The Self-targeting Ability of Cancer-derived Exosomes to their Parental Cells in a Time and Concentration-Dependent Manner

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Research Article

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Abstract

**Background/Aim:** Exosomes have recently attracted research interest as exploitable nano-carriers. However, cell specificity of exosomes toward their targets still needs to be addressed. This study, for the first time, deals with the natural tropism of cancer-derived exosomes towards HER2 positive cells compared to targeted and untargeted HEK-derived exosomes.

**Methods and Results:** Isolated targeted HEK293 and TUBO exosomes were successfully characterized. TUBO cells were treated with various concentrations of fluorescent labeled exosomes at different time points. The tropism of TUBO exosomes was assessed toward other HER2 positive breast cancer cells as well. TUBO-derived exosomes showed 40% uptake, whereas targeted HEK 293 exosomes had lower affinity. By increasing the incubation time, TUBO cells had more time to recognize TUBO exosomes. Fluorimetry analysis revealed that by increasing the dosage of exosomes on HER-2 positive cells, the prominent factor in the attachment was nonspecific physical absorption.

**Conclusion:** Cancer-derived exosomes might be a promising alternative to targeted exosomes as the delivery agent in targeting cancer cells.

**Introduction:**

Exosomes, the leading players in long-distance intercellular communication [1], are the endogenous extracellular vesicles in the size range of 30–150 nm [2]. They are produced by many cells including immune, tumor, epithelial, B, and T cells [3]. These cargos contribute to different biological processes such as cell-to-cell communication and signal transduction [4]. Exosomes have unique characteristics. They are more biocompatible with lower immunogenicity, have higher in vivo stability, and cause less cytotoxicity than synthetic nano-carriers like liposomes and polymeric nanoparticles [5, 6].

Due to the distinctive role of exosomes in cell-cell communication, especially at the tumor site, cancer-derived exosome can be a promising nano-based delivery system [7]. As exosomes enclose the contents of the parental cells, choosing the right donor cells is very important. It is suggested that exosomes derived from some cells such as NK or T cells, MSCs, and cancerous cells express some receptors on their surface, which recapitulates their tropism towards their parental cells [8–10]. Due to the expression of the same surface markers as parental tumor cells, cancer-derived exosomes interact with the parental cells in a better way than non-parental derived exosomes do [11]. Several studies have investigated the potency of tumor-derived exosomes as the delivery agent in cancer treatment [11–14].

The inherent ability of exosomes to deliver their specific repertoires to the recipient cells combined with the targeting strategies make exosomes as a potential delivery agent in the future clinical settings. Engineered targeted exosomes have been used in extensive studies for encapsulation and delivery of chemotherapeutic drugs and microRNA [15, 16]. Previously, our team engineered HEK293 cells to produce designed ankyrin repeat proteins (DARPin) ligand against the HER2 receptor [17]. The ligand was expressed on the surface of exosomes with lysosomal associated membrane (Lamp-2B) fusion protein.
DARPin is a class of synthetic peptides with high affinity to overcome the limitations of other targeting agents [18]. The efficiency of DARPin-positive exosomes has been ensured in our recent publications [18, 19].

Although reaching a comprehensive and accurate understanding of the tropism and uptake of exosomes is not within the scope of the present research, its informative findings will contribute to a better understanding of the mechanism of exosome internalization. Apart from their prognostic value, tumor-derived exosomes could be employed as therapeutics. Following the previous well-characterized literature, this study investigated the efficient delivery of exosomes to the recipient cells. We accurately quantified the uptake of cancer-derived exosomes by HER-2 positive cell lines and compared the results with the adherence of targeted and untargeted exosomes released by HEK293 cells. Targeted exosomes had the highest delivery to HER2 positive cells behind cancer-derived exosomes, while blank HEK exosomes showed little, if any, cell association, indicating that TUBO cells did not take up exosomes nonspecifically. To the best of our knowledge, this is the first time that the tropism and homing ability of cancer-derived exosomes have been compared with the specific affinity of targeted exosomes in a time- and concentration-dependent manner. Understanding the extent of affinity would pave the way for selecting the right donor cells in exosome-based therapeutics. We purified exosomal fractions from TUBO and HEK293 with well-established and widely used methods. Then, we could accurately characterize size distribution and distinct surface markers for the resulting particles. We exposed TUBO cell cultures to the fluorescently labeled exosomes and evaluated cellular uptake by fluorescence microscopy, flowcytometry, and fluorimetry.

**Material And Methods:**

**Cell culture:**

HEK293 cells were obtained from the Iranian biological resource center. TUBO breast cancer cell lines which overexpress HER2/neu protein was kindly provided by Dr. Pier-Luigi Lollini (Department of Clinical and Biological Sciences, University of Turin, Orbassano, Italy). All of the mentioned cell lines cultured in DMEM medium supplemented with 10% fetal bovine serum (Gibco, Waltham, MA, USA) and 1% penicillin/streptomycin solution (Gibco).

**Transduction of HEK293 cells**

Targeted HEK-293 cells expressing DARPin were produced according to the method previously described [20]. Briefly, the pLEX-LAMP-DARPin lentiviral vector was used to transduce seeded HEK 293T cells by MOI = 5 for 16 h. Afterwards, the supernatant was replaced with new medium. Puromycin antibiotic was used to select the transduced cells. The 1.5 μg/ml of puromycin (Roche) into the medium killed the non-transduced cells whereas, the transduced cells were resistant up to 6 μg/ml concentration of antibiotic.
Exosome Isolation:

The supernatants of TUBO, HEK293, and engineered HEK293 cells were harvested on a weekly basis from 80% confluent cells in FBS depleted media and conditioned for 48 hours. The dead cells were eliminated by centrifugation at 300 g, 10 min followed by filtration through a 100 KDa amicon filter (Merck Millipore) to concentrate the media containing exosomes and eliminate albumins. Afterward, the concentrated conditioned media filtered by a 0.2-micron filter (Merck Millipore, Burlington, MA, USA) to remove apoptotic cells and microvesicles. Then, exosomes were isolated using Cell Guidance Systems (Cambridge, UK) exosome isolation kit according to the manufacturer's instruction. The purified exosomes were diluted in 200 µl PBS. The protein content of exosomes was quantified using ELISA and Bradford microassay.

Exosome Characterization:

Size distribution and zeta potential of exosomes

Exosomes size distribution was measured by the Zetasizer Nano ZS90 (Malvern); exosomes were diluted in PBS first (1/1000). Zetasizer software v7.11 (Malvern Corp) was used to analyze the results.

Western blotting:

To detect exosome specific markers, exosomes were lysed in RIPA buffer and protease inhibitor (Roche, Mannheim, Germany). Samples were added to 4X loading buffer, heated 5 min 95°C, and then separated in a 12% SDS-PAGE. The resolved protein was transferred to the nitrocellulose membrane. After 1 hour blocking, the membrane was washed three times with washing buffer (TBS-T 0.1%) and incubated overnight at 4 °C with primary antibodies: Anti CD63, anti CD9 and after washing three times with TBST, incubated with secondary antibody for 1 hour. At last, membranes were visualized by enhanced chemiluminescence (ECL) detection system.

Flow cytometry and cellular uptake

PKH67 (Sigma-Aldrich) was used to label exosomes according to the manufacturer's instructions. In brief, exosomes pellet and PKH67 were separately diluted in 100 µL Diluent C according to the manufacturer's datasheet. Exosomes were resuspended in PKH67 solution and incubated at room temperature for 5 min. labeling was quenched by adding 1% BSA, and for removing the unbound dye, the mixture was transferred to a 100 kDa Amicon filter column. 6×10^4 TUBO cells were seeded in 24 well plates. The day after, 5 µg of PKH67 labeled exosomes were added to the cells; after overnight incubation, the cells were rinsed twice with PBS and trypsinized, then uptake was measured by flow cytometry (BD Biosciences,
Franklin Lakes, NJ, USA). The uptake data were quantified using Flow Jo (FlowJo, LLC, Ashland, OR, USA) and Flowing software 2.4.1. For statistical analysis, a t-test was used to measure differences between different cell lines.

**Fluorescent Microscopy and uptake:**

TUBO cells were seeded in a 96 well plate. 5 µg of labeled exosomes incubated with cells for 12 hours. Cells were rinsed with PBS twice and fixed using 4% paraformaldehyde. The nuclei were stained by DAPI fluorescent stain (Sigma-Aldrich); Microscopy was performed by an inverted fluorescence microscope (Nikon TE300; Nikon, Tokyo, Japan).

**Fluorimetry:**

TUBO cancer cells were seeded on the microtiter plate well. The day after, TUBO cells were treated with different concentrations of PKH-67 labeled exosomes following overnight incubation at 37°C. The next day, after removing unbound exosomes, the association of labeled exosomes was evaluated by reading the fluorescence intensity of each well using microplate readers (BioTek, Winooski, VT, USA) at 520 nm (excitation at 480 nm). All of the experiments were done in triplicates.

**Statistical Analysis**

Data were evaluated by ANOVA and differences between groups were analyzed by the Student’s t-test. P values \( \leq 0.05 \) were considered significant.

**Results:**

**DARPin ligand expressed by transduced HEK293 cells**

Total RNA was extracted from transduced HEK293 cells and subjected to RT-PCR by DARPin specific primers. Production of 430 band confirmed the successful integration (Fig 1a). Subsequently, western-blot was performed on the purified exosomes from transformed HEK cells using the anti-His tag antibody. The presence of 70 KDa protein (LAMP2B-DARPin) was identified on the targeted exosomes (Fig 1b).

**Characterization of exosomes:**

Exosomes were purified from TUBO cells (breast cancer), HEK293 (epithelial), and targeted HEK293 cell lines using exosome isolation kit. The purified exosome density was determined through two methods, namely Bradford and signal intensity from anti-CD9 ELISA assays. Initially, the density of exosomes was measured by Bradford assay. As commercial kits precipitate some protein serums together with
exosomes, performing the colorimetric assays was impractical. Hence, in order to exclude the effect of other precipitated proteins, the concentration of exosomes was measured by anti-CD9 ELISA assays (data are not provided here). HEK293 isolated exosomes made less intense signals than TUBO cells, which secreted large amounts of exosomes, in both CD9 and Bradford assays. The common exosomal surface marker proteins (CD9 and CD63) were confirmed by immune blotting. These canonical markers determined whether the purified exosomes were different from other endogenously derived vesicles (Fig. 2a). Next, the average size distribution of TUBO exosomes over three replicates was measured, as illustrated in Fig 2b, which was estimated in zeta sizer results at 120 nm.

**In vitro targeting of exosomes by fluorescent labeling and uptake assay**

Flow cytometry analysis was conducted to determine the association of various exosomal preparations by comparing the binding affinity of PKH67 labeled exosomes with the HER2 receptor on breast cancer cells. The labeling efficiency of PKH67 fluorescent dye was near 90%. The uptake of TUBO exosomes was relatively the same as that of targeted HEK293 exosomes with a positive rate of 28 ± 1.05%. The DARPin positive exosomes derived from HEK293 cells were bound to the recipient cells in an HER-2 dependent manner. The rate of TUBO cells co-cultured with targeted exosomes was 26 ± 1.89%. For nonspecific interaction analysis, we used HEK exosomes and achieved 6% uptake. The TUBO exosomes were found more efficiently absorbed by TUBO cells than by unmodified HEK exosomes. This reaffirms the higher interaction of exosomes with the parental cells (Fig 3a). Afterwards, we considered the incubation time as a factor that would affect the absorbance and uptake of exosomes. TUBO cells were treated with various exosomal preparations for 4, 12, and 24 h to check whether the extended exposure to exosomes would increase the uptake. We observed that while targeted exosomes uptake was already greater within 4 h, TUBO cells preparation needed more time to show a comparable uptake. However, we observed reversed results and increase in TUBO cell derived exosomes uptake after 12 h. After 4 h, TUBO exosomes uptake and internalization was 4 ± 1.05% and 6 ± 1.36% for targeted HEK 293, while it was near 1.5% for control HEK cells. Therefore, we concluded that the primary mechanism of exosome uptake was most likely receptor and ligand interaction, which was facilitated by the high affinity of DARPin ligand with HER-2 receptor in targeted exosomes. However, in the following, other mechanisms such as membrane fusion and lipid mixing increased the efficient uptake of TUBO exosomes. To extend the observation, after 24 h incubation, TUBO cells showed increased uptake and entrance (40%), but targeted HEK exosomes uptake did not experience any significant difference (33%), indicating different uptake kinetic characteristics of the exosomal fractions (Fig 3b).

Next, to compare the affinity of tumor derived exosomes with their counterparts derived from epithelial cells, the labeled exosomes released from SKBR3 cells, which is a well-established HER2 over-expressing breast cancer cell line, were added to TUBO seeded cells. Flow cytometry analysis after overnight incubation (24 h) indicated the higher binding of SKBR3 exosomes than HEK-derived exosomes. However, this time, the specific binding of DARPin to HER2 positive TUBO cells showed the
highest affinity. This finding confirmed the tropism of tumor-derived exosomes towards nonparental tumor cells (Fig 3c).

Based on the hypothesis of using naturally targeted exosomes as a candidate carrier for targeted delivery, and to determine if exosomes adhere/internalize with cancer cells (independent of origin), TUBO-derived exosomes have been added to SKBR3 seeded cells. TUBO-derived exosome were efficiently incorporated into other cancer cells as well but the efficiency of their uptake appears to be significantly higher in parental cells than in other cells (Fig 4).

To further confirm, microscopy analysis was subsequently performed on TUBO cells treated with PKH-67 labeled exosomes, which are depicted in Fig 5. TUBO and targeted exosomes exhibited the accumulation patterns within TUBO cells, the signals of targeted and TUBO derived exosomes were dispersed within the cytoplasm, which suggests that exosomes were taken up by the cells.

To support the observations above and evaluate the binding and migration of exosomes from different sources to the HER2 receptors, the fluorimetric analysis was performed. The different concentrations of PKH-67 labeled exosomes were added to TUBO seeded cells. Interestingly, TUBO and targeted exosomes showed the highest affinity to the HER2 receptor than untargeted exosomes. Targeted HEK and TUBO exosomes are similar in size and morphology, and there was no significant difference in uptake efficiency between them, which indicates DARPin ligand interaction increased the binding capability of targeted exosomes. Fluorimetry demonstrated that TUBO exosomes bound to HER-2 positive cells more efficiently than blank exosomes suggesting that the parental homing considerably improves the binding efficiency of exosomes. This variation in a lower concentration of exosomes is more significant, confirming nonspecific binding of untargeted exosomes in higher concentrations. Thus, it is essential to consider the dosage of administrating exosomes. Taken together, these data suggest that cancer-dreived exosomes can shuttle better into the cancer cell types that they are released (Fig 6).

**Discussion:**

The innate ability of exosomes to target tissues of interest besides their unique characteristics make them as an intriguing delivery agent in nanomedicine. Naïve exosomes accumulate within tumors due to the enhanced permeability retention (EPR) effect on leaky vasculature of tumors [21]. The goal of this study is whether exosomes have preferential association and uptake by parental cells. Since exosomes are shuttling information between cells, the components and biological functions are considerably dependent on the donor cells. As tumor-derived exosome contents mimic parental cell constituents, thus may be cancer-derived exosomes effectively migrate to the same source they are released. Here, we hypothesized that the natural tropism of cancer-derived exosomes could be beneficial to design an exosome-based delivery system. Our results clearly showed that exosomes derived from TUBO cells exhibited higher binding to TUBO cells than HEK-derived exosomes. This is consistent with Kim et al. study in which delivery potency of epithelial-derived exosomes (HEK293) was compared with tumor-derived exosomes in SKOV3 xenograft. SKOV3 exosomes demonstrated better targeting efficiency due to
the self-targeting of cancer-derived exosomes [14]. On contrary, Smyth et al. reported that exosomes released by MCF-7, MDA-MB-231, and PC3 cancer cell lines did not preferentially interact with their “parent” cell lines. Most notably, their results further suggested that the exosomal lipid components are more responsible for increased cell association [22]. Additionally by cleavage the surface proteins of exosomes they confirmed the importance of proteins in incorporation of exosomes. The involved mechanism for exosomes uptake after association with target cells are both endocytic [23] and phagocytic pathways [24]. Al-Nedawi et al. demonstrated the transfer of the mutated epidermal growth factor receptor (EGFRvIII) to cancerous cells by exosomes derived from highly aggressive brain gliomas [25]. The short circulation half-life of exosomes have been reported in numerous studies presumably due to recognition by immune cells. To enhance the circulation time of exosomes and thereby increase the affinity of exosomes towards tissue of interest, previously we introduced targeting ligand on the surface of exosomes by fusion proteins. Indeed, targeting ligand resulted in an effective internalization by tumor cells, it appears that this elevated affinity is mediated by ligand and receptor reaction which in our targeted exosomes is conferred by DARPin ligand to HER2 receptors. Moreover, fusion with the cell surface lipid is another mechanism for adherence and internalization of exosomes [1]. Yuki Toda et al. hypothesized that parental cell tropism is a critical factor for efficient exosome intercellular communication. They reported the incorporation of intact glioblastoma-derived exosomes into the parental and some other cancer cells. Furthermore, they demonstrated the underlying mechanism for the selective tropism and preferential uptake of exosomes can largely be attributable to the lipid component of U251-MG cell-derived extracellular vesicles [26]. Due to the difference of plasma membrane lipid components of various cells, the extracted exosomes show variation [27]. They attributed the higher uptake of U251 exosomes to the higher polyethylenimine (PE) and lower percentages of sphingomyelin (SM) in U251 exosomes, and this difference is due to the lipid composition of the parental cell membranes they have derived. The cone-shape structure of PE promotes fusion by increasing negative membrane curvature [28]. The abundance of PE in the plasma membrane of recipient cell and exosomes may be a key factor in self tropism. Smyth et al. investigated that apart from lipid composition, the distinct protein structures of exosomes also facilitate the internalization of exosomes in cancer cells [22]. They confirmed the role of lipids in preferential association by interaction of liposome composed of exosomal lipids. The enrichment of exosomes by cholesterol, sphingomyelin (SM), and anionic phospholipids, most notably phosphatidylserine (PS) make their surface lipid components unique for better association. The high stability and rigidity of exosomes is owing to the presence of high cholesterol and SM concentrations. From the same group studies revealed that the cholesterol domains promote delivery both in vitro [29, 30] and in vivo [31] implicating the effect of lipids in exosome trafficking and likely extend exosomes circulation half-life [32]. The enrichment of exosomes by trans membrane proteins such adhesion proteins most notably integrins [33, 34], tetraspanins [35], and the ICAM family of proteins [36] augment exosome adherence/internalization. Morelli et al discussed the presence of milk fat globule (MFG)–E8/lactadherin, CD11a, CD54, phosphatidylserine, and the tetraspanins CD9 and CD81 on the targeting of exosomes to DCs [37]. They demonstrated that exosomes interaction with their parental cells significantly decreased when their surface protein has been cleaved. Therefore, the higher uptake of TUBO exosomes compared to HEK exosomes may be due to particular
components of exosomal surface reportedly lipids and proteins involved in their uptake and association. Li Qiao et al. proved that isolated exosomes from Hela and HT1080 cancerous cell lines had preferential homing to their parent cancer cells. Furthermore, they found that exosomal integrin expression patterns might be responsible for the exhibited homing process [11]. According to our results, fluorimetry analysis revealed the unspecific binding of blank exosomes in higher concentrations. Importantly, we observed that with increasing the dosage of exosomes on HER-2 positive cells, the prominent factor in the attachment would be nonspecific physical absorption [19]. Here we could demonstrate that in lower concentrations of exosome, the uptake and affinity are selectively related to the exosome surface ligands and components, not to physical adsorption, which took part in the higher concentration of exosomes, thus decreasing the variation between two groups.

Here, again we reaffirmed the importance of incorporation of ligands to have better targeting efficiency. The most widely used method to produce targeted exosomes is the engineering of parental cells and expressing the desired ligand on the surface of exosomes beside a fusion protein [16, 38]. Although effective, some concerns about the engineering of exosome producing cells to express targeting ligand have been addressed. One of the main challenges in using fusion proteins, especially Lamp-2B, is the possible degradation of peptides fused to the N-terminal of lamp-2B protein. Hung et al. enhanced the half-life of Lamp-2B fusion protein by introducing a glycosylation motif [39]. Moreover, the improper expression of the transgene and the difficulties in the transfection of some cell lines are the other limitations in exosome redecoration. Nevertheless, despite efficient genetic manipulation of some cells like HEK293, the low yield of produced exosomes hinder their translation in clinical therapeutic applications.

Here in this study, our aim was to employ the natural and intrinsic targeting ability of exosomes to overcome the drawbacks of exogenously added ligands for therapeutic purposes. As aforementioned, cancer-derived exosomes resemble the signature of the cells that they are released. Although cancer-derived exosomes might have the possibility to stimulate immune responses by packaging some cancer antigens, they can play major role in tumor progression too. Therefore, owing to these controversial roles of cancer-derived exosomes, care should be taken in designating an exosome-based delivery system [40]. However, proteome array analysis are under progress in our team to unravel the involved mechanisms in preferential uptake by donor cells. We believe that understanding the contents of tumor derived exosomes is a promising avenue to overcome the unwanted side effects in cancer therapy. Several hurdles such as the clinical safety of cancer-derived exosomes required to be addressed prior to clinical translation.

As the physical property such as the size of exosomes might have a significant effect on cells ability to internalize them [41], the enhanced adherence of unmodified cancer-derived exosomes could be due to the greater size and density of cancer exosomes resulting in increased contact on the cell surface. Federica Caponnetto et al. determined the effect of physical properties of exosomes on their uptake and addressed the question whether the method of preparation of enriched exosomal fractions can affect their uptake by cells and their ability to trigger a response [42]. They compared the polymer-based precipitation purification method of exosomes with ultracentrifugation extracted exosomes and confirmed
that the smaller size of exosomes resulted by polymer-based method and led to higher and faster uptake rather than exosomes obtained by ultracentrifugation. This is corroborated with our previous study which we have isolated exosomes by differential ultracentrifugation procedure, and it yielded the lower amount of exosomes and bigger particle size distributions (172 nm) compared to the commercial kits precipitation method (120 nm). Here we confirmed the previous findings that NTA failed to recognize the smaller particles [42]. Having efficient clinical translation of exosomes requires a reliable isolation method, rendering in highly pure exosomes without contamination. The gold standard method for exosomes isolation and purification is differential ultracentrifugation [43]. But due to the high cost of ultracentrifugation, and protein aggregation we tried to purify exosomes using a commercial kit which precipitates exosomes by size exclusive chromatography. We assessed the size of various preparations of exosome by zeta sizer, and we could confirm that they had a similar size. Additionally, we homogenized the size of exosomes by filtering the supernatants. In fact, engineered exosomes may show different properties such as uptake efficiency, kinetics and the internalization mechanism due to increase in size after introducing the transgene [44]. But in case of our study, as the DARPin is a small ligand, the effect of this protein on the size of engineered exosome is negligible. The yield of exosomes depends on the isolation method and donor cells. It is believed that cancer cells generate more exosomes rather than healthy cells, which may be due to an enhanced growth rate or as a result of stimulation in response to stressful conditions. Here, TUBO cells secreted much higher exosomes than HEK cells according to the obtained results by Bradford and CD9 ELIZA assay. It has been already reported that exosome canonical markers are not equally expressed by different exosome subpopulations [45]. But here TUBO and HEK293 exosomes relatively expressed CD9 in the same amount. We have used CD9 measurement due to the overestimation of exosome density by co-precipitation of proteins along with exosomes which makes the colorometric assays like Bradford impractical. By this alternative assay we can measure directly exosome population and avoid the risk of overestimation in future clinical studies. However, as different populations do not express the markers equally it would be ideally to measure the exosome density of the same source because exosomal secretion likely differs among cells. Taken together the innate tumor tropism of cancer-derived exosomes along with the high production rate, suggest that exosomes derived from cancer cells may represent a target for therapeutic applications. Here we compared the tropism and uptake of tumor-derived exosomes with engineered exosomes considering different factors such concentration and incubation time. To our knowledge our work is the first study comparing specific binding of tumor and engineered cells. Further studies are under investigation to unravel the mechanism of entry to establish whether the targeting of tumor derived exosomes would boost the efficiency of uptake in tumor models. Tumor cells regulate the movement of their exosomes via the modulation of exosomes components. The molecular components involving in specific uptake of exosomes are not fully elucidated and depends to the donor and recipient cells. The initial step on the recognition of exosomes by recipient cells is ligand receptor interaction which was the primary key element of targeted exosomes recognition by TUBO cells in the first 4 hr but with increasing the incubation time, TUBO cells had more time to recognize TUBO exosomes and facilitate their effective internalization. The increased absorbance and internalization of TUBO exosomes inside SKBR3 cells reveals that cancer cells have a mechanism to recognize cancer-derived exosomes.
In the pilot *in vivo* study we administered TUBO-derived exosomes into TUBO xenograft mice model to assess whether they are effective at traveling back to the parent cell line that produced them. To address this question, we studied the systemic biodistribution of tumor-derived exosomes, remarkably, we observed that the tropism exhibited by tumor-derived exosomes can be utilized in the future to shuttle the cancer therapeutic agents with no specific targeting capacity, to tumor tissues.

**Conclusion:**

We found the preferential higher incorporation of cancer-derived exosomes compared to epithelial derived exosomes by donor cancer cells. Future studies will focus on investigating the homing mechanism of cancer-derived exosomes *in vivo*. A better understanding of exosomal tropism to target cells will lead to the emerging role of exosomes in theranostics.

**Declarations**

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**Disclosure**

To the best of our knowledge, none of the above-suggested persons have any conflict of interest, financial or otherwise...

**Ethical approval**

This article does not contain any studies with human participants or animals performed by any of the authors.

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**Conflicts of interest/Competing interests:**

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**Availability of data and material:**
Not applicable

**Code availability:**

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**Consent to participate:**

Not applicable

**Consent for publication:**

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Figures
Figure 1

The expression of DARPin in transduced HEK cells. (a) RT-PCR on transduced HEK cells. Lane 1: nontransduced HEK cells as control negative. Lane 2: 430 band represents the presence of DARPin in purified mRNA. lane 3: 1 kb ladder. (b) Western blotting confirmed the expression of LAMB-2B-DARPin on targeted exosomes surface. Lane 1: 70 KDa LAMB-2B-DARPin was recognized in the exosome lysates. Lane 2: nontransfected exosomes as a negative control.
Figure 2

Characterization of cancer-derived exosomes by western blotting and size distribution. (a) The presence of exosomal markers on the surface of exosomes. Lane 1: putative band confirms the presence of 50 KDa CD63. Lane2: Protein Marker. Lane3: 25 KDa CD9 band was observed in exosomes isolated from TUBO cells. (b) The size of cancer-derived exosomes was measured by zeta-sizer. One sharp peak shows the homogenous isolation of exosomes around 120 nm.
Figure 3

(a) Cancer-derived exosomes preferably migrate to TUBO cells. Uptake analysis by flow cytometry. Exosomes derived from TUBO cells showed 28% binding and attachment to the HER2 receptor on TUBO cells while targeted HEK exosomes revealed 26% affinity. Exosomes from untargeted HEK cells used as control. The shift in signals from the control indicates higher uptake of the TUBO exosomes. (b) The effect of incubation time on the attachment and internalization of above-mentioned exosomes at different time points. Targeted exosome exhibited increased association with TUBO seeded cells. But it reversed after 24 h incubation. (c) Treatment of SKBR3 cancer-derived exosomes by flow cytometry have revealed the higher uptake to TUBO cells rather that epithelial-derived exosomes (HEK 293), but targeted exosomes extracted from HEK cells resulted the highest uptake due to the high affinity of DARPin ligand toward HER-2 receptor.
Figure 4

Binding measurement of different exosomal fraction to SKBR3, HER2 positive breast cancer cells. Targeted exosomes indicated the highest interaction to the SKBR3 cells compared to TUBO exosomes and untargeted HEK-derived exosomes by flowcytometry.
Figure 5

Representative micrograph showing the attachment and internalization of PKH-67-labelled exosomes to TUBO cells. (a) The binding of TUBO derived-exosomes to the HER2 receptor on TUBO cells. (b) Targeted exosomes showed relatively efficient targeting and affinity to TUBO cells.
Figure 6

Binding measurement of exosomes to TUBO cells. Fluorimetry analysis demonstrated that exosomes in 0.5 and 0.1 µg/µl showed significant differences in attachment ability. Fluorescence emission was measured after rinsing the unbound exosomes. TUBO-derived exosomes showed the highest targeting efficiency in TUBO cells. Each error bar represents the mean ±SD of three replicates *P<0.05