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Extracellular vesicles from osteosarcoma cell lines contain miRNAs associated with cell adhesion and apoptosis.

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Abstract

Osteosarcoma is the most common primary bone tumor during childhood and adolescence. Several reports have presented data on serum biomarkers for osteosarcoma, but few reports have analysed circulating microRNAs (miRNAs). In this study, we used next generation miRNA sequencing to examine miRNAs isolated from microvesicle-depleted extracellular vesicles (EVs) derived from six different human osteosarcoma or osteoblastic cell lines with different degrees of metastatic potential (i.e., SAOS2, MG63, HOS, 143B, U2OS and hFOB1.19). EVs from each cell line contain on average ~300 miRNAs, and ~70 of these miRNAs are present at very high levels (i.e., more than 1,000 reads per million). The most prominent miRNAs are miR-21-5p, miR-143-3p, miR-148a-3p and 181a-5p, which are enriched between 3 to 100 fold and relatively abundant in EVs derived from metastatic SAOS2 cells compared to non-metastatic MG63 cells. Gene ontology analysis of predicted targets reveals that miRNAs present in EVs may regulate the metastatic potential of osteosarcoma cell lines by potentially inhibiting a network of genes (e.g., MAPK1, NRAS, FRS2, PRCKE, BCL2 and QKI) involved in apoptosis and/or cell adhesion. Our data

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Authors' contributions

MG and AJvW conceived and directed the study. MG, AJvW, RT, CEI and SJ designed of experiments. SJ, HA and DH performed experiments and contributed to the data acquisition. SJ, AJvW and MG performed data analyses and computational studies, and provided interpretation of the results. SJ, AJvW and MG wrote and prepared the manuscript with comments from all authors. All authors reviewed the manuscript and approved the final version.

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indicate that osteosarcoma cell lines may selectively package miRNAs as molecular cargo of EVs that could function as paracrine agents to modulate the tumor micro-environment.

Keywords

Cancer; Osteosarcoma; Metastasis; Cell aggressiveness; Exosomes; MicroRNA

1. Introduction

Bone malignancies are the third leading cause of cancer-related death in children and adolescents (Siegel et al., 2018) . Osteosarcoma is the most frequent primary tumor in bone and is the fourth most frequently treated solid tumor in pediatric patients (Mirabello et al., 2009b; Ward et al., 2014). Worldwide osteosarcoma incidence rates have a bimodal age distribution with a primary peak in incidence occurring in children and adolescents ages 0 to 24 years, followed for a plateau of very low incidence (25 to 59 years) and a secondary peak in elderly (60 to 85 years) (Mirabello et al., 2009a; Mirabello et al., 2009b). The earlier incidence osteosarcoma peak observed in children and adolescent is affecting roughly between 200 to 2,000 patients on different continents across the globe (Mirabello et al., 2009a). Although osteosarcomas are not very common, osteosarcoma patients which do not received chemotherapy treatment will develop metastatic disease after surgical resection at rates close to 80%, thus showing the aggressiveness of this bone cancer (Harris et al., 1998; Marina et al., 2004).

One of the parameters predicting the clinical outcome of osteosarcomas is the presence of metastasis at diagnosis, which counts for about 10-20% of all patients, while 30-40% of patients without metastasis at diagnosis will relapse and develop metastasis during progression of disease independently of treatment (Bhattasali et al., 2015; Daw et al., 2015; Isakoff et al., 2015). Standard clinical care for the treatment of osteosarcomas is dose-intensive multi-agent chemotherapy (pre- and post-operatively) combined with limb-sparing surgery or amputation (Daw et al., 2015; Guillon et al., 2011). Osteosarcoma derived metastasis are mostly located within the lungs (85-90%) and are the leading cause of death from this disease (Bhattasali et al., 2015; Isakoff et al., 2015) . Metastatic osteosarcoma is associated with poor prognosis with overall survival rates remaining at ~20% after five years (Allison et al., 2012; Friebele et al., 2015; Saraf et al., 2018; Zhu et al., 2013). Therefore, it is of considerable interest to identify new molecular therapeutic targets that specifically interfere with osteosarcoma tumor progression within their tumor niche to reduce the incidence of lung metastasis.

Previously, our laboratories have analyzed several cellular and molecular processes related to normal osteoblast growth and differentiation, as well as osteosarcoma progression and metastasis. For example, we have investigated molecular mechanisms mediating cell cycle control and osteoblast phenotype retention in osteoblasts or osteosarcoma (Galindo et al., 2005; Lucero et al., 2013; Pratap et al., 2003; Varela et al., 2016), pathways controlling cell motility and migration (van der Deen et al., 2012), paracrine signalling and Wnt-related cell signalling (Araya et al., 2018; Bravo et al., 2018; Galindo et al., 2007; Vega et al., 2017), as

well as DNA sensitivity to radiation (Mamo et al., 2017). We also have examined oncogenic deregulation of mechanisms controlling the activities of tumor suppressor (e.g., p53 and pRB) (Pereira et al., 2009; San Martin et al., 2009; van der Deen et al., 2013), as well as mechanisms of tumor cell adhesion and metastasis (Villanueva et al., 2019). We have also examined miRNA expression in primary osteosarcomas and osteoblastomas (Riester et al., 2017), similar to other studies that examined osteosarcoma related non-coding RNAs (e.g., lncRNAs, circRNAs and miRNAs (Lin et al., 2017; Otoukesh et al., 2018; Sun et al., 2014; Zhang et al., 2017). Recently, we investigated molecular components of the osteosarcoma secretome to understand paracrine effects of secreted proteins or extracellular vesicles in the microenvironment that are associated with osteosarcoma tumor progression and metastasis (Jerez et al., 2017; Villanueva et al., 2019).

In many cancer cell types, secreted factors such as soluble proteins, macromolecular complexes and extracellular vesicles (EVs) have been associated with tumor progression and poor prognosis (Makridakis and Vlahou, 2010; Paltridge et al., 2013). EVs may act as tumor messengers carrying proteomic, genomic and transcriptomic information from one cell to another within the same tumor or to other locations distal from the primary tumor (Lobb et al., 2017) to facilitate the preparation of new niches for future metastasis. Because EVs may convey information from the primary tumor, we performed proteomic analysis of osteosarcoma EVs (Jerez et al., 2017) and showed that EVs carry proteins involved in tumor progression process. In this work, we examined the small non-coding RNA content of EVs from five distinct osteosarcoma cell lines compared to the corresponding parent cells, because miRNAs may control protein expression through effects on mRNA translation and/or stability (Hammond, 2005; Romero-Cordoba et al., 2014; Ul Hussain, 2012) . MiRNAs have been implicated in cancer progression (Croce, 2009; Wang and Wang, 2012; Zhang et al., 2010) either by reduced expression of miRNAs that suppress oncogene expression (Negrini et al., 2009) or by elevated expression of oncomirs (Esquela-Kerscher and Slack, 2006) that inhibit the activity of tumor suppressor genes that normally block tumor suppression by preventing excessive cell proliferation or migration. Identification of EV specific miRNAs in osteosarcomas could lead to new miRNA based therapeutic strategies that increase or decrease miRNA levels in bone cancer patients (Bader et al., 2010; Nana-Sinkam and Croce, 2011).

To establish osteosarcoma specific gene expression patterns and identify new osteosarcoma biomarkers with diagnostic or prognostic potential, several studies have examined miRNA expression during normal osteoblast differentiation or in osteosarcomas focusing on intracellular or circulating miRNAs as part of macromolecular complex (Kushlinskii et al., 2016; Lulla et al., 2011; Maire et al., 2011; Ouyang et al., 2013; van Wijnen et al., 2013). Our study characterizes miRNAs from EVs derived from osteosarcomas to identify potential secreted/circulating miRNA molecules that may participate in osteosarcoma tumor progression or metastasis.

2. Methods

2.1. Cell lines

Human osteosarcoma cell lines (SAOS2, MG63, U2OS, HOS, and 143B) and an immortalized human fetal osteoblast cell line (hFOB1.19) were obtained from the American Type Culture Collection (ATCC) and maintained as recommended. SAOS2 and U2OS cells were cultured in McCoys 5A medium (Sigma–Aldrich, St Louis, MO, USA) supplemented with 15% and 10% of fetal bovine serum (FBS) (HyClone Laboratories Inc, Logan, UT, USA), respectively. MG63, HOS, and 143B cells were maintained in DMEM (Gibco, Grand Island, NY, USA) with 10% FBS. HFOB1.19 cells were maintained in DMEM/F12 with 10% FBS. All culture media were supplemented with L-glutamine (2mM) and penicillin-streptomycin cocktail (100 units/mL).

2.2. EVs and RNA Isolation

EVs were isolated from conditioned media by ultracentrifugation as previously described (Jerez et al., 2017) . Briefly, 48-hrs serum-free conditioned media were clarified by centrifugation to remove cell debris, filtered and concentrated through 0.22 μm, and ultra-centrifuged at 100,000g. RNA for RNA sequencing was isolated using Total Exosome RNA and Protein Isolation Kit from Invitrogen (Thermo Fisher Scientific, Waltham, MA, USA). Small RNA for qPCR was isolated using mirVana miRNA Isolation kit from Invitrogen (Thermo Fisher Scientific). As exogenous control, a heterologous microRNA (i.e., cel-miR-39-5p) was included before RNA isolation in all samples at 20 pM. RNA was quantified using a MaestroNano Micro-Volume Spectrophotometer (Maestro-Gen, Taiwan) and Bioanalyzer 2100 with 2100 expert software (Agilent Technologies, Santa Clara, CA, USA).

2.3. EVs size and concentration analysis

The size distribution and concentration of EV preparations were directly determined using a NanoSight N300 nanoparticle tracking analysis (NTA) device (Malvern Instruments, Malvern, UK). Before each session, NanoSight equipment was calibrated by determining the size and concentration of latex polystyrene beads (Malvern Instruments, Cat. No.: NTA4088, 100 nm). The camera level was set to 9, the temperature to 20°C and an arbitrary threshold of 5 was used. Serum-free culture medium (5μL) was diluted 500 times in PBS to obtain a solution free of extracellular microvesicles (ECMVs) as negative control. For each sample, 2 videos of 60s with more than 200 detected tracks per video were taken and analyzed using NTA software 2.3 with default settings that apply the Stokes-Einstein Equation to determine the size of the particles from their Brownian motion.

2.4. miRNA sequencing

MicroRNAs were sequenced using the NEBNext Small RNA library prep kit on an Illumina HiSeq 2000 as previously described (Martin, 2011) (Riester et al., 2015; Riester et al., 2017). Short reads were trimmed of adapters with Cutadapt. Trimmed miRNA sequences greater than 17 nucleotides in length were then aligned to the reference genome and miRBase reference sequences using Bowtie (Langmead et al., 2009). Expression of known

miRNAs and prediction of novel miRNAs prediction was performed using miRDeep2 (Friedlander et al., 2008) with the CAP-miRSeq analysis pipeline (Sun et al., 2014).

2.5. Data Analysis

All in silico data analyses were carried out using miRNAs with expression of at least 10 normalized reads per million (RPM) for further analysis. Unsupervised hierarchical clustering was performed using the Pearson correlation method with the Morpheus matrix visualization platform (Broad Institute, Cambridge, MA, USA). Target prediction for individual miRNAs was carried out using TargetScan (Lewis et al., 2005). Prediction of target genes for miRNA sets for selected cell line was also performed using the computational online tool ComiR for combinatorial microRNA target prediction (Coronnello and Benos, 2013). Gene Ontology analysis for biological processes associated with miRNA target genes was performed using DAVID 6.8 (Huang da et al., 2009a; Huang da et al., 2009b), and gene networks of predicted miRNA targets were created with STRINGv11 (Szklarczyk et al., 2019).

2.6. Real-Time qPCR validation

Selected miRNAs that were differentially expressed based on RNA-seq data were validated using TaqMan® microRNA assays (Life Technologies, Carlsbad, CA, USA). Real-time qPCR reactions were performed using the stably expressed hsa-miR-103a-3p as endogenous reference (Riester et al., 2015; Riester et al., 2017). MiRNA expression levels were quantified using the 2ΔCt method. TaqMan® microRNA assays were used for hsa-miR-103, hsa-miR-21, hsa-miR-26a, hsa-miR-30, hsa-miR-143, hsa-miR-148a, and hsa-miR-181a and cel-miR-39 (Life Technologies, Carlsbad, CA, USA).

3. Results

3.1. Isolation and characterization of EVs derived from osteosarcoma cells

EVs were isolated from conditioned culture media of five human osteosarcoma cell lines. Two of these lines are considered metastatic (SAOS2 and 143B), whereas three are non-metastatic (MG63, U2OS and HOS) based on data from a recent study (Ren et al., 2015). We also examined the non-tumorigenic immortalized osteoblastic hFOB1.19 cell line. As previously reported, the EVs we derived from osteosarcoma cells resemble exosomes, because they present as cup-shaped by transmission electron microscopy and express characteristic exosomal markers (i.e., CD9, CD63 and CD81) (Jerez et al., 2017). We analyzed the diameter sizes and concentrations of isolated EVs by nanoparticle tracking analysis (NTA) using a NanoSight device. NTA shows that size distributions for EVs derived from all six cell lines are unimodal and generally range from 30 to 250nm (Fig. 1A). EVs preparations from each of the cell lines show differences in size distribution (MG63, 80-120nm; SAOS2, 40-120nm; HOS, 110-160nm; 143B, 30-220nm; U2OS, 60-200 nm; hFOB1.19; 30-250nm). The most frequently occurring diameter sizes (mode) of EVs derived from osteosarcoma cells (Fig. 1B) show less variation and ranges between 84 to 126 nm (i.e., SAOS2: 84nm; MG63: 105nm; HOS: 109 m; 143B: 115nm; U2OS: 119nm; hFOB1.19: 126nm). The amount of secreted EVs normalized per million cells shows that hFOB1.19, SAOS2 and MG63 produce significantly more EVs (respectively, 526×10^6

EVs, 465 × 10^6 and 418 × 10^6 EVs per million cells) than HOS, U2OS and 143B cells (respectively, 166 × 10^6, 153 × 10^6, and 100 × 10^6 EVs per million of cells) (Fig. 1C). In addition, the total amounts of RNA packaged into EVs ranges between 7 and 36ng RNA per cell line (per 10^6 cells, respectively, MG63: 36ng, SAOS2: 35ng, hFOB1.19: 19ng, HOS: 12ng, U2OS: 10ng, and 143B: 7ng) (Fig. 1D). The measured range and mode of diameter sizes for the EVs, the amount of secreted EVs per cell, as well as the amount of RNA packaged into EVs is in accordance with expected values for each of these descriptive parameters of EVs. We note that the five osteosarcoma cell lines produce a smaller number of EVs, which are typically smaller in size than EVs produced by non-tumor derived hFOB1.19 cells. These differences may perhaps reflect distinctions in biological properties between the various cell lines. Furthermore, neither the number and size of EVs, nor the amount of RNA packaged into EVs correlates with the biological status of osteosarcoma cell lines as metastatic or non-metastatic, similar to previous observations we made for the amount of protein delivered into EVs from osteosarcoma cells (Jerez et al., 2017)

3.2. Next generation RNA sequencing and identification of EV miRNAs

Analysis of RNA size distribution for RNA preparations from EVs revealed presence of small RNAs (~20 nucleotides) (Fig. 2). Therefore, we performed next generation sequencing to identify miRNAs enriched in EVs in our panel of six cell lines. Sequencing of small non-coding RNAs by miRNA sequencing revealed a total of 400 to 600 distinct miRNAs in EVs from each of the cells lines (listed in Supplementary file S1). We detected 237 miRNAs that were exclusively detected in EVs for any of the osteosarcoma cell lines and 77 miRNAs were unique for hFOB1.19 cells. The majority of these miRNAs have previously described in EXOCARTA as present in EVs from other cell types (Fig. 3A), and this is particularly evident if only the most abundant miRNAs are considered (i.e., RPM>1,000) (Fig. 3B). Thus, our analysis appears to detect EV miRNAs with a high degree of confidence. We examined the number of miRNAs exclusively detected in EVs from metastatic (SAOS2 and 143B) versus non-metastatic cells (MG63, U2OS and HOS). This analysis reveals that SAOS2 and 143B cells secrete 50 versus 209 exclusive miRNAs, and 7 versus 43 of these miRNAs are highly expressed (Figs. 3C and D). Comparison of miRNAs in EVs secreted by metastatic 143B cells (i.e., derived from non-metastatic HOS cells by vKi-ras transformation) versus HOS cells, shows that 143B and HOS cells secrete, respectively, 24 versus 225 exclusive miRNAs, and 8 versus 34 of these miRNAs are highly expressed. Thus, a trend emerges suggesting that metastatic osteosarcoma cells may perhaps secrete a more restricted set of cell line-specific miRNAs compared to non-metastatic osteosarcoma cells (Figs. 3C and D). Unsupervised hierarchical clustering of expression patterns of EV miRNAs detected in our panel of six cell lines revealed that metastatic SAOS2 and 143B cells cluster differently from non-metastatic MG63 and hFOB1.19 cells (Figs. 3A and E). The same results were found regardless of whether all expressed miRNAs were examined, or whether the analysis was limited to strongly expressed miRNAs (>1000 RPM) (Figs. 3B and 4A). Taken together, the detection of many cell line specific miRNAs in the six cell lines indicates considerable heterogeneity in the presence of miRNAs in EVs, consistent with the distinct biological phenotypes of each of these cell types.

3.3. Predicted target genes of EV miRNAs relate to tumor progression and metastasis

MiRNAs in cancer cells act as post-transcriptional regulators of their mRNA targets to control expression of genes involved in tumor progression. To understand the biological activities of highly abundant EV miRNAs for each cell line, we performed gene ontology analysis of potential target genes (Figs. 4A and B). We observed that the different sets of predicted target genes for each miRNA converge toward similar annotation clusters. Clusters with enrichment scores >1 for each of the miRNAs contain essentially the same biological gene ontology categories that are directly or indirectly related to tumorigenesis or metastasis (Fig. 4B). For example, putative target genes for miRNAs in EVs from metastatic SAOS2 cells form annotation clusters for a large number of biological categories, including glycoprotein biosynthesis and protein transport/localization process, cell adhesion, regulation of apoptosis, lung development, lymphoid organ and immune system development, lymphocyte differentiation and cytokine production, as well as the TGFβ receptor signalling pathway and regulation of kinase activity (Fig. 4B). These ontology categories are related to tumor progression properties such as immune evasion, survival and lung metastasis, consistent with the general idea that miRNAs in EVs may contribute to the metastatic potential of osteosarcoma cells.

3.4. Identification of specific EVs miRNAs relate to tumor progression and metastasis

To identify specific miRNAs that may support tumor progression and metastasis, we compared the top 50 most abundant EV miRNAs from metastatic SAOS2 cells with those from non-metastatic MG63 cells. Six miRNAs with the highest fold changes (FC) of greater than 5 fold between these cell types were selected (Figs. 5A and B): miR-21-5p, miR-30d-5p, miR-26a-5p, miR-143-3p, miR-181a-5p and miR-148a-5p. We also monitored specific enrichment of each miRNA in EVs by comparing miRNA abundance in EVs with expression of these miRNAs in the corresponding cell type (Figs. 5C and D) using RT-qPCR with Taqman probes. Expression levels of intracellular miRNAs were normalized using miR-103-3p, which is a validated endogenous control for osteosarcoma samples (Riester et al., 2017). Spike-in methodology using cel-miR-39 at the moment of the RNA isolation was used to normalize EV-miRNA expression levels. The results show that SAOS2 cells have much higher intracellular expression levels of miR-143-3p, miR-21-5p, miR-181a-5p and miR-148-5p than MG63 cells (>3 fold). Similarly, these same miRNAs are also higher in EVs from SAOS2 cells compared to MG63 cells (>2 fold). Because these four miRNAs exhibit higher expression levels in metastatic SAOS2 cell line compared to non-metastatic MG63 cells, we focused our studies on the putative targets of this set of four microRNAs.

3.5. EV miRNAs in SAOS2 cells are predicted to target metastasis related genes.

We investigated the putative mechanisms by which miRNAs enriched in EVs could potentially support the metastatic potential of SAOS2 cells. We retrieved target genes for miR-143-3p, miR-21-5p, miR-181a-5p and miR-148-5p from the TargetScan database (Figs. 6 and 7). We first prioritized genes that are targeted by three or more of these four miRNAs (Fig. 6A). Remarkably, as many as 75 distinct predicted targets are shared by these four miRNAs group. This set of 75 genes was then subject to network analysis using STRINGv11, and 31 of these genes form a cohesive network (Fig. 6B) with a core of six

very well connected genes (Fig. 6C). Gene ontology (GO) analysis using DAVID 6.8 revealed that this set of 31 networked genes is involved in cell adhesion and apoptosis (Fig. 6D). Examination of all putative targets for each of the four miRNAs is consistent with this conclusion (Fig. 6E). Hence, the four most abundant miRNAs enriched in metastatic SAOS2 cells are predicted to suppress genes required for cell adhesion and apoptosis.

EVs may affect the tumorigenic properties of adjacent osteosarcoma cells through auto-paracrine mechanism. We tested the hypothesis whether miRNAs expressed in metastatic osteosarcoma cells and transmitted in EVs have the potential to control tumor suppressor proteins either within the parent cell or through transmission to adjacent cells via EVs. We investigated which of the predicted miRNA targets for the four selected EV miRNAs (mir-21-5p, mir-143-3p, mir-181a-5p and mir-148a-5p) may target known tumor suppressor genes that are actively expressed in osteosarcoma cells. First, we identified the most probable target genes for robustly expressed miRNAs in EVs from SAOS2 and MG63 using ComiR. This analysis yielded a list of 1,000 most probable targets based on miRNA expression levels. A similar analysis was made for the non-tumor cell line hFOB1.19 to permit elimination of miRNAs involved in normal cellular processes that are unrelated to tumorigenesis. This subtraction yielded a list of 343 target genes for SAOS2 EV-miRNAs and 571 target genes for MG63 EV-miRNAs. We filtered this gene list for Tumor Suppressor Genes (TSG) from the TSG Data base (https://bioinfo.uth.edu/TSGene/) (Zhao et al., 2016; Zhao et al., 2013) (Fig. 7A). This filtering revealed multiple predicted tumor suppressor genes that targeted by the four most abundant EV-miRNAs from SAOS2 cells (Figs. 7A and B). We note that one of the miRNAs target the collagen-crosslinking enzyme LOX, while two miRNAs target the MMP inhibitor TIMP3. The possibility arises that SAOS2 related miRNAs may influence metastatic potential of osteosarcomas in part by modulating ECM remodelling. The results presented in Figures 6 and 7 suggest that the miRNA cargo present in EVs from metastatic osteosarcoma cells may promote both tumorigenic and metastatic properties (Fig. 8).

4. Discussion

The content of tumor-derived EVs represents a potential source of possible tumor biomarkers (Duijvesz et al., 2011; Taylor and Gercel-Taylor, 2008) which may be used as diagnostic or prognostic markers, or perhaps represent a source of potential therapeutic targets (Hu et al., 2012; Viaud et al., 2010). Recent studies suggest that in EVs from tumors may modulate tumor growth and/or metastasis either positively or negatively (Aigner, 2011; Al-Nedawi et al., 2009; Trang et al., 2008). Proteomic analysis of osteosarcoma derived EVs revealed that these vesicles contain different proteins involved in tumor progression (Jerez et al., 2017). In this study, we characterized the miRNA content of derived EVs from six distinct osteosarcoma or osteoblastic cell lines. EV preparations from these lines differ to some degree in physical and biochemical properties (e.g., diameter and number of EVs per cell, RNA content), suggesting heterogeneity in the types of EVs that are produced by different cell types. Analysis of miRNAs from osteosarcoma derived EVs by next generation sequencing revealed that EVs from each cell type contains at least 300 distinct miRNAs. Unsupervised hierarchical clustering of the most abundant miRNAs (>1000 RPM) revealed qualitative differences in miRNAs that appear to be preferentially present in metastatic cell

types (e.g., SAOS2 and 143B) as opposed to non-metastatic cell lines (e.g., MG63 and hFOB1.19).

Quantitative RT-qPCR of the four most relevant miRNAs (miR-181a-5p, miR-143-3p, miR-21-5p and miR-148a-3p) showed that those are highly abundant in EVs from SAOS2 cells compared to MG63 cells. Of these, miR-21 represents a classical oncomir and is present at elevated levels in many different tumors compared to normal tissue (Ren et al., 2016). MiR-181a-5p and miR-148a-3p are very abundant in EVs from metastatic SAOS2 cells and have been characterized as circulating miRNAs in serum samples from gastro-intestinal cancer patients (Ghaedi et al., 2019; Jacob et al., 2017). MiR-181a-5p has been reported to play a role in tumor progression, because it is overexpressed in gastric cancer (Kim et al., 2011; Zhang et al., 2012) , breast cancer (Mansueto et al., 2010; Taylor et al., 2013), and colorectal cancer (Takahashi et al., 2012). In addition, miR-181a-5p may represent a biomarker for endometrial cancer (Yanokura et al., 2010). MiR-148a-3p is overexpressed in osteosarcoma biopsies (Ma et al., 2014; Maire et al., 2011) and is a potential serum biomarker for thymic ephitelial cancer (Bellissimo et al., 2016). Less is known about miR-143-3p, but its elevated expression in SAOS2 cells is incongruent with recent studies suggesting that this miRNA may counter metastatic properties of squamous cell carcinoma (Han et al., 2018). Our gene ontology analysis of predicted target genes for miR-181a-5p, miR-143-3p, miR-21-5p and miR-148a-3p suggests that these miRNAs control proteins that regulate apoptosis, angiogenesis, cell adhesion and regulation of cell migration. Taken together, the four most abundant miRNAs in EVs from metastatic SAOS2 cells have previously established biological roles in different cancer types.

5. Conclusions

Characterization of metastatic and non-metastatic osteosarcoma cell lines reveals that they each produce EVs with distinct miRNA cargo. These miRNA are predicted to target a network of genes that may suppress cell adhesion and apoptosis, thus perhaps increasing the metastatic potential of osteosarcoma cells. Because miR-21-5p and miR-143-3p show higher levels in the metastatic osteosarcoma cell lines, it is possible that these miRNAs could find use as prognostic biomarkers for osteosarcoma. We conclude that miRNAs from osteosarcoma cell lines that are packaged into EVs may be transmitted to cells within the osteosarcoma micro-niche as part of as paracrine mechanism to promote osteosarcoma growth or metastasis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

miRNA	microRNA
EV	extracellular vesicle
RPM	Reads Per Million mapped reads

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Highlights: Extracellular vesicles from osteosarcoma cell lines contain multiple microRNAs, including miR-21-5p, miR-143-3p, miR-148a-3p and 181a-5p that are abundant in SAOS2 cells. Common target genes for these miRNAs may form a gene network that could control the metastatic potential of osteosarcoma cells. The data suggest that osteosarcomas produce extracellular vesicles with molecular cargos that are predicted to modulate the osteosarcoma microenvironment.

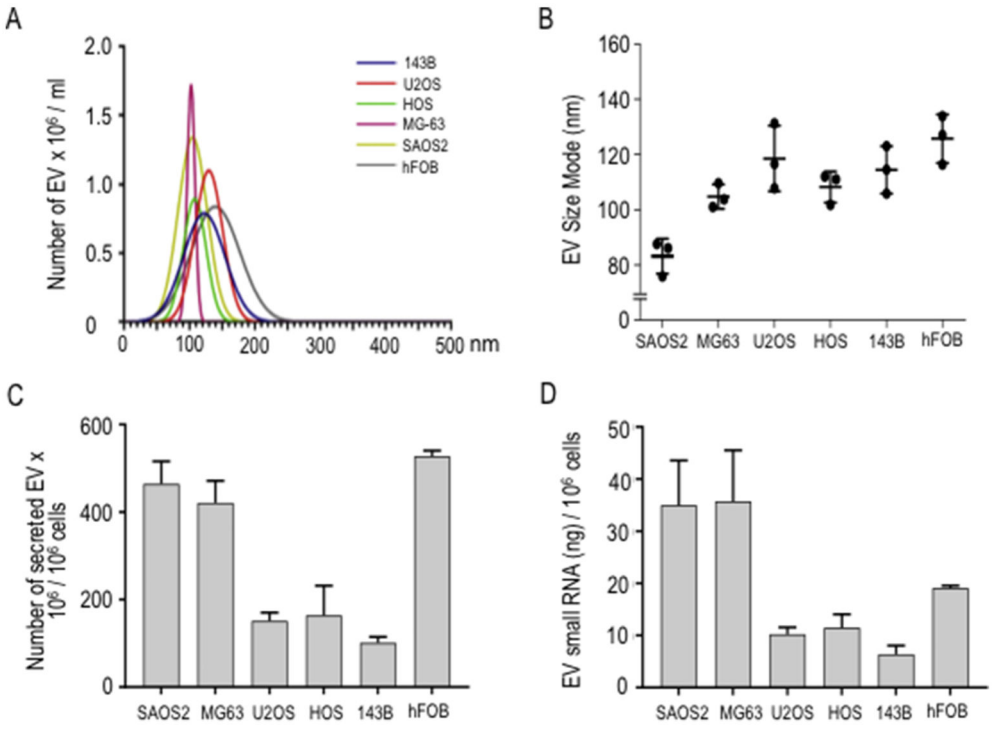


Fig. 1.

Characterization of exosome-rich extracellular vesicles (EVs) derived from human osteosarcoma or osteoblastic cell lines. EVs were isolated from 24 hours conditioned culture medium obtained from osteosarcoma (SAOS2, MG63, U2OS, HOS, and 143B) and osteoblastic (hFOB1.19) cells. Diameter size (nm) distribution and concentration of purified EVs were determined by NTA (**A**), and mode size (nm) of three replicates were plotted (**B**). Relative number of secreted EVs contained in equal volume aliquots of purified EVs was normalized to 1 million of cells (**C**). Relative concentration of small RNA (ng) into EVs contained in equal volume aliquots of purified EVs was determined and normalized to amount of EVs secreted by 1 million cells (**D**).

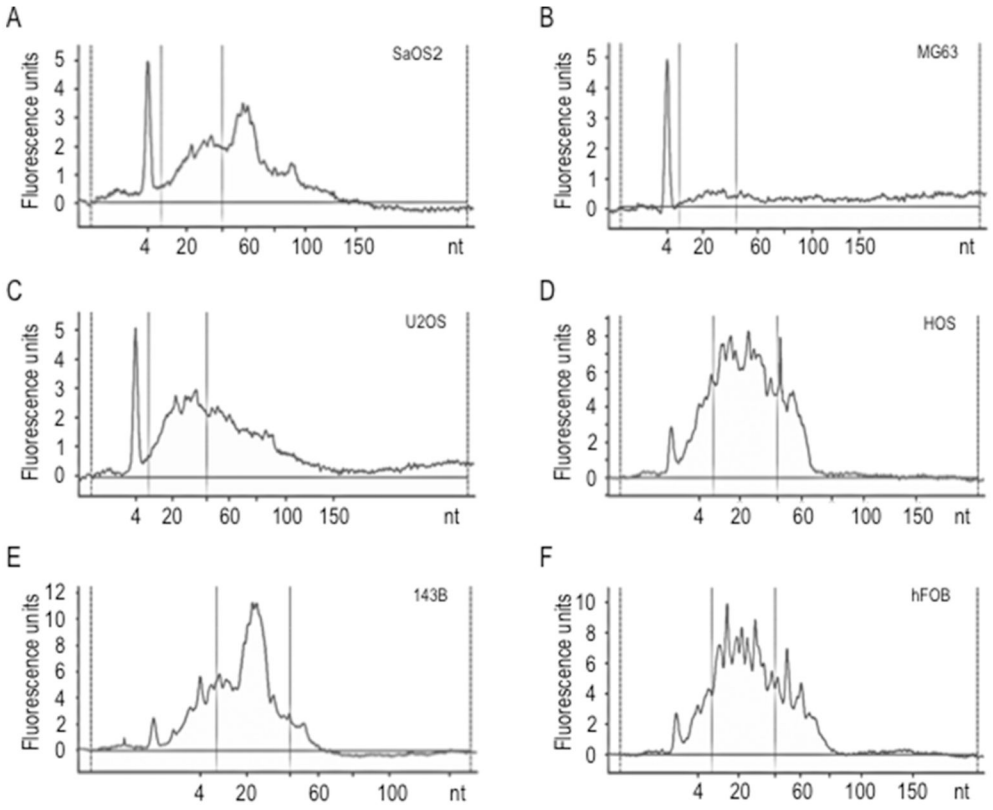


Fig. 2.

Characterization of small RNAs purified from EVs derived from human osteosarcoma or osteoblastic cell lines. Bioanalyzer electropherograms for each cell line (**A**, SAOS2; **B**, MG63; **C**, U2OS; **D**, HOS; **E**, 143B; **F**, hFOB) show EVs small RNA molecules abundance, expressed as Fluorescence Units [FU] according to molecule nucleotide [nt] extension.

A

Total exosomal miRNAs derived from osteosarcoma or osteoblastic cell lines annotated in Exocarta					
Cell line	Exocarta annotated		Not described in exocarta		Total
SAOS2	280	90.3 %	30	9.7 %	310
MG63	159	90.3 %	17	9.7 %	176
U2OS	205	87.6 %	29	12.4 %	234
HOS	389	82.2 %	84	17.8 %	473
143B	239	87.9 %	33	12.1 %	272
hFOB	382	90.5 %	40	9.5 %	422

B

Exosomal miRNAs >1,000 RPM derived from osteosarcoma or osteoblastic cell lines annotated in Exocrta					
Cell line	Exocarta annotated		Not described in exocarta		Total
SAOS2	73	100 %	0	0 %	73
MG63	72	97.3 %	2	2.7 %	74
U2OS	78	96.3 %	3	3.7 %	81
HOS	90	100 %	0	0 %	90
143B	64	100 %	0	0 %	64
hFOB	77	100 %	0	0 %	77

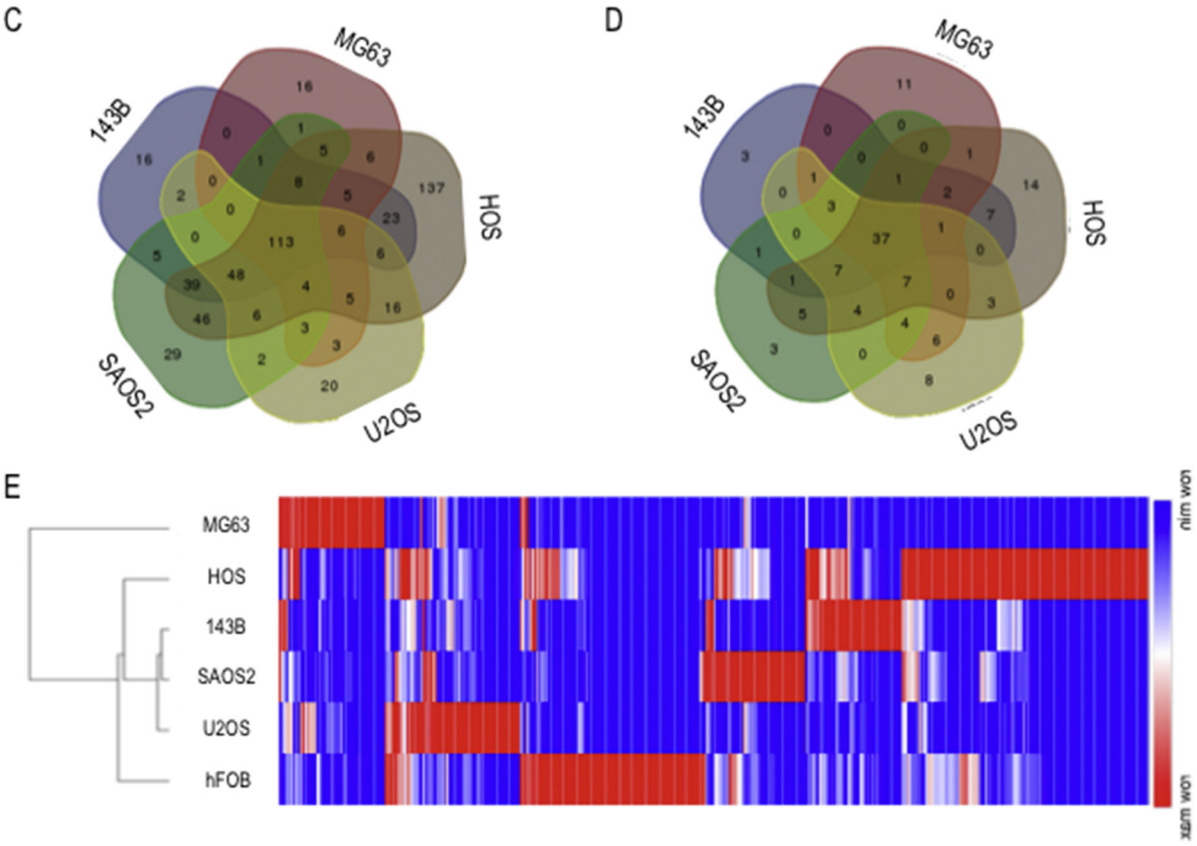


Fig. 3.

Analysis of EVs miRNAs derived from osteosarcoma or osteoblastic cell lines. Total miRNAs identified by NGS **(A)** and only robustly expressed miRNAs with more than 1000 RPM **(B)** were compared to those previously annotated in Exocarta data base (http://www.exocarta.org/), that include miRNAs previously identified on exosomes. 5-sets venn diagrams of total miRNAs **(C)** and robustly expressed miRNAs **(D)** show distribution of miRNAs among osteosarcoma cell lines. Unsupervised hierarchical clustering analysis (Pearson correlation method) of all EVs miRNAs detected in osteosarcoma or osteoblastic cell lines **(E)**.

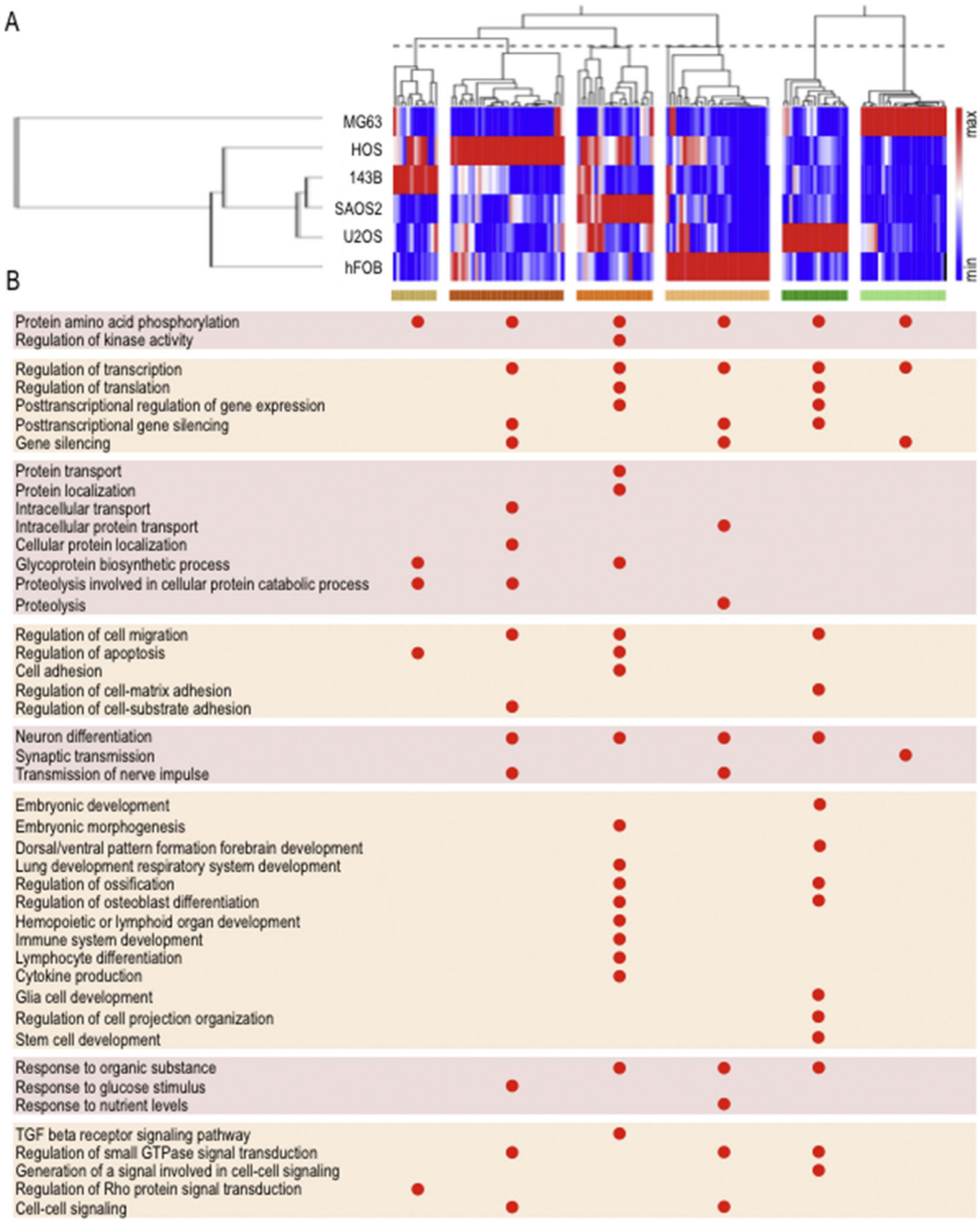


Fig. 4.

Gene ontology analysis of predicted target genes of EV miRNAs revealed biological categories related to tumor progression and metastasis. Unsupervised hierarchical clustering analysis (Pearson correlation method) of robustly expressed EVs miRNAs in osteosarcoma or osteoblastic cell lines show miRNA clusters according to their high relative expression on each cell line **(A)**. Gene ontology analysis of predicted target genes, revealed by TargetScan (http://www.targetscan.org/), associated to miRNA clusters showed in A was assessed using DAVID 6.8 (http://david.ncifcrf.gov/) to identify pathways and biological processes functionally linked to tumor progression and metastasis **(B)**.

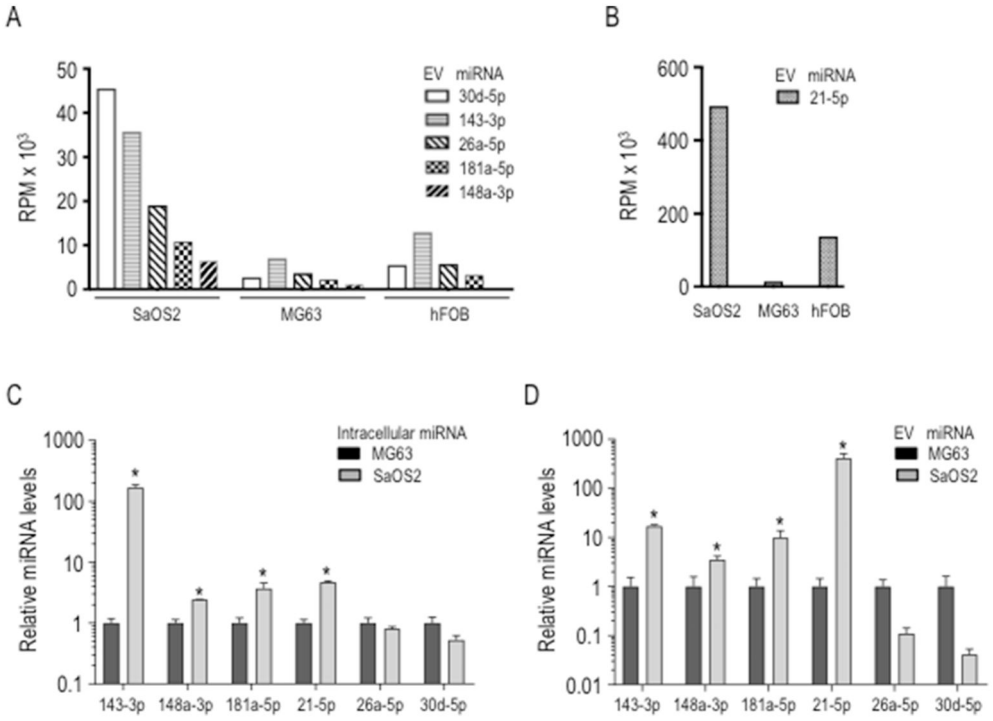
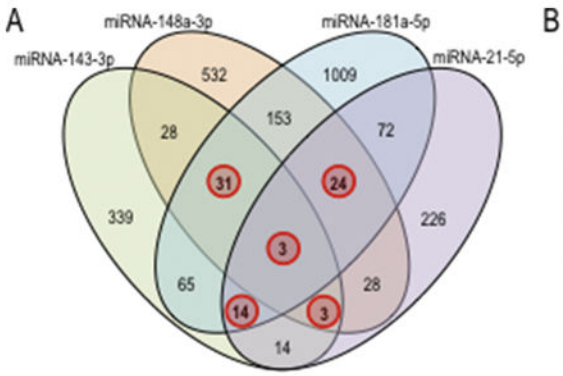
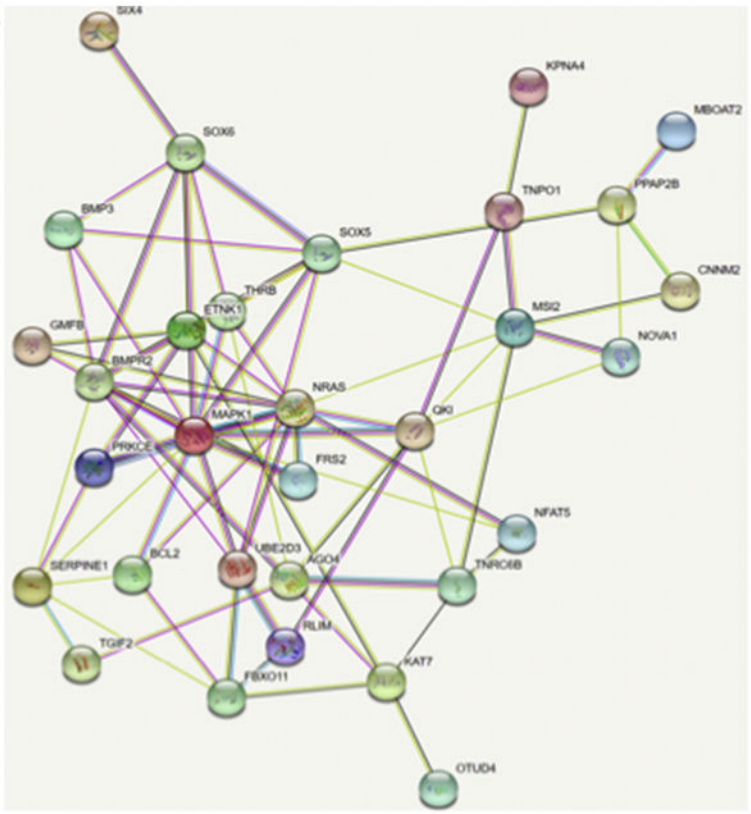


Fig. 5.

Analysis of EVs miRNAs differentially expressed in metastatic versus non-metastatic osteosarcoma cell lines. Expression levels of specific EVs miRNAS: miR-30d-5p, miR-143-3, miR-26a-5p, miR181a-5p, miR-148a-5p **(A)**, and miR-21-5p **(B)** as determined by RNA sequencing in the metastatic (SAOS2) and non-metastatic (MG63) osteosarcoma cell lines as well as the osteoblastic cell line hFOB1.19. MiRNAs expression was validated by qPCR using Taqman probes for both intracellular **(C)** and EV **(D)** compartments.. QPCR values were normalized to endogenous expression of miR-103-3p. Expression levels in SAOS2 cells are shown in Log10 scale relative to MG63.





D

Gene Ontology Terms related to gene core network		
Cluster	Enrichment Score	GO term
1	1.80	PI3K-Akt signaling pathway
2	1.76	MAPK cascade
3	1.57	Apoptotic process
4	1.45	Nucleus
5	1.11	Protein ubiquitination
6	0.92	Kinase
7	0.43	Zinc-finger
8	0.13	Membrane
9	0.05	Glycoprotein

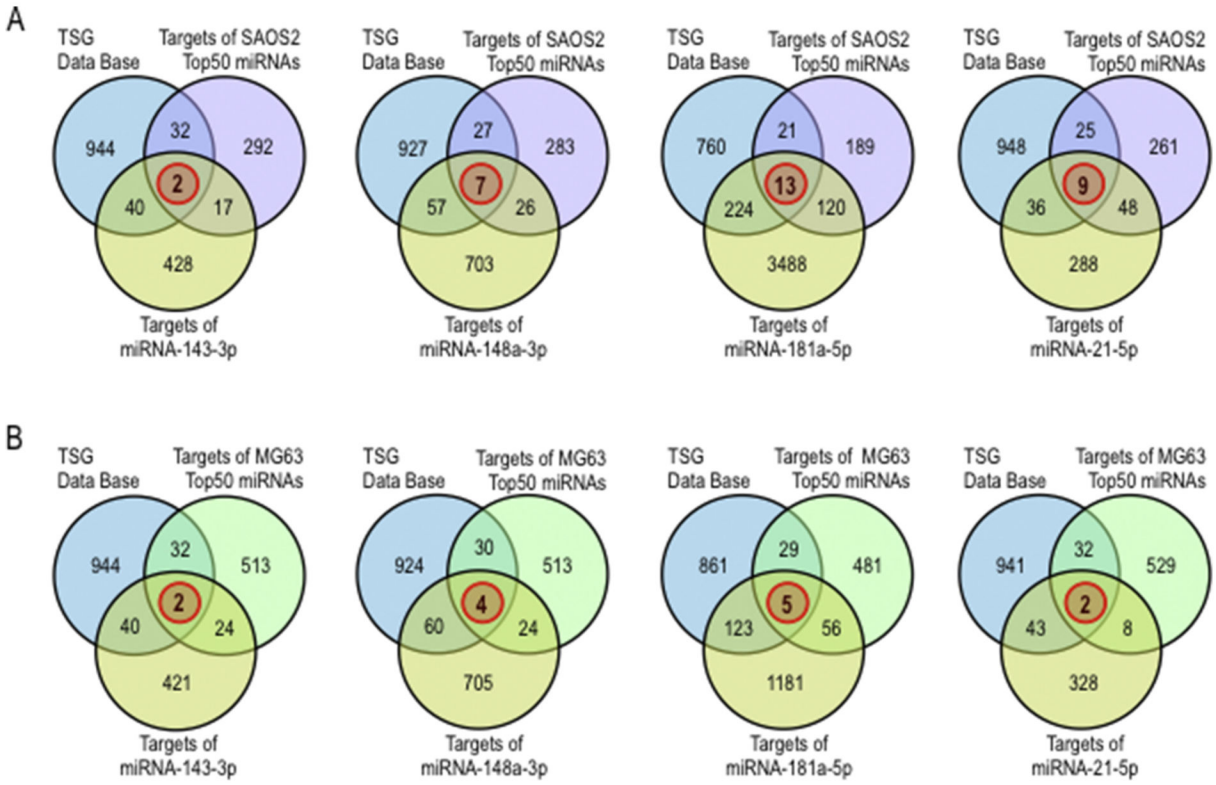
E

GO terms related to Tumor Progression enriched in target genes from measured miRNAs.				
	Enrichment Score (Number of genes)			
GO term	miRNA-143-3p	miRNA-148a-3p	miRNA-181a-5p	miRNA-21-5p
Negative regulation of cell migration	0.52 (6)	1.56 (4)	0.52 (4)	1.59 (5)
Cell migration	2.55 (14)	0.72 (8)	3.45 (27)	1.34 (14)
Regulation of Apoptosis	1.82 (82)	1.33 (34)	2.40 (55)	1.33 (34)
Angiogenesis	2.37 (17)	4.38 (19)	2.42 (10)	1.49 (8)
Adhesion	1.43 (67)	1.63 (10)	1.59 (13)	1.63 (10)

Fig. 6.

Identification of biological processes related to tumor progression and metastasis reveled by physical interaction network analysis of proteins encoding for genes targeted by EVs miRNAs differentially expressed for metastatic osteosarcoma cells. Target prediction for selected EVs miRNAs: miR-143-3, miR181a-5p, miR-148a-5p, and miR-21-5p, differentially overexpressed in the metastatic SAOS2 osteosarcoma cell line, was carried out using TargetScan. 4-Set venn diagram show number of genes (red circle) that are targeted for multiple EVs miRNAs **(A)**. The resulting 75 multiple miRNA target genes were further analysed with STRINGv11 (http://string-db.org/) to identify functional interaction networks containing 31 of these target genes **(B)** and a high confidence core network of six target genes (**C**). The gene network components showed in C (**D**) as well as gene target for each

EVs miRNA (**E**) were analyzed with David 6.8 to identify specific pathways and biological processes functionally linked to tumor progression and metastasis.



C

Candidate Tumor Suppressor Genes targeted by SaOS2 EV miRNAs			
miRNA-143-3p	miRNA-148a-3p	miRNA-181a-5p	miRNA-21-5p
TMEM127	TMEM127	TMEM127	SASH1
AMER1	AMER1	ADARB1	SUZ12
	KLF6	KLF6	KLF6
	RASSF8	RASSF8	RNF111
	DCUN1D3	PGRMC2	DCUN1D3
	MAP3K4	TIMP3	TIMP3
	ZDHHC2	STAT3	STAT3
		COPS2	KRIT1
		ATM	SKP2
		LOX	

Fig. 7.

Identification of predicted tumor suppressor genes targeted by EVs miRNAs differentially expressed for metastatic osteosarcoma cells. Venn diagrams show the significant overlap (red circle) of the target genes of each selected EVs miRNAs: miR-143-3, miR181a-5p, miR-148a-5p, and miR-21-5p, and tumour suppressor genes listed in Tumor Supressor Gene database (TSGene) (http://bioinfo.uth.edu/TSGene/) as well as target genes predicted by ComiR (http://www.benoslab.pitt.edu/comir/) for combinatorial microRNA target prediction for the set of more expressed top 50 EVs miRNA derived from metastatic SAOS2 (A) and

non-metastatic MG63 (B) cell lines, along with the list of tumor supressor genes commonly targeted by these EVs miRNAs in SaOS2 cells **(C)**.

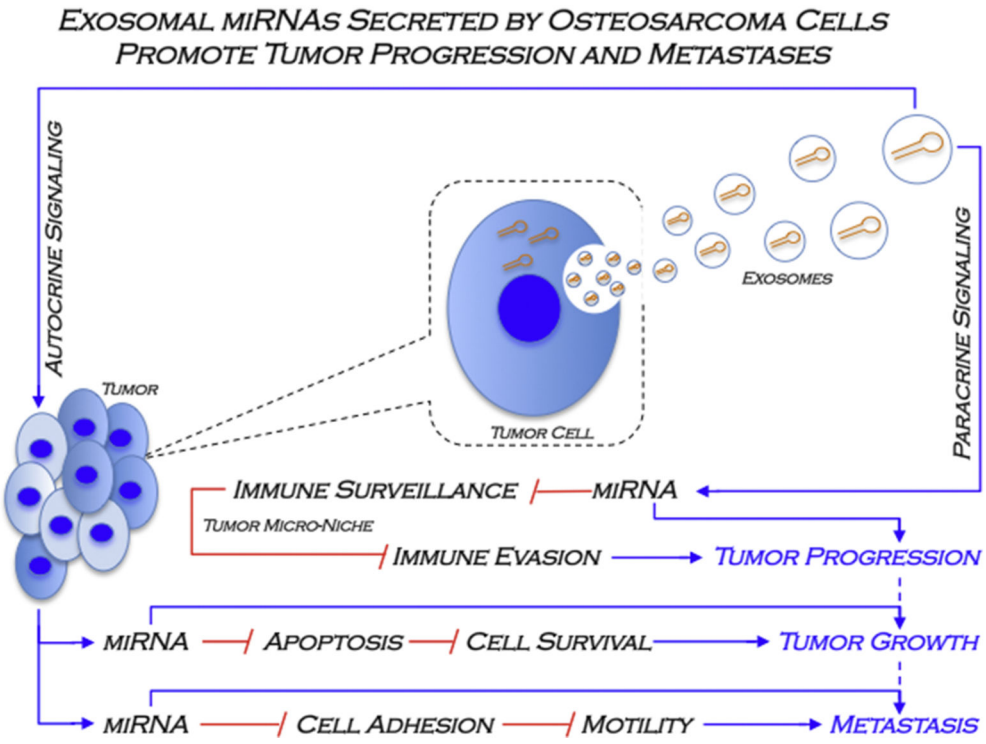


Fig. 8.

A model of the miRNAs secreted into EVs by metastatic osteosarcoma cells in tumor progression and metastasis. Previous studies provided evidence that SAOS2 cell, which exhibit metastatic properties in pre-clinical osteosarcoma animal models, secrete EVs that were characterized as exosomes. The current study shows that intracellular miRNAs can be packaged in EVs (exosomes), which are secreted to the extracellular milieu to establish cell-cell communication at the tumor microenvironment. These EVs miRNAs potentially modulate expression of target genes related to different gene ontology, defining putative pathways and biological categories related to the autocrine (e.g. apoptosis, cell adhesion, cell migration) and paracrine (e.g. immune evasion) control of tumor progression and metastasis in osteosarcoma.