Breast Cancer Research and Treatment (2019) 174:129–141
https://doi.org/10.1007/s10549-018-5043-0

PRECLINICAL STUDY

Plasma exosomes stimulate breast cancer metastasis through surface interactions and activation of FAK signaling

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Received: 4 July 2018 / Accepted: 8 November 2018 / Published online: 27 November 2018

Abstract

Purpose The interaction between malignant cells and surrounding healthy tissues is a critical factor in the metastatic progression of breast cancer (BC). Extracellular vesicles, especially exosomes, are known to be involved in inter-cellular communication during cancer progression. In the study presented herein, we aimed to evaluate the role of circulating plasma exosomes in the metastatic dissemination of BC and to investigate the underlying molecular mechanisms of this phenomenon.

Methods Exosomes isolated from plasma of healthy female donors were applied in various concentrations into the medium of MDA-MB-231 and MCF-7 cell lines. Motility and invasive properties of BC cells were examined by random migration and Transwell invasion assays, and the effect of plasma exosomes on the metastatic dissemination of BC cells was demonstrated in an in vivo zebrafish model. To reveal the molecular mechanism of interaction between plasma exosomes and BC cells, a comparison between un-treated and enzymatically modified exosomes was performed, followed by mass spectrometry, gene ontology, and pathway analysis.

Results Plasma exosomes stimulated the adhesive properties, two-dimensional random migration, and transwell invasion of BC cells in vitro as well as their in vivo metastatic dissemination in a dose-dependent manner. This stimulatory effect was mediated by interactions of surface exosome proteins with BC cells and consequent activation of focal adhesion kinase (FAK) signaling in the tumor cells.

Conclusions Plasma exosomes have a potency to stimulate the metastasis-promoting properties of BC cells. This pro-metastatic property of normal plasma exosomes may have impact on the course of the disease and on its prognosis.

Keywords Breast cancer · Metastasis · Exosomes · Surface interaction · Mass spectrometry · FAK signaling

Introduction

Metastasis is a multistep process in which cancer cells escape from the primary tumor, penetrate into blood vessels, survive shear forces and immune destruction in the circulation, seed at distant sites, and proliferate. Each of these stages involves interactions between tumor cells and non-malignant cells of the tumor microenvironment. Communication between these two types of cells can be performed by extracellular vesicles called exosomes, that transfer molecules such as mRNAs, microRNAs, and proteins between cells [1].

Paracrine exosomal signaling can occur between tumor and stromal cells supporting tumor growth [2]. For example, highly metastatic BC cells release exosomes that promote migratory activity of less invasive cancer cells [3]. In addition, cancer-associated fibroblasts secrete exosomes that promote breast cancer cell protrusive activity and motility by activating WNT-planar cell polarity (PCP) signaling in breast cancer cells [4]. Mesenchymal stem cell-derived exosomes can induce early stages of cancer stemness as well as motility and invasiveness of breast cancer cells by activating a critical signaling axis within

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10549-018-5043-0) contains supplementary material, which is available to authorized users.

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the tumor cells [5, 6]. Exosomal cross talk between BC cells and tumor-associated immune cells was investigated in detail revealing various underlying mechanisms (miRNA transfer, Toll-like receptor activation, and others) [7, 8]. Thus, vesicular exchange via exosomes is considered as one of the essential mechanisms of communication between primary tumors and associated stroma.

Metastatic dissemination of BC cells is enhanced by exosome-mediated tumor–stroma interactions beyond the primary tumor niche. For instance, exosomes derived from tumor cells can interact with immune effector cells in the circulation and thereby facilitate tumor escape from the immune system [9]. Exosomes that are secreted from primary tumors and are delivered via the circulation can induce pre-metastatic niche formation in distant organs [10, 11]. It was recently reported that the tissue tropism of metastatic tumor cells is determined by tissue-specific integrins that are included in the exosomes which are up-taken by cells in the targeted pre-metastatic niche and are associated with specific organs in which metastasis occurs [12]. By analogy with primary tumor site, bi-directional vesicular exchange between cancer cells and environment might play a role in the bloodstream or in the distant metastatic niches at the advanced stages of the disease. Circulating tumor cells undergo influence of heterogeneous "cocktail" of plasma exosomes produced by platelets, endothelium, circulating immune cells, and other cells. This mixture of vesicles might have impact on the fate of circulating tumor cells. The stimulatory activity of plasma exosomes towards BC cells was first demonstrated by Ochieng et al. in an in vitro model of anchorage-independent growth [13]. The ability of plasma-circulating exosomes to potentiate tumor malignant properties was observed in other studies as well [14, 15]. Thus, plasma exosomes appear to promote the dissemination of cancer cells, but the exact mechanism of this phenomenon is poorly understood at present.

Here, we report that exosomes purified from plasma of healthy donors induce adhesive properties and motility of BC cells, as demonstrated by their increased anchorage-independent growth capability in vitro, as well as their in vivo metastatic dissemination in a zebrafish model. Mass spectrometry analysis of exosome surface proteins followed by motility and invasion assays revealed that the ability of human plasma exosomes to stimulate breast cancer cell invasiveness is mediated, at least in part, by interactions between surface exosome proteins and BC cells. Moreover, this interaction leads to stimulation of FAK-mediated signaling within tumor cells. Collectively, our findings provide a novel insight into the mechanism by which plasma exosomes induce motility and invasiveness of BC cells, and may suggest a new opportunity for prevention or/and therapy of BC dissemination.

Materials and methods

Cell lines

MDA-MB-231 human breast adenocarcinoma cells were obtained from the American Type Culture Collection. MCF-7 cells were obtained from the Cell Culture Collection, Institute of Cytology of the Russian Academy of Science. Cells were cultured in RPMI-1640 containing 10% exosome-depleted FBS (Exo-FBS, System Biosciences, USA) at 37 °C, 5% CO_2. MDA-MB-231 stably expressing control pSUPER-Retro plasmid, FAK shRNA (FAK-KD), or GFP were previously described [16].

Plasma collection and isolation of extracellular vesicles

Blood samples were collected in the department of blood transfusion of N.N. Petrov National Medical Research Center of Oncology from healthy female donors at the age of 35–40 years. Blood was collected in EDTA-coated vacutainers, plasma was isolated immediately and stored at − 80 °C. Exosomes were isolated by differential ultracentrifugation methods according to standard procedure [17] and as previously described [18, 19]. Briefly, plasma (diluted 1:5 with PBS) was centrifuged in three consecutive steps: 2000 g for 30 min, 17,000 g for 60 min, and 110,000 g for 90 min. For mass spectrometry analysis, exosomes were washed by PBS and ultra-centrifuged again (110,000 g for 90 min). Exosome containing pellet was dissolved in PBS and filtered through a 0.22-μm filter for sterilization and used immediately or stored at + 4 °C for no more than 3 days. Before using exosomes for in vitro assays, their average size and concentration were measured by NTA. For in vitro and zebrafish in vivo experiments, freshly isolated vesicles were dissolved in exosome-free culture medium in concentration of 2×10^{10}/ml (100%) or 1×10^{10}/ml (50%), which corresponds to the concentration of exosomes in human plasma [20].

Exosome analysis

Size determination of isolated exosomes was performed by using Zetasizer Nano particle analyzer (Malvern, UK) according to the manufacturer's instructions. The size and concentration of isolated exosomes were evaluated by NTA using NanoSight NS300 (Malvern, UK) at several dilutions according to manufacturer's instructions. The presence of exosomal markers was assayed with Exo-FACS ready-to-use kit for plasma exosome analysis (HansaBioMed, Lonza) supplied with primary antibodies against CD9 or CD63 and secondary Alexa488-labeled antibodies. Additionally,

bead-coupled exosomes were assayed using FITC-conjugated Anti-CD9 antibody (ab34162) with corresponding isotype control, mouse FITC-conjugated IgG2b (ab91368). Analysis was performed with CytoFlex instrument (Beckman Coulter, USA). Cryo-electron microscopy was used for direct visualization of exosomal particles as described previously [21, 22]. Briefly, exosome solution in PBS was diluted 50 times in deionized water, and 10 µl put on freshly cleaved mica. After 1 min incubation at room temperature, that mica surface was thrice washed with water to remove salt [23]. Topography measurements of samples were performed in semi-contact mode using atomic force microscope NT-MDT-Smena B. An NSG03 probe was used (NT-MDT, Russia). Images were analyzed using the Gwyddion software [24].

Exosomal protein profiling by mass spectrometry

Shotgun mass spectrometry analysis of exosomal proteins was performed according to the filter-aided sample preparation (FASP) protocol. In short, proteins were digested by trypsin (Promega Trypsin Gold) and MS/MS analysis of resulting peptides was performed in duplicates using Orbitrap Fusion Lumos MS (Thermo Scientific, USA) [25–27]. Data were searched by using Mascot 2.4.1 (http://www.matrixscience.com), and neXtProt (October 2014) was used as a protein sequence database. The exponentially modified form of protein abundance index (emPAI) defined as the number of identified peptides divided by the number of theoretically observable tryptic peptides for each protein was used to estimate protein abundance [28, 29].

Gene ontology functional annotation and pathway analysis

Gene ontology analysis of exosomal proteins was performed with the functional annotation tool of the Database for Annotation, Visualization and Integrated Discovery (DAVID) [30, 31]. Human molecular pathways were extracted from the following databases: BioCarta (https://cgap.nci.nih.gov/Pathways/BioCarta_Pathways), KEGG [32], NCI [33], Reactome [34], and SABiosciences Pathway Central (http://www.sabiosciences.com/pathwaycentral.php). The Pathway Activation Score (PAS) for each pathway was calculated using the above-mentioned databases. PAS of the pathway P is the number of exosome surface genes, which are involved in the pathway P.

Western blot analysis

For western blotting, anti-FAK (ab40794), anti-β actin (ab8227), anti-FAK (phospho Y397) antibody (ab24781), goat anti-rabbit (ab6721), and anti-mouse (ab97023) HRP antibodies were obtained from Abcam, USA. Total protein concentration was determined using BCA Protein Assay Kit (Thermo Scientific, USA), and equal amounts were loaded on SDS–PAGE, transferred to polyvinyl membrane, blocked in 5% skim milk in PBS/0.05% Tween-20, incubated with primary and secondary antibodies, incubated in western blotting Luminol reagent (sc-2048; Santa Cruz, USA), and imaged using Image Quant LAS-4000 imaging system (General Electric, USA).

Anchorage-independent growth

To analyze anchorage-independent cell growth, tissue culture plates were pre-coated with poly-2-hydroxyethyl methacrylate/poly-HEMA (Sigma–Aldrich, USA) as previously described [35]. Cells were plated on poly-HEMA coated plates in medium supplemented with exosome-free FBS with or without plasma exosomes in concentration of 2×10^{10}/ml for 48 h, and imaged using MIB-R microscope with C-Mount (0,5×) Adaptor (LOMO, Russia).

In vitro random cell migration and transwell invasion assays

The 2D random cell migration assay was performed using In Cell Analyzer HCA System (GE Healthcare Life Sciences, USA); cells were plated on adherent plastic, allowed to adhere for 3 h, and automatically imaged every 5 min for 8 h. The data obtained were analyzed using the ImageJ software (NIH, USA) and Chemotaxis and Migration Tool (Ibidi GmbH, Germany) that allows the estimation of length of a single-cell movement trajectory.

Invasion through extracellular matrix was measured as previously described [36] with slight modifications. The upper surface of permeable Transwell supports were covered with a thin layer of Matrigel (BD Biosciences, USA). The bottom chamber was filled with medium containing 10% exosome-free FBS and plasma exosomes. 2000 cells were resuspended in medium containing 0.5% exosome-free FBS, plated in the upper chamber, and allowed to invade for 24 h. Fixation and staining of cells were performed as previously described [36]. Intact membranes were imaged using MIB-R microscope (LOMO, Russia) with C-Mount (0,5×) Adaptor. Data were analyzed using ImageJ.

In order to test the effect of plasma exosomes, freshly isolated vesicles were dissolved in culture media in concentration 2×10^{10}/ml (100%) and 1×10^{10}/ml (50%), that nearly corresponds to concentrations of exosomes in complete and 50% donor's plasma, based on our previous and others' published data [20]. Random migration and Transwell invasion assays were performed in triplicates, and results of independent experiments were averaged. Statistical evaluation

of results was performed by GraphPad Prism software using non-parametric Kruskal–Wallis test.

In vivo invasion assay in Zebrafish

MDA-MB-231/GFP cells were harvested, washed twice with PBS, and resuspended in PBS with or without 2×10^{10}/ml exosomes. Cells were incubated in the suspension of exosomes for one hour before injection. Zebrafish (Danio rerio) were maintained in an aquatic system at 28 °C with 14/10 day/night cycle environments. We used the transparent "casper" zebrafish line, which is characterized by reduced number of pigment cells as a result of two homozygous mutations [37]. Embryos at 48 h post fertilization (hpf) were anesthetized with 0.003% tricaine solution (Sigma, USA) for 5–10 min before injection. Suspensions of cells with exosomes (or cells and PBS as a control) were injected into the yolk sac of embryos using an M33 micromanipulator and an automatic PV 830 Pneumatic Pico Pump microinjector (both from World Precision Instruments, USA). Approximately 250–300 cells per embryo were injected using a borosilicate glass needle (tip ø15 µm). The efficacy of injection and embryo viability were assessed following 30 min incubation at room temperature under a Nikon SMZ 1500 fluorescent stereomicroscope (Nikon, Japan). Normal embryos bearing a visible fluorescent cells mass in the yolk sac were transferred into the wells of a 96-well plate and incubated for 48 h at 35 °C. At 96 hpf, embryos were euthanized by immersion into high concentration solution of tricaine and observed under Nikon SMZ 1500 microscope with GFP filter set. Experimental and control groups consisted of 30 embryos each, and experiments were repeated twice. Observed differences between experimental and control groups were reproducible.

Results

Extracellular vesicles isolated from human plasma share characteristics of exosomes

Extracellular vesicles were isolated from human plasma by ultracentrifugation according to a standard procedure that was described in several previous studies, and the isolated vesicles were characterized in accordance with International Society for Extracellular Vesicles (ISEV) guidelines [38]. To assess the size of the isolated plasma vesicles, we used dynamic light scattering. Our analysis revealed vesicle size within the range of 70–200 nm and an average hydrodynamic radius of 140–150 nm (Fig. 1a). Presence of exosomal markers (CD9, CD63) was confirmed by flow cytometry (Fig. 1b). Specificity of CD9 staining was additionally

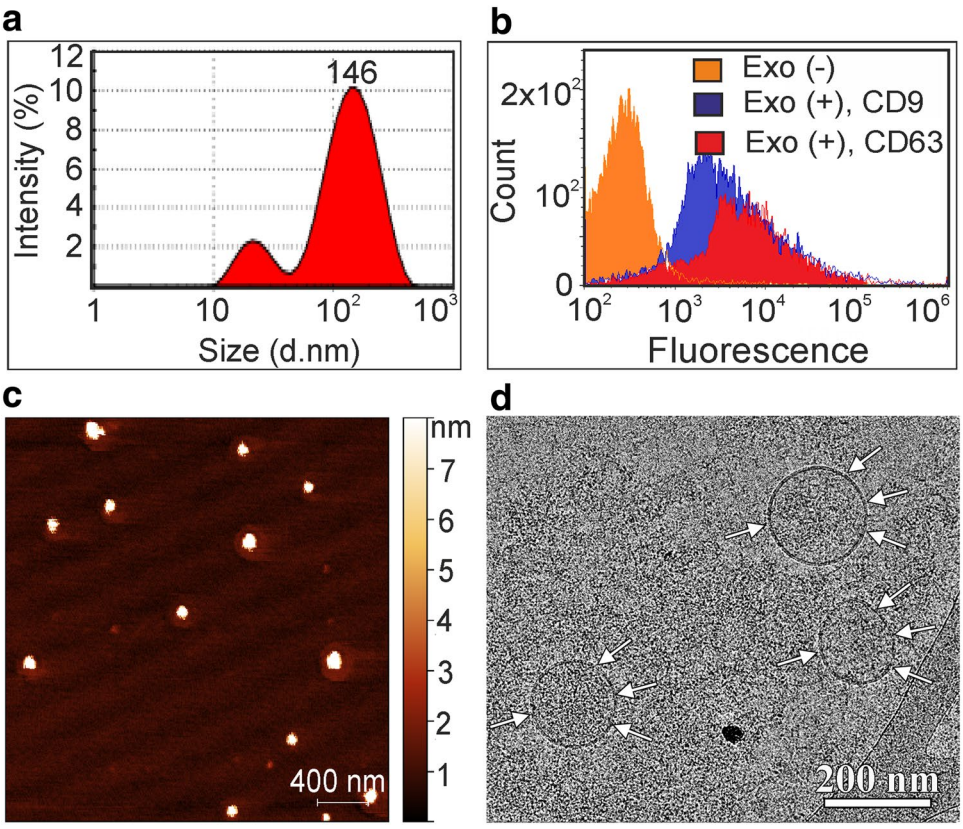

Fig. 1 Characterization of exosomes from human plasma. **a** Quantification of exosome size by dynamic light scattering (DLS). Small peak (particles size around 20 nm) reflects trace of plasma proteins, large peak (particles size around 140 nm) corresponds to plasma exosomes. **b** Isolated exosomes were analyzed by flow cytometry for surface expression of the exosomal markers CD9 and CD63. A solution without exosomes (Exo (−)) was used as a negative control. **c** Atomic force microscopy (AFM) of the surface of human plasma exosomes. Pseudocolor ruler on the right indicates the height of particles in nanometers. Scale bar, 400 nm. **d** Cryo-electron microscopy of exosome pellet derived from human plasma. Scale bar, 200 nm

controlled using FITC-conjugated mouse IgG2b against vegetarian protein as isotype control (Supplementary Fig. 1). The homogeneity of isolated vesicles was demonstrated by atomic force microscopy (AFM) (Fig. 1c). The morphology of single plasma vesicles was examined by cryo-electron microscopy, and demonstrated spherical vesicles with a bilayer membrane (Fig. 1d). Collectively, our data demonstrate that the vesicles isolated from human plasma contain all of the characteristics of exosomes.

Plasma exosomes induce adhesive activity of breast cancer cells in vitro

To evaluate the effect of human plasma-derived exosomes on anchorage-independent growth of BC cells, we plated MDA-MB-231 and MCF-7 cells on tissue culture plates that were coated with poly-HEMA, a polymer that prevents cell attachment, in medium supplemented with exosome-depleted serum, and in the presence or the absence of isolated plasma exosomes. First, exosomes from individual donors (n.5) were applied after normalization of their concentration to the level equivalent to their concentration in human plasma (2×10^{10}/ml) [20]. Then, pool of three samples was tested at the concentration 2×10^{10} particles/ml. Cultivation of BC cells in medium containing plasma exosomes (individual or pooled samples) resulted in the formation of multi-cellular conglomerates in both cell lines tested (Fig. 2a, c), which were more prominent in the MDA-MB-231 cell line, probably due to its higher malignant potential compared to MCF-7 cell line. Adhesive activity was significantly less prominent in cells that were grown without plasma exosomes (Fig. 2b, d). Human plasma exosomes also induced anchorage-independent proliferation of both BC cell lines; however, this effect was not statistically significant (data not shown). These results indicate that exosomes isolated from human plasma can increase the tumorigenic characteristics of BC cells as reflected by their ability to form inter-cellular contact and grow as multi-cellular conglomerates.

Exosomes from human plasma induce migratory activity of breast cancer cells in Zebrafish

To explore whether human plasma exosomes could contribute to the invasive capability of breast cancer cells in vivo, we used the zebrafish model (*Danio rerio*). MDA-MB-231 cells expressing GFP, which enables their tracking in vivo, were incubated with plasma exosomes or PBS as control for 1 h before their injection into the yolk sac of zebrafish embryos. Experiments were performed with exosomes isolated from plasma of three donors. Before incubation with tumor cells, samples of exosomes were pooled and concentration of vesicles was adjusted to 2×10^{10} particles/ml. The presence and localization of GFP-labeled tumor cells was examined by fluorescence microscopy at 48 h post injection. Fluorescently labeled tumor cells that were pre-incubated with human plasma exosomes migrated out of the yolk sac

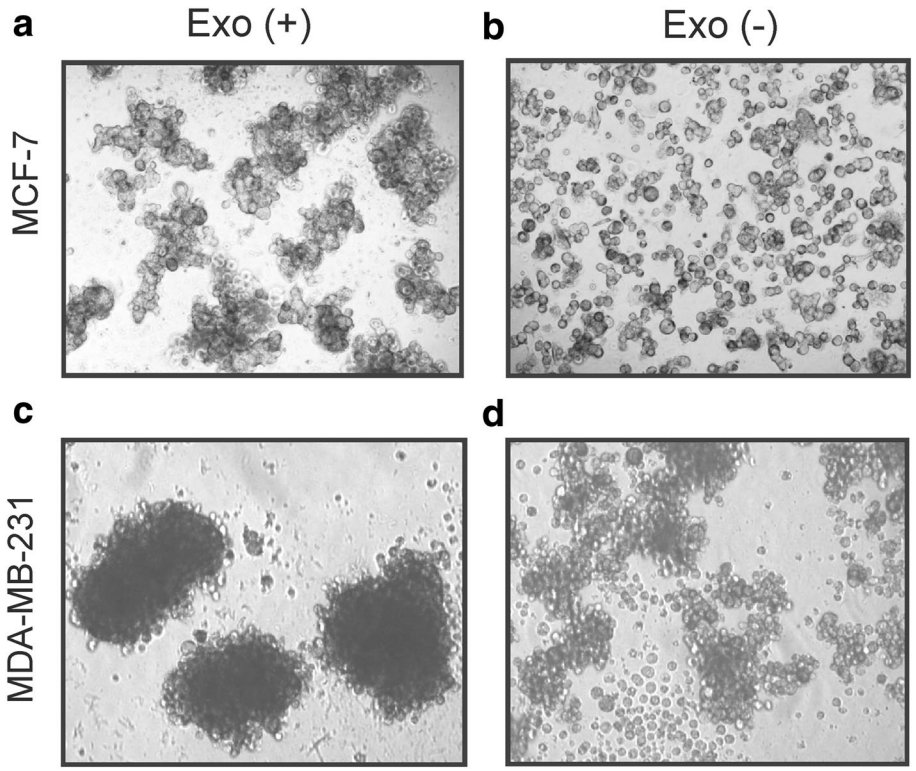

Fig. 2 The effect of plasma exosomes on anchorage-independent growth of breast cancer MDA-MB-231 and MCF-7 cells. Representative images of MCF-7 cells plated on Poly-HEMA-coated culture plates in the presence of exosomes pooled from 3 individuals (**a**) or in exosomes-free medium (**b**). Representative images of MDA-MB-231 cells plated on Poly-HEMA-coated culture plates in the presence of exosomes pooled from 3 individuals (**c**) or in exosomes-free medium (**d**). Imaging was performed following 48 h of cultivation by phase contrast microscope "MIB-R" supplied with digital camera (LOMO, Russia) using ×10 objective lens

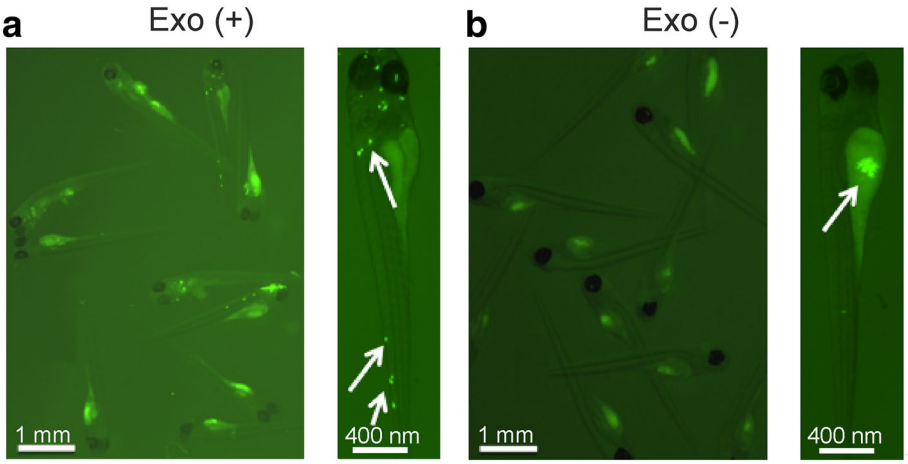

Fig. 3 Plasma exosomes increase the in vivo migratory activity of MDA-MB-231 breast cancer cells. A suspension of fluorescently labeled MDA-MB-231/GFP cells was incubated either with human plasma exosomes or with PBS (control, Exo(−)) or for 30 min and then injected into the yolk sac of 2-day-old zebrafish embryos. Cells were allowed to migrate for 48 h, after which images were acquired using a fluorescent microscope. Representative images of low zoom and high zoom magnification of embryos injected with cells pre-incubated with exosomes. **a** Representative images of low zoom and high zoom of embryos injected with cells that were pre-incubated with PBS as control. **b** Scale bars, 1 mm (low zoom) and 400 nm (high zoom). Experiments were repeated twice with similar results

to different parts of the embryos. Representative results of experiment with pooled exosomes samples are shown in Fig. 3a. In contrast, tumor cells that were incubated in PBS did not show any dissemination outside place of injection (Fig. 3b). These data demonstrate that exosomes isolated from human plasma can stimulate the in vivo invasiveness and dissemination of BC cells in zebrafish embryos.

Enzymatic modification of plasma exosomes changes their surface protein composition

The enhancement of invasiveness of BC cells by exosomes could be resulted by their surface interaction with motility-related receptors on the membrane of cancer cells. To determine whether contact interaction of exosomes with tumor cells is sufficient to stimulate their motility, we performed enzymatic digestion of plasma exosome surface proteins by limited trypsinization. Exosomes isolated from fresh plasma by ultracentrifugation were treated with 0.25% trypsin solution for 1 h, washed with PBS, and centrifuged again. To identify surface proteins that were cleaved from exosome surface by trypsinization, we performed mass spectrometry analysis of native and trypsin-treated exosomes (Supplementary Table 1). Using this method, we identified more than 350 human plasma exosomal proteins. Trypsinization led to a partial or complete loss of certain proteins from the exosomal proteome. Gene ontology functional annotation approach revealed that majority of proteins removed from exosomes surface by enzymatic digestion are components of circulating plasma implicated in extracellular signaling and immunity [39]. Further pathway analysis demonstrated the involvement of some of these exosomal surface proteins, such as extracellular matrix protein 1 (ECM1), fibrinogen (FGA), vitronectin (VTN), fibronectin (FN1), thrombospondin (THBS1), and talin-1 (TLN1) (Table 1) in the focal adhesion kinase (FAK) signaling pathway which is known to regulate cellular adhesion and motility.

Plasma exosomes stimulate migratory and invasive activities of BC cells in dose- and surface composition-dependent manner

To confirm that plasma exosomes induce cancer cell motility by interaction of exosome surface proteins with the tumor cells, we first performed 2D random motility assays of MDA-MB-231 and MCF-7 cells in the presence or the absence of purified plasma exosomes in the medium. To estimate the dose-dependent effect of exosomes on 2D motility, these experiments were performed using medium without exosomes (Exo (−)), containing exosomes in a concentration that is equivalent to 50% (Exo (50%)) or 100% (Exo (100%)) in human plasma. To confirm that interaction of surface exosome proteins with tumor cells stimulates motility, we also included a sample of exosomes in a concentration equivalent to 100% in human plasma, that were pre-treated with trypsin as above (Exo (100%) Tryp). As demonstrated in Fig. 4, plasma exosomes stimulated two-dimensional random motility of MDA-MB-231 cells, and to a lesser extent also of MCF-7 cells, in a dose-dependent manner. The enhancement of cell motility by exosomes was dependent on their surface proteins, as enzymatic modification of these exosomes decreased their stimulatory capability. Observed

Table 1 List of exosome surface proteins involved in FAK signaling

Gene symbol	Name	UniProt ID	Percentage of protein removed by trypsinization*		ExoCarta ID	Involvement in FAK signaling
			%	SD		
ECM1	Extracellular matrix protein 1, Iso1	Q16610	90.0	1.5	1893	[47]
FGA	Fibrinogen alpha chain, Iso 1	P02671	89.3	7.1	2243	[48]
VTN	Vitronectin, Iso 1	P04004	86.8	4.3	7448	[49]
FN1	Fibronectin, Iso1	P02751	92.7	1.5	2335	[50]
THBS1	Thrombospondin-1	P07996-1	82.4	13.7	7057	[51]
TLN1	Talin-1, Iso 1	Q9Y490-1	43.2	13.7	7094	[52]

*Averaged results of three independent experiments and standard deviations (SD)

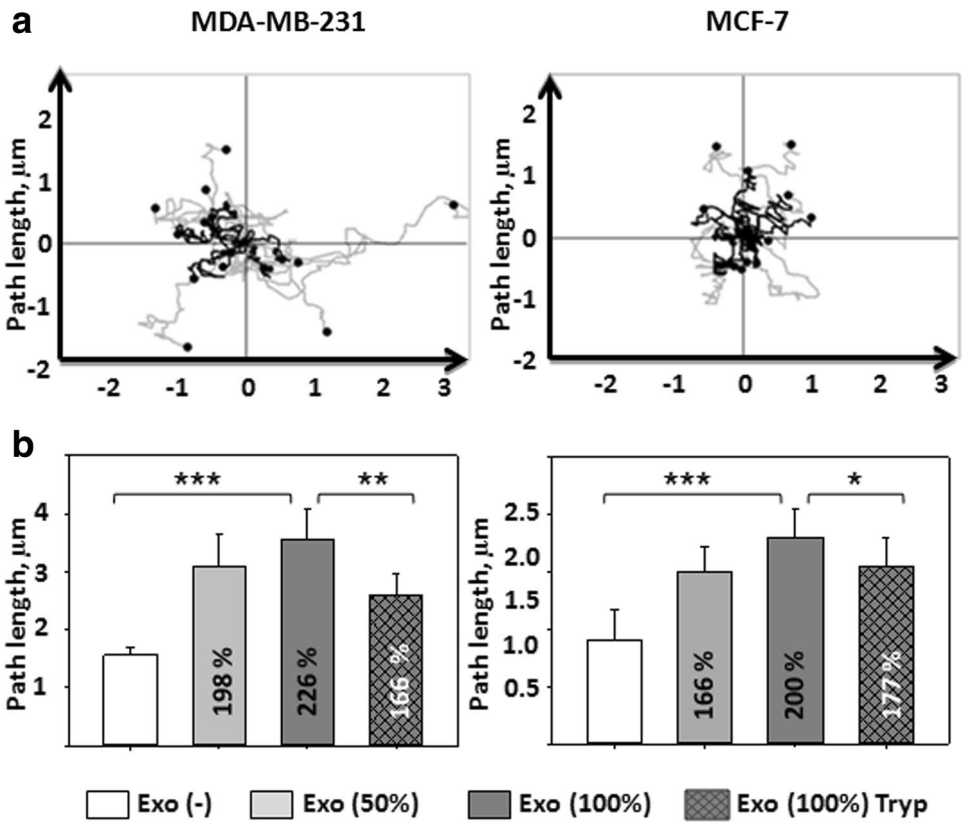

Fig. 4 Plasma exosomes induce breast cancer cell motility in a dose-dependent manner. **a** Trajectory plots of single-cell migration experiments of MDA-MB-231 and MCF-7 cells plated in presence (black lines) or absence (gray lines) of exosomes. **b** Quantification of accumulated distance (total path length) of MDA-MB-231 of MCF-7 migrating cells. Four groups of cells were compared in each experiment: cells that were cultured in medium without exosomes (Exo(-)), medium containing isolated plasma exosomes in a concentration which is equivalent to 50% in human plasma (Exo (50%)), medium containing isolated exosomes in a concentration which is equivalent to 100% in human plasma (Exo (100%)), and medium containing limited trypsin-treated plasma exosomes in a concentration which is equivalent to 100% in human plasma (Exo (100%) Tryp). Error bars present averaged results of three independent experiments (±SD) with number of the cells observed in each experiment = 8. Percentage numbers on graph bars represent increase in cell migration compared to Exo (−) condition. Statistical significance of observed difference between three groups (Exo(-), Exo(50%), and Exo(100%)) is determined by Kruskal–Wallis test (***$p < 0.0005$) and between two groups (Exo(100%) vs. Exo(100%)Tryp) is determined by Mann–Whitney test (**$p < 0.005$, (*$p < 0.05$))

difference of cellular migration was statistically significant for both MDA-MB-231 and MCF-7 cell lines.

Cancer cell invasiveness is regulated by both focal adhesion-mediated motility and by invasiveness through extracellular matrix (ECM). To test whether surface interaction of human plasma exosomes with BC cells affects their invasion through ECM, we examined the ability of MDA-MB-231 and MCF-7 cells to invade through Matrigel-coated membranes towards complete medium containing exosomes in different concentrations or exosomes pre-treated with trypsin. As demonstrated in Fig. 5, plasma exosomes stimulated Matrigel invasiveness of MDA-MB-231 cells, and to a lesser extent also of MCF-7 cells, in a dose-dependent manner. The enhancement of cell invasiveness by exosomes was dependent on their surface proteins, as enzymatic modification of these exosomes significantly decreased their ECM invasiveness stimulation capability. Observed difference of invasive activity was statistically significant for MDA-MB-231 cell line. In case of MCF-7 cell line, difference in dose-dependent stimulatory effect of exosomes was statistically significant, whereas difference between effects of intact

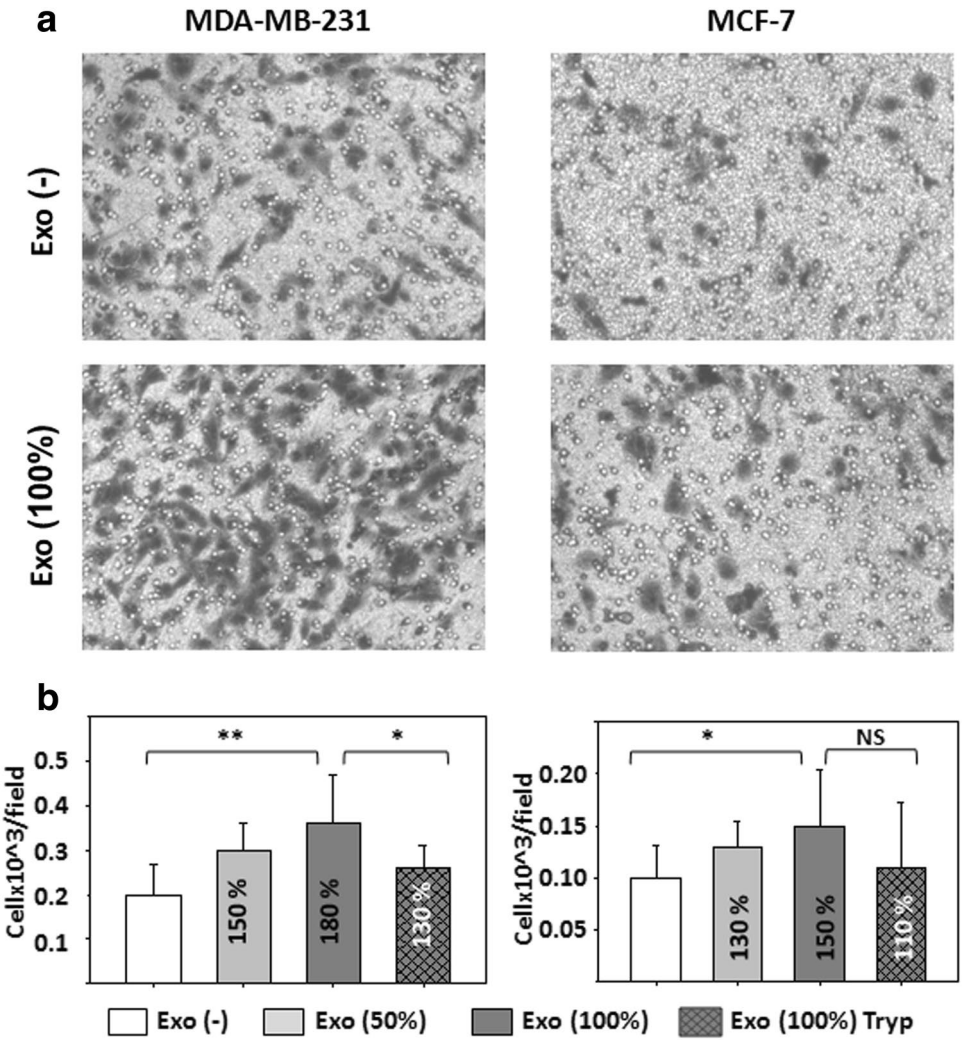

Fig. 5 Transwell invasion of breast cancer cells is increased by plasma exosomes. **a** MDA-MB-231 and MCF-7 cells were plated on Matrigel-coated membranes in medium with Exo (100%, bottom panels), or without plasma exosomes (Exo (−), top panels), allowed to invade for 24 h, fixed, and stained. **b** Quantification of cell invasion through Matrigel-coated membranes in medium without exosomes (Exo (−)), medium containing isolated plasma exosomes in a concentration which is equivalent to 50% in human plasma (Exo (50%)), medium containing isolated exosomes in a concentration which is equivalent to 100% in human plasma (Exo (100%)), and medium containing limited trypsin-treated plasma exosomes in a concentration which is equivalent to 100% in human plasma (Exo (100%) Tryp). Error bars presents averaged results of three independent experiments (± SD) with number of fields of view evaluated in each experiment = 8. Percentage numbers on graph bars represent increase in cell migration compared to Exo (−) condition. Statistical significance of observed difference between three groups (Exo (−), Exo(50%), and Exo(100%)) is determined by Kruskal–Wallis test (**$p < 0.005$, *$p < 0.05$) and between two groups (Exo (100%) vs. Exo (100%)Tryp) is determined by Mann–Whitney test (*$p < 0.05$, NS $p > 0.05$)

vesicles and vesicles pre-treated with trypsin was visible while not statistically significant.

Collectively, these results suggest that plasma exosomes stimulate dose-dependently both 2D motility and 3D invasiveness of BC cells by surface interaction of their membrane-anchored proteins with the cancer cells.

Plasma exosomes stimulate BC cell movement via FAK pathway activation

Pathway analysis of the mass spectrometry data revealed that several proteins which were removed from exosome surface by enzymatic digestion are upstream regulators of FAK-mediated adhesion and migration signaling pathways (Table 1). To further examine whether signaling via FAK is involved in the surface interaction-mediated effect of plasma exosomes on breast cancer cell motility and invasion, we used MDA-MB-231 cells that were stably knocked down for FAK [16]. FAK knockdown was almost completed while incubation with exosomes induced tyrosine phosphorylation of FAK at Tyr 397 position clearly detectable in control cells and slightly visible in FAK-KD cells (Fig. 6a). Indeed, knockdown of FAK significantly reduced the stimulatory effect of exosomes on both 2D random migration (Fig. 6b) and 3D transwell invasion (Fig. 6c) of MDA-MB-231 cells. Together, these data indicate that human plasma exosomes enhance cancer cell motility and invasion by stimulation of FAK-mediated signaling in BC cells. The significant but incomplete reduction in motility and invasiveness of FAK knockdown BC cells that were stimulated by human plasma exosomes suggests that additional mechanisms and signaling pathways by which exosomes induce migration and invasion of BC cells may exist.

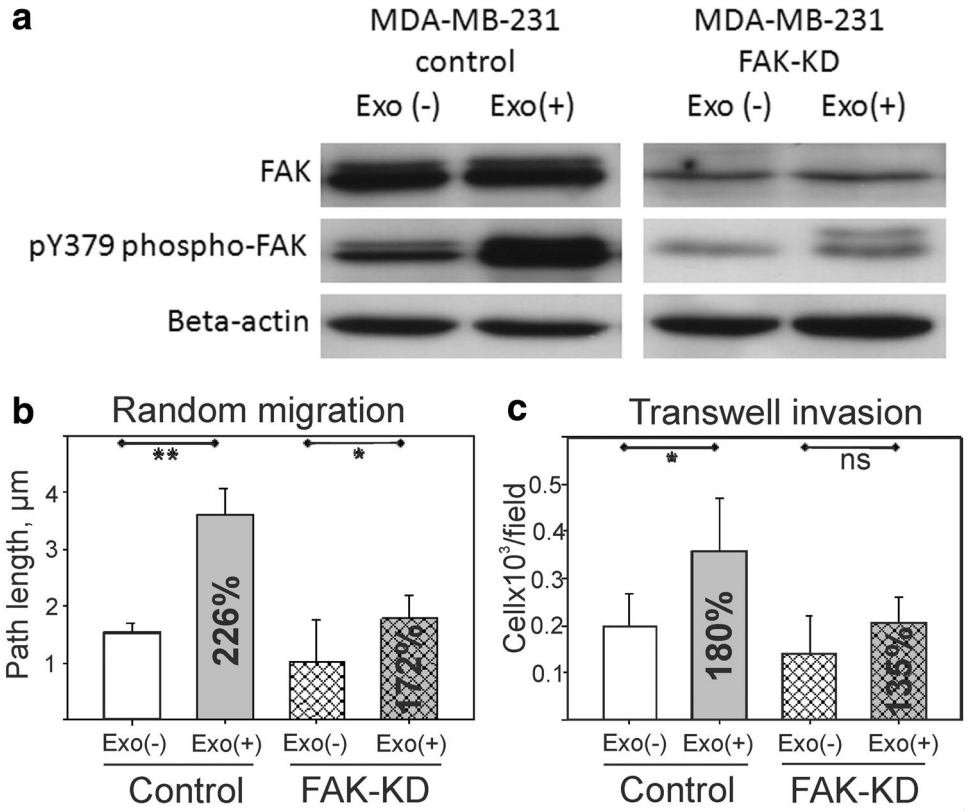

Fig. 6 Knockdown of FAK decreases the sensitivity of breast cancer cells to stimulation by plasma exosomes. **a** Representative immunoblot of MDA-MB-231 cells stably expressing FAK shRNA (FAK-KD) and control cells. Whole cell lysates were blotted for either total amount of FAK analysis, analysis of activated FAK (phosphorylated at Tyr 397 position), and analysis of β-actin as loading control. **b** Quantification of accumulated distance (total path length) of FAK-KD and control cells in random migration assay conduced in the presence or the absence of plasma exosomes in medium. Error bars presents averaged results of three independent experiments (±SD) with number of cells observed in each experiment = 8. **c** Quantification of invasion through Matrigel-coated membranes of FAK-KD cells and control in the presence or the absence of plasma exosomes. Error bars presents averaged results of three independent experiments (±SD) with number of fields of view evaluated in each experiment = 8. Statistical significance was determined by Kruskal–Wallis test (*$p < 0.05$, **$p < 0.005$, $p > 0.05$ (non significant)

Discussion

The ability of exosomes to promote cell motility and invasiveness has been extensively studied over the past few years. It was demonstrated that exosomes can stimulate cell protrusion formation [42], cell adhesion [41], and cell polarity [43]. Exosomes are involved in matrix remodeling and invasive behavior [44]. Recently, it was reported that exosomes secretion is essential for chemotaxis of cancer cells [40]. However, all these studies refer to exosomes that are secreted by tumor cells or cells of the tumor microenvironment, and are involved in regulation of local tumor growth and invasion. However, the interaction of metastasizing tumor cells with plasma exosomes which may affect later stages of the metastatic process has only been vaguely explored until present.

Metastatic dissemination of tumor is associated with pathological influence of tumor cell-derived exosomes on tissue of pre-metastatic niche [10, 11]. Involvement of exosomes secreted by other normal cells and circulating in plasma into the metastatic process can be supposed as well. However, evaluation of the possible pro-metastatic role of plasma exosomes is challenged by their heterogeneity. For instance, plasma of healthy donors and melanoma patients contains exosomes secreted by platelets, antigen-presenting natural killer (NK) cells, while disease progression is associated with reactive changes of composition of plasma exosome pool [45]. Herein, specific populations of plasma exosomes were not separated, but the functional properties of this complex mixture of vesicles were evaluated as a whole. We report that plasma exosomes may induce adhesive properties, motility, and invasiveness of BC cells and consequently support their metastatic dissemination. Further research aiming to distinguish specific populations of circulating exosomes and their functional diversity may provide deeper insights into the pro-metastatic effects of plasma exosomes.

Another aspect challenging analysis of pathological effects of plasma exosomes is the complex composition of plasma. Vesicles circulating in the plasma interact with plasma proteins and glycoproteins that may attach to exosomal surface, modify it, and mask tissue-specific surface molecules. For example, fibronectin, one of the major plasma proteins, is attached to the surface of exosomes [46] and is also loaded into exosomes [41] and plays a critical role in promoting cell motility. Using a simple approach, we modified the surface of plasma exosomes and demonstrated that exosomes with trypsin-modified surface partially lost their adhesive and invasive stimulatory activity on BC cells. These results may suggest a novel approach of anti-metastatic therapy involving modification of circulating exosomes or isolation of specific fraction of active exosomes; however, the efficacy of such approach has to be experimentally proven.

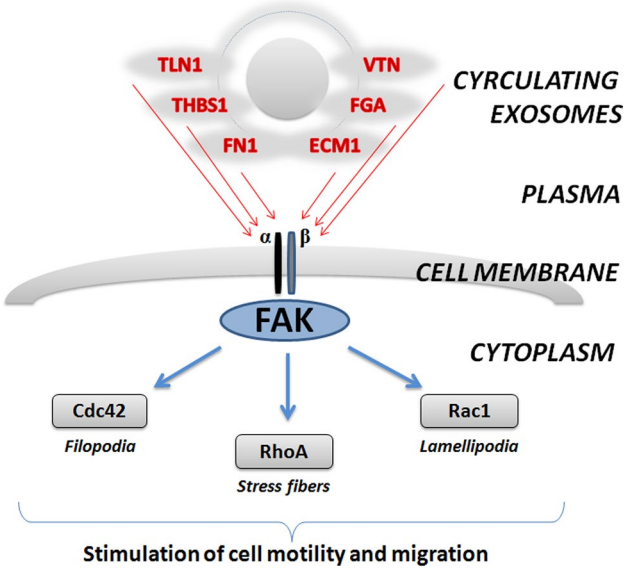

Fig. 7 Schematic presentation of biological effects induced by proteins attached to the surface of plasma exosomes and affecting FAK-signaling cascade in BC cells

Our work demonstrates the involvement of FAK and FAK-mediated signaling in regulation of exosomes-mediated BC cell motility and invasion. Indeed, FAK is well known to play a critical role in integrin- and growth factor-mediated signaling and control of cellular adhesion and migration [16]. Our analysis of mass spectrometry data from intact and trypsin-treated plasma exosomes revealed several exosomal surface proteins, that can stimulate FAK-mediated signaling within BC cells (Table 1; Fig. 7). In line with these findings, knockdown of FAK in MDA-MB-231 tumor cells significantly reduced their plasma exosome-mediated 2D cell motility and 3D invasiveness capabilities. However, the reduction in exosome-dependent motility and invasion was not complete. This suggests that other signaling pathways or alternatively, other compensating proteins, may be involved in plasma exosome-mediated enhancement of BC cell motility.

Acknowledgements This study was supported by RFBR (grant numbers 15-54-12380 and 18-015-00289), by Russian Science Foundation (grant number 17-14-01416), by the Ministry of Education and Science of the Russian Federation (ID RFMEFI62117×0017) (to Anastasia Malek), as well as by the Israel Cancer Association and Estee Lauder Companies (grant number 20180089), by the Israel Cancer Research Foundation (grant number 17-902-AG), and by the Israel Science Foundation (grant number 1462/17) (to Hava Gil-Henn).

Compliance with ethical standards

Conflict of interest The authors declared no conflicts of interest.

Ethical approval The design of study has been approved by Ethical Committee of N.N. Petrov National Medical Research Center of Oncology. All procedures were in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki Declaration. All applicable international, national, and institutional guidelines for the care and use of animals were followed.

Informed consent Informed consent was obtained from all individual participants included in the study.

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