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In vitro and *in vivo* characterization of stem-like cells from canine osteosarcoma and assessment of drug sensitivity

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ABSTRACT

Osteosarcoma is an aggressive bone tumor which displays biological and molecular heterogeneity, largely dependent on cancer stem cells (CSCs). CSC self-renewing and drug resistance cause treatment failure and tumor recurrence. CSC identification of in osteosarcoma and their efficient targeting are still open questions. Spontaneous canine osteosarcoma shares clinical and biological features with the human tumor, representing a model for translational studies. Here we enriched in CSCs three canine osteosarcoma cultures. These CSC cultures grow, in serum-free conditions, as anchorage-independent spheroids, show mesenchymal-like properties and *in vivo* tumorigenicity, recapitulating the heterogeneity of the original osteosarcoma. Osteosarcoma CSCs express stem-related factors (Sox2, Oct4, CD133) and chemokine receptors and ligands (CXCR4, CXCL12) involved in tumor proliferation and self-renewal. Standard drugs for osteosarcoma treatment (doxorubicin and cisplatin) affected CSC-enriched and parental primary cultures, showing different efficacy within tumors. Moreover, metformin, a type-2 diabetes drug, significantly inhibits osteosarcoma CSC viability and self-renewal, and in co-treatment with doxorubicin and cisplatin enhances drug cytotoxicity. Collectively, we demonstrate that canine osteosarcoma primary cultures contain CSCs exhibiting distinctive sensitivity to anticancer agents, as a reliable experimental model to assay drug efficacy. We also provide proof-of-principle of metformin, alone or in combination, efficacy as pharmacological strategy to target osteosarcoma CSCs.

Keywords: osteosarcoma, cancer stem cell, tumorigenicity, metformin, self-renewal

Abbreviations

OSA, osteosarcoma; CSCs, cancer stem cells; CXCR4, C-X-C motif chemokine receptor 4;CXCL12, C-X-C motif chemokine ligand 12; CD117, KIT proto-oncogene receptor tyrosine kinase; STAT3, signal transducer and activator of transcription 3; Oct4, POU class 5 homeobox 1; Sox2, SRY-box 2¸CI, combination index; CFE, colony formation efficiency; DAPI, 4,6-diamidino-2-phenylindole dihydrochloride; MTT, (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide); EGF, epidermal growth factor; bFGF, basic fibroblast growth factor; H&E, hematoxylin and eosin staining.

Conflicts of interest: none.

1. INTRODUCTION

Osteosarcoma (OSA) is the most common primary bone malignancy in young people, predominantly occurring in long bones, in areas characterized by proliferating cells and active bone growth [1]. OSA is classified into different histopathologic subtypes (e.g. osteoblastic, fibroblastic, chondroblastic), which, however, have not prognostic or therapeutic relevance [2, 3]. Despite the relative low incidence, OSA accounts for high morbidity and mortality. The therapy of these tumors is extremely complex [4] and the outcome influenced by several factors such as tumor and metastasis site, presence of undetectable micro-metastases at diagnosis (in more than 80% of patients), extent of lesion and feasibility of complete surgical resection [5]. Neo-adjuvant chemotherapy for localized OSA includes methotrexate, doxorubicin, and cisplatin (MAP), followed by surgery and adjuvant therapy (doxorubicin) to eliminate micro-metastases [6, 7]. Patients bearing localized OSA, and treated with MAP regimen, reach more than 50% overall survival at 5 years, which however did not improve in the last decades [8]; up to 30% of patients with newly diagnosed OSA have already metastatic spread, commonly in the lungs, making the treatment challenging and survival expectancy very poor (5-year survival of about 20%) [9]. No new chemotherapeutic drugs demonstrate effectiveness in resistant or relapsed OSA (pulmonary recurrence occurs in about 30% of patients) for which no standard second-line strategies are available; thus intense investigations are currently needed to identify both innovative agents active in this disease and cellular/molecular mechanisms that sustain OSA cell resistance and recurrence.

Different drug resistance mechanisms have been observed in OSA: impairment of drug cellular uptake, enhanced DNA repair system activity, evasion of apoptosis, adaptive signaling from tumor microenvironment, and the presence of cancer stem cells (CSCs) [10]. OSA, as other solid tumors, is cellularly heterogeneous, containing hierarchically organized cells, sustained by CSCs, acting as tumor-initiating cells. CSCs are a tumorigenic cell subset with intrinsic drug resistance, able to persist in the tumor mass by self-renew and to differentiate into non-tumorigenic cell populations composing the bulk of the tumor [11, 12]. In OSA, several cell types have been proposed as stem-like population, including committed osteo-progenitors or undifferentiated mesenchymal stem cells (MSCs), carrying genetic and/or epigenetic alterations [13-15], which enable these cells to escape the control of proliferation and to differentiate into mature bone-forming osteoblasts [16]. Self-renewal properties of CSCs, which allow the isolation and enrichment *in vitro* of this cell subset, are evaluated by the formation of tumor spheres under non-adherent and serum-free culture conditions [17] while, *in vivo*, tumorigenicity of putative CSCs can be assayed by serial transplantation into immunocompromised mice [18]. Beside functional assays, the evaluation of stem markers, such as CD133, CD117, Sox2 and Oct4, was also proposed to identify CSCs, being claimed as possible specific marker candidates [19-21]; however, a definite phenotype to precisely identify tumor stem-like cells is still lacking in OSA [12] as well as in other cancers. Importantly, cell populations endowed with

stem-like properties were isolated from several pet tumors including canine and feline mammary carcinomas, canine prostate and hepatocellular carcinomas, and OSAs, among others [22-30] representing a high translational value model for human tumors. In particular, naturally occurring OSA is the most common primary bone tumor in adult dogs and represents an excellent model for the human disease, sharing similar biological and clinical behavior (invasiveness and spreading to lungs, poor median survival time, frequent metastatic disease) [31-33]. In oncology, the dog model of spontaneous OSA, due to its development in a shorter time than in humans, allowed relevant studies on genomics, diagnosis and drug sensitivity [31]. Moreover, genomic (e.g. *TP53, RB1* alterations) and molecular features (dysregulated expression of Met and STAT3, and the chemokine receptor CXCR4, proposed as putative prognostic markers or indicators of metastatic disease) of canine OSAs closely resemble the human disease [34]. Furthermore, the same current therapeutic strategies are used in both humans and dogs, and the new drugs under study are proposed to represent valuable approaches for both kind of patients [31, 35].

Both human and canine OSAs contain CSCs that grow in anchorage-independent conditions as sarcospheres and possess tumor-initiating properties, expressing common putative CSC-related markers and stem-like signatures [30, 36-38].

Therefore, understanding the heterogeneous biology of OSA, likely driven by CSCs, is important to elucidate its pathogenesis and therapeutic response, representing the critical target for treatments eliciting tumor growth arrest and avoiding relapses.

Several *in vitro* studies report the antiproliferative efficacy of repurposed drugs against CSCs from different tumor types [39, 40]. In particular, metformin, an oral biguanide commonly used as first-line treatment for type 2 diabetes, selectively affects CSC viability in several human tumors [41-48]. Metformin inhibits OSA cell proliferation, invasiveness, and self-renewal [49], and sensitizes OSA CSCs to cisplatin [50, 51]. However, in these studies CSC-like cells were mainly derived from continuous cell lines grown in the presence of high fetal bovine serum, an experimental conditions causing the loss of original CSC biological features [52]. To overcome this problem, in this study we performed phenotypic and *in vitro* and *in vivo* functional characterization of three primary cultures enriched with tumorigenic CSCs, derived from canine OSAs. Using this model, we investigated the cytotoxic activity of anticancer drugs (cisplatin, doxorubicin) commonly used for OSA treatment, comparing the sensitivity of OSA CSCs and the respective non-stem cell counterparts. In addition, we studied the efficacy of metformin on OSA CSC viability and self-renewal, and the effects achievable by combined treatment with standard drugs and metformin.

2. MATERIALS AND METHODS

2.1. Primary cell cultures of canine OSA and cancer stem cell (CSC) enrichment

Three primary canine OSA cell cultures, named OSA1, OSA2 and OSA3, were isolated from post-surgical tumor specimens at the Department of Animal Pathology of the University of Torino [53]. OSA1 cells derived from osteoblastic productive grade III OSA, while OSA2 and OSA3 cells derived from chondroblastic grade III OSAs. The osteoblastic origin of the cultured cells was assessed by morphological observation and alkaline phosphatase activity [53]. Cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM, GIBCO®),) supplemented with 10% fetal bovine serum (FBS, EuroClone), penicillin/streptomycin (EuroClone) and maintained at 37°C in a humidified atmosphere with 5% CO_2.

Enrichment of OSA primary cultures with stem-like cell subpopulation was performed exploiting their low attachment to tissue culture plastic when cultured in "stem cell medium" containing IMDM, 1% FBS, N2-supplement (100X, GIBCO®), epidermal growth factor (EGF, 10 ng/ml, Miltenyi Biotec) and basic fibroblast growth factor (bFGF, 10 ng/ml, Miltenyi Biotec) [38, 54]. This *in vitro* procedure allows the selection of tumor cells displaying defining stem cell properties of self-renewal and differentiation as prospectively identifiable subpopulations of tumor cells. CSCs from OSA1 and OSA2 have been already partially characterized *in vitro* in a previous study [30], while the third one (OSA3) has been used to select putative CSCs for the first time.

2.2. Sarcosphere formation assay

Single cell suspensions of primary cultures were seeded in 6-well ultra-low attachment plates (Corning) at a density of $6X10^4$ cells/well in stem cell medium. After 7 days of incubation, primary spheres (sarcospheres) were obtained. The persistence of self-renewal after long-lasting *in vitro* culturing was shown by dissociating primary spheres to single cells, which were allowed to generate secondary sarcospheres under the same culture conditions. Serial passaging of first generation spheres, was performed every 7 days of incubation, harvesting the spheres by centrifugation, and dissociation of the pellet to single cells using TrypLE Express Enzyme (GIBCO®), and then expanding them again. To test the effect of metformin on sphere growth, after 7 days of culture to allow sphere generation, sarcospheres were treated with 3 mM metformin and counted after 5 days. Only spheres with at least 40-50 µm were considered. To further demonstrate the activity of metformin, sphere-formation capacity was monitored in the absence or presence of the drug (3 mM): every 7 days sarcospheres formed were disaggregated, cells were counted and re-plated in fresh medium in the same conditions (in the presence of vehicle or metformin). The number of viable cells was evaluated at every passage.

2.3. Immunohistochemistry (IHC)

IHC was performed on 4-µm sections of formalin-fixed paraffin-embedded tissue derived from 12 OSA (9 males and 3 females with mean age 7.7 years, range 0.5-14). As previously described [55], deparaffinized/rehydrated sections were incubated in citrate-antigen retrieval buffer, nonspecific immunoreactivity was blocked with 10% normal goat serum (Sigma-Aldrich) and primary antibodies applied

overnight at 4 °C. The following antibodies were used: Oct4 (Abcam), CXCR4 (Sigma-Aldrich), CXCL12 (Abcam), CD117 and STAT3 (SantaCruz). Real Envision Detection System Peroxidase/DAB+, mouse/rabbit (Dako) was used for the detection according to the manufacturer's instructions. Counterstaining with hematoxylin was performed. For each staining, a negative control, was included without the primary antibody or with rabbit/mouse IgG. Images were captured using a Nikon Coolscope microscope. The intensity of immunoreaction and the percentage of positive cells were evaluated, and a score ranging from 0 to 3 was assigned (0 = negative, 1 = low positivity, 2 = positivity, 3 = high positivity), independently by two pathologists (A.R. and C.C.).

2.4. Immunofluorescence (IF)

OSA spheres growing on glass coverslips were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 in PBS, and blocked with NGS (all from Sigma-Aldrich) before adding the following primary antibodies: Oct4 and CXCL12 (AbCam), CXCR4 (Sigma-Aldrich), osterix (Santa Cruz Biotechnologies), type I collagen (SP1.D8) and osteonectin (AON-1) from Developmental Studies Hybridoma Bank, vimentin (Dako), and Sox2 (Millipore), all diluted 1:100, and CD133 (Milteny Biotec) diluted 1:10, at r.t for 1 h. Fluorochrome-conjugated secondary antibodies (goat anti-mouse and anti-rabbit Alexa Fluor-568 or Alexa Fluor-488, Molecular Probes) were applied, and nuclei were counterstained with DAPI (Sigma-Aldrich) [56]. Negative controls omitting primary antibodies were included in all the experiments. Coverslips were mounted with Mowiol (Sigma-Aldrich) and images were obtained with a DM2500 microscope equipped with a DFC350FX digital camera (Leica Microsystems).

2.5. *In vitro* multilineage differentiation analysis

To study the ability of cells from sarcospheres to differentiate into adipogenic, osteogenic and chondrogenic lineages and verify the enrichment of OSA primary cultures with stem-like cell subpopulation, differentiation analyses were performed. First, to assess the ability to differentiate into non-stem tumor cells, sarcospheres were collected, dissociated into single cells, shifted to IMDM+10%FBS (without growth factors) and cultured for at least 10 days [18, 57].

To differentiate OSA CSCs towards mesenchymal lineages (osteoblast, chondrocyte and adipocyte), sarcospheres were disaggregated into single-cell suspension and induced to phenotype change using the StemPro® differentiation kits (GIBCO®) according to the manufacturer's instructions. Cells were seeded into StemPro® Adipogenesis Differentiation medium (GIBCO®) for 21 days, replacing medium every 3 days. Adipogenesis was using Oil Red O (Sigma-Aldrich) staining to detect intracellular lipid vacuoles. Osteogenic differentiation was assessed after 10 days of incubation in StemPro® Osteogenesis differentiation medium (GIBCO®) by 2% alizarin red S (Sigma-Aldrich) staining to detect the formation of calcium deposits.

Chondrogenic differentiation was monitored after 20 days in StemPro® Chondrogenic differentiation medium (GIBCO®) by staining cultures with 1% Alcian Blue (Sigma-Aldrich) for proteoglycan matrix production.

2.6. Tumorigenicity

All animal procedures were carried out under project license in compliance with guidelines approved by Ethical Committee for animal use in cancer research at IRCCS-AOU San Martino-IST (Genova, Italy) and Italian Ministry of Health (n° 327, Dl.vo 116/92 and 412, in accordance with EU Directive 2010/63/EU). Four NOD-SCID mice (Charles River) aged 6–8 weeks were used to test their ability to grow in vivo. Xenografts were established by pseudo-orthotopic inoculation of $1.5x10^5$ OSA1, OSA2 and OSA3 cells re-suspended in 100 µl Matrigel™ (BD Bioscience) and 100 µl medium, derived either from cells selected in by growing in stem cell medium and cultured as sarcospheres or the respective parental primary cells. Mice were monitored for disease symptoms and sacrificed by CO_2 asphyxiation when weight loss or any other sign of suffering was observed. Excised tumors were divided in two parts: one was fixed in 10% buffered formalin and embedded in paraffin for IHC analysis, the second was cut into small fragments, and cell suspension was collected for *in vitro* culture. Tumor-derived single cells forming spheres were re-injected into new recipient mice to verify tumor-forming ability upon serial transplantation.

2.7. MTT assay

Cells (4,000/well) were plated into 96-well plates, allowed to attach for 24h, and treated with 1-30 mM metformin, 0.03-30 µM doxorubicin and 0.3-30 µM cisplatin, for 48h. All drugs were dissolved in sterile water and purchased from Sigma-Aldrich. Mitochondrial activity, as index of cell viability, was evaluated by incubation with MTT (Sigma-Aldrich, stock solution 2 mg/ml) for 2h, formazan crystals were dissolved in DMSO and absorbance spectrophotometrically measured at 570 nm [58]. IC_{50} values were calculated using nonlinear regression curve fit analysis selecting the log (drug) *vs*. response-variable slope (three parameters) using Prism 5.02 (GraphPad San Diego CA, USA). Each treatment was analyzed in quadruplicate, and the experiments were repeated at least three times.

2.8. Colony formation assay

Viable single cells were plated in 100mm dishes at a density of 450 cells/dish in IMDM+10% FBS medium in the presence or absence of metformin, and allowed to grow for 2 weeks monitoring colony formation [59]. Colonies were fixed and visualized by May-Grunewald Giemsa staining (Sigma-Aldrich). Colony formation efficiency (CFE %) was calculated as (number of colonies per dish/number of cells seeded per dish) × 100.

2.9. Statistical analysis

All experiments were repeated at least three times. Data from quantitative experiments are expressed as mean ± s.e.m. Statistical significance between groups was assessed by t-test (unpaired, two-tailed) or one-way ANOVA using Prism 5.02. Statistical significance was established at p values <0.05. For the evaluation of drug interaction, data form MTT assay (as described above) using doxorubicin or cisplatin alone or in combination with metformin, were analyzed using the median-effect method [60] and expressed as the combination index (CI) calculated using CompuSyn software (ComboSyn Inc., Paramus, NJ, USA) where CI<1, =1, and >1 indicates synergism, additive effect and antagonism, respectively. Based on CI, synergy is further refined as moderate synergism (combination index = 0.7-0.9), synergism (combination index = 0.3-0.7), strong synergism (combination index = 0.1-0.3), and very strong synergism (combination index < 0.1) [61, 62].

3. RESULTS

3.1. Isolation and *in vitro* expansion of cancer stem-like cells from canine osteosarcoma

Three primary cell cultures were derived from one osteoblastic productive OSA grade III (OSA1), and two chondroblastic OSAs grade III (OSA2 and OSA3) [53]. All cultures were able to proliferate as adherent monolayers in FBS-containing medium (Figure 1A); however, when cultured as single cell suspension in low serum concentration (1% FBS) supplemented with EGF and bFGF, two growth factors required for the *in vitro* expansion of normal and cancer stem cells [54], OCS cells formed within 7-10 days non-adherent, multi-cellular spheroids (sarcospheres) (Figure 1B). Tumor spheroids showed different morphology: OSA1 formed spherical, compact aggregates, while OSA2 and OSA3 generated less dense and uniform shape and circular spheres (Figure 1C). In growth factor-supplemented medium, sarcosphere cells were able to proliferate and survive for long term. Moreover, in these culture conditions OSA cells are able to generate secondary sarcospheres after dissociation, and re-formation passages could be repeated several times (data not shown). This selection allowed the *in vitro* expansion of an OSA cell subpopulation with morphological and biological characteristics different from the original cell cultures and, being *in vitro* spherogenic activity considered an indirect index of self-renewal ability, we named these cell subpopulations as OSA1 CSC, OSA2 CSC, and OSA3 CSC (see below for the complete characterization).

3.2. Osteosarcoma CSC spheres express osteogenic markers

To test whether OSA CSCs within sarcospheres retain osteogenic potential, we characterized their phenotype by IF for bone-specific markers (Figure 1D). Osterix, one of the early osteogenic transcription factors and vimentin, a constituent of the intermediate filament protein family and typically a mesenchymal

cell marker, were expressed by almost all cells within spheres of the three OSA CSCs. Osteogenic differentiation was explored by detection of late osteogenic lineage proteins such as collagen type I and osteonectin that showed a non-homogeneous staining pattern: in particular, OSA 1 and OSA 2 CSC spheres contained few scattered type I collagen positive-cells and were negative for osteonectin, contrarily OSA3 CSCs were almost negative for type I collagen and osteonectin-positive (Figure 1D). These results indicate that OSA1, OSA2 and OSA 3 spheres contained stem-like cells committed to osteogenic differentiation (maturation), although a complete differentiation was still not detectable (as demonstrated by the low levels of bone extracellular matrix gene osteonectin, and type I collagen).

3.3. Osteosarcoma CSC spheres express stemness markers

Being CSC pluripotency associated with the ability to maintain the expression of specific proteins and transcription factors, to further characterize the putative stem-like phenotype of sarcosphere cells, by IF we analyzed the expression of Sox2 and Oct4, two intracellular transcription factors overexpressed by stem cells, and CD133, a stem-cell surface marker, (Figure 2). OSA1, OSA2 and OSA3 CSC express, although not homogeneously, all the above mentioned markers, thus indicating the prevalence of cells with stem-like phenotype within spheroids (Figure 2). In addition, we tested the immunoreactivity for the chemokine receptor CXCR4 and its ligand CXCL12, both overexpressed in several human [63-65] and canine tumors [55] including bone sarcomas, and contributing to CSC maintenance, migration, and homing in metastatic sites [66]. IF analysis revealed that both proteins are expressed in the majority of sarcosphere cells (although CXCL12 was detected in less OSA2 cells) (Figure 2) confirming the relevance of the CXCL12/CXCR4 axis in OSA likely involved in the control and/or modulation of cell stemness. Overall, these analyses corroborated the stem cell properties of the spherogenic cells derived from all canine OSA cultures.

3.4. *In vitro* differentiation capacity of osteosarcoma sphere cells

Since serum depletion promotes CSC-like phenotype, we tested whether this process was reversible by shifting sarcosphere-derived single cells from OSA1, OSA2 and OSA3 CSCs to the original culture condition, in serum-containing medium. After 10-15 days, CSCs lost anchorage-independent proliferation, grew as monolayers (Figure 3), and re-acquired the morphology and shape resembling parental primary OSA cultures (see Figure 1), likely suggesting that OSA sarcospheres harbor stem-like cells able to *in vitro* (re-)differentiate.

Next, based on the recent evidence suggesting that the cell-of-origin in OSA most likely derives from mesenchymal stem cells, the precursor of osteoblasts, rather than osteoblast-committed cells [13], to explore the differentiation potential of sarcosphere cells, we induced our cultures to differentiate along

mesenchymal lineages. Therefore, the putative mesenchymal origin of OSA CSCs should grant this cell subset the ability to differentiate into osteocytes, chondrocytes, and adypocytes.

We evaluated the potential of OSA1, OSA2 and OSA3 CSCs to undergo tri-lineage differentiation by culturing them in specific standardized differentiation media (Figure 3). Foci of mineralization were visualized by Alizarin Red staining in osteogenic-cultured conditions after 21 days in all three OSA CSCs, although at different level: OSA1 CSC formed a stronger deposited mineralized matrix, detectable by more consistent staining than OSA2 and OSA3 cultures (Figure 3). Chondrogenic differentiation was demonstrated from Alcian Blue staining after 21 days in chondrogenic medium: OSA1 and OSA2 CSCs showed solid proteoglycan deposits as index of chondroblastic matrix, while OSA3 CSCs scarcely grew in this culture conditions and differentiation was less evident (Figure 3). After 14 days of adipogenic differentiation, OSA1 CSC were stained with Oil Red and showed several visible intracellular vacuoles containing lipid drops in most of the cells, whereas in OSA2 and OSA3 CSCs only few cells survived the treatment; however residual adherent cells exhibited intracellular lipid droplets (Figure 3). These data demonstrate that OSA CSCs from all the three tumors studied retain differentiation capacity as expected for both normal and tumor stem cells. Moreover, we can speculate that, in line with predictable individual differences among primary tumors, OSA1 has a more marked mesenchymal phenotype, while OSA2 and OSA3 cells are likely progenitors somewhat more committed to osteogenic rather than chondrogenic or adipogenic differentiation.

3.5. Osteosarcoma sphere cells initiate tumors *in vivo*

Tumor propagation *in vivo* is a distinctive hallmark of CSCs (tumor-initiating cells). Thus the gold standard method to characterize CSC subpopulations is the tumorigenic assay after serial transplantation in animal models. To examine the tumorigenic potential, we tested the ability of the three OSA CSCs and the corresponding primary cultures to initiate and support tumor growth in mice. Cells ($15x10^4$) from OSA1 CSC, OSA2 CSC and OSA3 CSC were injected subcutaneously into immunodeficient mice. To minimize inter-individual variation and the number of animals to be used, CSCs and non-CSCs obtained from the same OSA were injected into the left and right flank of the same mouse, respectively. Tumor development was daily monitored, and animals sacrificed when signs of suffering were observed. OSA1, OSA2 and OSA3 non-CSC did not develop tumor during the observation period, except for OSA1 which formed a very small tumor mass (Figure 4A). In contrast, the CSC counterpart developed tumors, which become visible after about 1 month from the grafting. Due to OSA CSC tumor development, mice were sacrificed within approximately 50-60 days (OSA1, 53 days; OSA2, 63 days; OSA 3, 56 days). The weight of xenograft tumors collected at the end of the experiments, is reported in Figure 4A. These results confirm that sarcospheres are enriched with

CSCs exhibiting enhanced tumorigenic potential and that parenteral cell cultures might contain a too small fraction of stem cells, not able to generate tumors.

3.6. Osteosarcoma CSC xenografts recapitulate the original canine tumor histology

One of the key characteristic of CSCs is the ability to reproduce in xenotransplants the characteristics of the tumor from which they were isolated. We therefore compared the histology of tumors developed in mice with that of canine original OSA. As shown in Figure 4B, H&E analysis revealed that OSA1, OSA2 and OSA3 CSC-derived tumors closely reproduced the histotype of the original OSA: tumor osteogenic (OSA1) and chondroblastic areas (OSA2 and OSA3) were observed and confirmed the resemblance with original lesion in dogs, including the presence of extensive areas of osteoid matrix formed by tumoral osteoblasts or atypical cartilage in OSA of chondroblastic pattern.

To further characterize the phenotype of xenografts , we assessed, by semi-quantitative IHC score (see Materials & Methods) the immunoreactivity for relevant markers associated with OSA tumorigenesis and/or stemness, including the mesenchymal stem cell markers CD117, the transcription factor Oct-4, STAT3 frequently overexpressed in human and canine OSA cell lines [67, 68], and the tumor metastasis-associated chemokine/receptor pair CXCR4/CXCL12 [69], (Table in Figure 5A). The analysis showed that all markers are expressed in mice tumors formed by OSA1, OSA2 and OSA3 CSCs, although at different levels of abundance and distribution throughout the tissues (Figure 5B). Following these observations, to verify whether the OSA CSC tumor model we developed reflects the general histological and immunohistochemical features of canine OSAs, we evaluated the expression of these markers in a series of canine OSAs. To this aim, sections of 12 canine OSAs, including the three initial tumors from which we isolated the cell cultures, were studied. Animal and tumor characteristics are reported in Supplementary Table 1. The analysis of the expression pattern of CXCR4 (11/12 score 1 and 1/12 score 2), CXCL12 (7 score 1 and 5 score 2), Oct4 (9 score 1 and 3 score 2) and STAT3 (9 score 1 and 2 score 2) showed a marked immunopositivity, although detectable at different levels, whereas CD117 (1 score 2, 4 score 1 and 7 score 0) was detected in a lower number of samples (5/12). Overall, these data confirm that tumors obtained by CSC xenografts maintain the general phenotype of canine OSAs.

3.7. Osteosarcoma CSCs retain tumor-forming potential during serial *in vivo* transplantation

Tumorigenicity of OSA CSCs after serial transplantation should be retained or even increased due to *in vivo* enrichment in tumor-initiating cell subpopulation. Since a small tumor was obtained only after xenograft of OSA1 cells non enriched in CSCs, we analyzed the tumorigenicity of OSA1 CSC and OSA1 cells isolated from first xenografts into new recipient mice. OSA1 cells isolated from mice tumors were re-cultured in stem cell

permissive medium (containing EGF and bFGF), confirming that cells from tumors originated from OSA CSC can proliferate *in vitro* as spheroids (data not shown). Xenografted OSA1 not enriched in CSC were collected and cultured again in medium with serum. Both cell cultures re-implanted in mice developed tumors (Figure 6A), which at the sacrifice of animals (after 56 days) were much larger than those developed after the first *in vivo* passage (see Figure 5A), suggesting an increased tumorigenic potential likely induced by an enrichment of tumor-initiating CSCs. Importantly, also the cells derived from OSA1 non-CSC (original culture) xenografts, which developed very small tumors in the first round of *in vivo* growth (Figure 5), gave origin to large tumors in the second round (Figure 6A), further confirming that the *in vivo* growth contribute to the selection and enrichment in tumor-initiating cells also in cells with initial low tumorigenic potential. Tumors of both first and second transplants showed the same histology (data not shown). Comparing immunohistochemistry results on sections from first and second injection, we observed that scores of CD117, STAT3 CXCR4 and CXCL12 increased, while Oct4 levels did not change in OSA1 non CSC-derived tumors and decreased in OSA1 CSC xenografts (Figure 6C). Altogether these findings suggest that *in vivo* growth not only preserves but also enhances CSC content within OSA cell population.

3.8. Doxorubicin and cisplatin decrease cell viability of osteosarcoma CSCs

The sensitivity toward drugs commonly used in clinics (doxorubicin, cisplatin) between OSA CSCs and non-CSCs, was tested using the MTT cell viability assay. In order to allow a better cellular and biochemical evaluation, experiments were performed on short-term monolayer cultures obtained after disaggregation of sarcospheres and seeding cells on Matrigel to facilitate temporary adhesion prior drug exposure, that was previously reported not interfere with CSC features [18, 57].

OSA1, OSA2 and OSA3 CSCs, and their respective parental cultures, were treated with increasing concentrations of doxorubicin (range 0.03-30 μM) and cisplatin (range (0.3-30 μM) for 48h (Figure 7). Dose-response curves reported in Figure 7A indicate that doxorubicin caused a concentration-dependent reduction of cell viability, in all the three cultures, however very slight differences were observed among CSC subsets and original cultures being IC_{50} values similar (OSA1 CSC 0.8 μM *vs*. OSA1 1 μM; OSA2 CSC 0.2 μM *vs*. OSA2 0.1 μM; OSA3 CSC 0.2 μM *vs.* OSA3 0.1 μM) (Figure 7A).

The cytotoxic effect of cisplatin (Figure 7B) showed the IC_{50} for OSA2 CSC and OSA3 CSC higher (12.5 μM and 12.8 μM, respectively) than respective original non-CSCs cells (OSA2 4.3 μM; OSA3 3.1 μM), showing the expected increased drug resistance (about 3-4 fold) which characterizes CSCs. On the contrary, OSA1 parental cells and CSCs exhibited similar dose-response curves and IC_{50} values (9.2 μM and 9.1 μM, respectively) (Figure 7B).

3.9. Metformin inhibits survival, self-renewal and proliferation of OSA CSCs, and enhances doxorubicin and cisplatin cytotoxicity

Recent evidence indicates that metformin, an oral hypoglycemic drug, may reduce cell growth and survival selectively affecting CSCs in different tumors including OSA [30, 49, 51]. Therefore, we compared sensitivity to increasing concentrations of metformin (0.3-30 mM, for 48h) between OSA CSCs and non-stem OSA cells (Figure 8A). Metformin reduced cell viability in a dose-dependent manner (about -75% for the highest concentration tested), reaching IC_{50} values of 2.4 mM and 1.8 mM for OSA1 CSC and OSA2 CSC, respectively, with a slightly more potent response as compared to non-stem OSA1 and OSA2 that showed IC_{50} of 7.2 mM and 2.2 mM, respectively. Conversely, both OSA3 and OSA3 CSCs showed lower sensitivity to metformin (-35% viability at 30 mM concentration), with IC_{50} not evaluable.

The effect of metformin on stemness and proliferation rate was further detailed only on sensitive OSA CSC cultures. In particular, we performed *in vitro* assays to evaluate the effects of metformin on clonal growth ability, measuring the colony-forming efficiency (CFE), as well as testing self-renew potential assessing the ability to generate spheroids after serial *in vitro* passages (Figure 8B-C-D).

CFE of OSA1 and OSA2 CSCs, was tested using 3 mM metformin, representing a concnetration close to the IC_{50} for both OSA1 and OSA2, to limit the direct cytotoxicity induced by higher concentrations of the drug. The evaluation was performed after 14 days of drug exposure to allow non-responsive cells to proliferate and generate visible colonies. metformin treatment induced a marked reduction in colony formation as compared to control cultures in both OSA1 (CFE 19% *vs.* 43%) and OSA2 (CFE 1% *vs.* 29%) (Figure 8B).

To determine whether metformin impairs self-renewal, we analyzed metformin effect on the ability of OSA CSCs to grow as sarcospheres under serum-free medium culturing conditions (using low adhesion plates). Within 7 days from the plating OSA1 and OSA2 CSCs formed spheroids that were treated with metformin (3 mM) for further 5 days: metformin reduced the number of spheres in both cultures as compared to untreated cells (Figure 8C). In order to study the long-term effects of metformin on self-renewal ability, primary spheres were dissociated into single cells, treated with vehicle or metformin (3 mM for 48h) and allowed to grow for further 7 days until formation of secondary spheres. This procedure was repeated for up to 6 cycles of dissociation/formation in the presence or absence of metformin, and total cells composing spheres were counted: untreated OSA1 CSC and OSA2 CSC showed a constant ability to self-renew at all the time points as shown by the observation that cells isolated from spheroids were able to generate new sarcospheres with basically the same efficiency even after 6 passages (Figure 8D). Conversely, metformin treatment completely impaired self-renewal of OSA1 CSCs that after only two passages were no longer able to form new spheroids, and progressively reduced this ability in OSA2 CSCs over passages, only few very small sarcospheres present after the last dissociation cycle (Figure 8D).

Collectively, these results indicate that metformin significantly inhibited both the growth of primary spheroids and sphere generations in OSA1 CSC and OSA2 CSC, suggesting the depletion of the stem subpopulation within OSA cultures.

Metformin was reported to increase CSC sensitivity to standard anti-cancer agents, likely circumventing drug resistance [70, 71]. Thus we evaluated whether metformin acts as OSA CSC sensitizer toward the cytotoxic effects of doxorubicin and cisplatin. Metformin-sensitive OSA CSCs were exposed to each drug alone or to combinations of doxorubicin or cisplatin with metformin. After 48h cell viability was assayed by MTT test. Cisplatin- and doxorubicin-induced cytotoxicity was comparably potentiated by co-treatment with metformin, in OSA1 and OSA2 CSCs, resulting in approximately 10-40% potentiation of the cytotoxicity of both drugs (Figure 9). Quantitative determination of combination effect was evaluated using the Chou-Talalay's CI method where CI<1 is index of synergistic activity between the drugs analyzed [60]. We observed that for concentrations corresponding to the IC_{50} values CIs were 0.78 and 0.70 for the association of doxorubicin and metformin, and 0.68 for the association of cisplatin and metformin, in OSA2 CSCs, respectively. These results support that the administration of metformin induces a moderate synergism with both cytotoxic drugs.

4. DISCUSSION

OSA is a mesenchymal malignancy of bones that shares genetic and molecular alterations, histopathology, frequency of microscopic metastases at diagnosis, and clinical behavior in humans and dogs [31]. Conventional chemotherapeutic agents (including cisplatin and doxorubicin) prolong OSA patient's lifespan without increasing survival rate which remains poor due to development of microscopic or diffuse chemotherapy-resistant metastases [72]. Therefore, search of alterative adjuvant therapies for improving management of OSA progression is clinically warranted. CSC model proposes that a subset of tumorigenic cells possessong stem-like properties drive tumor initiation, metastasis, drug resistance, and recurrence. This model gained increasing relevance in many solid cancers, including OSA, likely representing a biological correlate for the aggressive clinical behavior of this tumor. Isolation and characterization of CSCs represents a key step to study OSA pathogenesis and biology and develop more efficacious therapies. In particular, the identification of human and canine CSCs derived from surgical tissues of OSA contributed to clarify their stem-like features and, prospectively, to develop targeted drugs to eradicate this cell subpopulation [30, 73, 74]. In the current study we assessed the biological and pharmacological properties of three patient-derived canine OSA cultures.

All cultures showed ability to grow as spheroids when grown in growth factor-containing medium, forming either compact spheres or loose aggregates, which retained self-renewal ability for multiple passages.

Spherogenesis has been indeed exploited as one of the methods to identify CSCs in vitro, reflecting the capacity of cells endowed with stem-like properties to grow under non-adherent conditions. In particular, spherogenesis was used to identify CSC subsets cell lines and tumors by self-renew ability in human and canine OSA [11, 38, 74], as well as in several other cancer types [75]. *In vitro* self-renewal properties is not exclusive for CSCs [76], thus *in vivo* tumorigenicity of OSA CSC cultures after various initial enrichment techniques (expression and activity of aldehyde dehydrogenase, ALDH, side population isolation, spherogenesis, CD133, CD117 (c-kit), Stro-1 expression) [77-80], was also reported, representing the crucial method to validate putative CSCs. In particular, the serial transplantation of low number of CSC candidates into immunocompromised mice (tumor-initiating activity) is, to date, considered the only definitive prove of the presence of CSCs in a given culture. We report that cells isolated from canine primary OSA, and grown *in vitro* as sarcospheres, possess high tumorigenic capacity *in vivo*. OSA1, OSA2 and OSA3 cultures xenotransplanted in mice, achieved 100% engraftment and developed tumors recapitulate the original tumor histology. Mouse xenograft generation has been obtained at low number of cells ($15x10^4$), as compared to other studies (e.g. $3x10^6$ cells of human osteosarcoma 3AB-OS CSCs isolated from human osteosarcoma MG63 cells, [81], or a subset of OSA cells sorted from OS99-1 cell line for ALDH enzymatic activity, in which tumor formation was highly dependent on the number of injected cells, likely suggesting that tumorigenic cells were extremely rare [82]). The tumor-initiating ability of sarcosphere cells, also considering that the parental primary cultures were not able to consistently give rise to tumors in the same experimental conditions, definitely demonstrates the stem nature of our OSA cultures.

Moreover, cells obtained from excised xenograft tumors re-cultured *in vitro* exhibited functional characteristics such as spherogenesis and proliferation confirming stem-cell like properties of these OSA CSC cultures. Second transplant of OSA1 CSCs successfully engraft and initiate tumor in new recipient mice, preserving the original histopathologic appearance of original canine OSA, also observed in the primary xenografts, indicating that the *in vitro* experimental conditions we adopted do not impair tumor-initiating potential of these cells. OSA1 non-CSCs derived from primary xenograft tumors formed secondary neoplasms with a higher efficiency than the first injection (in which only small tumors developed) suggesting that *in vivo* growth favors the selection of CSC-like cells, as also shown by the increased detection of stem cell markers in histological preparations from the second injection.

The observation that the first injection of non-CSCs failed to form consistent tumors as done by CSCs, suggests that sarcosphere cells are essentially different from their original counterparts, and initial OSA primary cultures contain a limited number of CSC cells within the large number of non-CSCs; conversely, CSCs are enriched *in vitro* by stem-permissive culture conditions and *in vivo* by mice transplantation, and can be found in both conditions after the second mouse transplant. By enriching the cultures in OSA CSC subpopulation, xenografts enhance the aggressive and self-renewing potential of these cells, and amplify the

proportion of cells expressing proteins able to sustain tumor growth and stemness (*i.e.* Oct4, CD117, STAT3, CXCR4, CXCL12). The differential tumor-initiating ability between CSCs and non-stem tumor cells was previously reported, nevertheless a direct confrontation between the two populations derived from the same primary tumor was rarely performed: Murase *et al.* detected higher tumorigenic ability of sorted side population cells from the bone malignant fibrous histiocytoma cell line MFH2003 compared to the main population cells [78]. Mesenchymal tumor (1 osteosarcoma, and 2 malignant fibrous histocytomas, 1 synovial sarcoma) transplantation into NOD/SCID mice comprising only showed that higher frequency tumors were generated with smaller numbers of side population cells as compared with main population cells [83].

Although a definitive panel of antigens unequivocally identifying CSCs in OSA and other solid cancers is not available [84], we combined tumorigenic and spherogenic activities with the analysis of the expression of some stem-related markers, to assess changes in CSC content after *in vivo* growth. We show the concomitant expression of CD133, Oct4, and Sox2 in OSA spheroids, suggestive of stem-cell like properties in tumor cells [79, 85, 86], including canine OSA [74, 87]. In particular, CD133 expression has been correlated with the gain of tumorigenic activity by OSA cells [80], although it has to be remarked that both CD133-positive and -negative cells are able to propagate tumors *in vivo* [88, 89]. On the whole, these results support that a combination of CD133+ with other pluripotency factors characterize the stem-like phenotype of OSA CSCs.

CSCs are also responsible for the formation of metastasis. Among proliferation- and metastasis-associated proteins in CSCs, the cytokine CXCL12 and its receptor CXCR4 contribute to bone sarcoma progression [90], in which the metastatic diffusion represents a major life-limiting factor for both humans and dogs. Few studies explored the biological relevance of the CXCL12/CXCR4 axis in canine OSA [91], showing high expression similarly to other canine solid tumors [55]. In the present study, CXCL12/CXCR4 expression within sarcospheres well-matches with their diffuse detection in tumor xenografts and original OSA tissues of the corresponding dogs (see Tables), suggesting that the regulation of CSC invasive behavior by this chemokine system involve autocrine/paracrine activity besides the chemokinergic stimuli produced by tumor microenvironment.

The complexity of OSA CSC characterization also resides in the originating cell types: mesenchymal cells are likely one of the cell populations which drive OSA development [13], since these cells are involved in normal bone formation by differentiation into osteoblasts. This implies a high mesenchymal differentiating capacity of OSA CSCs, allowing the generation of adipocytes, chondrocytes. We show that canine OSA sarcosphere cells possess multipotency, with OSA1 CSCs able to differentiate in all the three lineages, and OSA2 and OSA3 CSCs showing higher propensity to differentiate into osteoblasts than chondrocytes and adypocytes. Overall these findings suggest that OSA CSCs retain a different degree of mesenchymal features, correlated

with the stage of differentiation of parental cells or the histologic subtype, highlighting that intrinsic heterogeneity of individual tumors may reflect the diverse properties of CSCs. The mesenchymal phenotype of canine OSA CSCs is also suggested by vimentin expression, the most widely accepted mesenchymal marker that is down-regulated in fully differentiated osteoblasts [92]. Moreover, all OSA sarcosphere cells also express osterix, an early/middle osteogenic regulator, which indicates that canine OSA CSCs are likely derived by osteogenic progenitors [93]. The presence of scattered positive cells for type 1 collagen, a late osteogenic marker, in OSA1 and OSA2 spheroids, further confirms the predominance of OSA cells not terminally differentiated. We therefore propose that CSC cultures isolated form the three OSAs are composed of tumor-initiating cells at distinct differentiation stage, but showing a common, although not identical, phenotype.

CSCs are responsible of drug resistance and tumor recurrence [94]; while the prediction of treatment response in aggressive tumors is still an unmet need, cytotoxicity *in vitro* assays routinely performed on established cancer cell lines or, rarely, on primary cultures deriving from bulk tumor cells, still have a low translational value. In particular, since these models neglect the chemo-sensitivity profile of the minority CSC population which may significantly differ from the other tumor cell populations. Indeed, although chemo-sensitivity is not a defining feature for CSC isolation, it could be considered as a relevant CSC property [95]. This issue is even more critical in OSA whose lethality is mainly due to the early onset of metastases and drug resistance. In our study, IC_{50} calculations of doxorubicin and cisplatin cytotoxicity, the chemotherapy regimen used in both humans and dogs, showed that canine OSA CSCs are generally less sensitive than the corresponding parental cells, although the difference was higher for cisplatin than doxorubicin. Previous studies reported that CSCs isolated from OSA cell lines are more resistant to doxorubicin [96]. The originating cell type may contribute to the drug response of CSCs (*e.g*. CSCs isolated from established, long-term culture cell lines tend to be more resistant to drugs, because of the more differentiated phenotype or adaptation to in vitro culture conditions) as also observed in glioblastoma CSCs [97]. On the contrary, our CSC cultures exhibited a marked resistance to cisplatin, as observed in stem-like populations from human sarcoma cell lines [96], and in agreement with the observation that cisplatin-resistant OSA cells exhibited stem-like characteristics both *in vitro* and *in vivo,* which are not observed in cisplatin-responsive cells [98].

Repositioning of metformin as antitumor drug [40] was related to the ability to preferentially affect CSC proliferation and survival [45-47]. AMPK activation and the consequent inhibition of mTOR signaling was the first proposed mechanism of metformin activity on CSCs [99], but several AMPK-independent pathways contributing to its antitumor activity have been also suggested [45, 100]. Here we report that metformin significantly impairs survival and spherogenesis of canine OSA1 and OSA2 CSCs, indicating, besides an antiproliferative effect, also a direct inhibition of self-renewal. Similar results were obtained in stem-like cells derived from the MG63 cell line, canine mammary carcinoma, human hepatocellular carcinoma or human

glioblastoma [28, 47, 49, 101]. Unexpectedly, OSA3 CSCs were less sensitive to metformin. We do not have an explanation for this difference, but it is noteworthy that these individual pharmacological diversities are evidenced only using cells isolated from primary tumors, which thus should represent the more reliable model to translate preclinical data to clinical trials. Differently from other tumor cells [47], parental OSA1 and OSA2 were only slightly less sensitive to metformin than respective CSCs. The high level of intra-tumor heterogeneity of OSA and plasticity of CSCs which dynamically can undergo differentiation and dedifferentiation [102], highlights how ideal therapeutic compounds or drug combination must suppress the survival and proliferation of both CSCs and non-CSCs, and metformin, targeting both cancer cell subsets, prospectively provides a rationale for novel therapeutic options against cancer progression. The chemo-sensitizing/adjuvant role of metformin has been postulated in various cancers however, beside preliminary encouraging data, its impact on tumor response is still under investigation [103]. In OSA cells, metformin co-treatment enhanced anti-proliferative and pro-apoptotic effects of trichostatin A and simvastatin [104, 105]. In our experimental model, the treatment with metformin sensitizes OSA1 and OSA2 CSCs to cisplatin and doxorubicin, as also reported in stem-like cells derived from human OSA cell lines [50, 51]. Thus these data support the development of novel clinical trials in which the adjuvant activity of metformin on cytotoxic drug activity may represent a novel therapeutic approach for the treatment of OSA.

5. CONCLUSIONS

In summary, we demonstrate here that OSA naturally-occurring in dogs contain tumorigenic stem-like cells with distinct phenotypes from the non-tumorigenic cells composing the tumor mass. These peculiar features, including the inter-tumor heterogeneity, are preserved in long-term culture *in vitro* and determine distinct biological behaviors in term of stem properties and response to conventional drugs, but are reconciled in the common essential ability to recapitulate the original tumor *in vivo*. Metformin suppresses viability of OSA CSC and parental cells *in vitro*, and combination treatment with metformin and conventional drugs improve cytotoxic drug response, suppressing both CSC self-renewal and proliferation. Conceivably results obtained in canine OSA support their relevance in veterinary oncology and serve as preclinical basis for future human therapy strategies.

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FIGURE LEGENDS

FIGURE 1. Characterization of canine OSA cultures enriched in cancer stem cells.

A) Morphological appearance of OSA1, OSA2 and OSA3 primary cultures. **B-C)** Phase-contrast microphotographs of sarcosphere formation observed after 7 days of culture in OSA1, OSA2 and OSA3 cells grown in serum-free medium (OSA CSC). **D)** Immunocytofluorescence assessment of osteogenic lineage markers in OSA sarcospheres. Analysis of the expression of osterix and type I collagen, which appear in cells committed to osteogenic lineage, vimentin, which regulates osteoblast differentiation, and osteonectin, up-regulated by osterix and generally used as later marker of differentiated osteoblasts. Photomicrographs represent merged images (green: marker-positive cells; blue: DAPI counterstained nuclei). Scale bars: 50 μm.

FIGURE 2. Immunocytofluorescence assessment of stem markers in OSA sarcospheres

Analysis of the expression of markers commonly used to identify cancer stem cells (CD133, Sox2, Oct4, CXCR4 and CXCL12) in OSA sarcosphere cells (CSCs) by fluorescence microscopy. Photomicrographs represent merged images (green: marker-positive cells; blue: DAPI counterstained nuclei). Scale bars: 50 μm.

FIGURE 3 Analysis of the pluripotency of OSA sarcosphere cells: differentiation into osteoblasts, chondrocytes and adipocytes.

Appearance of monolayer culture obtained by shifting OSA sarcosphere cells (CSCs) from the stem-permissive growth factor medium to serum-containing medium.

OSA CSCs were induced to differentiate into osteoblasts, chondrocytes or adipocytes, using appropriate defined media, and differentiation ability was assessed by specific staining:

-Alizarin Red S staining highlights extracellular calcium deposits indicating osteogenic differentiation.

-Alcian Blue staining indicates proteoglycan synthesis forming cartilage extracellular matrix by chondrocytes.

-Oil Red O stains cytoplasmic lipid vesicles in adypocytes.

Representative microphotographs are shown from independently performed experiments.

FIGURE 4. OSA sarcosphere cells are tumorigenic *in vivo* and xenografts recapitulate the original canine tumor

A) OSA1, OSA2 and OSA3 primary cultures and sarcosphere cells grown in stem conditions (OSA CSCs) were xenografted in immunodeficient mice, and tumor development was monitored. After animal sacrifice tumors were excised and weighted. Mean weights ± s.d. are reported in histograms.

B) Histopathologic examination of mice tumors: representative H&E staining showing that xenografts obtained from OSA1, OSA 1 CSC, OSA2 CSC and OSA3 CSC, recapitulate histological characteristics of the corresponding original canine tumor, as evaluated on post-surgical samples formalin-fixed, paraffin-embedded tissue sections.

FIGURE 5. Osteosarcoma CSC xenografts recapitulate the original canine tumor marker expression.

A) Immunoistochemical (IHC) scores indicating the intensity and distribution of immunopositive labellling for CD117, Oct4, STAT3, CXCR4 and CXCL12 in the original canine OSA and corresponding OSA CSC xenografts.

B) Representative images of antibody localization done using horseradish peroxidase: brown staining indicates the presence of the specific antigen. Sections were counterstained with Mayer's haematoxylin (blue). Negative controls (Neg) were run in parallel by omitting the primary antibody and by using rabbit/mouse IgG.

FIGURE 6. OSA1 CSCs exhibit enriched tumor-forming ability in secondary xenografts.

A) Single cell suspension isolated by dissociation of excised xenografts formed by OSA1 and OSA CSC cells, were grown under stem conditions as sarcospheres, and subsequently re-injected into new recipient mice. Secondary tumor initiation and development was monitored and mean weights are reported in the histograms ± s.d.

B) Variation of IHC scores (0=negative; 1=low positivity; 2= positivity; 3=high positivity) for CD117, Oct4, STAT3, CXCR4 and CXCL12 among tumors tissues derived from first (OSA1 CSC 1^{st}) and second injection (OSA1 CSC 2^{nd}).

FIGURE 7. Doxorubicin and cisplatin impair cell viability of osteosarcoma CSCs.

Sensitivity to doxorubicin and cisplatin was evaluated in OSA CSCs in the same condition (2D, monolayer on Matrigel) as the parental OSA cells, after dissociation of sarcospheres into single cells, seeded in a 96-well plate, and incubated for 24h. Cells were treated with increasing concentrations of doxorubicin **(A)** and cisplatin **(B)** or vehicle for 48h (the same condition applied for the parental cells) and cell viability was measured by MTT assay. Dose-response curves represent mean ± s.e.m. from three independent experiments each performed in quadruplicate.

FIGURE 8. Metformin inhibits the cell viability and self-renewal of osteosarcoma CSCs.

A) Sensitivity to metformin was evaluated in parental OSA cells and OSA CSCs (cultured as monolayer on Matrigel), seeded in a 96-well plates for 24h prior to the treatment with the drug or vehicle for 48h. Cell viability was measured by MTT assay. Dose-response curves represent mean ± s.e.m. from three independent experiments each performed in quadruplicate.

B) Colony forming efficiency (CFE) in OSA1 CSC and OSA2 CSC was evaluated in the presence of 3 mM metformin or vehicle: CFE (%) was significantly lower in treated cells *vs.* controls (* p= 0.05; ** p =0.01,). Representative photographs of colonies are reported. Data represent mean ± s.e.m.

C). Sarcospheres formed by OSA1 CSC and OSA2 CSC were treated with 3 mM metformin or vehicle, and counted after 5 days of treatment: the number of sarcospheres was significantly reduced in treated cells *vs.* controls (* p= 0.05). Data represent mean ± s.e.m.

D) Sphere-forming of OSA1 CSC (left panel) and OSA2 CSC (right panel) capacity was evaluated in the absence or presence of metformin (3mM): every 7 days sarcospheres were dissociated to single cell suspension and cells were counted and re-plated in fresh medium in the same. The number of viable cells composing spheres was counted for up to six passages. Data represent mean ± s.e.m. (* p= 0.05; ** p =0.01; ***P=0.001). Metformin impaired self-renewal of OSA1 CSCs after two passages, and progressively and reduced this ability in OSA2 CSCs.

FIGURE 9. Cisplatin- and doxorubicin-induced cytotoxicity is enhanced by co-treatment with metformin.

OSA1 CSC and OSA2 CSC were treated with doxorubicin or cisplatin alone or as combination with metformin, (at concentrations corresponding to the IC_{50} for each drug) or vehicle, for 48h. Cell viability was evaluated by MTT assay. Data represent the mean ± s.e.m., as % of controls taken as 100% (* p= 0.05; ** p =0.01; ***P=0.001).

Figure 1

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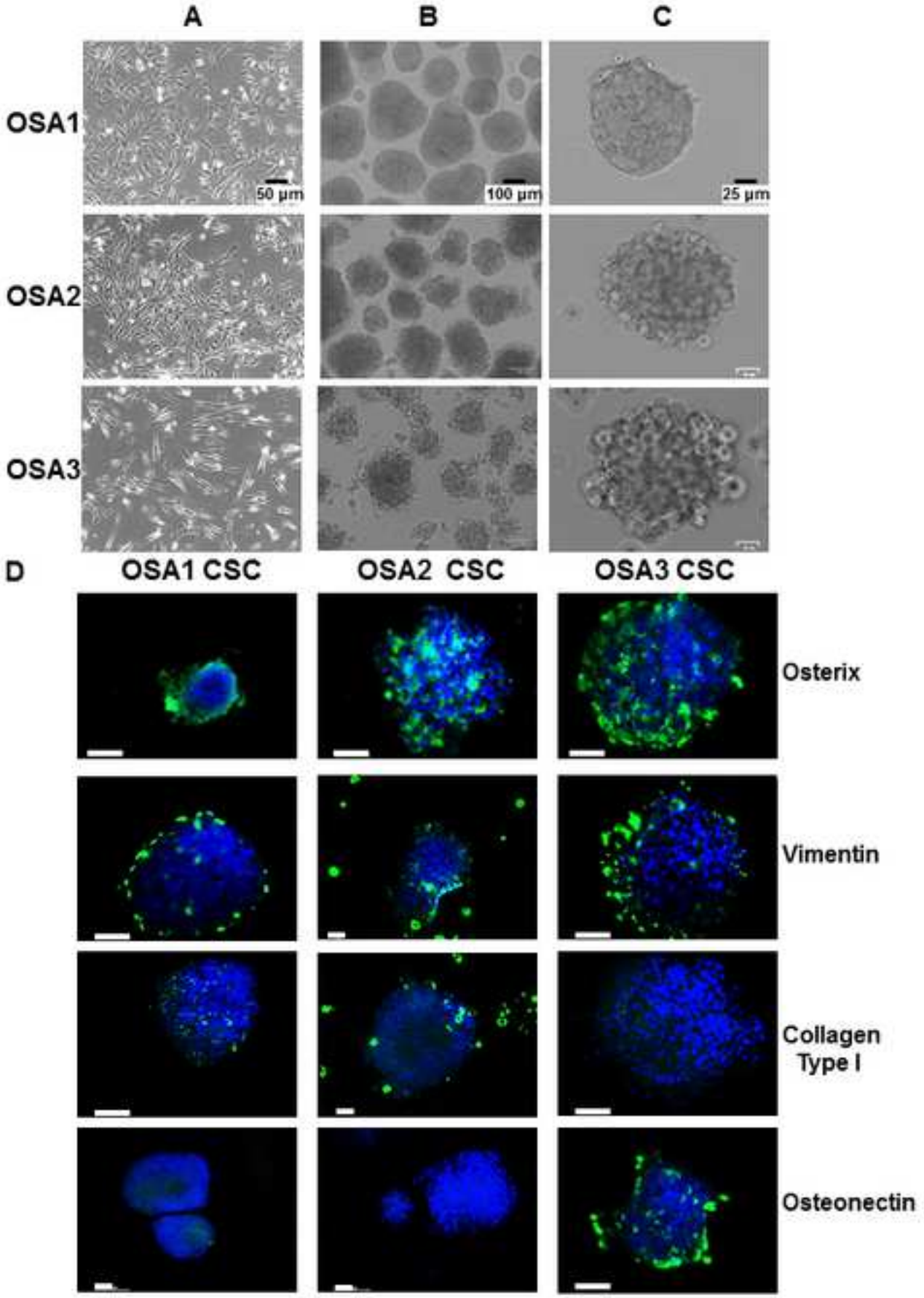

Figure 1

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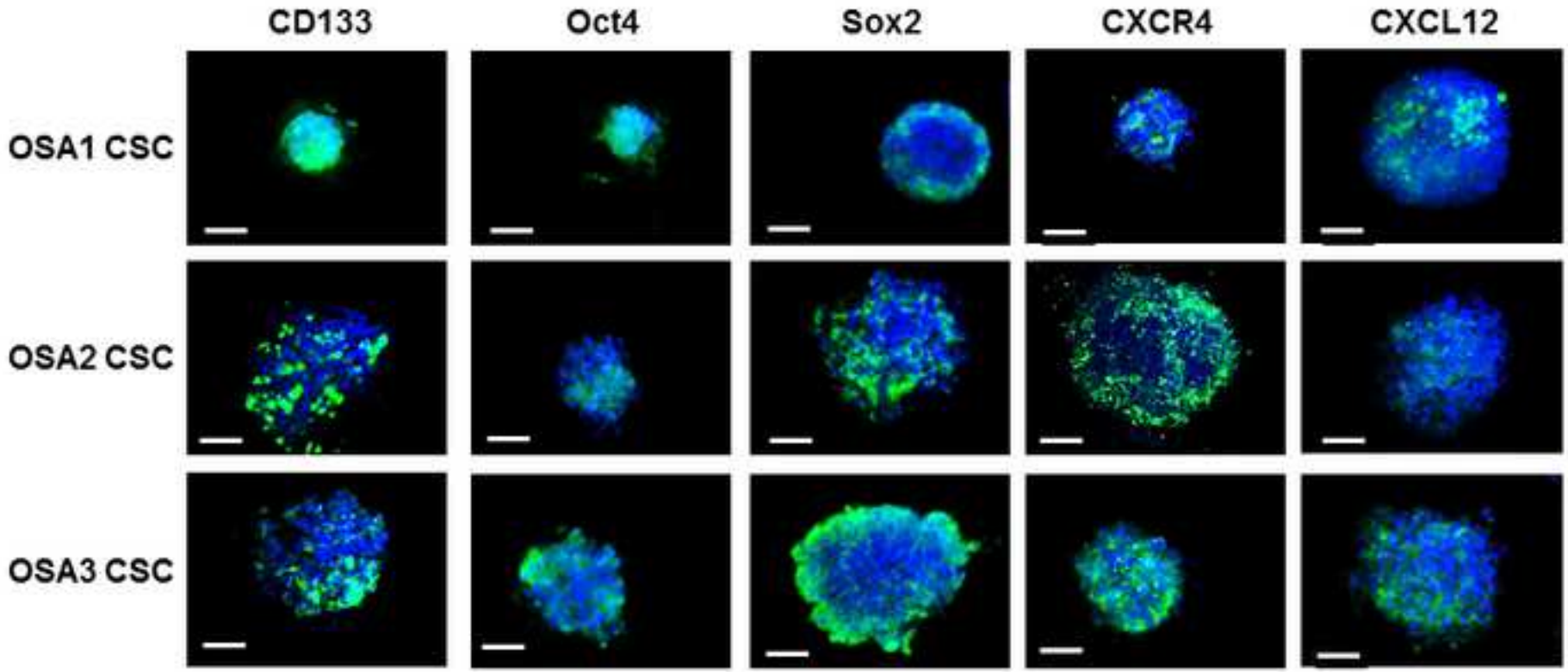

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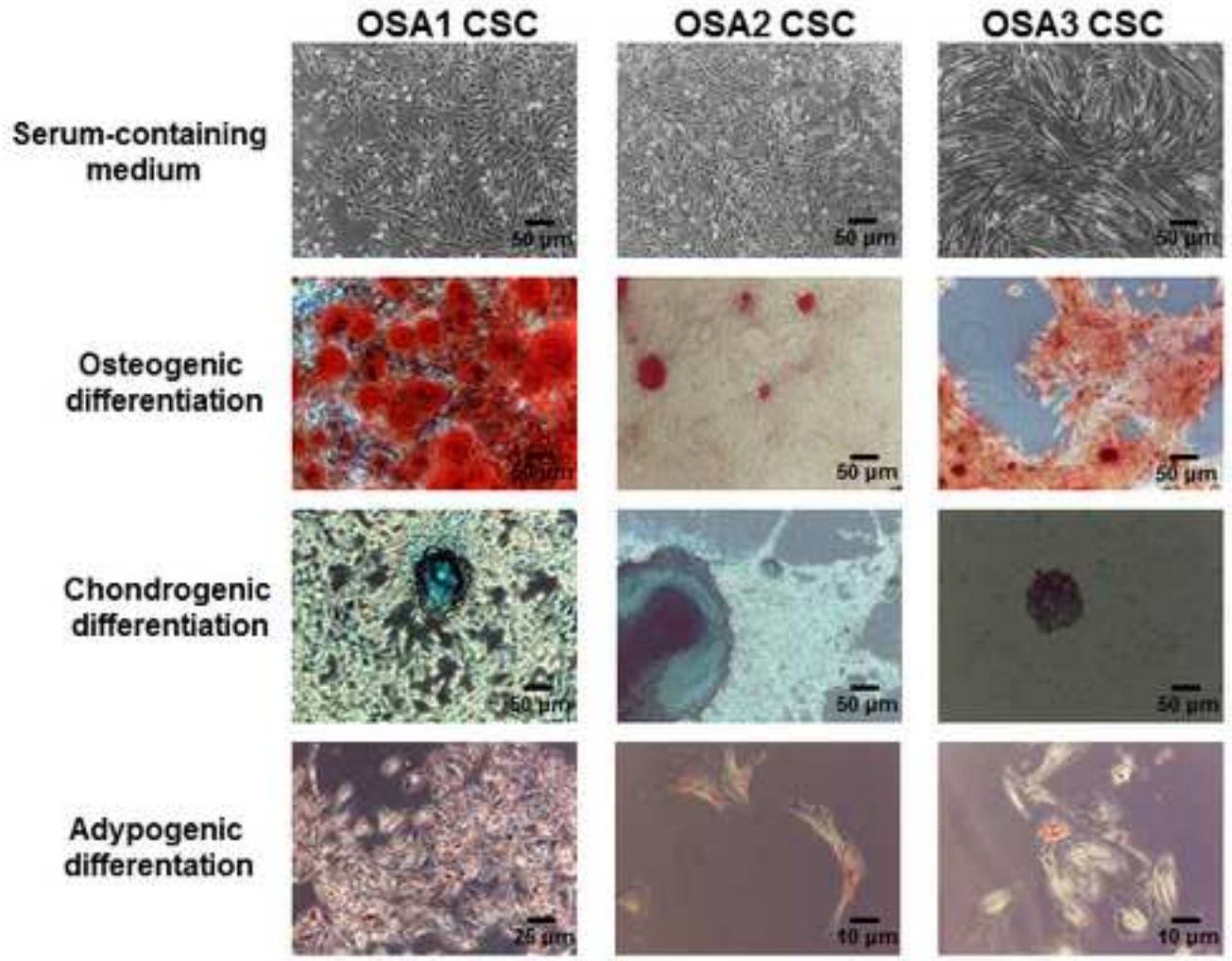

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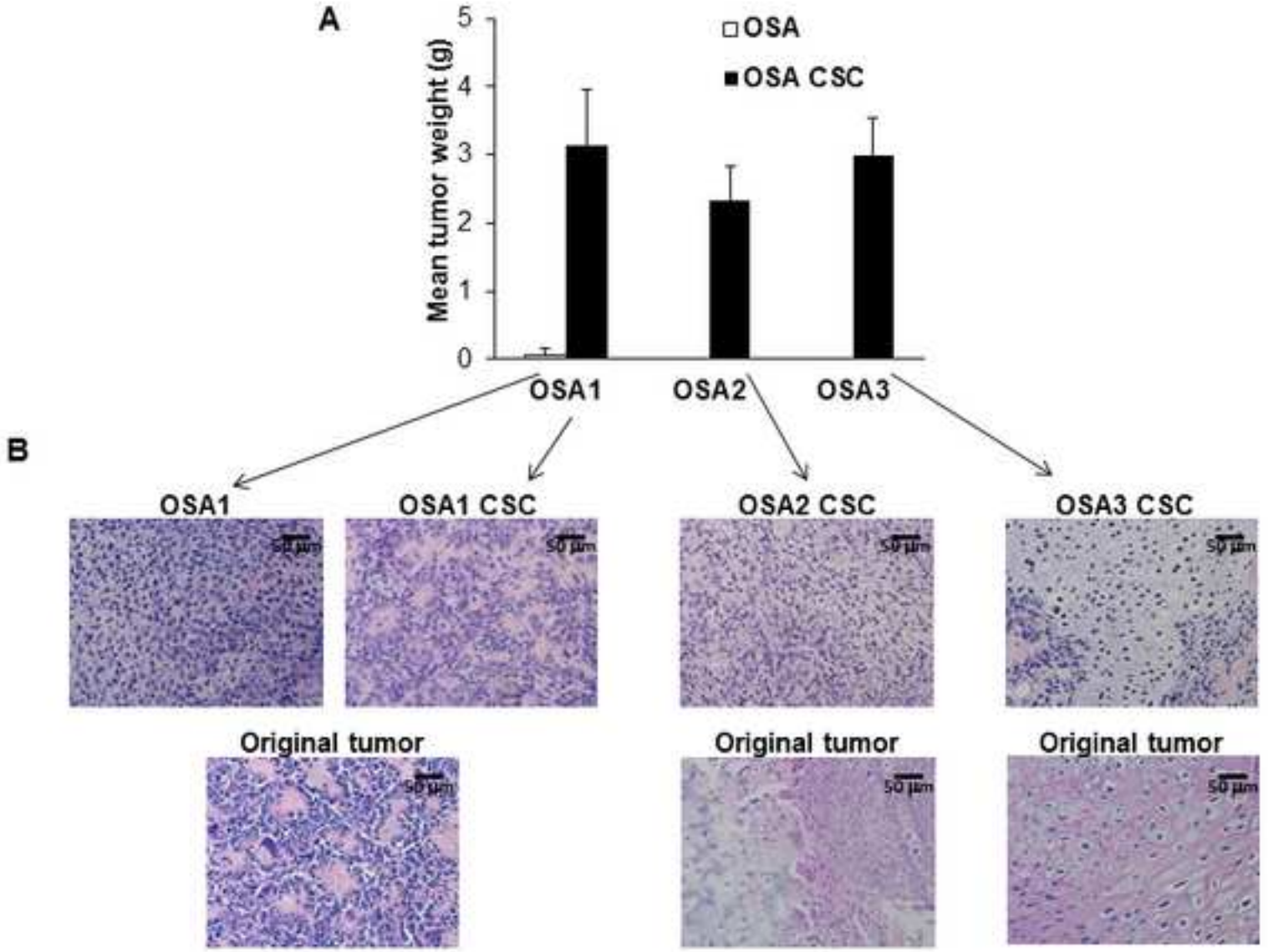

Figure 4

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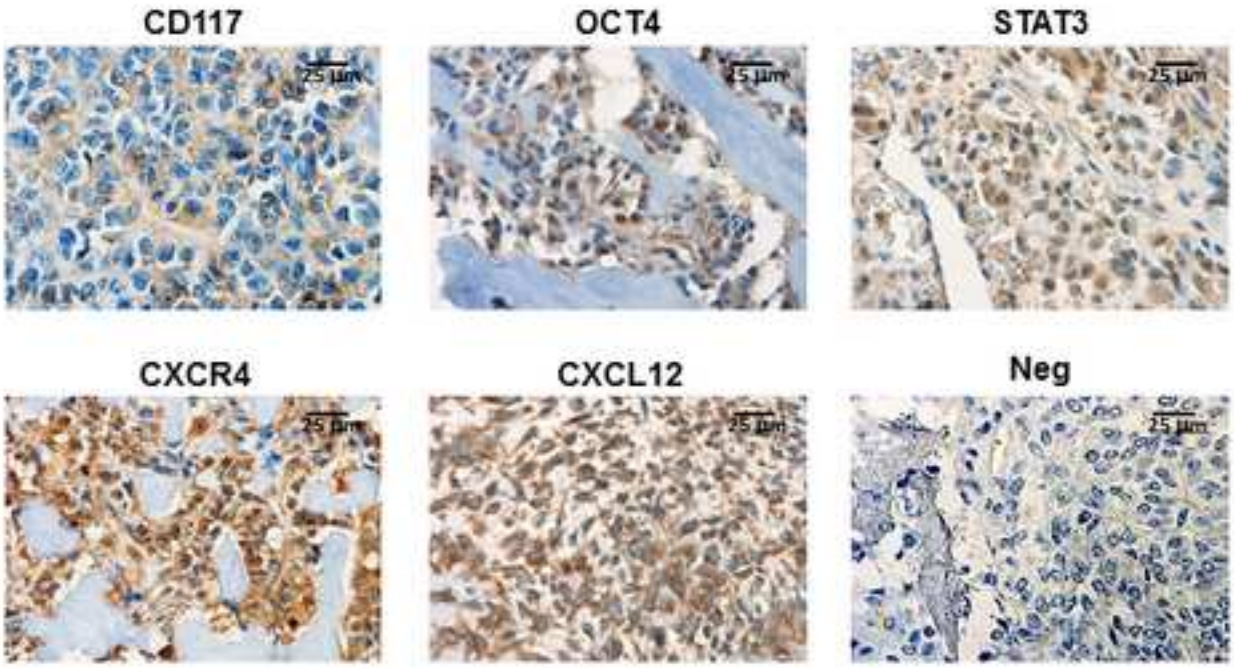
A

	OSA1		OSA2		OSA3	
	Original tumor	CSC xenograft	Original tumor	CSC xenograft	Original tumor	CSC xenograft
	IHC score		*IHC score*		*IHC score*	
CD117	2	1	1	1	0	3
Oct4	1	2	2	3	1	2
STAT3	1	1	1	1	1	1
CXCR4	1	1	1	2	1	2
CXCL12	2	1	1	1	1	2

IHC score: 0 = negative, 1 = low positivity, 2 = positivity, 3 = high positivity

B



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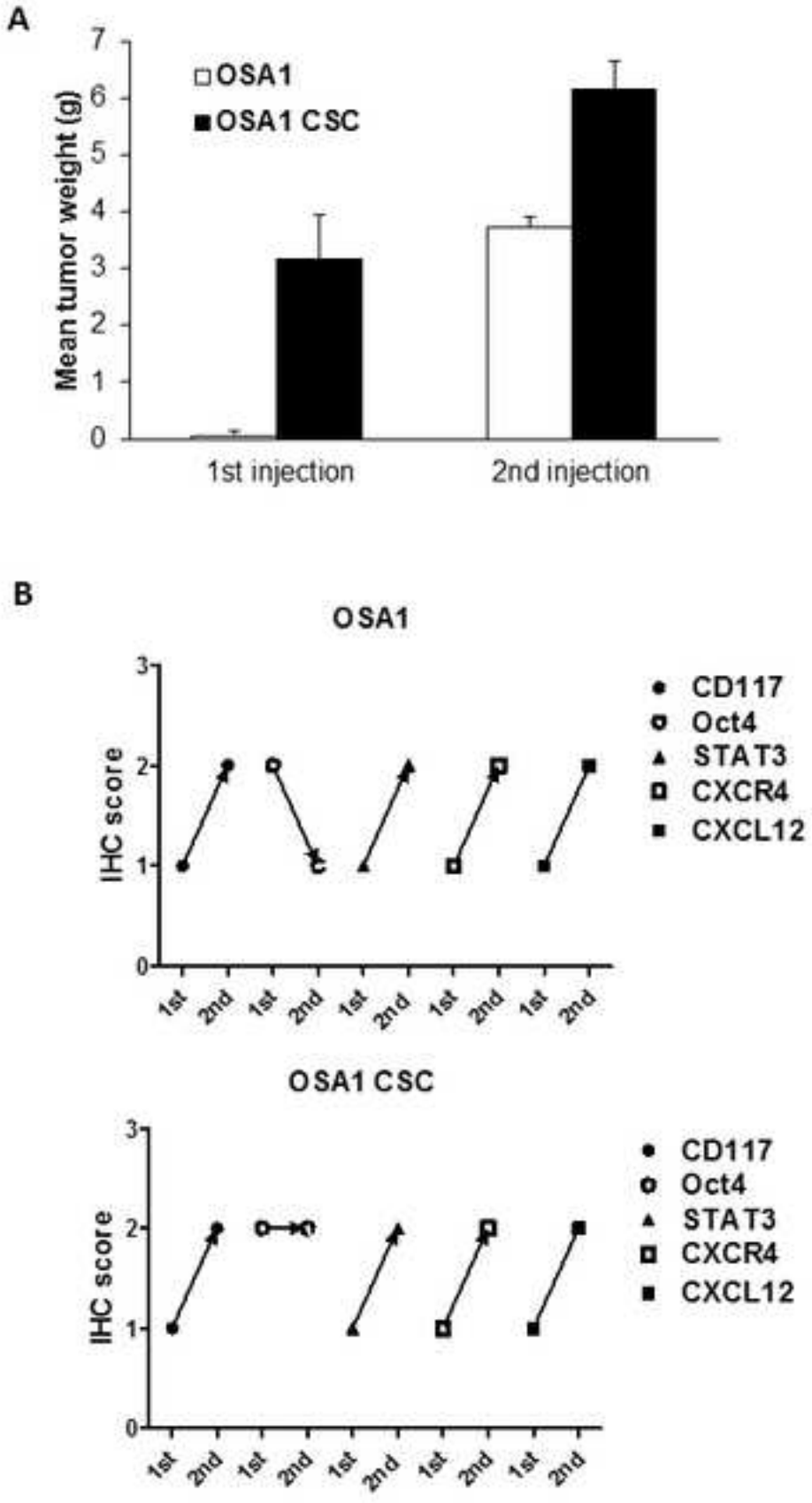

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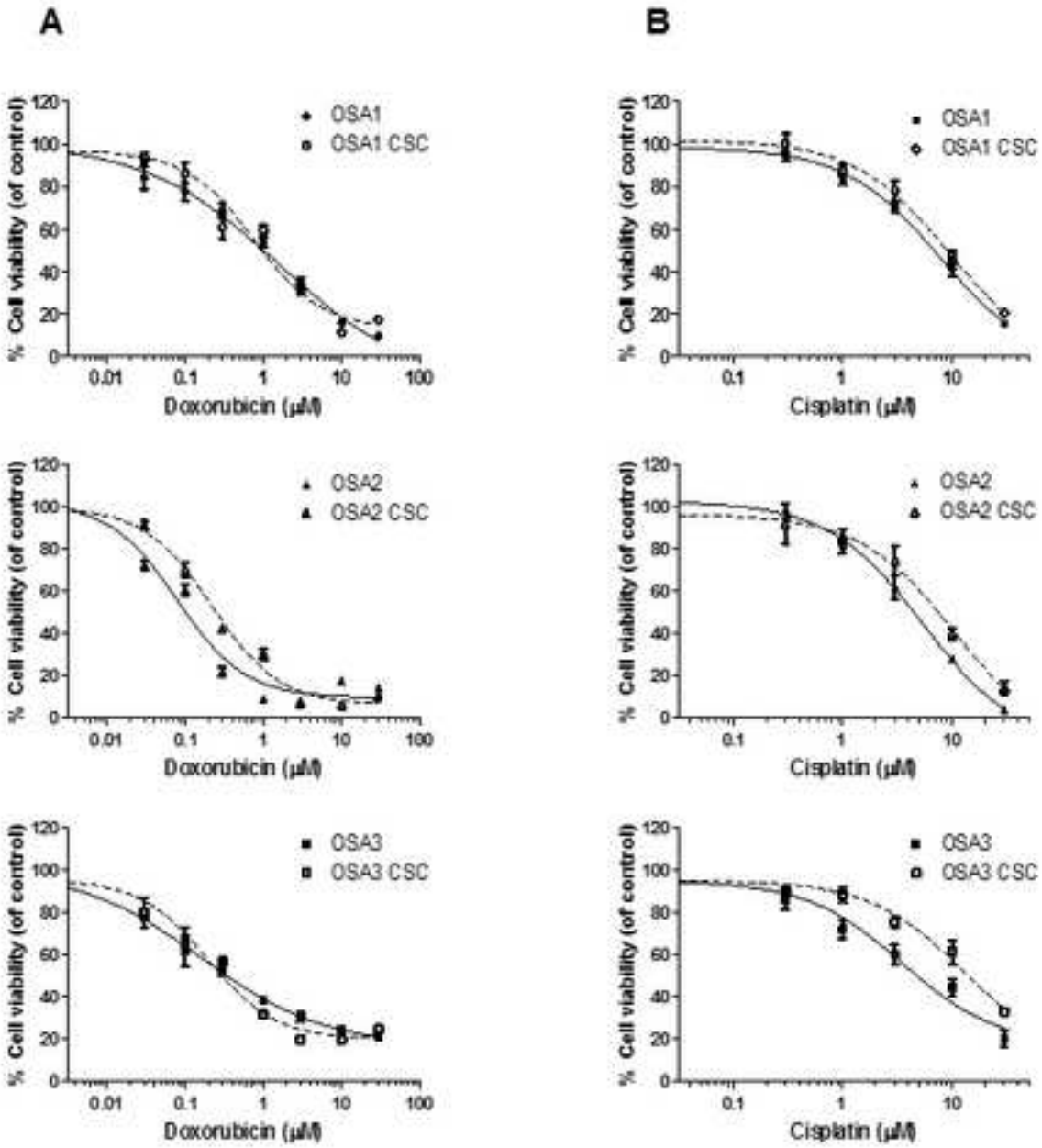

FIGURE 6

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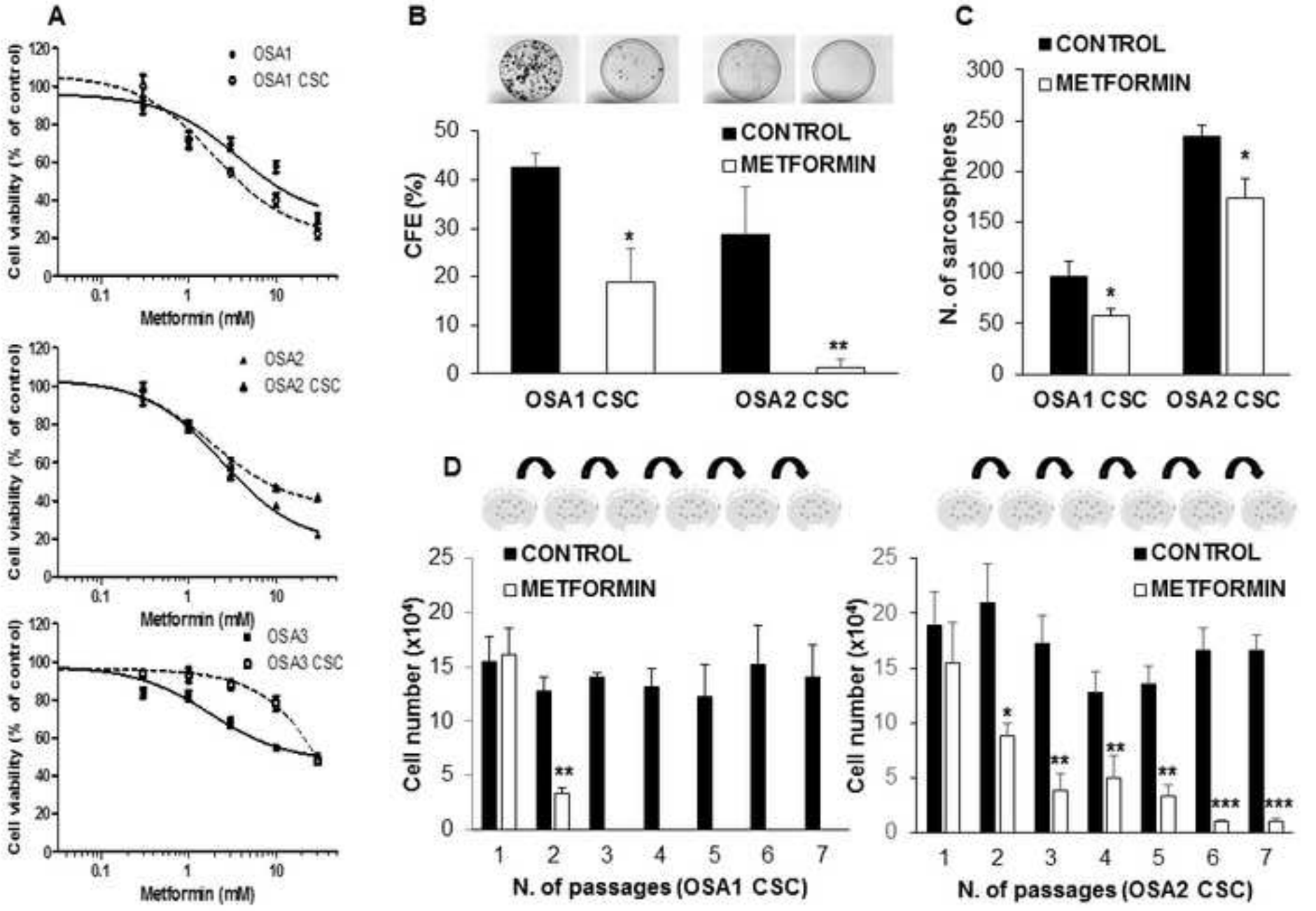

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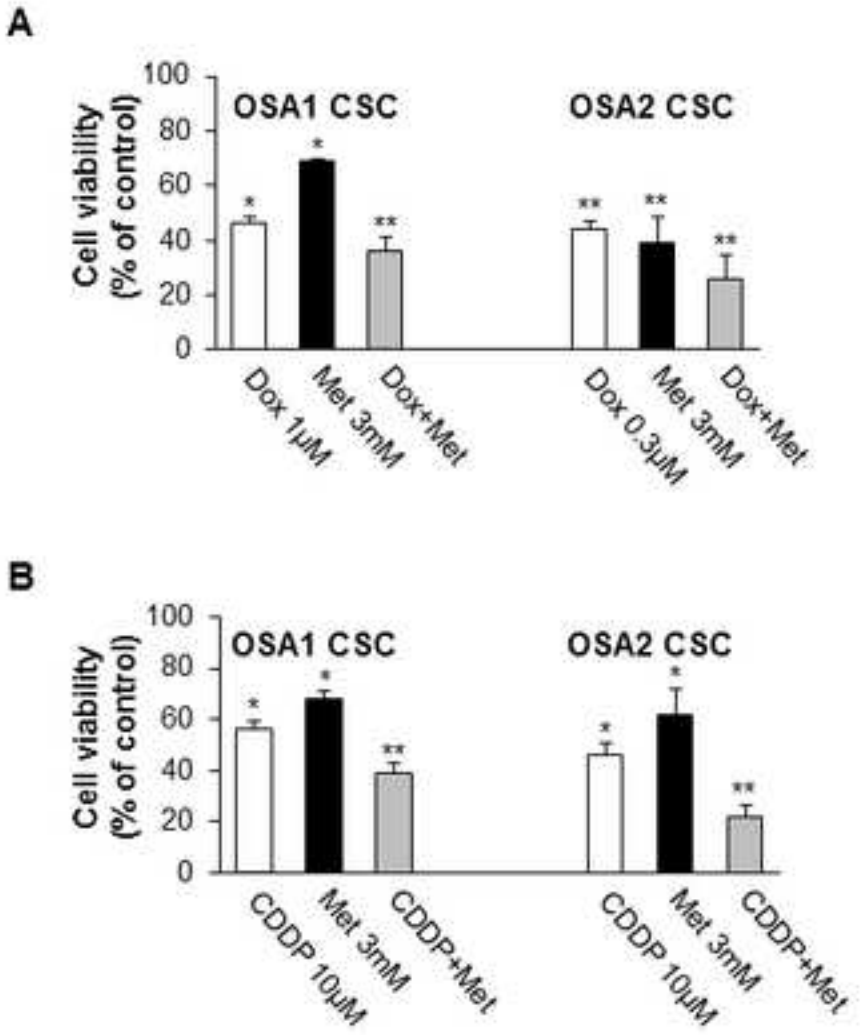

Figure 9

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