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Abstract	Osteosarcoma (OS) is the most frequent malignant bone tumor, affecting predominantly children. Metastases represent a major clinical challenge and an estimated 80% would present undetectable micrometastases at diagnosis. The identification of metastatic traits and molecules would impact in micrometastasis management. Microvascular endothelium tube formation and in vivo angiogenesis assays, adhesion assays, apoptosis assays, proteomic analysis, RT-qPCR. We demonstrated that OS LM7 metastatic cells secretome was able to induce microvascular endothelium cell rearrangements, an angiogenic-related trait. A proteomic analysis indicated a gain in angiogenic-related pathways in these cells, as compared to their parental-non-metastatic OS SAOS2 cells counterpart. Further, factors with proangiogenic functions like VEGF and PDGF were upregulated in LM7 cells. However, no differential angiogenic response was induced by LM7 cells in vivo. Regulation of the Fas–FasL axis is key for OS cells to colonize the lungs in this model. Analysis of the proteomic data with emphasis in apoptosis pathways and related processes revealed that the percentage of genes associated with those, presented similar levels in SAOS2 and LM7 cells. Further, the balance of expression levels of proteins with pro- and antiapoptotic functions in both cell types was subtle. Interestingly and of relevance to the model, Fas associated Factor 1 (FAF1), which participates in Fas signaling, was present in LM7 cells and was not detected in SAOS2 cells. The subtle differences in apoptosis-related events and molecules, together with the reported cell-survival functions of the identified angiogenic factors and the increased survival features that we observed in LM7 cells, suggest that the gain in angiogenesis-related pathways in metastatic OS cells would relate to a prosurvival switch rather to an angiogenic switch as an advantage feature to colonize the lungs. OS metastatic cells also displayed higher adhesion towards microvascular endothelium cells suggesting an advantage for tissue colonization. A gain in angiogenesis pathways and molecules does not result in major angiogenic potential. Together, our results suggest that metastatic OS cells would elicit signaling associated to a prosurvival phenotype, allowing homing into the hostile site for metastasis. During the gain of metastatic traits process, cell populations displaying higher adhesive ability to microvascular endothelium, negative regulation of the Fas–FasL axis in the FasL (−) lung parenchyma and a prosurvival switch, would be selected. This opens a new scenario where antiangiogenic treatments would affect cell survival rather than angiogenesis, and provides a molecular panel of expression that may help in distinguishing OS cells with different metastatic potential.

Keywords (separated by '-')	Osteosarcoma - Prosurvival phenotype - Apoptosis - Metastasis - Angiogenesis
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Up-regulation of pro-angiogenic molecules and events does not relate with an angiogenic switch in metastatic osteosarcoma cells but to cell survival features

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Abstract

Osteosarcoma (OS) is the most frequent malignant bone tumor, affecting predominantly children. Metastases represent a major clinical challenge and an estimated 80% would present undetectable micrometastases at diagnosis. The identification of metastatic traits and molecules would impact in micrometastasis management. Microvascular endothelium tube formation and in vivo angiogenesis assays, adhesion assays, apoptosis assays, proteomic analysis, RT-qPCR. We demonstrated that OS LM7 metastatic cells secretome was able to induce microvascular endothelium cell rearrangements, an angiogenic-related trait. A proteomic analysis indicated a gain in angiogenic-related pathways in these cells, as compared to their parental-non-metastatic OS SAOS2 cells counterpart. Further, factors with proangiogenic functions like VEGF and PDGF were upregulated in LM7 cells. However, no differential angiogenic response was induced by LM7 cells in vivo. Regulation of the Fas–FasL axis is key for OS cells to colonize the lungs in this model. Analysis of the proteomic data with emphasis in apoptosis pathways and related processes revealed that the percentage of genes associated with those, presented similar levels in SAOS2 and LM7 cells. Further, the balance of expression levels of proteins with pro- and antiapoptotic functions in both cell types was subtle. Interestingly and of relevance to the model, Fas associated Factor 1 (FAF1), which participates in Fas signaling, was present in LM7 cells and was not detected in SAOS2 cells. The subtle differences in apoptosis-related events and molecules, together with the reported cell-survival functions of the identified angiogenic factors and the increased survival features that we observed in LM7 cells, suggest that the gain in angiogenesis-related pathways in metastatic OS cells would relate to a prosurvival switch rather to an angiogenic switch as an advantage feature to colonize the lungs. OS metastatic cells also displayed higher adhesion towards microvascular endothelium cells suggesting an advantage for tissue colonization. A gain in angiogenesis pathways and molecules does not result in major angiogenic potential. Together, our results suggest that metastatic OS cells would elicit signaling associated to a prosurvival phenotype, allowing homing into the hostile site for metastasis. During the gain of metastatic traits process, cell populations displaying higher adhesive ability to microvascular endothelium, negative regulation of the Fas–FasL axis in the FasL (−) lung parenchyma and a prosurvival switch, would be selected. This opens a new scenario where antiangiogenic treatments would affect cell survival rather than angiogenesis, and provides a molecular panel of expression that may help in distinguishing OS cells with different metastatic potential.

Keywords Osteosarcoma · Prosurvival phenotype · Apoptosis · Metastasis · Angiogenesis

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Introduction

Osteosarcoma (OS) is the most common malignant bone tumor, arising during metaphyseal rapid growth in adolescents and children [1]. Pulmonary metastases exist in early stages during OS progression. While lung metastases are detected in 20% of patients at diagnosis, an 80% is estimated to carry undetectable micrometastasis at that time [2, 3]. Patients with pulmonary metastases at diagnosis have a 25–30% five-year survival rate with no substantial changes in the last three decades [2, 3]. OS etiology is unclear, with osteogenic precursors accumulating not well-defined oncogenic events which hinders the use of potential markers associated to progression and metastasis [4]. Complex signaling occurs during OS onset involving a bidirectional communication between tumor cells and the bone niche. Thus, OS arises because of imbalanced bone homeostasis in the bone marrow environment. OS progression involves profound bone homeostasis deregulation, extracellular matrix remodeling and biochemical signaling that affect the stromal compartment [4]. Lungs represent the most frequent target organ for metastatic OS. Fas ligand (FasL) is constitutively expressed by alveolar and bronchial epithelial cells [5]. Our model comprises the parental, human OS Fas^+ (CD95, APO1) SAOS2 cells that are cells unable to colonize the lungs when intravenously injected into immunodeficient mice, and the SAOS2-derived LM7 cells, which are able to establish secondary tumor growth into the lungs and express significantly lower Fas levels [6]. LaFleur et al. [7] have previously demonstrated that Fas^+ OS cells are eliminated by the $FasL^+$ lung epithelium while Fas^- OS cells escape this surveillance establishing pulmonary metastases. Thus, the gain in metastatic traits involves the absence of Fas or molecular changes necessary to downregulate its expression as a critical step in this disease. The lack of this feature turns OS cells unable to survive in the lung environment [8]. Clinical specimens corresponding from OS lung metastases express inappreciable Fas levels, while the primary bone tumor counterpart was demonstrated to express high Fas levels, making this model clinically relevant to understand underlying mechanisms that favor OS cells colonization into the lungs and allowing the search for novel therapeutic approaches [6]. Further, the complex modifications in the stromal primary tumor compartment could consequently exert a selection pressure over previously residing OS subpopulations with differential abilities, thus favoring cells with metastatic traits to *leave the nest* towards future pulmonary metastatic sites [9]. Metastasis results in a complex process, with variable routes and interlinked steps [10, 11]. For metastasis to occur, the tumor cell must leave the primary site, intravasate, adhere at the metastatic site and left the circulatory system by extravasation [12]. Requirements for this are a microvessel network, and the ability of the tumor cell to survive both in the circulation and at the target site [13].

Angiogenesis is a multistep process constituting the angiogenic cascade, involving complex signaling among several participating actors, inducing the formation of new vessels from preexisting ones. This process includes the degradation of the basal membrane mediated by proteolytic enzymes like metalloproteinases and cathepsins, and the proliferation and migration of endothelial cells (ECs), followed by the proliferation and differentiation/maturation of ECs [14]. The last step involves other cellular populations, pericytes and smooth-muscle cells, which are recruited by the new vessel stabilizing it. Angiogenesis is mediated by the coordinated action of various cytokines and growth factors. Angiogenic factors such as platelet-derived growth factors (PDGFs) and vascular endothelial growth factor (VEGF) are necessary for the establishment of new vessels in physiological conditions and in tumors [15, 16].

We demonstrated that critical steps and events related to the angiogenic cascade like EC re-organization, and biological pathways and processes like VEGF and PDGF signaling were upregulated in metastatic OS cells secretome. However, this did not result in a net differential vascular bed formation distinguishing metastatic from non-metastatic cells. Given that molecules associated with the identified gene ontology (GO) terms through a proteomic approach such as VEGF, PDGF, endothelins, are also related to survival features, we further analyzed the proteomic data with emphasis in prosurvival related proteins and other molecules arose as relevant. Given our results, we conclude that even when angiogenesis is a tumor-progression associated feature and a tumor cannot develop without this, the process itself and the molecular functions associated with it, would not be determinant in the lung metastatic features in OS, but instead, a prosurvival function of these molecules would allow OS cells to colonize a hostile environment surviving the adverse circulation. This finding shed light into multiple functions for a given molecule/les, a feature that adds complexity and multiple advantages to a given tumor to progress.

Cancer progression involves multistep functional events, which may ultimately lead to the acquisition of a metastatic phenotype [17]. We describe for the first time a functional and molecular comparison between a parental non-metastatic OS cell line and its derived cell line selected by its metastatic behaviour, highlighting a differential molecular pattern that may relate to angiogenic induction potential but also to favour survival in a hostile environment, such as the pulmonary metastatic niche. Pulmonary metastases remain as a major OS mortality determinant, and identification of mechanisms and differentially expressed genes associated

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with metastasis would help in discovering promising markers and targets for therapeutic approaches for OS metastatic spread.

Materials and methods

Cell lines

SAOS2 and LM7 cells human OS cell lines were supplied by Dr. Kleinerman, MD Anderson Cancer Center (MDACC). Cells were grown in Dulbecco's Modified Eagle Medium: Nutrient Mixture F12 (DMEM:F12) supplemented with non-essential amino acids (NEAA), 2 mM L-glutamine, 100U/mL penicillin, 100 mg/mL streptomycin (Invitrogen), 10% fetal bovine serum (FBS; Natocor), at 37 °C, 5%CO_2. SAOS2 are OS cells that do not possess the capacity to form secondary tumor growth sites in the lungs, while LM7 cells have been selected from parental SAOS2 cells by their metastatic ability through lung cyclic circulation, ability associated to avoidance of apoptosis and apoptosis-resistance mechanisms [6, 18]. Human Microvascular Endothelial cells HMEC-1 (Dr. Candal, Centers for Disease Control, Atlanta, GA, USA) were grown in high-glucose DMEM (DMEM high, Invitrogen), 10% FBS (Natocor), 2 mM L-glutamine, 100U/mL penicillin, and 100 mg/mL streptomycin [19]. Verification of mycoplasma species was carried out (MycoAlert Mycoplasma Detection Kit, Lonza Inc.).

Cell conditioned medium

The cells´ secretome compartment is represented by their conditioned medium (CM). Cells were seeded on 100 mm culture dishes until 80% confluence, washed with phosphate basic solution (PBS) and cultured during twenty-four hours with basal medium (DMEM:F12). After this, the CM was collected, centrifuged for 5 min (1100 rpm), aliquoted and stored at −80 °C until use.

Tube formation assay

Tube formation was assayed using Geltrex® LDEV-Free reduced growth factor (GF) basement membrane matrix (ThermoFisher). Forty µL Geltrex/well were seeded in 96-well plates (JET Bio-Filtration) allowing polymerization (37 °C, 30 min). HMEC-1 cells (2×10^4, FBS-starved during 24 h) were seeded on 50 µL of FBS free DMEM high and stimulated with CM (50 µL) from SAOS2 and LM7 cells (6 h, 37 °C). After this, cells were fixed (2% PFA) three pictures were taken from every well to allow for quantification (100× magnification (10× objective/10× eyepiece), Nikon). The number of loops/well was quantified using Image J software, NIH, MD).

In vivo angiogenic assay

Animal experiments were approved by the Institutional Animal Care and Use Committee (MDACC IACUC #00001633-RN00). For in vivo angiogenesis assay, athymic male nude mice were subcutaneously (s.c., right flank, midline section) injected with a pre-mixed solution of SAOS2 or LM7 cells in Geltrex® LDEV-Free reduced GF basement membrane matrix (5×105 cells/40 µL PBS/500 µL Geltrex). One week after inoculation, plugs were excised, fixed (PFA 4%), embedded in Optimal cutting temperature compound (OCT), frozen (liquid nitrogen) and processed for cryostate sectioning. CD31 was detected using rat anti-mouse CD31 (BD Biosciences PharMingen, San Diego, CA, USA) as primary antibody and goat anti-rat Texas Red (Jackson ImmunoResearch, PA, USA) as secondary antibody. Nuclei were stained using Hoechst 33342 solution (1 µg/mL in PBS, Sigma) [20, 21]. Microvessel density was assessed as previously described by Weidner et al. [22], briefly microvessel density was analyzed in areas with high density of capillaries and small venules (vascular "hotspots") and microvessels were counted at 200× magnification fields. Any endothelial cell cluster or vessel positive for CD31 and clearly separated from an adjacent capillar was considered to be a single microvessel [23, 24].

Cell adhesion assay

HMEC-1 cells were seeded at 2×10^5cells/96-well, allowing the establishment of a monolayer. OS cells were stained with DiO (fluorescent cell tracker, Molecular Probes) to allow visualization; 5.0×10^3 DiO^+ cells were seeded over the microvascular endothelium monolayer. Cell adhesion was allowed (30 min, 37 °C). Attached cells were fixed (4% PFA), visualized (fluorescence microscope, Nikon) and five representative visual fields were counted at 100× (DiO^+ cells, ImageJ software, NIH, MD, USA) [25]. The microvascular endothelium cell line was used as it was shown to be of clinical relevance in experimental approaches [26, 27]. Disrupted microvascular endothelium areas were not included.

Acridine orange/ethidium bromide (AO/EB) fluorescence staining

OS cell lines were cultured in culture medium with 2,5% or without FBS at 37 °C in a 4% CO_2 atmosphere and apoptosis was evaluated at 6 h. Morphological changes associated with apoptosis were assessed by acridine orange-ethidium

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bromide mixture staining (Sigma). Briefly, cell pellets were resuspended in dye mix (100 µg/mL acridine orange plus 100 µg/mL ethidium bromide in PBS) and visualized by fluorescence microscopy (Nikon Eclipse E800). A minimum number of 200 cells were counted and the number of cells presenting fragmented nuclei, enlarged cytoplasm and condensed chromatin were determined. The percentage of apoptotic cells was calculated as total number of cells with apoptotic nuclei/total number of cells counted × 100 as previously described [28].

Chromatin condensation assay

OS cell lines were grown on gelatin-coated glass coverslips, with basal medium in the presence or absence of doxorubicin (0,1 and 1 µM) and chromatin condensation and nuclear fragmentation was evaluated at 24 h after treatment. Cells were washed with PBS and fixed with 4% PFA. As previously described, nuclei were stained with 0.01 mg/mL Hoechst 33342 (15 min) to allow for nuclear morphology visualization at 100× and 400× magnification (10× and 40× objective/10× eyepiece, Nikon Eclipse E400 fluorescence microscope) [29].

Reverse transcription-polymerase chain reaction (RT-qPCR) and real Time polymerase chain reaction (qPCR)

Total RNA from OS cells (Trizol Reagent, Molecular Research Center, USA) was reverse transcribed (2 µg) with 200 U of *EasyScript* Reverse Transcriptase (Transgenbiotech) using Oligo (dT) primers (500 ng). cDNAs were subjected to qPCR (CFX96 Touch TM Real-Time PCR Detection System, Bio-Rad). Fas-associated factor 1 (FAF1), VEGF, PDGFA, PDGFB, PDGFC, PDGFD, mRNA levels were quantified (SYBR Green, Roche)using the primers: FAF1 forward 5' GACCAGCTTTGGAGCTCTTG3', reverse 5' TGCGGGAAATAAAGATCTGG3'; VEGF forward 5'ATCTTCAAGCCATCCTGTGTGC 3', reverse 5'GCTCACCGCCTCGGCTTGT 3';PDGFA forward 5' CCTGCCCATTCGGAGGAAGAG 3', reverse 5' TTG GCCACCTTGACGCTGCG 3';PDGFB forward 5' TCC CGAGGAGCTTTATGAGA 3', reverse 5' ACTGCACGT TGCGGTTGT 3'; PDGFC forward 5' GGAGCACCATGA GGAGTGTGA 3', reverse 5'GAGCTGCTGGTGGTGATG C 3'; PDGFD forward 5' CCCAGGAATTACTCGGTCAA 3', reverse 5' ACAGCCACAATTTCCTCCAC 3'. PCR amplification was carried out using a 95 °C for 10 min cycle and 40 cycles under the parameters: 95 °C for 20 s, 60 °C for 1 min, 72 °C for 40 s and a 95 °C for 20 s cycle. At the end the temperature was increased from 60to 95 °C (2 °C/min rate), and fluorescence was measured every 15 s to construct the melting curve. Values were normalized to levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels, forward 5' GGGGCTGCCCAG AACATCAT 3', reverse 5' GCCTGCTTCACCACCTTC TTG 3'. Data were processed by the DDCt method. A non-template control (NTC) was run in every assay; all determinations were performed as triplicates in three separated experiments.

Proteomics and proteomic data analysis

To analyzed the proteomic profile of the cellular and secretome components of OS tumor cells, cell pellets (4×10^7cells) and CM (12 mL) were lyophilized for storage and transport. Later, samples were resuspended in lysis buffer (100 mM Tris–HCl, pH 7.5, 4%SDS, 100 mM DTT and H2O 18.2 MΩ cm at a ratio of 1:1 (v/v) for 15 min at 94 °C. The samples were subjected to sonication (30 min), centrifuged at 16,000×*g* for 5 min and separated by 10% SDS-PAGE. Once the electrophoretic run was finished, the gel was stained with Coomassie blue and the lanes were excised and cut into small pieces of equal size and treated with trypsin. The resulting peptides were processed, and analyzed with a tandem system of nanocapillary liquid chromatography-mass spectrometry (Thermo Scientific Easy-nLC 1000 system connected to an LTQ Orbitrap XL ETD) as previously described [30].

For the identification, quantification (label free) and validation of the proteins, the MaxQuant platform (version 1.5.2.8) was used, which includes the Andromeda algorithm fordatabase search. Uniprot was the database used for protein search and complemented with the elimination of frequent contaminants (porcine trypsin) and also reverse sequences. To validate the assigned protein identity, a minimum of seven amino acids was established for each peptide and a Q value cut-off of 0.01 was also established at the level of peptides and proteins [30, 31]. To obtain the gene ontology terms (GO) of the identified proteins, an enrichment analysis was carried out with the software Funrich, of the gene groups corresponding to the secretome and the intracellular compartment. To select the categories with statistical significance, p values were taken at 0.05. The focus was on the analysis of GO terms related to angiogenesis, survival and processes related to angiogenic potential. To assess relative expression of individual proteins in each compartment, a label free approach was performed as previously described. The normalized label free quantification (LFQ) protein values were expressed as relative intensity values and for normalization the LFQ media intensity of all proteins were used. LFQ (Label-free quantification) intensities are based on the (raw) intensities (sums of all individual peptide intensities, peaks in a MS

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spectra, belonging to a particular protein) and normalized on multiple levels to ensure that profiles of LFQ intensities across samples reflect the relative amounts of the proteins. [30].

Database search

Data about the expression of PDGFA, PDGFB, PDGFC, PDGFD, VEGF and FAF1 in OS samples were obtain from the Gene Expression Omnibus (GEO) database number GSE42352. The data set called mixed Osteosarcoma-Kuijjer-127-vst-ilmnhwg6v2 data set, has 127 samples originally. Genome-wide gene expression analysis was performed using pretreatment high-grade diagnostic OS biopsy samples. The R2: Genomics analysis and visualization platform (http://r2.amc.nl) was used to generate Kaplan–Meier metastasis-free survival curves, omitting from the analysis 39 samples that lacked survival data.

Statistical analysis

Ninety-five percent (95%) of confidence intervals (CI) were determined by calculating arithmetic mean values and variance (standard deviation, SD) of three independent experiments. Unpaired 2-sided Student's t test (two groups comparisons) and analysis of variance (ANOVA) followed by post-tests Kruskal–Wallis and Dunn's post-tests (more than two experimental groups comparisons) (GraphPad Prism Software, San Diego, CA, USA) were used for statistical analyses, considering p value < 0.05 as statistically significant.

Results

Microvascular endothelium cells rearreagements and in vivo angiogenic response induced by OS cells

Neovessel formation, which is associated with cancer progression in a variety of tumor models, involves the coordinated occurrence of several steps leading to new functional vessels. We evaluated the capacity of SAOS2 and LM7 OS cells secretome to exert morphogenic rearrangements in microvascular endothelium cell monolayers, a step associated to the angiogenic cascade. To this end we performed in vitro tube formation assays on HMEC-1 cells. LM7 cells secretome resulted as the major tube inducer as compared to the tube-inducing capacity of parental cells secretome, basal medium and serum-supplemented basal medium, producing a 1.3-fold increase in microvascular endothelium cell rearrangement as compared to the response exerted by SAOS2 cells (Fig. 1a, b). When in vivo angiogenesis assays were performed with OS cells, the density of $CD31^+$ microvessels induced by SAOS2 and LM7 cells were similar. Further, no qualitative differences were observed in the vasculature induced by OS cells (Fig. 1c, d).

Osteosarcoma cells adhesive behavior towards microendothelium

Cell adhesion to endothelium is critical for intravasation and extravasation during the metastatic cascade. We analyzed the adhesive behavior of OS cells to microvascular endothelium cells (HMEC-1) and also analyzed proteomic data with emphasis on proteins related to adhesion. To this end, SAOS2 and LM7 cells were subjected to an adhesion assay on HMEC-1 cells. We observed that LM7 cells displayed significantly higher adhesiveness to HMEC-1 cells (30 min, 1.6-fold increase, Fig. 2a ,b). Proteomic analysis with emphasis in adhesion-related molecules, revealed that both cell lines expressed proteins implicated in this biological process like integrins, catenins and cell adhesion molecules (CAM). Analysis of protein relative levels revealed an overall higher expression of adhesion related proteins in SAOS2, with LM7 cells expressing high levels for ALCAM (activated leukocyte cell adhesion molecule, Fig. 2c).

Expression of molecules related to angiogenesis and pro-survival signaling pathways

Analysis of biological pathways indicated that PDGF signaling was increased in LM7 cells. PDGF was demonstrated to have angiogenic and cell-survival properties [32]. Validation through qPCR indicated an eightfold and threefold increase for PDGFB and D respectively in LM7, with no appreciable differences in PDGFA and C expression (Fig. 3a). We observed a gain in biological pathways associated with angiogenesis (PECAM1 interactions, VEGF and VEGF receptor signaling, endothelins, integrins in angiogenesis and angiopoietin receptor tie2 mediated signaling among others) (Supplementary material Table 1), but a lack of a net in vivo angiogenic response difference between the cell types (see Fig. 1).Of interest, expression analysis of VEGF, a factor with pro-angiogenic and pro-survival reported functions [33], showed a twofold increase in expression by qPCR in LM7 cells (Fig. 3b).Given that apoptosis and cell survival exert a role in this model, these results could point to a scenario where PDGF and VEGF would be related to their reported cell-survival functions rather than to a differential angiogenic response. Further, of the exclusive LM7 proteins identified, FAF1, a FAS interactor [34], (see in "Proteomic analysis of apoptosis pathway and related processes" section) demonstrated a 17-fold expression increase in LM7 cells as compared to their non-metastatic counterpart

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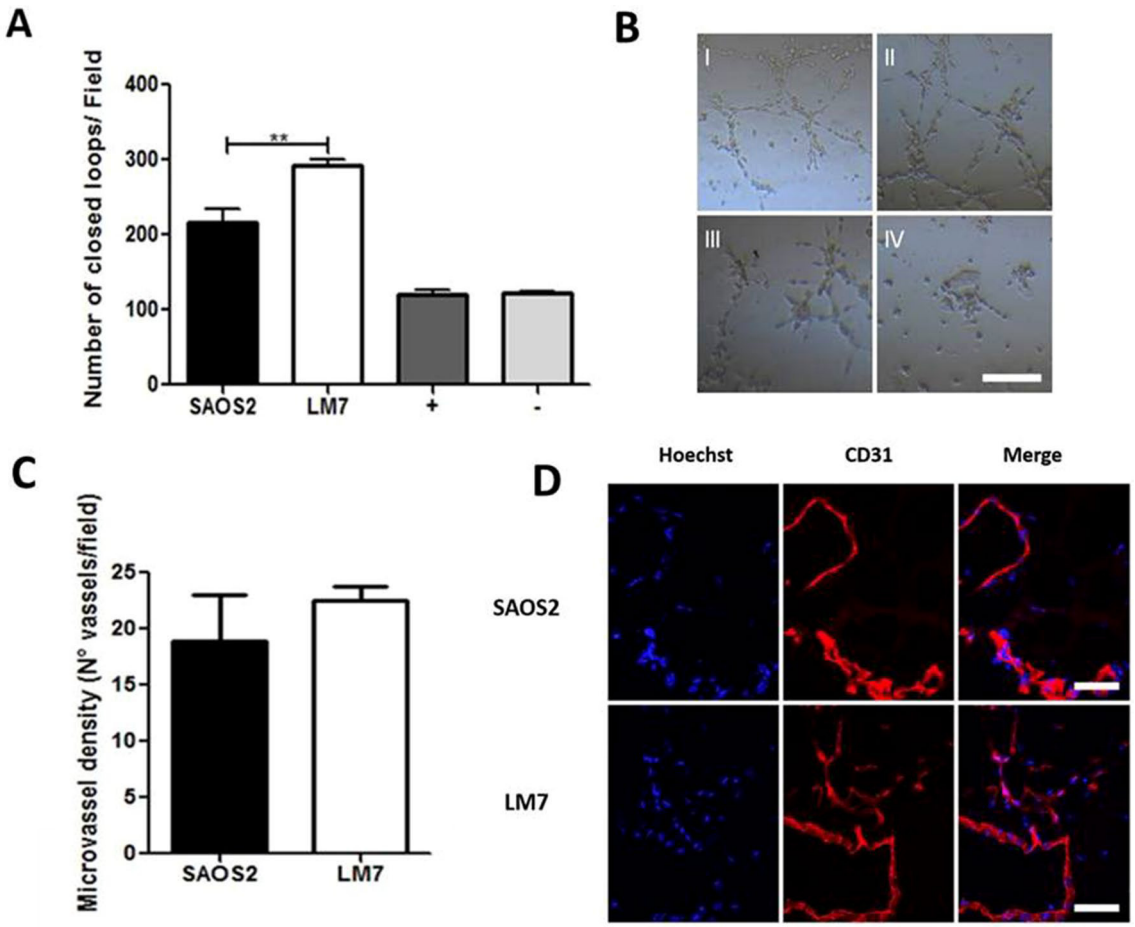

Fig. 1 Angiogenic effect exerted by OS human cells or their secretome. **a** In vitro tube formation assay to test the ability of OS cells secretome to induce morphogenic rearrangements. HMEC-1 microvascular endothelium cells were treated with SAOS2 (parental cells) or LM7 cells-derived conditioned medium (CM). DF12 supplemented with 10% FBS and DF12 basal medium were used as positive (+) and negative (−) stimuli, respectively. One-way ANOVA, $^{**}p < 0.01$. Data are representative of three independent experiments. **b** Representative images of in vitro tube formation assays with SAOS2 or LM7-derived CM; (*i*) HMEC-1 cells treated with SAOS2 CM; (*ii*) HMEC-1 cells treated with LM7 CM; (*iii*) HMEC-1 cells treated with DF12 medium with 10% SFB; (*iv*) HMEC-1 cells with DF12 basal medium. Scale bar represent 0.2 mm. (C) In vivo angiogenesis assay with Geltrex plugs containing SAOS2 or LM7, showing the quantification of microvessel density by$CD31^+$ microvessels detection after 7 days. (D) Representative images of the plugs of the in vivo angiogenesis assay. Nuclei were stained with Hoechst and vessels detected with CD31 antibody. Scale bar represent 50 µm

(Fig. 3c). We complemented this data by analyzing a genome-wide gene expression dataset (The R2: Genomics Analysis and Visualization Platform) of high grade OS pre-chemotherapy biopsies (88 pre-treatment high-grade osteosarcoma diagnostic biopsies). Of relevance, we observed that PDGF isoforms, FAF1 and VEGF shared a common feature in patients, with higher expression of these proteins related with a worst overall survival as confirmed by Kaplan–Meier curves (Supp. Figure 1).

Proteomic analysis of apoptosis pathway and related processes

Apoptosis is a cell death mechanism, where a cascade of mediators triggered by different ligand mediated signals like Fas/FasL, induce the release of caspases from the mitochondria and conclude in cell death. Since our model involves a Fas^+ SAOS2 and Fas^- LM7 cell model, which allows LM7 cells to survive in the $FasL^+$ lung parenchyma [6], we analyzed our proteomic data with emphasis in apoptosis pathways and related processes. The percentage of genes associated with apoptotic signaling pathways, apoptotic processes in general and regulation of apoptotic processes presented similar levels in SAOS2 and LM7 cells (Table 1). Interestingly and of relevance to the model, when looking into proteins associated to apoptosis, Fas associated Factor 1 (FAF1), which participates as an enhancer of Fas signaling [34, 35], was present in LM7 cells (2,876200e+007, normalized LFQ value) and was not detected in SAOS2 cells, which was validated through

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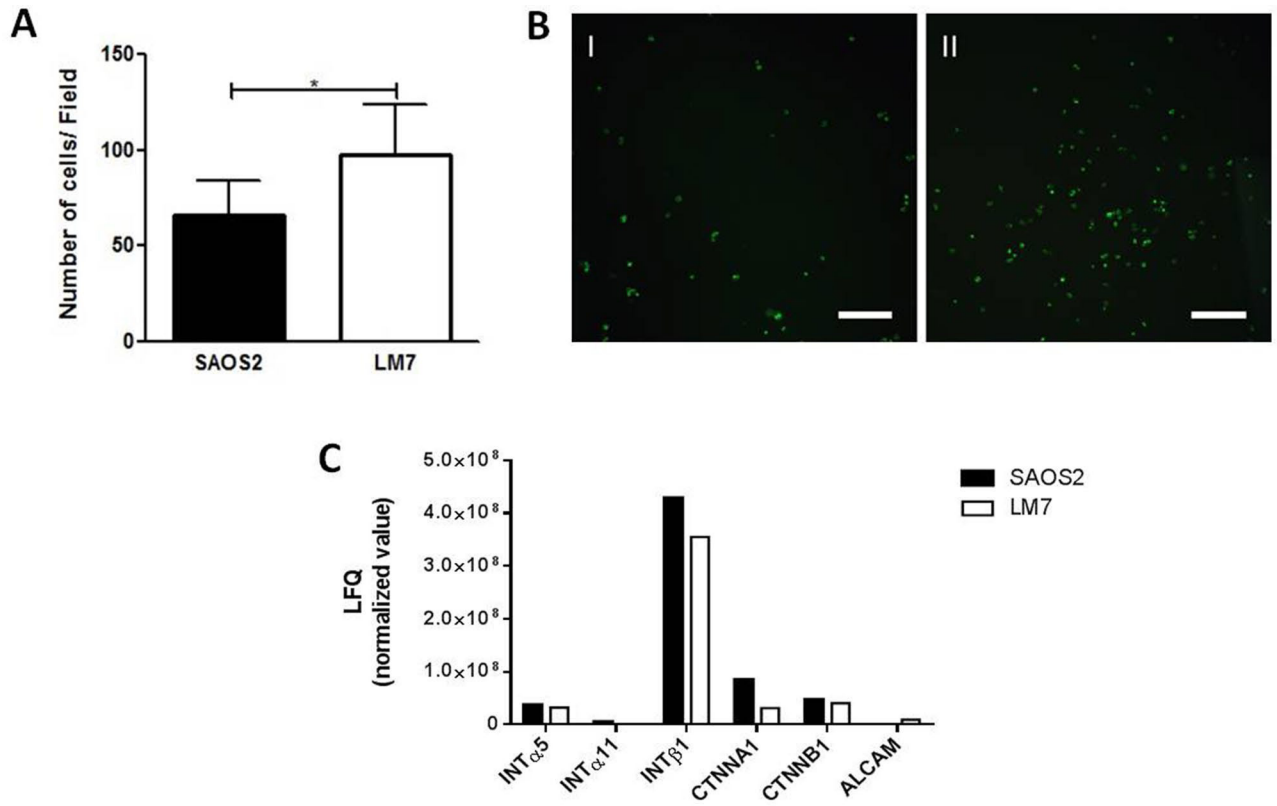

Fig. 2 Osteosarcoma cells adhesive behavior to microvascular endothelium. **a** Adhesion of SAOS2 and LM7 cells on HMEC-1 cells monolayers at 30 min, represented as number of cells per field. *t* test, *p < 0.05. **b** Representive pictures of SAOS2 cells (panel I) and LM7 cells (panel II) stained with DiO, over the HMEC-1 monolayer. Pictures were taken at ×100, with fluorescence microscope to allow visualization of pre-labelled OS cells. Scale bar represents 0.2 mm. Data and images are representative of three independent experiments. **c** The graph bars show the LFQ normalized values of adhesion-related proteins identified by analysis with Funrich program, for both OS cell lines proteomes. INTα5: integrin alpha 5; INTα11: integrin alpha 11; INTβ1: integrin beta 1; CTNNA1: catenin alpha 1; CTNNB1: catenin beta 1; ALCAM: activated leukocyte cell adhesion molecule. Notably, ALCAM was only present in LM7 cells

qPCR, with LM7 showing significantly higher levels of expression as compared to SAOS2 cells (see Fig. 3c). The balance of expression levels of other proteins with pro- and antiapoptotic functions in both cell types was subtle, with SAOS2 displaying higher expression of the proapoptotic BAG2 and BAG6 and of the antiapoptotic AATF, BCL2L13 and API5 molecules, while LM7 showed increased expression of the proapoptotic BLAF1, AIFM1 and CASP3, and of the antiapoptotic BAG3 and BAG5 proteins (Supplementary material table 2).The subtle differences in apoptosis-related events and molecules together with our previous results showing that LM7 are more resistant than SAOS2 cells to cytotoxic agents like doxorubicin [36], could suggest that cell survival-related mechanisms would be of relevance in this model. Interestingly, under starvation conditions SAOS2 cells had a 6.3-fold increase in apoptosis levels (without FBS supplementation), and a 6.0 fold increase in apoptosis with 2.5% FBS supplementation, as compared to LM7 cells (Fig. 4a). After treatment with 0.1 and 1 μM doxorubicin for 24 h, an increasing number of SAOS2 cells started to display nuclear features compatible with apoptotic cells, like chromatin condensation or nuclear fragmentation, while LM7 cells showed similar levels of apoptotic-like nucleus in the control and treated groups as compared to SAOS2 (Fig. 4b).

Discussion

Despite therapeutic combinations, the five-year survival rate for OS remains in 60–70%, and patients with pulmonary metastases at diagnosis present a 25–30% five-year survival rate for the last thirty years [3]. Human OS LM7 cells, which are able to establish secondary tumor growth into the lungs and express significantly lower Fas levels, are derived from the non-metastasic, Fas^+ SAOS2 cells [6]. Fas^+ OS cells are eliminated by the $FasL^+$ lung environment and Fas^- OS cells are able to establish pulmonary metastases as previously demonstrated in this model [7]. Of relevance, this has

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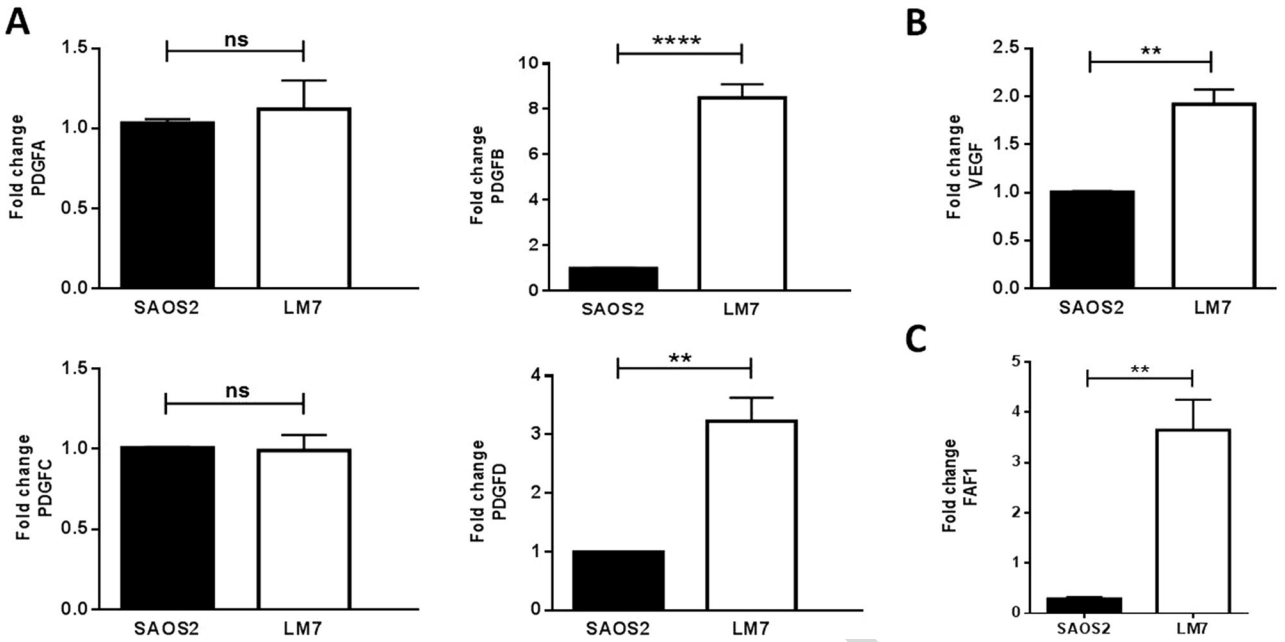

Fig. 3 RT-qPCR analysis of OS human cells. **a** PDGFA, PDGFB, PDGFC and PDGFD. **b** VEGF and **c** FAF1. The results represent the average expression + SD. PDGFA: platelet derived growth factor A; PDGFB: platelet derived growth factor B; PDGFC: platelet derived growth factor C; PDGFD: platelet derived growth factor D; VEGF: vascular endothelial growth factor; FAF1: Fas-associated protein 1. *t test*, *ns* not significant; ** $P < 0.01$; **** $P < 0.0001$. Data are representative of three independent experiments

Table 1 OS cells apoptosis pathway and related processes

Biological process	SAOS2			LM7		
	Percentage of genes	Fold enrichment	P-value	Percentage of genes	Fold enrichment	P-value
Negative regulation of apoptotic process	4.78	1.76	***	4.59	1.69	***
Positive regulation of apoptotic process	2.72	1.51	**	2.77	1.53	**
Regulation of apoptotic process	1.60	1.38	ns	1.53	1.32	ns
Apoptotic signaling pathway	0.46	1.41	ns	0.51	1.54	ns
Apoptotic process	3.39	1.09	ns	3.57	1.15	ns

Percentage of genes represent the relation between the number of expressed genes related to a specific GO term and the number of genes of the GO. Fold enrichment represents the comparison between the frequency of genes annotated in a specific GO term against the frequency of genes that fall into the same GO term. Analyses considering the relative abundance of the proteins (LFQ normalized intensities) were carried out using Funrich software

LFQ label free quantification, *GO* gene ontology, *Ns* not significant

**$p < 0.01$; ***$p < 0.001$

a relationship with clinical observations, with the primary OS tumor expressing high Fas levels and inappreciable Fas levels in OS lung metastases [6]. The tumor niche is established through the interplay between tumor cells, cancer stem cells, stromal cells and the extracellular matrix [37, 38], and the use of this clinically relevant model would help in addressing fundamental mechanisms that allow for OS lung metastasis establishment.

Tumor progression is a complex biological process that involves a gain in several biological mechanisms such as angiogenesis, adherence, and cell survival, among others. In order to elucidate if metastatic abilities acquired and/or selected in LM7 cells were accompanied with other hallmarks, we performed functional assays and analyzed proteomic data with emphasis on biological pathways and processes involved in the different functional abilities that

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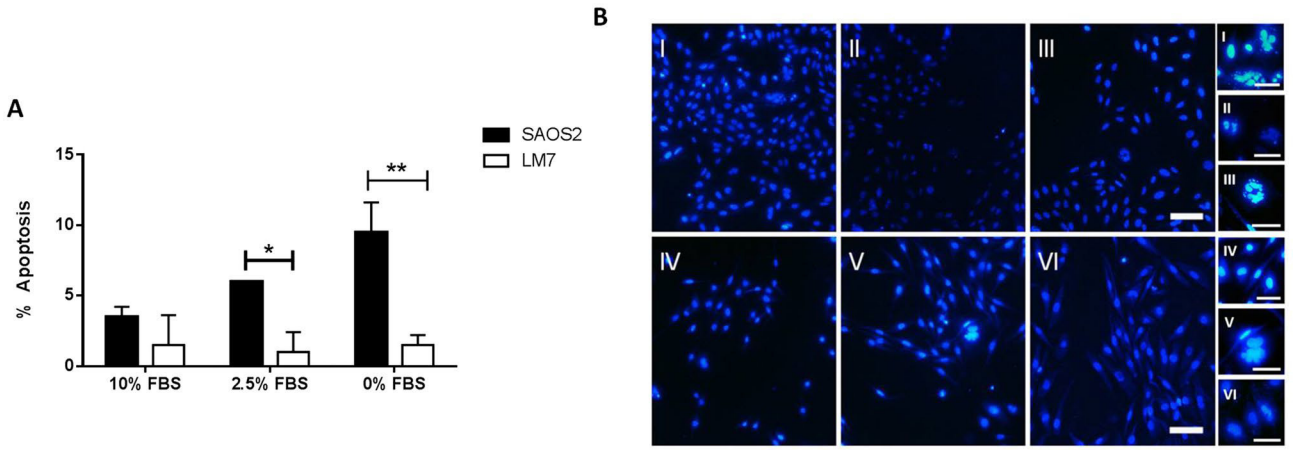

Fig. 4 Apoptosis analysis by Acridine orange/ethidium bromide staining and analysis of nuclear morphology. **a** Acridine orange/ethidium bromide assay on OS cells treated with 2.5% FBS or without FBS for 6 h. Columns show the percentage of apoptotic cells as the total number of cells with apoptotic nuclei/total number of cells counted 100× Data are representative of three independent experiments. Two-way ANOVA, * $P<0.05$; ** $P<0.01$. **b** Analysis of nuclear morphology by chromatin condensation assay. OS cells were treated with 0.1 or 1 μM doxorubicin for 24 h, followed by staining with Hoechst 33342. Representative fluorescence images of SAOS2 and LM7 OS cells; (*I*) SAOS2 control (basal medium); (*II*) SAOS2 treated with 0.1 μM doxorubicin; (*III*) SAOS2 treated with 1 μM doxorubicin; (*IV*) LM7 control (basal medium); (*V*) LM7 treated with 0.1 μM doxorubicin; (*VI*) LM7 treated with 1 μM doxorubicin. Scale bar represents 100 μm. Panels on the right correspond to representative images of nucleus at higher magnification, scale bar represents 50 μm. Images are representative of four independent assays at ×100 and higher magnification images at ×400

were evaluated on both OS cell types. GO terms associated with cell survival, angiogenesis and signaling through PDGF/VEGF were relevant in metastatic cells. Pathways related to the angiogenic response were upregulated in LM7 cells (PECAM interactions, integrins in angiogenesis and endothelins (Supp. Table 1), and we demonstrated that LM7 cells secretome induced a higher cell rearrangement on microvascular endothelium cells, although no significant increase in the in vivo angiogenic response was observed. The quality of microvessels such as an increase in vessel diameter may determine tumor progression success [24]. Related to this, no quality difference in metastatic cells-induced vessels that may account for a microcirculation advantage was observed. This points that although there is a difference associated to a remodeling stage related to the angiogenic cascade, both cell lines attain similar levels of in vivo angiogenic response, suggesting that LM7's secretome could be acting as a remodeling process inducer. In this scenario and in accordance to our results, an enhanced angiogenic response would not represent an advantage to any of the cells lines. HMEC-1 cells were established from foreskins, and given the heterogeneity of microvascular endothelium [39] it may represent a limitation of the study, although they are a model of functional human microvascular endothelium widely used as opposed to the use of cells derived from human umbilical veins (HUVEC) to dissect tumor cells and stromal cells overall interaction [40–43].

Angiogenic related growth factors have been also related to cell survival pathways, given that they boost survival, proliferation and overall cellular state [44]. VEGF and PDGF are known master regulators of angiogenesis, but also present reported properties as survival factors acting through different mechanisms. VEGF, for example, has been implicated in the inhibition of apoptosis, promoting the survival of ECs in a direct-manner, under adverse conditions and also promoting tumor cell survival [45, 46]. The cytoprotective effect of VEGF resulting in apoptosis inhibition, involves signaling through VEGFR2, which leads to the up-regulation of members of the anti-apoptotic machinery such as Bcl-2, Bcl-2A1, XIAP (X-chromosome linked inhibitor of apoptosis) and survivin [47, 48]. Endothelial permeability, which is induced by VEGF, allows the intra and extravasation of cells by disrupting the vascular barrier and allowing the widespread of metastatic cells, with previous reports analyzing the relationship between VEGF expression in OS cells and vascular permeability [49–51]. Related to this and associated to our identification of VEGFR signaling as relevant in LM7 cells, it has been proved in other models that more aggressive OS cells have an autocrine VEGF loop which induce OS-tumor growth in vivo [52], pointing that VEGF could be exerting both pro-angiogenic and pro-survival features [53]. PDGF pro-survival properties were reported to involve the downregulation of pro-apoptotic factors such as Bad, and the upregulation of anti-apoptotic factors like Bcl-2l. PDGF has been described as an inducer factor of cell proliferation, survival and migration primarily for cells with a mesenchymal lineage origin [54]. Given the observed presence of various pro- and antiapoptotic proteins in both

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OS cell types evaluated, without a significant net balance in either cell type, cell protective effects exerted by PDGF and VEGF could be counteracting pro-apoptotic effects and thus promoting OS LM7 cell survival. It was demonstrated that the activation of caspase 3 was significantly reduced by PDGF-BB pretreatment in cells challenged with gp120 [55] and VEGF was shown to promote cell survival through the inhibition of caspase 3 cleavage [56]. In this regard, the significant higher expression levels of caspase 3 in LM7 cells, despite cell-death resistant features observed in these cells, could be related to signaling through PDGF and VEGF promoting a switch to cell survival despite the presence of pro-apoptotic proteins. As mentioned, OS metastasizes predominantly to the lungs emphasizing the importance of the microenvironment. Associated to its function, lungs are very well vascularized [57, 58], and although there are reports of angiogenic-independent tumor growth in this organ [59, 60], in OS the high vascularization in the lungs may provide a suitable scenario where molecules like VEGF and PDGF would switch into a pro-survival function rather to a pro-angiogenic function. Our results showed that PDGFB and PDGFD were upregulated members of the PDGF family (see Fig. 3). Of interest, analysis of metastasis-free survival data of OS patients revealed that both PDGF isoforms upregulated in our model, were of importance in a clinical scenario, with high expression associated to worst overall survival as confirmed by Kaplan–Meier curves (Supplementary material Fig. 1). Pertinent to this, there is a correlation between PDGF and VEGF networks, exemplified in the potent VEGF secretion induced by PDGF-B in an ovarian cancer model [61]. Of relevance, Langley et al. [62] have demonstrated that the PDGFBB isoform functions as a survival factor for bone-derived microvascular endothelial cells. Cell stress conditions can lead to cell survival or to cell death [63]. In this regard we showed that LM7 cells were more resistant to apoptosis under starvation conditions (see Fig. 4a), and doxorubicin treatment induced in LM7 cells diminished nuclear features compatible with apoptosis (see Fig. 4b). In this context we have recently shown that OS metastatic cells have an increased capacity to modify the intracellular localization of chemodrugs, further emphasizing the idea of a gain in pro-survival mechanisms in LM7 cells [36]. Worth mentioning in this scenario, the angiogenic-related endothelins that we identified in pathways upregulated in LM7 cells, have also been reported as multifunctional proteins with prosurvival and chemoprotective properties [64, 65]. Altogether, our results suggest that augmented PDGF and VEGF could relate, in metastatic OS cells, to signaling carrying increased surviving properties. Relevant to the model, when looking into proteins associated to apoptosis, the Fas signaling enhancer FAF1 protein was expressed in high levels in LM7 cells (see Fig. 3). Initially recognized as a member of the FAS death-inducing signaling complex, subsequent work revealed FAF1 functions in diverse biological processes, playing an important role in development and neural survival [34], thus adding to pro-survival features. Recent evidence shows that AKT can induce FAF1 phosphorylation through the action of growth factors or oncogenic mutations, ultimately inducing pro-metastatic functions induced by TGF-β [66]. FAF1 overexpression in pre-osteoblastic cells resulted in suppression of endogenous Wnt-induced genes and decreased osteoblast differentiation, and in relation with this our group has reported that LM7 cells present lower osteoblastic differentiation potential in contrast to SAOS2 cells [36, 67]. This evidence poise a novel advantage for FAF1 expressing OS cells irrespective of its role in the FAS-mediated apoptosis response and adds to the picture as a possible regulator of tumor cells survival upon lung arrival.

We demonstrated an increase in cell adhesion towards microvascular endothelium in LM7 cells. Proteomic analysis revealed that both cell types expressed proteins associated with this biological process like integrins, catenins and cell adhesion molecules (CAM), with protein relative levels overall higher in SAOS2. This would point to a higher adhesive behavior of non-metastatic cells at a primary tumor site, but the selective advantage of metastatic cells to highly adhere to endothelium would relate to the ability of being retained in the lung' microvessels and to colonize the target site [68]. Of interest, ALCAM, a molecule involved in mechanisms associated to cell intravasation was identified as an upregulated protein in metastatic cells (see Fig. 2c), supporting this notion. Further, ALCAM was associated to metastasis to bone in a primary prostate model, associating an antiapoptotic function to this protein based on the intracellular signaling that implicates ALCAM [69]. Relevant to a role in metastases, antibody neutralization of ALCAM was demonstrated to significantly reduce tumor cells colonization into the brain using metastatic breast carcinoma models [70], and the expression of this molecule could relate to an overall function in favoring migration of mobile cells like metastasizing cells, mediating cell–cell-interactions in general [71].

From our results, a picture emerges that depicts a heterogeneous OS tumor site of pathologic bone remodeling with selection of advantageous properties in bone residing cells allowing lung colonization. An overall molecular balance may shift into one or the other side of survival or death, which may co-occur independently of the presence or absence of Fas. We identified proteins that are pro-apoptotic in a context where Fas is present like FAF1, but its participation also in prosurvival pathways could present a scenario in which not only Fas negative OS cells could colonize the lungs. To our knowledge, this notion is novel and little explored, deserving more investigation to allow for the manipulation of the permissive soil for metastasis to occur.

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Interestingly, Kaplan–Meier curves for FAF1 predict that a higher expression is associated to lower metastasis-free survival (Supplementary material Fig. 1). A pathological analysis involving new vessel formation would not be clinically useful as indication of metastatic potential. Further, a molecular pattern associated to apoptosis and survival was presented in cells with divergent metastatic potential. Identification of novel molecules in OS cells with metastatic features would allow for a prompt validation of molecules with biomarker usefulness in a disease where the existence of non-detectable lung micrometastases remains as a critical clinical challenge.

Availability of data

The authors confirm that the data supporting the findings of this study are available within the article or its supplementary information.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10495-021-01677-x.

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Author contributions LMG and MVA performed experiments, analyzed data and designed figures; FS, MJ., MGG and YY performed experiments; AC conducted proteomic analysis and analyzed data; ESK, MGG and LA contributed with essential reagents and analyzed data; ESK provided OS cells. ESK and AC contributed with paper revision; MB conceived research and experiments and analyzed data. Authors discussed and commented the results on the manuscript.

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Declarations

Conflicts of interest Authors declare that no competing financial interests or conflicts of interest exist.

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