, Miltenyi Biotec) and basic fibroblast growth factor (bFGF, 10 ng/ml, Miltenyi Biotec)[14,2]. Cell morphology changes was monitored daily using an inverted contrast microscope.

Sphere formation assay: To address self-renewing ability of OSA stem-like cells, dissociated single sphere-forming cells were diluted to a density range of 1,000–60,000 cells/well and plated in ultra-low attachment 6-well plates (Corning) in stem-permissive, serum-free medium. After 7 days, primary spheres were collected by gentle centrifugation, dissociated with TrypLE (Invitrogen) and mechanically disrupted with a pipette to obtain single cell suspensions and then re-seeded in the same conditions to re-form spheres. Sphere formation was daily monitored and sphere were passaged every 7–14 days, for up to 7 weeks.

Immunocytofluorescence (ICF): To analyze the expression of relevant markers, OSA primary cultures and OSA stem-like cells, grown on glass coverslips, were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 in PBS, blocked with normal goat serum (all from Sigma-Aldrich) and immunostained with primary antibodies for Oct4 (Abcam), CXCR4 (Sigma-Aldrich) and osterix (Santa Cruz Biotechnologies) diluted 1 : 100, osteocalcin (Takara Bio), osteonectin (AON-1, Developmental Studies Hybridoma Bank, Iowa City, IA, USA), pro-collagen type I (SP1.D8, Developmental Studies Hybridoma Bank), CD133 (Milteny Biotec) diluted 1 : 50 in PBS for 1 h at r.t. Fluorochrome-conjugated secondary antibodies (goat anti-mouse and anti-rabbit Alexa Fluor-568 or Alexa Fluor-488, Molecular Probes-Invitrogen) were applied, and nuclei were counterstained with DAPI (Sigma-Aldrich)[3]. Coverslips were mounted with Mowiol (Calbiochem) and images were obtained with a DM2500 microscope equipped with a DFC350FX digital camera (Leica Microsystems).

Cell viability assay: Mitochondrial activity, as index of cell viability, was evaluated by measuring the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich). Viable cells (5,000) were plated into 48-well plates and assayed for growth rate every 48 h for up to 7 days. For cytotoxicity evaluation, cells were seeded over-night prior to exposure to metformin (Sigma-Aldrich) at increasing concentrations (range 1–90 mM) for 48 h. Cells were incubated with MTT (stock solution 2 mg/ml) for 1h, and formazan crystals, derived by the cleavage of MTT by mitochondrial dehydrogenase, were dissolved in DMSO and absorbance spectrophotometrically measured at 570 nm[27].

Western Blotting: Cells were lysed in a buffer containing 1% Igepal, 20 mM Tris-HCl, pH 8, 137 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 10 mM NaF (Sigma-Aldrich), and

the "Complete" protease inhibitor (Roche) for 10 min at 4°C and proteins quantified using the Bradford assay (Bio-Rad Laboratories). Proteins (40 μg) were resuspended in Laemmli buffer, size-fractionated by 10% SDS-PAGE, electroblotted onto PVDF membrane (Bio-Rad Laboratories) and probed with Oct4 antibody (Abcam) and α-tubulin (Sigma-Aldrich). Immunocomplexes were detected using a chemiluminescence system (BioRad Laboratories), and quantified by the ChemiDoc XRS apparatus (Bio-Rad Laboratories).

Statistical Analysis: Statistical analysis and IC_{50} values, calculated using nonlinear regression curve fit analysis selecting the log (drug) vs. response–variable slope (four parameters) equation, were done using GraphPad Prism version 5.02 (GraphPad Software, San Diego CA, USA). Data analysis for normal distribution and similar variance between groups was performed by using GraphPad Prism version 5.02. Two-tailed Student's t-tests was used for comparisons between 2 groups. Statistical significance was established at p values <0.05. All experiments were repeated at least thrice, data are presented as mean $\pm$ s.e.m.

Results

Two primary canine cell cultures (Fig. 1A) were obtained from post-surgical tumor specimens with histologically confirmed diagnosis of appendicular osteoblastic productive (OSA1) and chondroblastic (OSA2) osteosarcomas (Table 1)[23]. Cells were obtained according to the methodology previously described[8] (Fig. 1A). Osteoblast origin was confirmed by phosphatase alkaline immunohistochemistry (Fig. 1A).

To confirm the origin of OSA cultures, the expression of osteoblastic differentiation markers, osteonectin, osteocalcin and type I pro-collagen, also used for OSA pathological diagnosis[10] was analyzed by ICF. All markers were detectable in both cell cultures, however differences in the pattern of expression were observed. In particular, a homogeneous presence of pro-collagen and osteocalcin was found in almost all OSA2 cells, while only scattered osteonectin-immunopositive cells were present. OSA1 showed lower levels of expression for all markers (Fig. 1B).

The analysis of the proliferative activity of OSA cultures *in vitro*, as evaluated by MTT assay in time-course experiments, revealed that OSA1 cells possess a higher growth rate than OSA2 cells at all the experimental time points (Fig. 1C).

Actively proliferating cells were shifted from standard culture conditions (10% FBS) to a FBS-free medium supplemented with growth factors (EGF and bFGF), that selectively allows the growth of stem cells. Cultures were daily analyzed by microscopy: in these conditions, after about 2 weeks, OSA1 and OSA2 stem-like cells grew as non-adherent aggregates and appeared as rounded-shaped cells forming sarcospheres, long lasting *in vitro* (Fig. 2A).

To verify whether OSA-stem like cells maintain their proliferative potential *in vitro*, MTT assay was carried out for up to 7 days. A constant but slow proliferation rate, (Fig. 2B), was observed. In these conditions cell proliferation was much slower than the corresponding cultures maintained in FBS-containing medium (Fig. 1C). Moreover, OSA-stem like cells, when transferred in the standard medium, containing 10% FBS, are able to grow as adherent monolayers with the same characteristic of corresponding OSA cell line of origin (data not shown) suggesting the retaining of ability to differentiate in non-stem cancer cells. Conversely, when kept in stem cell-selective growth conditions (Iscove's medium, N2 supplement, 1% FBS, bFGF and EGF), OSA cells do not enter into differentiation program as demonstrated by the stable expression of stem cell markers (see below).

We characterized the putative OSA-stem-like population evaluating the expression of relevant CSC markers. OSA1 and OSA2 stem-like cultures were analyzed by immunocytofluorescence for the

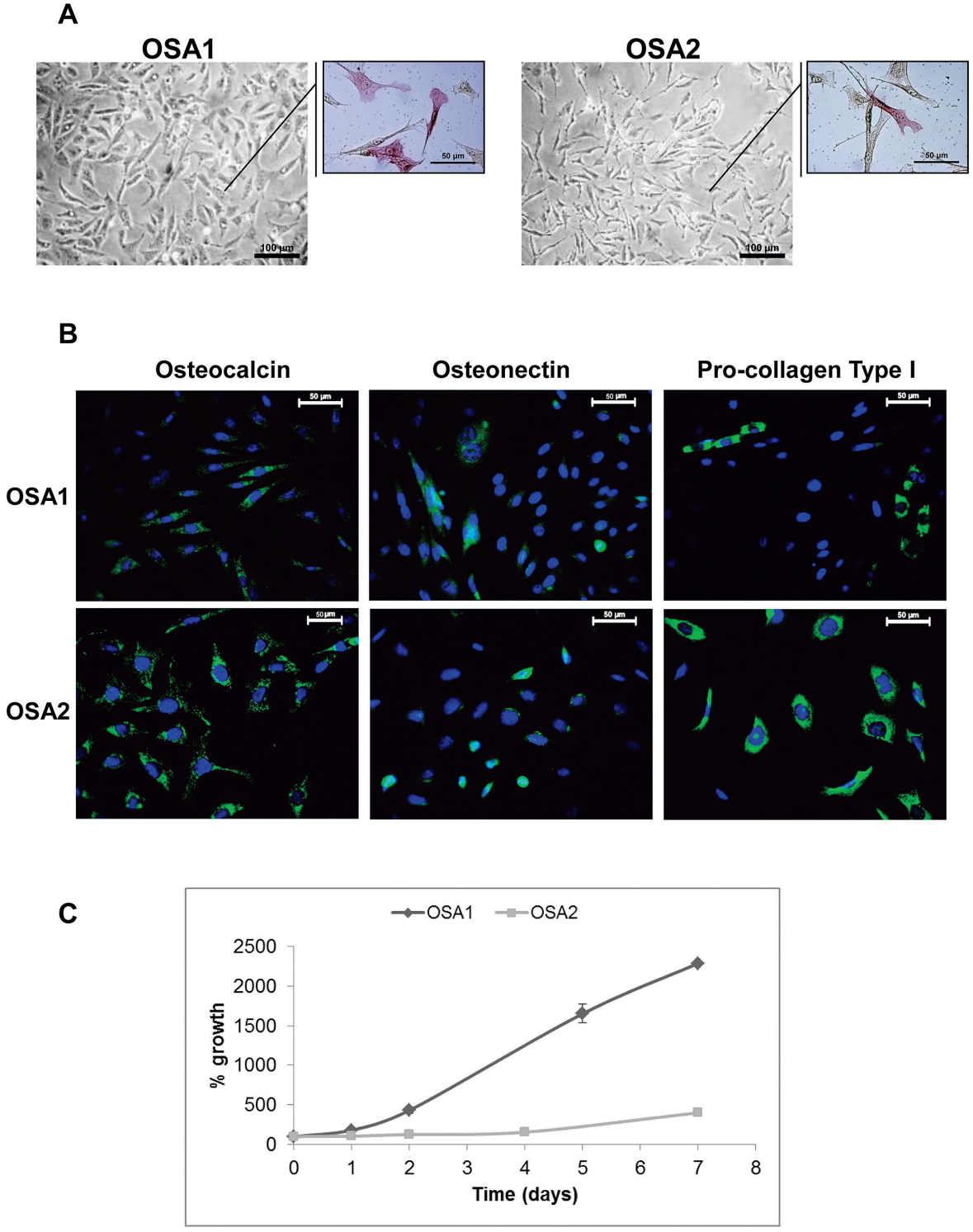

Fig. 1. A. Phase-contrast microphotographs of OSA primary cultures. Representative morphological appearance of canine primary OSA monolayers in FBS-containing medium. Magnification 10X. Inserts show the alkaline phosphatase staining of cells. B. Analysis of the expression of osteonectin, osteocalcin and type I procollagen in OSA primary cultures by fluorescence microscopy. Photomicrographs represent merged images (marker-positive, green; DAPI counterstained nuclei, blue). Magnification 40X. Scale bar = 50 µm. C. Growth curves of OSA1 and OSA2 primary cell cultures. Cell viability was assessed by MTT assay, at different time-points. Data are shown as mean ± SE from three separate experiments.

Table 1. Primary osteosarcoma cell cultures: clinico-pathological features of canine tumors

Tumor code/ Cell line	Age (years)	Sex	Histology	Grade	DFI (days)	OS (days)
OSA1	0.5	m	Osteoblastic productive OSA	III	No relapse	Still alive
OSA2	8	f	Chondroblastic OSA	III	70	70

DFI: disease free interval; OS: overall survival

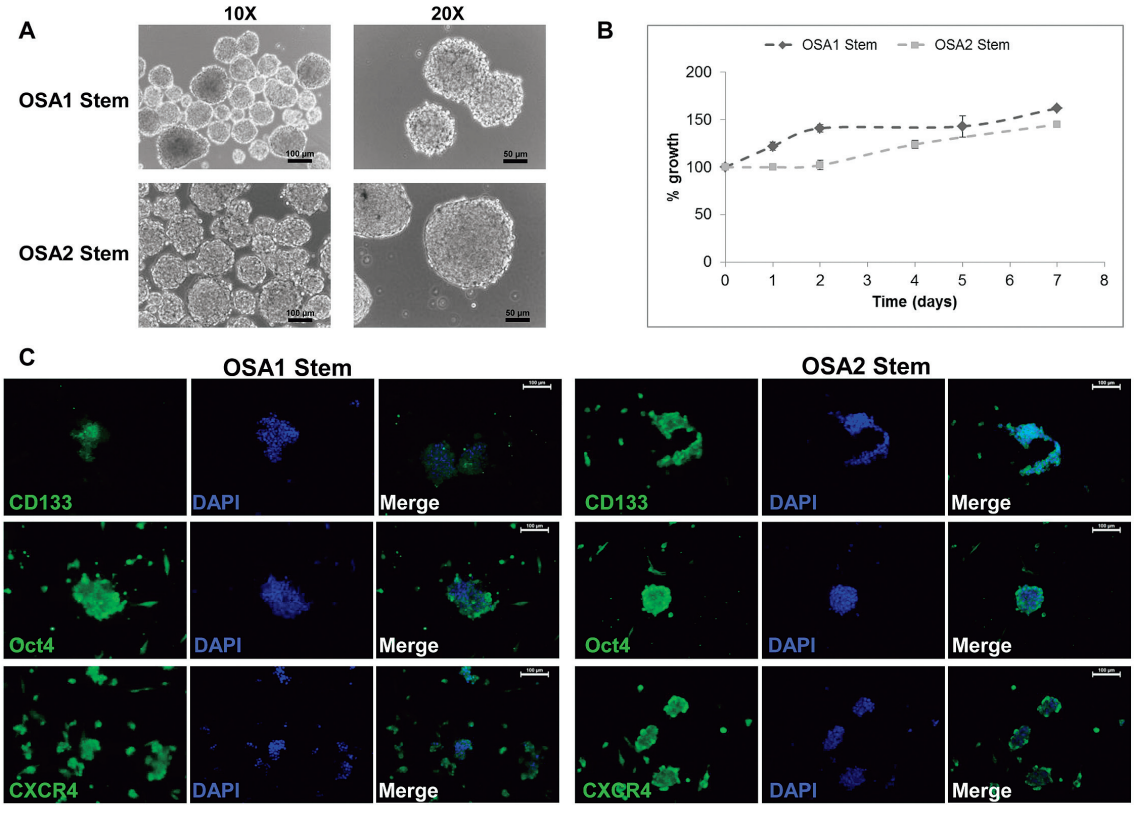

Fig. 2. A. Phase-contrast microphotographs of spheroids formed by OSA stem-like cells. Representative images of stem-enriched cultures (sarcospheres) obtained by shifting primary cultures into selective stem culture medium. Magnification 10X and 20X. B. Growth curves of OSA1 and OSA2 stem-like cells. Cell viability was assessed by MTT assay, at different time-points. Data are shown as mean ± SE from three separate experiments. C. Analysis of the expression of CD133, Oct4 and CXCR4 in OSA stem-like cells by fluorescence microscopy. Photomicrographs represent marker-positive cells (green), DAPI counterstained nuclei (blue) and merged images. Magnification 20X. Scale bar = 100 μm.

expression of CD133, a marker for normal pluripotent and cancer stem cells, Oct4, an embryonically expressed transcription factor, previously identified in CSCs from canine mammary carcinoma tissues[38] and essential for the maintenance of cell pluripotency, and CXCR4, a chemokine receptor involved in OSA malignancy and linked to metastatic ability of CSCs[33,21]. The results depicted in Fig. 2C demonstrated that both OSA1 and OSA2 stem-like cells express CD133, although the expression levels was rather modest, but they are consistently positive for Oct4 and CXCR4. No significant variation of the expression of these CSC antigens was observed over time (data not shown), thus suggesting that CSC population was stable during *in vitro* culturing within the OSA1 and OSA2 cultures (Fig. 2C).

A key property of CSCs is their unique ability to self-renew. One of the methods to determine

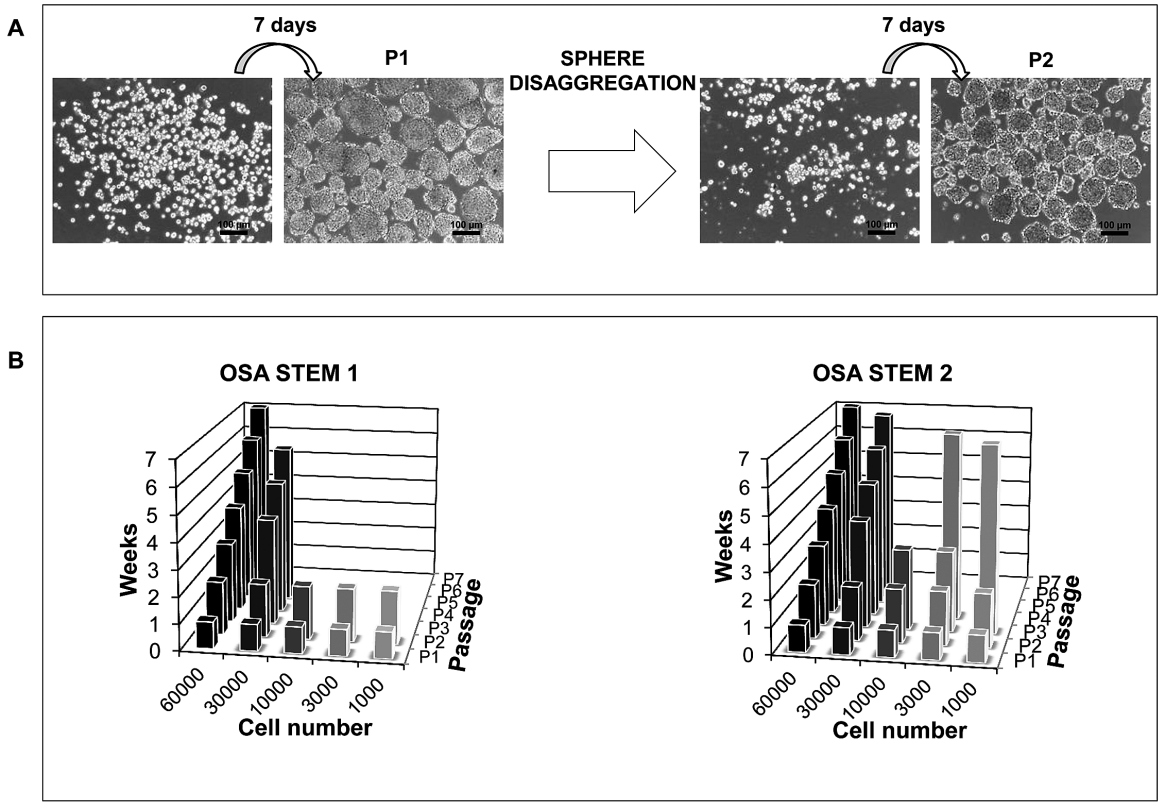

Fig. 3. A. Sphere-forming protocol. Figure depicts cell plating (time 0), and formation of primary spheres, followed by subsequent passages (P1, P2, ...) to obtain secondary sheres, repeated every 7–14 days. B. Sphere formation and self-renewal ability of OSA1 and OSA2 stem cells. Histograms represent the number of passages (sphere disaggregation/replating as single cells) throughout time (for up to 7 weeks), and their relationships to sphere-formation time intervals The number of primary (P1), secondary (P2, generated from dissociated primary spheres), tertiary (P3, generated from dissociated secondary spheres) and subsequent (up to P7) sphere passages, obtained from different number of cells is shown.

this ability is to test CSC capability of serial passage *in vitro*, thus to more deeply investigate the self-renewing ability of OSA stem-like cells, OSA1 and OSA2 spheroids were dissociated to single cells and re-seeded at increasing cell densities, which allowed for the formation of separate non-adherent, three-dimensional sphere clusters, in stem permissive medium. The passages were repeated every 7–14 days when the spheres grew in diameter, by monitoring sphere formation efficiency (Fig. 3A). The cells from both OSA cultures formed spheres, however, at different sizes and efficiency, suggesting that each culture contained a different number of cancer stem-like cells. OSA2 sphere-forming cells grew faster and were capable of more extensive proliferation than OSA1 stem-like cells. Sphere-formation ability of OSA1 stem-like cells mainly depended on the number of seeded cells, being able to self-renew for up to 7 weeks only at higher cell number, retaining spherogenic potential for at least six passages. Conversely, OSA1 clones deriving from lower cell densities (1,000–10,000) were lost between passage 2 and 3, with no cells able to further sustaining sphere formation. On the contrary, also the lowest cell number of OSA2 stem-like cells (1,000) generated spheres that survived throughout the experimental time, although sphere dissociation/replating was done only 3 times. High cell numbers (30,000 and 60,000) of OSA2 CSCs retained sustained spherogenic potential for about 6–7 passages (Fig. 3B).

Overall these results confirmed the presence

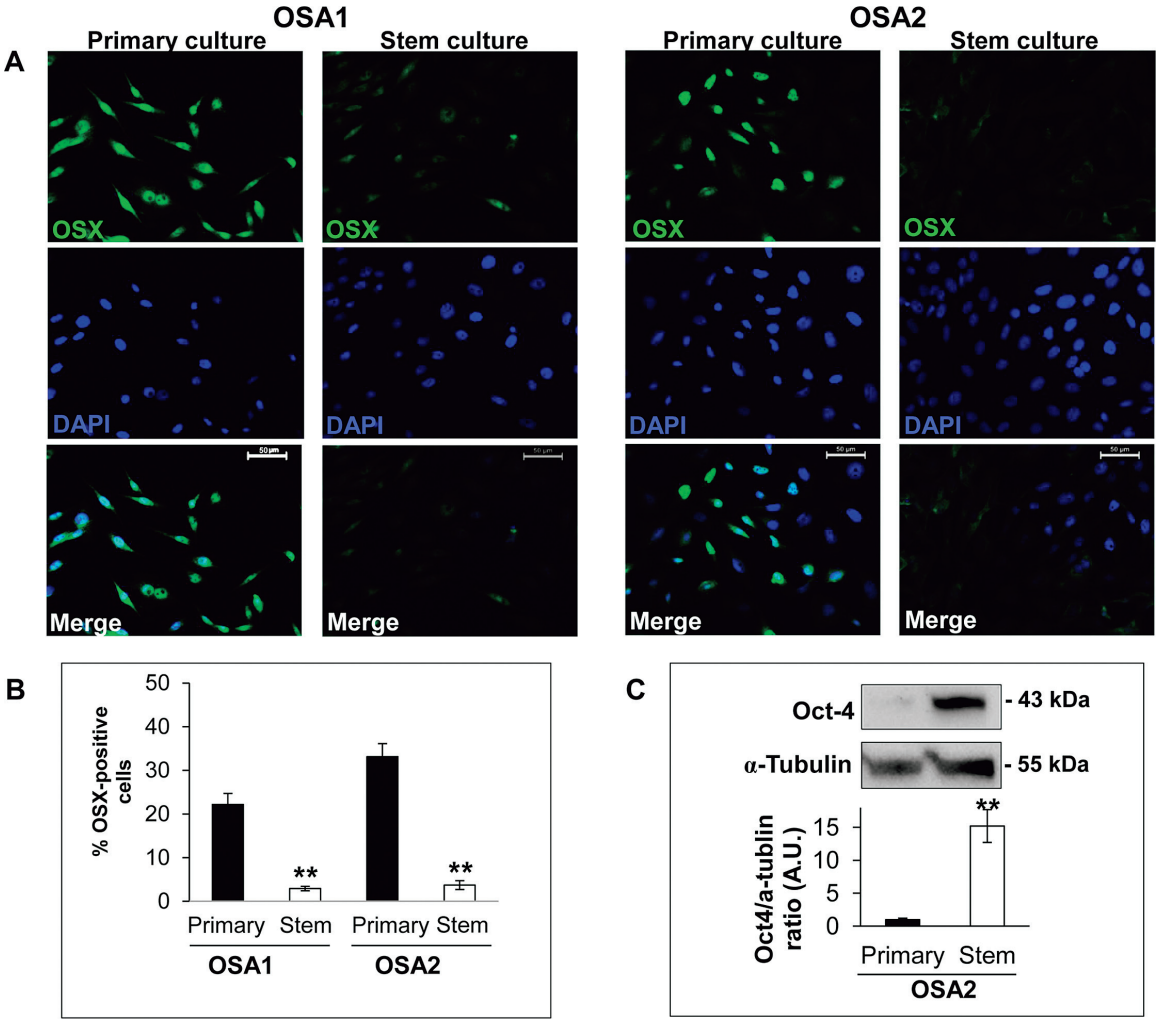

Fig. 4. A. Analysis of the expression of osterix (OSX) in OSA primary cultures and stem-like cells by fluorescence microscopy. Photomicrographs represent marker-positive cells (green), DAPI counterstained nuclei (blue) and merged images. Magnification 40X. Scale bar = 50 μm. B. OSX expression in OSA primary cultures and stem-like cells. The percentage of OSX expressing cells was determined by the number of nuclear immunopositive positive cells among the total number of counted DAPI-stained tumor cells (a minimum of 500 cells were counted per coverlips). A significant decrease in OSX-positive cells was observed in OSA stem cultures, compared to the respective primary cultures. (**$p < 0.01$). C. Western Blot analysis of Oct4 protein in OSA2 primary culture and stem-like cells. The histogram shows the quantitative densitometric analysis of Oct4 protein (normalized to α-tubulin) and reported as arbitrary units (A.U.), **$p < 0.01$, *vs.* primary culture set at 1 A.U..

of stem cell subpopulations within both cultures even if the CSC content differs between OSA1 and OSA2 (Fig. 3B).

To investigate whether OSA stem-like cells maintain the expression of differentiation protein, the presence of osterix (OSX), a zinc finger–containing transcription factor, essential for mature osteoblast differentiation, whose expression in mouse and human OSA cell lines has been described[7], was chosen as differentiation marker and quantified by ICF scoring the nuclear cell positivity (Fig. 4A).

OSX was more abundantly expressed in OSA1 and OSA2 primary cultures (22% and 33% of positive cells, respectively) than in corresponding CSC cultures (about 2% for both cultures) (Fig. 4B). Conversely, sarcosphere cells expressed the transcriptional factor Oct4, analyzed by western blot, at significantly higher levels (15-fold) in OSA2 CSC culture than in the

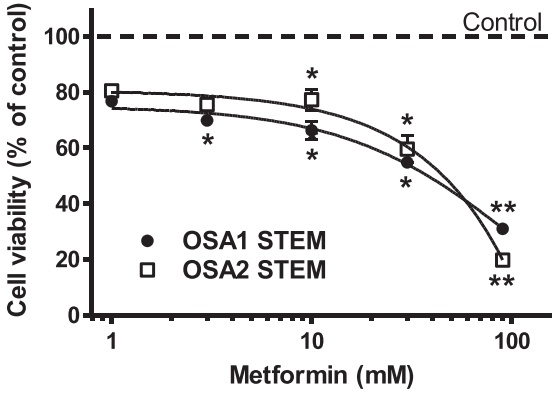

Fig. 5. Dose-response curves of OSA stem-like cells treated with metformin. Metformin exerts antiproliferative effects in cancer stem-like cells derived from OSA1 and OSA2 in a dose-dependent manner. Cells were plated in 48-well plates and treated for 48 h with increasing concentrations (1–90 mM) of metformin. Respective untreated controls are set as 100% and depicted as a broken line. Concentration is reported in a log scale and results, expressed as percentage ± SEM, are reported as means from triplicates. *$p < 0.05$, **$p < 0.01$, *vs.* untreated cells.

parental OSA2 cells (Fig. 4C).

Taken together these results, showing the loss of OSX positivity and the increase in Oct4 expression in the stem cell subset, suggest the stem cell enrichment of OSA primary cultures after growth in stem permissive medium.

Finally, we analyzed the sensitivity of OSA-stem like cells to metformin, the first-line drug for treating type 2 diabetes, that was proposed to selectively kill CSCs in different human cancer types. Metformin induced a powerful dose-dependent antiproliferative effect obtaining IC_{50} values, calculated after 48 h of treatment, of 6.40 mM and 17.03 mM for OSA1 and OSA2 stem-like cells, respectively (Fig. 5), closely matching the concentrations reported in previous studies[18,19].

Discussion

According to the current vision of comparative oncology, spontaneously occurring tumors of companion animals, such as canine OSA, provide interesting translational opportunities for human diseases.

Similarities in gene expression and clinical behavior of OSA in dogs and humans make the dog an excellent model for the study of the biology of this tumor, adequately representing many of the features that define human OSA, including heterogeneity of both tumor cells and microenvironment[29,30]. Indeed, the complex biology of cancer cells is not appreciably conserved in conventional cancer cell lines and mouse models commonly used in drug development.

We here demonstrated that it is possible to isolate CSCs from canine OSA cell cultures growing them in stem cell permissive medium. These cells, representing a subset of canine OSA cells possess stem cell-like features, retaining the ability to self-renew *in vitro*. Indeed, two stem-like cultures can be expanded *in vitro* in stem cell medium and repeatedly form spheres after multiple passages in long-term assays. However, different timing and efficiencies were observed between OSA1 and OSA2 CSCs, suggesting that each culture contains a different number of cancer stem-like cells. This observation supports the stemness of CSC-enriched OSA cultures as measured by their ability to form spheres when cultured in defined medium, according to first evidence showing that human OSA cell lines and primary OSA cells contain cells with CSC features[14,37], and further confirmed in canine cell models[26,39].

Indeed, these results provide evidence that a subset of OSA cells with stem-like properties do exists within canine OSA cultures we developed; as pluripotent cells they are characterized by the expression of a group of genes ("stemness" genes) required to maintain the stem cell-like feature such as Oct4, a transcription factor that regulates the suppression of differentiation genes[25] and, although less evident CD133, as also described in canine osteosarcoma biopsies,[17] the most commonly reported marker of CSCs, as also described in canine OSA biopsies. In addition, both OSA stem-like spheroids showed a marked expression of CXCR4, a chemokine

receptor that through the binding to its ligand CXCL12, plays a key role in controlling tumor cell proliferation and homing of stem cells and metastasis, thus suggesting relevant functions also in OSA development[1].

OSA development may be ascribed to altered osteogenic differentiation[34], and OSA cells exhibit osteogenic markers as OSX, controlling the differentiation into mature osteoblast. We verified the presence of OSX in OSA1 and OSA2 primary cultures evidencing a subset of immunopositive cells (20–30%) consistent with the presence of markers of differentiation (alkaline phosphatase, osteocalcin, osteonectin, pro-collagen) in both cultures, indicating an heterogeneous cell population not exclusively composed by completely mature cells but also by a more undifferentiated subpopulation.

Interestingly, OSX expression was undetectable in OSA cultures enriched in stem-like cells, revealing the selection under stem culture conditions of undifferentiated/progenitor cells, likely not yet committed to the osteoblast lineage.

As the marked expression of genes involved in stem cell maintenance such as Oct4 is a common characteristic of the CSCs derived from different human tumors, including osteosarcoma[20], consistent with previous findings[26] we demonstrate that OSA spheres had significantly higher expression of Oct4 as compared to the adherent primary culture. Therefore, in canine OSA cells, Oct4 expression likely contributes to the maintenance of the CSC pool. This observation, taken together with the loss of OSX upon stem culture conditions, further reinforces the achieving of isolation of CSC subpopulation from primary canine samples.

The main challenge in CSC pharmacological research is the identification of possible drugs effective on this otherwise elusive tumor cell population. To date only few molecules have been reported to be effective on these cells[41]. Several epidemiologic and pre-clinical studies support the potential antitumor effect of metformin, and its selective antiproliferative activity on CSC subpopulation[18,41,4]. Our results indicate that also canine OSA stem-like cells are sensitive to metformin with similar efficacy that identified in previous studies *in vitro* on different human and animal CSC cultures. *In vivo* studies are now required to confirm these results, but the data here reported due to the predictive value of the CSC model are extremely promising. Moreover, these cells represent a novel experimental model for pre-clinical *in vitro* evaluation of drug efficacy further confirming the potential for the dog as a model of human tumors.

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