Molecular Therapy
Nucleic Acids
Original Article

Identification of a novel RNA aptamer that selectively targets breast cancer exosomes

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Breast cancer is a leading cause of cancer mortality in women. Despite advances in its management, the identification of new options for early-stage diagnosis and therapy of this tumor still represents a crucial challenge. Increasing evidence indicates that extracellular vesicles called exosomes may have great potential as early diagnostic biomarkers and regulators of many cancers, including breast cancer. Therefore, exploiting molecules able to selectively recognize them is of great interest. Here, we developed a novel differential SELEX strategy, called Exo-SELEX, to isolate nucleic acid aptamers against intact exosomes derived from primary breast cancer cells. Among the obtained sequences, we optimized a high-affinity aptamer (ex-50.T) able to specifically recognize exosomes from breast cancer cells or patient serum samples. Furthermore, we demonstrated that the ex.50.T is a functional inhibitor of exosome cellular uptake and antagonizes cancer exosome-induced cell migration *in vitro*. This molecule provides an innovative tool for the specific exosome detection and the development of new therapeutic approaches for breast cancer.

INTRODUCTION

Breast cancer (BC) is the most common tumor type and one of the leading causes of cancer mortality in women, with >450,000 women dying annually.[1] The disease stage at diagnosis greatly influences the therapeutic success, and research on circulating biomarkers is fundamental for the early detection of the tumors in a non-invasive and cost-effective way.[2] At the same time, there is a need to develop new therapeutic options with improved efficacy and safety.[3]

In recent years, extracellular endosome-derived vesicles called exosomes have attracted growing interest as promising cancer biomarkers and therapeutic targets. Exosomes are vesicles of 50–150 nm diameter released by most cell types into the circulation and that contain nucleic acids and proteins.[4] It has been shown that tumor cells, including BC cells, release great amounts of cell-specific exosomes that, being stable and easily accessible from body fluids, may be used for specific cancer detection.[5,6] In addition, several studies have revealed that exosomes represent an important mechanism of communication between cancer cells and the tumor stroma, influencing tumor progression and dissemination, drug resistance, and immune surveillance.[7–11] Therefore, the removal of oncogenic exosomes from the bloodstream can result in important anti-cancer effects.[12,13] To date, several exosome-enriched proteins, including members of the tetraspanin family (CD9, CD63, and CD81) or of the endosomal sorting complexes required for transport (TSG101 and Alix) and heat-shock proteins (Hsp60, Hsp70, and Hsp90), have been characterized.[14] Some exosome proteins (i.e., HER2, CD47, Del-1), microRNAs (i.e., *miR-1246* and *miR-21*) and RNAs (i.e., *NANOG*, *NEUROD1*, and *HTR7*) have also been associated with BC tumor recurrence, metastasis, and patient survival, confirming the theranostic value of these vesicles.[15–17] In addition, several protocols for exosome recognition based on their enriched components are emerging in the literature.[18] However, specific tools to easily distinguish intact cancer cell-derived exosomes from exosomes normally present in the organism are mostly unknown.

A promising class of targeting molecules is represented by nucleic acid-based aptamers. They are high-affinity ligands of disease-associated proteins and possess many advantages for diagnostic and therapeutic applications, including low toxicity, cost-effectiveness, easy synthesis and modification, and no immunogenicity.[19] Aptamers are selected through an *in vitro* process called SELEX (systematic evolution of ligands by exponential enrichment), which has been successfully applied to a wide range of different targets, from small molecules to complex cells or tissues.[20] The specific targeting ability of aptamers has ensured their efficient use for biomarker discovery, detection, and therapy.[21–23]

In the present study, we explored the use of aptamers to specifically target BC-derived intact exosomes, discriminating them from those derived from normal cells. By developing a novel differential-SELEX (Exo-SELEX) approach, we selected a pool of nuclease-resistant

Received 27 July 2020; accepted 14 January 2021;
https://doi.org/10.1016/j.omtn.2021.01.012.

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sequences with improved binding for BC-derived exosomes. The best ligand (ex-50.T) specifically recognizes exosomes derived from BC cells and BC patient sera irrespective of the tumor subtype of origin. Interestingly, ex-50.T inhibits exosome uptake in breast cells, so it has anti-tumor activity, expressed as a reduction in cell migration. Furthermore, the aptamer shows no binding to human serum albumin (HSA), even at high doses, and good serum stability, providing a very promising tool for BC exosome targeting.

RESULTS

Exo-SELEX approach

To select aptamers able to specifically recognize the exosomes produced by BC cells, we developed a new SELEX variant, Exo-SELEX, using exosomes derived from either primary normal or cancer breast epithelial cells as complex targets for negative and positive selection, respectively. A library of 2′ fluoro-pyrimidines (2′F-Py) nuclease-resistant RNAs were used as a starting pool.

As schematized in Figure 1A, at each round of Exo-SELEX, the library was incubated on primary normal cell-derived exosomes conjugated with magnetic beads (counter-selection step). The unbound aptamers were recovered and incubated with bead-conjugated exosomes derived from primary BC cells (selection step). The bound aptamers were thus recovered and subjected to RNA extraction and amplification by reverse transcriptase polymerase chain reaction (RT-PCR). During the selection process, we used exosomes from different BC primary cells of two subtypes (Table S1) without introducing many subtypes whose handling would have been difficult. In particular, we decided to focus on the two most aggressive and heterogeneous BC subtypes—human epidermal growth factor receptor 2^+ ($HER2^+$) and triple-negative BC (TNBC)—that show a different expression of hormone receptors, biology, and treatment options. We performed eight rounds of SELEX, progressively increasing the selection pressure by changing incubation and washing conditions (Table S1).

Following the last round, we monitored the binding ability of the final pool (pool 8) as compared to the starting one (pool 0). Binding analyses by quantitative real-time PCR revealed that pool 8 had an enhanced discrimination (~8- to 10-fold increase) of exosomes derived from 2 different primary BC lines ($HER2^+$ Pt.37 and TNBC Pt.170 cell lines) used during the selection (Figure 1B). This result suggests that with respect to pool 0, pool 8 was enriched for aptamers against intact BC-derived exosomes.

Analyses of individual sequences

To isolate individual aptamers that may specifically recognize BC-derived exosomes, pool 8 from Exo-SELEX was cloned and a panel of 57 individual clones was sequenced and grouped in families based on their primary sequences (Figure 1C). We identified 3 distinct clusters of sequences with the 3 most-enriched aptamers (ex-50, ex-55, and ex-56, Figure 1C, boxed), covering, respectively, 5%, 3.5%, and 17.5% of the analyzed clones. We thus tested the ability of those aptamers and of 2 isolated sequences (ex-14 and ex-53) to discriminate normal versus BC exosomes by quantitative real-time PCR-based binding analyses. Aptamer binding was determined at 200 nM, comparing exosomes derived from Pt.72 (normal epithelial, NE), Pt.37 ($HER2^+$ BC), and Pt.170 (TNBC) primary cell lines. Among the screened sequences, we found that sequence ex-50 had the best binding for both BC-derived exosomes tested, reaching up to 6- to 9-fold increase with respect to NE-derived exosomes (Figure 2A). Preferential binding for Pt.170 (TNBC)-derived exosomes was detected for ex-55, while ex-56, ex-14, and ex-53 bound to a lower extend or were unable to discriminate BC-derived exosomes (Figure 2A).

Given the higher and wider binding ability of ex-50, we restricted further analyses to this sequence.

Binding validation and specificity of ex-50 aptamer

To demonstrate that the recognition capability of ex-50 was not limited to Pt.37 and Pt.170, we analyzed binding on exosomes derived from additional BC patient-derived cells with different subtypes (Pt. 46, luminal A; Pt.60, luminal B; Pt.148, TNBC). We also included exosomes derived from Pt.138 cells, isolated from a metastatic lymph node of a patient with luminal B tumor. As shown in Figure 2B, ex-50 retains the ability to bind all of the BC exosomes analyzed, irrespective of the tumor subtype of origin, as compared to normal-derived exosomes.

To further characterize ex-50 binding ability and specificity, we performed analyses on a panel of exosomes derived from continuous cell lines of BC or different cancer types, namely non-small cell lung cancer (NSCLC) and glioblastoma (GBM). Exosome preparations from different cell lines were comparable (mean particle size ~100–140 nm and D50 ~100–130 nm), as assessed by nanoparticle tracking analysis (NTA) (Figure S1). In accordance with the previous results, ex-50 had good binding ability on all of the BC-derived exosomes as compared to those from NE cells (Figure 2C). Notably, no binding was detected on exosomes derived from any of the NSCLC or GBM cell lines tested with respect to the control NE exosomes (Figures 2D and 2E).

In addition, ex-50 was able to discriminate BC primary cells (Pt.37 and Pt.170) from NE cells (Pt.72), suggesting that the recognized target is as well expressed on the surface of the target cells from which exosomes are derived (Figure 2F).

These data indicate that the ex-50 aptamer binds BC-derived exosomes and is able to discriminate them from those derived from normal BC and two other cancer types.

Ex-50 aptamer optimization for BC exosome detection

A key aspect of aptamer optimization is reduction to a length compatible with effective chemical synthesis. Thus, we decided to truncate ex-50 aptamer (80-mer) to a shorter version containing the active binding site. Based on the predicted secondary structure of the sequence (RNAfold), we designed a truncated version (33-mer), covering the central stem-loop portion, from nucleotide 27 to nucleotide 59, without the 5′ and the 3′ regions (Figure 3A). The resulting aptamer (indicated as ex-50.T) was then validated for binding ability.

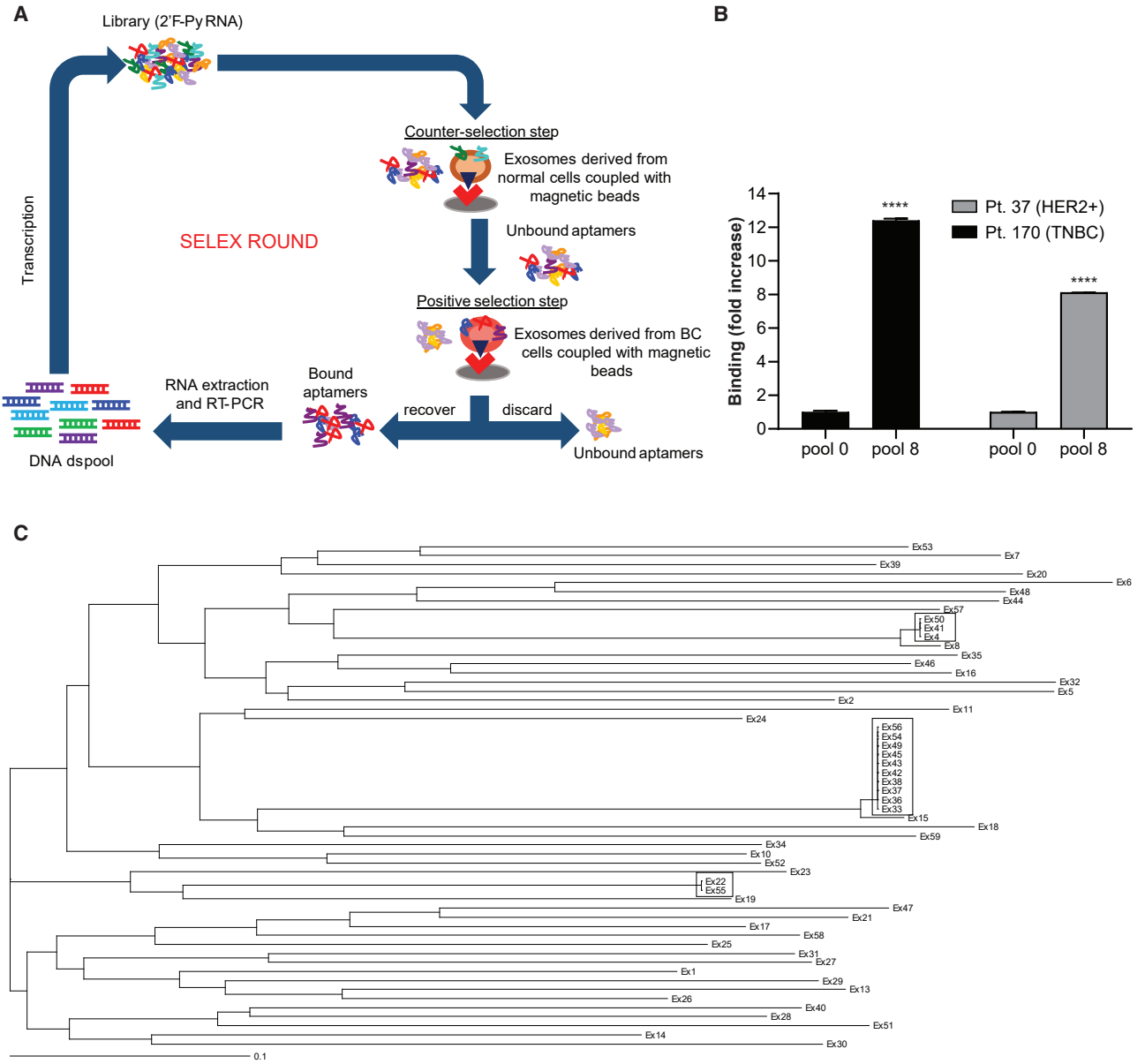

Figure 1. Exo-SELEX

(A) Scheme of the exo-SELEX strategy. (B) Binding by quantitative real-time PCR of the starting (pool 0) or the final (pool 8) pools from Exo-SELEX were analyzed on exosomes derived from primary breast cancer (BC) cells (Pt.37, HER^{+}; Pt.170, TNBC) at 200 nM concentration. Results are expressed as folding increase over pool 0. Error bars depict means ± standard deviations (SDs). Significance of pool 8 versus pool 0 was measured by t test: ***$p < 0.0001$. (C) Phylogenetic tree of the individual sequences cloned following the selection. The most represented sequences are boxed.

As shown in Figure 3B, the quantitative real-time PCR-based binding assay revealed that the ex-50.T aptamer preserves the ability to bind BC-derived exosomes.

To further characterize the binding ability of the truncated aptamer, we applied an aptamer-based system in a direct-enzyme-linked oligonucleotides assay (ELONA) format. Plates were coated with intact exosomes coming from NE or BC cells, and the correct coating was checked by using anti-CD63 antibodies (Figure S2). For aptamer-binding analyses, plates left uncoated (blank, "-") or coated with intact exosomes were incubated with the biotin-labeled ex-50.T. Signals were then detected with streptavidin-horseradish peroxidase (HRP) (Figure 3C). Using such an approach, we tested the binding of ex-50.T at 200 nM on a panel of different exosomes from NE or BC cells (Figure 3D) and compared the results with a control unrelated biotinylated aptamer (CtrlApt). Ex-50.T retained wide and

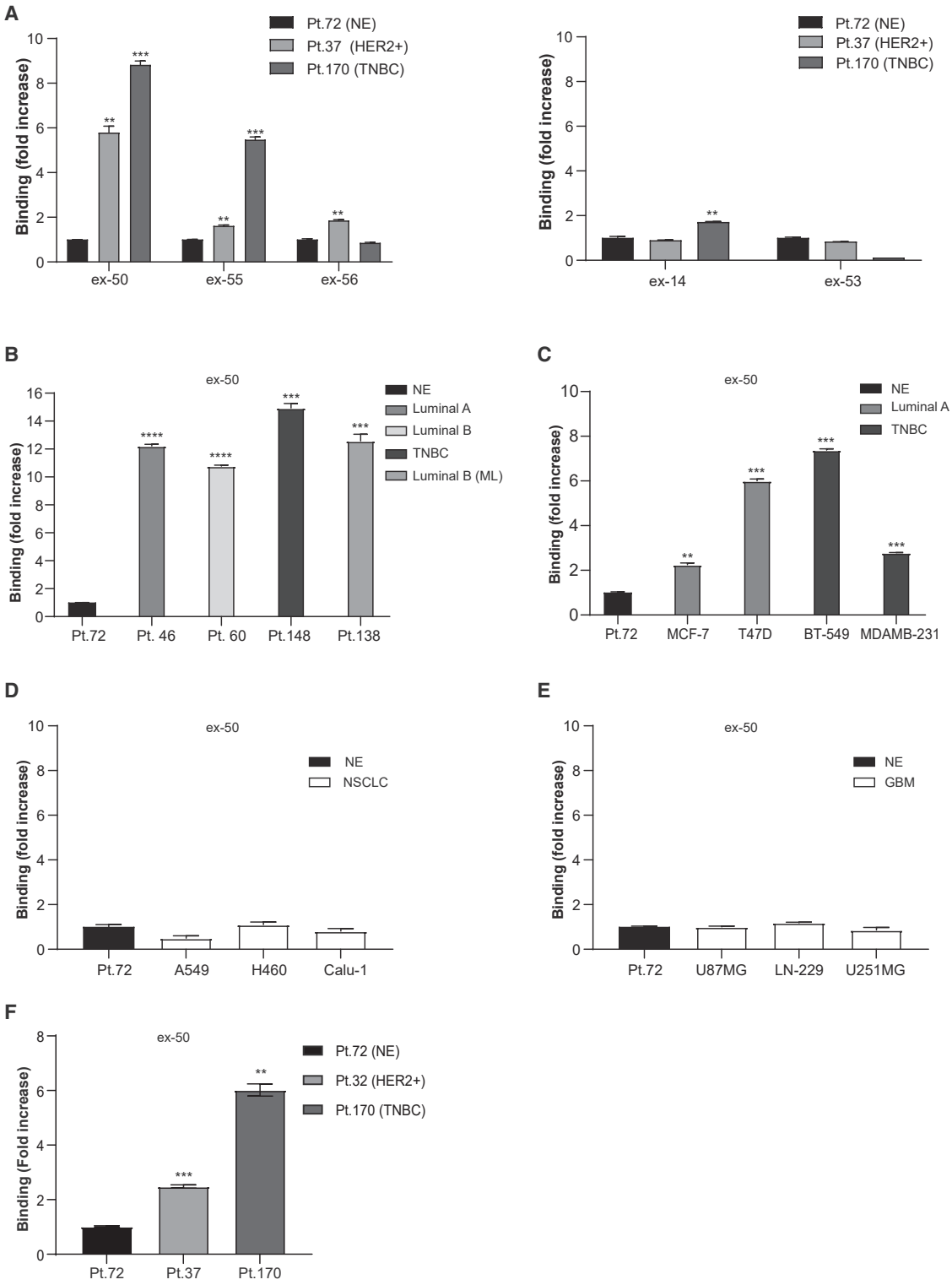

Figure 2. Binding analyses of individual sequences

(A) Binding ability of the 3 most enriched aptamers (ex-50, ex-55, and ex-56) and 2 isolated sequences (ex-14 and ex-53) on exosome derived from primary breast normal (Pt.72, NE) or cancer (Pt.37 and Pt.170) epithelial cells was analyzed by quantitative real-time PCR. (B) Analyses by quantitative real-time PCR of the binding of the ex-50

(legend continued on next page)

specific binding only on exosomes derived from primary BC cells. Notably, no binding was observed for the control aptamer, indicating that BC exosome recognition is specifically mediated by ex-50.T. Furthermore, the assay was applied to confirm ex-50.T binding specificity, by analyzing a panel of exosomes from non-related cancer cells (Figure 3E). As shown, ex-50.T maintains specific binding for BC-derived exosomes, while it is not able to discriminate other cancer cell line-derived exosomes (GBM and NSCLC).

These data confirm ex-50.T as a promising aptamer candidate for the detection of BC exosomes.

Ex-50.T affinity and serum stability

Next, we used the ELONA-based assay to measure the apparent dissociation constant (K_D) of ex-50.T on BC-derived exosomes. Plates were left uncoated or coated with MDA-MB-231 cell-derived exosomes and incubated with increasing amounts of biotinylated ex-50.T or CtrlApt (ranging from 0.1 to 200 nM). Specific binding analyses (Figure 4A) revealed that ex-50.T had high affinity for BC exosomes, with a K_D of ~0.8 nM. To further explore the aptamer detection ability, plates were left uncoated or coated with increasing concentrations of MDA-MB-231 cell-derived exosomes (ranging from 0.1 to 100 $\times$ 10^6 particles/mL) and incubated with 400 nM ex-50.T aptamer (Figure 4B). We found a good dose-dependent interaction of ex-50:T with exosome, and data fitting revealed an affinity of ~15 $\times 10^6$ particles/mL. These data strongly reinforce the utility of ex50.T for BC exosome detection, considering that a concentration of exosomes on the order of 10^8 particles/mL has been reported in serum or plasma.[24]

Next, we evaluated aptamer-binding HSA, the most enriched protein in human plasma and capable of binding nucleic acids due to its charge, thus limiting the applicability of oligonucleotides for patient sample analyses. To this purpose, biotinylated ex-50.T or CtrlApt aptamers were incubated at increasing concentrations on plates previously coated or not with HSA (Figures 4C and S3A). Compared with blank points, no aptamer binding was measured up to 1,000 nM, indicating that ex-50.T does not react with HSA. Binding was instead detected when ex-50.T was incubated with BC-derived exosomes used as a positive control (Figure S3B).

Ex-50.T aptamer contains 2′F-Py, which confers resistance to enzymatic degradation, providing a stable and easy-to-handle tool. We thus analyzed the serum stability of ex-50.T, incubating the aptamer in 85% human serum at 37°C for increasing times. RNA samples were then analyzed by denaturing polyacrylamide gel electrophoresis (Figure 4C, left panel). We found that the ex-50.T aptamer remains almost stable up to 24 h, and then is gradually degraded, with an approximate half-life of ~40 h in our experimental conditions (Figure 4C, right panel).

These data indicate that the ex-50.T aptamer represents a promising tool with great applicability potential for BC exosome targeting.

Ex-50.T binding on liquid biopsy of BC patient-derived exosomes

Exosomes derived from cells grown in culture are much more homogeneous than those expected in patient blood. We thus determined whether, despite the enormous complexity of blood samples, ex-50.T aptamer was able to recognize BC exosomes. To this end, we isolated exosomes from serum samples of patients suffering from BC (luminal A n = 2, luminal B n = 2, TBNC n = 1, and $HER2^+$ n = 2; Table S2). As control, exosomes were extracted from serum samples of patients with non-tumoral breast hyperplasia (n = 3) or suffering from different non-oncologic pathologies (n = 3) (Table S3). We thus used the more sensitive quantitative real-time PCR-based binding assay (as described above). Exosomes were incubated with CD-81 magnetic beads and subjected to a binding assay as reported in Materials and methods. As shown in Figure 5A, the aptamer discriminated between normal and tumoral exosomes, showing a good binding rate for all of the tumoral samples analyzed, with the exception of sample no. 143.

The binding on exosomes from patients was confirmed by ELONA assays by using plates directly coated with exosomes without CD-81 pre-cleaning (Figure 5B). The analysis was performed on exosomes from serum samples of 13 patients suffering from BC (luminal A n = 3, luminal B n = 4, TBNC n = 4, and $HER2^+$ n = 2), 8 patients with non-tumoral breast hyperplasia (n = 3) or suffering from different non-oncologic pathologies (n = 5), and 2 healthy donors (with no evident pathologies) (Table S3). The aptamer showed a good binding rate for all of the tumoral samples analyzed, irrespective of their BC subtype, as compared to a control aptamer. Notably, no binding was detected on normal exosomes either from patients with breast hyperplasia and non-oncologic pathologies or from healthy donors used as controls.

These data greatly reinforce the notion of ex-50.T being a promising tool for exosome targeting in BC.

Ex-50.T functional activity

To investigate its functional activity, we wondered whether the ex-50.T aptamer prevented exosome uptake by recipient cells. To this end, we treated TNBC MDA-MB-231 cells for 3 h with PKH26-labeled exosomes derived from the same cell line, previously incubated or not with ex-50.T aptamer or with a control aptamer (CtrlApt). After checking that the background resulting from the incubation of cells only with PKH26 (without exosomes or aptamers) was negligible (Figure S4), exosome uptake was monitored by confocal microscopy (Figure 6A, left panels) and the percentage of exosome-positive cells (red signal from PKH26) over total cells

aptamer on exosomes derived from Pt.72 (NE), indicated primary BC cultures, or from cells of a metastatic lymph node (ML) of a luminal B BC patient. (C–E) Binding by quantitative real-time PCR of the ex-50 aptamer on exosomes derived from indicated continuous cell lines. (F) Binding by quantitative real-time PCR of the ex-50 aptamer on the indicated primary cell lines. In (A)–(F), results are expressed as fold increase relative to control exosomes derived from NE cells. Error bars depict SD values. Statistics by t test (versus NE): $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$.

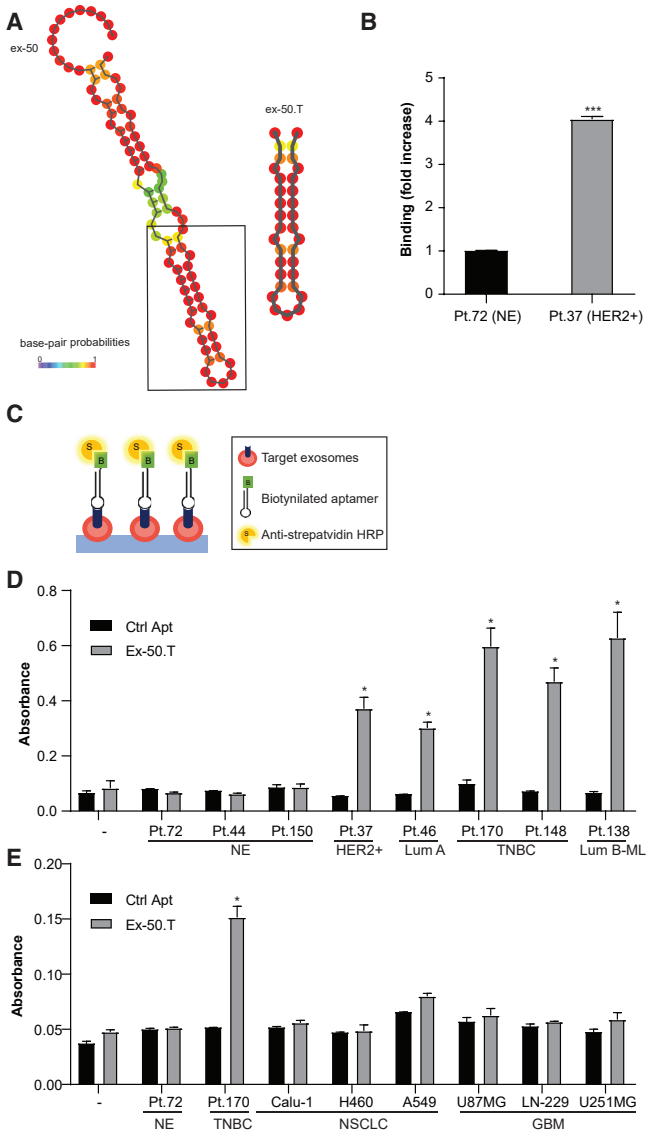

Figure 3. Ex-50 aptamer optimization

(A) Secondary structure prediction of ex-50 and its shortened version (ex-50.T). (B) Binding ability of the ex-50.T by quantitative real-time PCR on exosome-derived from primary epithelial normal breast (Pt.72, NE) or BC cells (Pt.37). Error bars depict means ± SDs. (C) Scheme of the ELONA-based detection system. Plates are coated with exosomes and incubated with biotinylated aptamers; signals are detected with streptravidin-HRP. (D and E) Binding of exo-50.T or unrelated control aptamers (CtrlApt) was measured at 200 nM by ELONA-based assay on plate left uncoated (–) or coated with exosomes derived from the indicated cell lines. Error bars indicate means ± SEMs. Lum, luminal. Statistics by t test (versus Pt.72, NE): *$p < 0.05$; ***$p < 0.001$.

(nuclei visualized with DAPI) was calculated (Figure 6A, right panel). As shown, ex-50.T aptamer inhibited exosome uptake by MDA-MB-231 cells by ~50% as compared with CtrlApt. These data were also confirmed by fluorescence-activated cell sorting (FACS) analyses of exosome (PKH26)-positive cells (Figure S5).

Since it has been demonstrated that tumor cell-derived exosomes act on the surrounding cell population, favoring tumor progression and cell transformation, we analyzed the aptamer ability to inhibit the pro-tumor effects of exosomes from highly tumorigenic BC cells. To this end, as a model, exosomes isolated from MDA-MB-231 cells were used to treat MCF-7 BC cells, which exhibit a lower metastatic potential than MDA-MB-231 cells. After 24 h of treatment, cell migration was evaluated with a wound healing assay. Pictures were taken immediately after wound creation (0 h) or following 24 h of exosome treatment (Figure S6, left panel); wound healing was calculated as a percentage of area closure (Figure S6, right panel). The migration of MCF-7 cells was significantly induced by MDA-MB-231-derived exosomes. Therefore, we explored whether the ex-50.T aptamer interfered with this induction. To this end, MCF-7 cells were treated for 24 h with MDA-MB-231-derived exosomes previously incubated or not with ex-50.T aptamer or with a control aptamer (CtrlApt), and cell migration was evaluated with a wound healing assay (Figure 6B). We found that the ex-50.T aptamer inhibited MCF-7 cell migration induced by MDA-MB-231-derived exosomes by ~30%, as compared with the CtrlApt.

Furthermore, the aptamer inhibition of cell migration induced by exosomes isolated from highly metastatic MDA-MB-231 BC cells was also evaluated on normal epithelial MCF10A breast cells. To this end, MCF10A cells were seeded into the upper chamber of 24-well Boyden chamber Transwells in the absence or presence of MDA-MB-231-derived exosomes pre-incubated or not with ex-50.T aptamer or CtrlApt. Pictures were taken following 24 h of treatment (Figure 6C, left panel), and the percentage of migrated cells was calculated and expressed relative to cell migration without exosomes (Figure 6C, right panel). The obtained results indicate that the exosome isolated from MDA-MB-231 cells strongly induced MCF10A cell migration and that this pro-migratory effect was significantly reduced in the presence of the ex-50.T aptamer.

On the contrary, exosomes from highly metastatic MDA-MB-231 BC cells did not provide a consistent induction of MCF10A cell viability after 24 or 48 h, and no effects on induced viability were detected in the presence of ex-50.T (Figure S7).

These results indicate that the ex-50.T aptamer, upon binding to target exosomes, inhibits their cellular uptake and blocks exosome-induced cell migration; therefore, it holds great potential for the development of future therapeutic approaches for BC.

DISCUSSION

Exosomes are an important mechanism of communication between cancer cells and the tumor stroma, representing ideal candidates for the development of innovative approaches for early cancer diagnosis and therapy.[5,7] Cancer exosomes are easily accessible, thus providing stable diagnostic targets. In addition, the inhibition of their uptake by surrounding tissues and organs can be an effective strategy to interfere with tumor spreading and development.[7,25] However, the exploitation of molecules able to distinguish cancer exosomes from normal

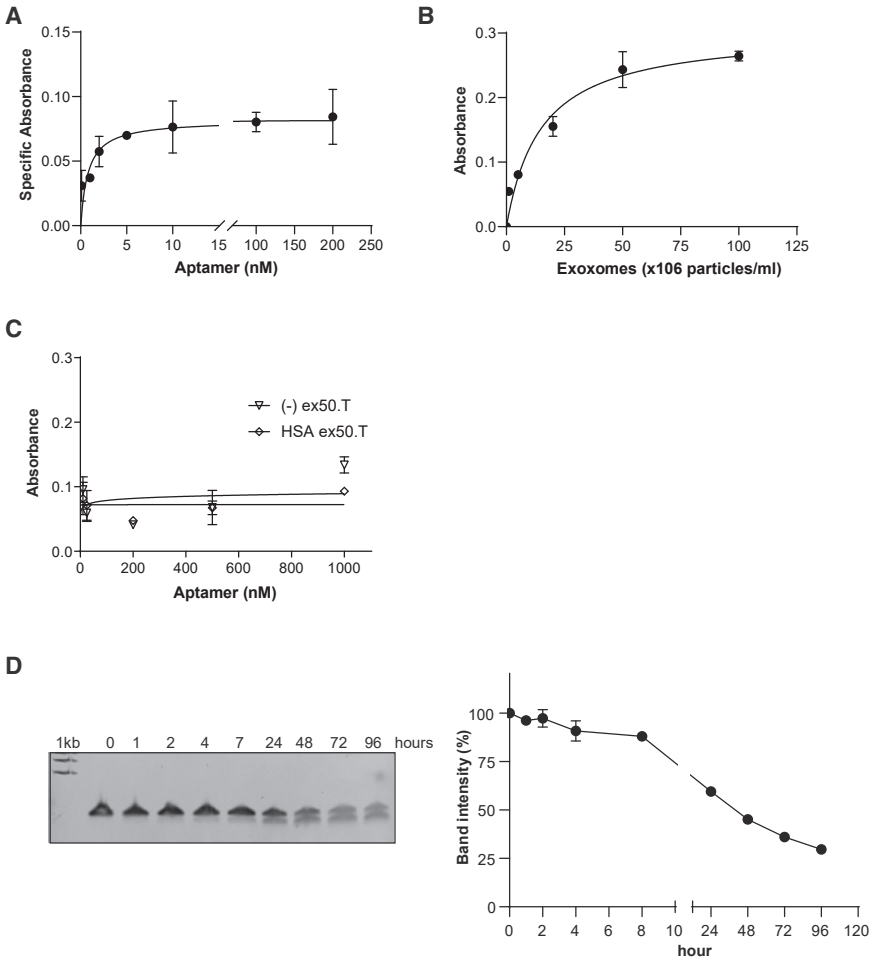

Figure 4. Ex-50.T aptamer affinity and serum stability

(A) Increasing concentrations of ex-50.T or control aptamer (CtrlApt) were incubated with plates left uncoated or coated with MDA-MB-231-derived exosomes. Specific binding of ex-50.T was measured subtracting the values obtained with CtrlApt. (B) Plates were coated with increasing concentrations of MDA-MB-231-derived exosomes and incubated with 500 nM ex-50.T. Background values obtained on uncoated wells were subtracted. (C) Increasing concentrations of ex-50.T were incubated with plates left uncoated (–) or coated with has-purified protein. In (A)–(C), error bars indicate means ± SEMs. (D) Ex-50.T was incubated at 4 μM in 85% human serum for the indicated times. Left, RNA-serum samples were evaluated by electrophoresis with 15% denaturing polyacrylamide gel. Right, the intensity of the bands was quantified by using the ImageJ program and expressed as the percentage relative to time 0.

ones as well as exosomes released from different cancer types is a critical aspect in the realization of their clinical utility.

Here, we used a new differential SELEX methodology (Exo-SELEX) to isolate a nuclease-resistant aptamer (ex-50.T) that can specifically bind BC-derived exosomes and inhibit cancer cell exosome uptake. This aptamer displays great potential for exosome targeting in diagnosis and therapy.

Given the attractive features of nucleic acid aptamers,[26] their application to exosome targeting has emerged in recent years.[27] An aptamer-based molecular profiling approach, called SOMAscan, has been applied to the proteomic analyses of prostate cancer-derived exosomes; the assay is based on the use of a set of chemically modified aptamers against known targets.[28] More recently, the possibility of applying combinatorial strategies for oligonucleotide-based profiling of cancer exosomes has been reported.[29,30] Taking advantage of the versatility of aptamers, a growing number of proof-of-principle studies on aptamer-based biosensors for exosome detection have been described.[18,31–33] In addition, it has been shown that aptamer-functionalized biomaterials can be used to eliminate blood exosomes and reduce exosome-induced pro-tumor effects.[34,35] Nevertheless, all of these studies use oligonucleotides against known commonly enriched targets (i.e., CD63, EpCAM, and epithelial growth factor receptor [EGFR]) suffering from the lack of selectivity.

In this context, our work describes an innovative selection strategy for the isolation of new aptamers specifically binding intact cancer exosomes, without any prior knowledge of their target. The approach was applied to exosomes derived from BC, which remains the major cause of death among women.[36] The developed protocol was effective in isolating molecules discriminating BC exosomes from those derived from NE cells or unrelated cancer cell lines. Among the selected sequences, we optimized the ex-50 aptamer, deriving a short sequence of 33 bases showing effective and high specific binding to BC-derived exosomes, irrespective of their subtype, as compared to exosomes from NE or from unrelated cancer cell lines (NSCLC and GBM). The aptamer is also able to recognize exosomes from the serum of BC patients.

Most important, ex-50.T inhibited exosome uptake by recipient cells and reduced MDA-MB-231 exosome-induced cell migration in non-metastatic MCF-7 BC cells and normal MCF10A epithelial cells, indicating that it could be an interesting molecule to block tumor spreading and cell transformation. Furthermore, the developed sequence retains important features, such as serum stability and high exosome detection (affinity at $\sim 15 \times 10^6$ particles/mL), and does not bind to HSA, enhancing its potential applicability.

The use of our sequence would thus allow the introduction within exosome targeting systems of a highly specific recognition element

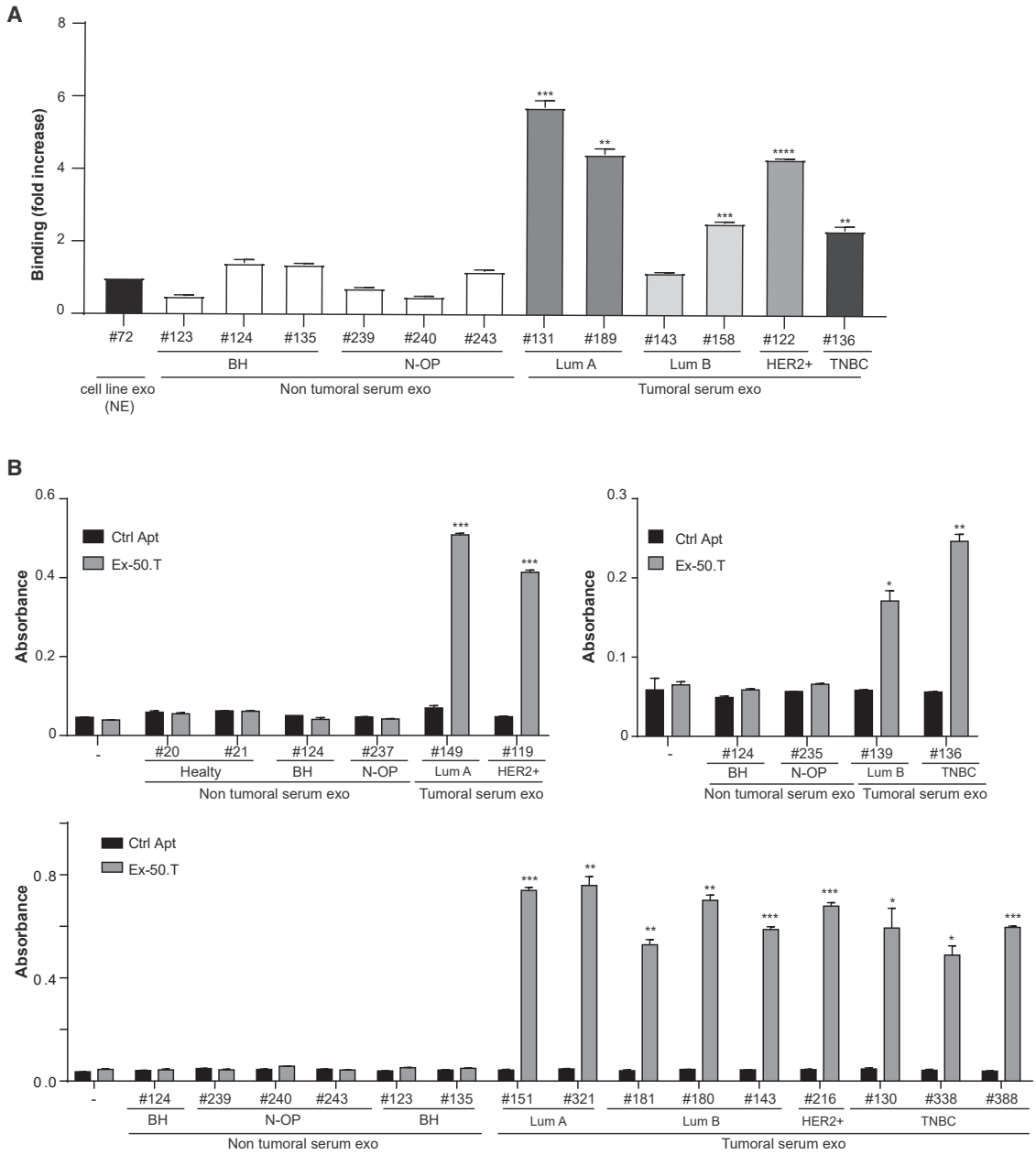

Figure 5. Ex-50.T aptamer binding of exosomes derived from patient serum samples

(A) Binding of ex-50.T on exosome from serum samples of 6 BC patients and 6 non-tumoral patients. Pt.72 normal epithelial breast cell-derived exosomes were used as a control. Error bars indicate means ± SEMs. Statistics by t test (versus NE): **p < 0.01; ***p < 0.001; ****p < 0.0001. (B) Binding of exo-50.T or unrelated control aptamers (CtrlApt) was measured at 200 nM by ELONA-based assay on plate left uncoated (–) or coated with exosomes derived from patient sera. Error bars indicate means ± SEMs. Statistics by t test (versus #124 non-tumoral sample): *p < 0.05; **p < 0.01; ***p < 0.001. In (A) and (B), BH, non-tumoral breast hyperplasia; Exo, exosomes; N-OP, non-oncologic pathologies.

that is required for their clinical applicability. The described ex-50.T aptamer shows great potential for BC exosome detection and can be optimized for its development within highly sensible aptamer-based biosensors for the detection of exosomes directly from serum samples for liquid biopsy.[37,38] At the same time, the aptamer can provide an innovative therapeutic tool for specific BC exosome removal. It has been demonstrated that tumor cell-derived exosomes acting on the surrounding cell population can favor tumor progression and spread. In this view, our results demonstrating the ability of aptamers to inhibit the cell migration of non-metastatic BC or normal breast

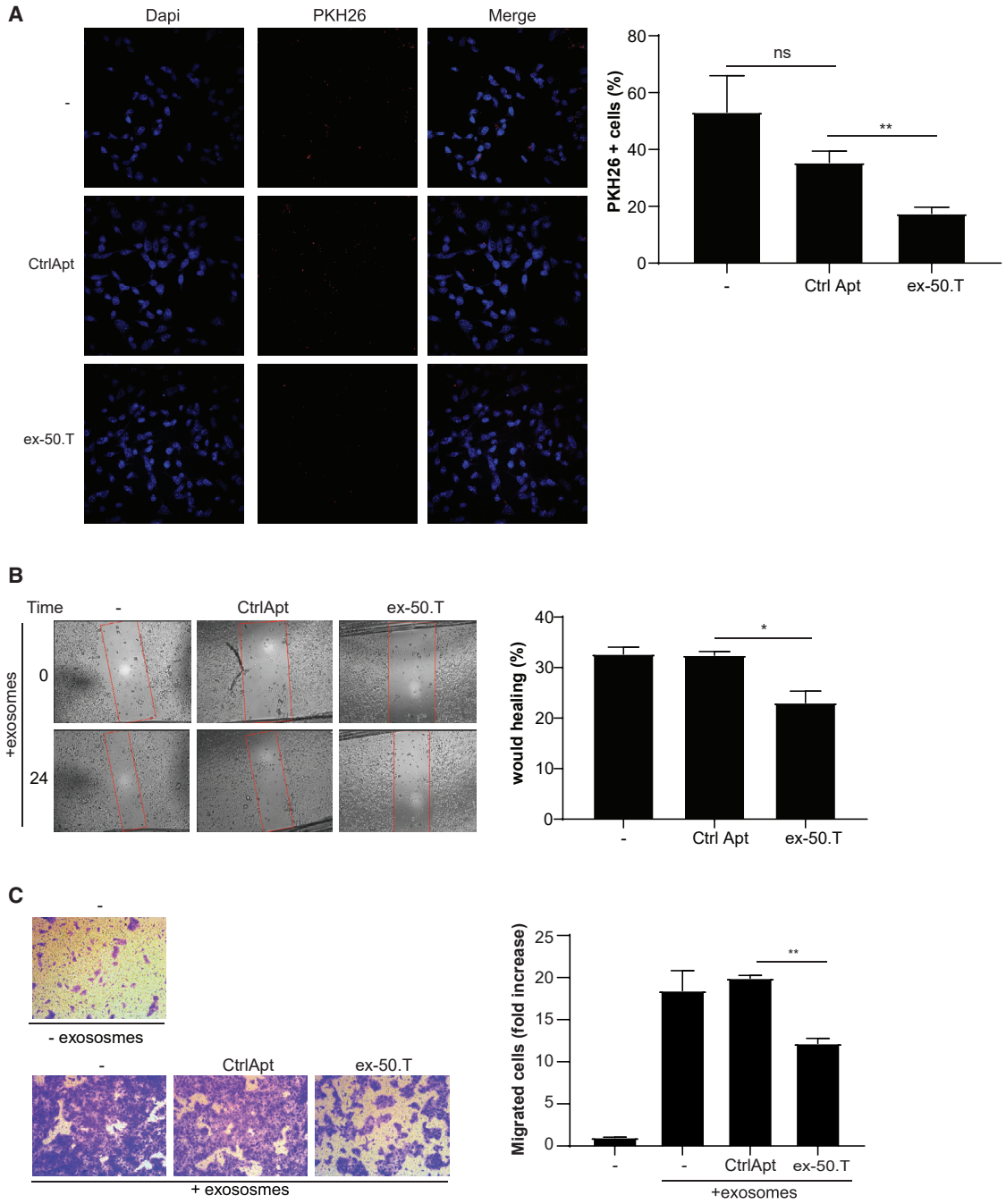

Figure 6. Ex-50.T aptamer functional activity

(A) MDA-MB-231 cells were treated for 3 h with MDA-MB-231-derived PKH26-labeled exosomes pre-incubated or not (–) with 400 nM ex-50.T or control aptamer (CtrlApt). Exosome internalization was visualized by confocal microscopy. Left, cell nuclei are visualized with DAPI (blue) and exosomes with PKH26 (red). Magnification 40×. Right, percentage of $PKH26^+$ cells (red) over total cells (blue) was calculated. (B) MCF-7 cells were grown in 10% FBS exosome-free medium and then scraped to induce a wound. Cells were maintained for 24 h with 50 μg MDA-MB-231-derived exosomes pre-incubated or not (–) with 200 nM ex-50.T or CtrlApt. Left panel, pictures were taken immediately after wound creation (0) or following 24 h (24). Right panel, percentage of wound healing area disclosure was calculated considering at least 2 different fields. (C) MCF10A cells were seeded into the upper chamber of a 24-well Transwell in the absence or presence of MDA-MB-231-derived exosomes pre-incubated or not (–) with ex-50.T or CtrlApt (200 nM). The percentage of migrated cells was evaluated after 24 h. Left panels, representative micrographs. Right panel, migrated cells were quantified and expressed relative to cell migration without exosomes. In (A)–(C), Error bars depict SDs, statistics by t test: *$p < 0.05$; **$p < 0.01$.

epithelial cells induced by exosomes derived from highly metastatic cells, suggest the potential of ex-50.T to impair tumor progression by reducing exosome exchange.

The developed strategy has a wide applicability to different tumor types as well as to different clinical problems, including cardiovascular diseases and neurological disorders that may benefit from exosome targeting.[39–41] Furthermore, it affords the possibility of identifying aptamer targets by finding new signatures specifically associated with a particular tumor type or condition. In conclusion, by using an innovative selection strategy, we isolated a molecule with the concrete potential to open new horizons for the development of innovative approaches for BC early detection and personalized therapy based on the selective cancer exosome targeting.

MATERIALS AND METHODS

Cell lines and primary cultures

Primary epithelial BC or normal (normal breast hyperplasia) cultures were prepared from human biopsy surgical samples (samples from the Mediterranea Cardiocentro), as previously reported.[9] Briefly, tissues were mechanically fragmented by cutting with sterile scissors and extracellular matrix was digested with collagenase (Sigma-Aldrich) overnight at 37°C with agitation. Cells were separated by centrifugation at 500 rpm for 5 min to obtain epithelial cell pellets. Cells were maintained in Dulbecco's modified Eagle's medium/Nutrient F12-Ham (DMEM-F12) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich).

Continuous cell lines were purchased from the ATCC (LG Standards) and grown in the following media: Roswell Park Memorial Institute (RPMI) medium for human breast T47D, BT549, and MDA-MB-231, and human NSCLC A549 and H460; DMEM for human breast MCF-7, human NSCLC Calu-1, and human GBM U87MG and U251MG; advanced DMEM for human GBM LN-229; and DMEM/F12 supplemented with EGF (20 ng/mL), hydrocortisone (0.5 μg/mL), cholera toxin (100 ng/mL), insulin (10 μg/mL), and horse serum (5%) for human breast MCF10A. All of the media were supplemented with 10% FBS from Sigma-Aldrich. All of the cells were grown at 37°C in 95% air/5% CO_2.

Exosome isolation

Exosomes were isolated from culture media of cells grown with 10% Exo-FBS (FBS depleted of exosomes, System Biosciences) in a 150-mm plate (15 mL medium volume) with ExoQuick-TC Exosome Isolation Reagent (System Biosciences), according to the manufacturer' s instruction.

RNA preparation and *in vitro* transcription

For the SELEX procedure, we used a library of 2′-F-Py-modified RNA library that was prepared from the correspondent DNA pool (from TriLink Biotech) containing a central random region of 40 nt flanked by 2 constant parts for amplification and transcription. Libraries or individual long sequences were PCR amplified using the following oligonucleotides: forward primer (with T7 RNA polymerase promoter), 5′-TTCAGGTAATACGACTCACTATAGGGAAGAGAAGGACATATGAT-3′, and reverse primer: 5′-TCAAGTGGTCATGTACTAGTCAA-3′. The program used was 10 cycles of 30 s at 95°C, 30 s at 64°C, and 1 min at 72°C.

In vitro transcription of library or individual sequences was performed in the presence of 1 mM 2′-F pyrimidines using a mutant form of T7 RNA polymerase (Epicenter Biotechnologies). DNA template was incubated at 37°C overnight in a transcription mix containing transcription buffer 1×, 1 mM 2′F-Py (2′F-2′-dCTP and 2′F-2′-dUTP, TriLink Biotech), 1 mM ATP, 1 mM guanosine triphosphate (GTP) (Thermo Fisher Scientific), 10 mM dithiothreitol (Thermo Fisher Scientific), 0.5 μg/μL RNase inhibitors (Roche), 5 μg/μL inorganic pyrophosphatase (Roche), and 1.5 μg/μL of the mutant T7 RNA polymerase. After transcription, any leftover DNAs were removed by DNase I (Roche) digestion, and the resulting sequences were purified by phenol:chloroform extraction and ethanol precipitation. The RNAs were run on a denaturing 8% acrylamide/7 M urea gel and the band of expected size was cut out from the gel and eluted with 0.3 M sodium acetate and 2 mM ethylenediaminetetraacetic acid at 42°C. The purified 2′F-Py RNA library was quantified with NanoDrop UV-Vis Spectrophotometer (Thermo Fisher Scientific). Short aptamers 2′-F-Py-modified were purchased from Tebu-bio srl. Aptamer sequences are reported in Table S4. Biotin was added at the 3′ end of the aptamer.

Exo-SELEX

For the SELEX strategy, the exosomes were isolated from cells (15 mL medium), quantified by standard Bradford (Bio-Rad) and 40 μg were conjugated with magnetic beads marked with anti-CD81 antibody (Thermo Fisher Scientific) at 4°C overnight with gentle rotation. Before each cycle of SELEX, 2′F-Py RNA pool was dissolved in PBS and subjected to denaturation/renaturation steps of 85°C for 5 min, ice for 2 min, and 37°C for 10 min. The pool was first incubated for 30 min at room temperature with exosomes from NE bound to magnetic beads with gentle rotation (counter-selection step). Unbound RNAs were recovered by using a magnetic separator (Millipore) and incubated with beads-conjugated tumor-derived exosomes for 30 min with gentle rotation (selection step). Aptamer-exosome complexes were recovered with the magnetic separator, washed with PBS, and suspended in euroGOLD TriFast (Euroclone) for RNA extraction. Recovered RNA was amplified by RT-PCR and transcribed for the following round of SELEX (see Table S1 for details). When exosomes from multiple lines were included in the cycle, an equal amount of exosomes (for a total of 40 μg) from each sample was pooled and used as target.

Reverse transcription was performed by using M-MuLV enzyme reverse transcriptase (RT; Roche). The obtained product was then amplified by error-prone PCR reaction in the presence of high $MgCl_2$ (7.5 mM) and dNTP (1 mM) concentrations to introduce random mutations. The PCR program was 30 s at 95°C, 1 min at 64°C, and 30 s at 72°C.

Cloning of individual aptamers

The final SELEX pool was cloned with the TOPO-TA Cloning Kit (Thermo Fisher Scientific), according to the manufacturer's protocol. Transformation was performed with the *Escherichia coli* DH5α strain (Thermo Fisher Scientific). Single white clones were grown, DNA was extracted with the plasmid MINIPREP kit (QIAGEN) and sequenced by Eurofins Genomics.

Sequence analysis

Individual aptamer sequences were aligned by using the Multiple Sequence Alignment tool (ClustalW2) and were clustered with TreeView to identify aptamer sequence similarities. Aptamer secondary structures were analyzed by the RNAfold algorithm (http://rna.tbi.univie.ac.at).

Binding assay by quantitative real-time PCR

For binding assays on exosomes, RNA sequences were dissolved in PBS, subjected to denaturation/renaturation steps, and incubated at a 200-nM concentration with exosomes bound (13 μg) to magnetic beads for 30 min with gentle rotation at room temperature. Unbound RNAs were removed with the magnetic separator by washing 4 times with PBS.

Binding assays on cells were performed by seeding 2×10^5 cells (in 6-well plates). Cells were incubated with 200 nM aptamer in serum-free medium for 30 min at 37°C. Unbound RNAs were removed by washing 3 times with serum-free medium.

Bound RNAs were recovered by TRIzol containing 0.5 pmol/mL of an unrelated aptamer (CL4: 5′-GCCUUAGUAACGUGCUUUGAUGUCGAUUCGACAGGAGGC-3′) used as a reference control and amplified. Briefly, recovered RNAs were reverse transcribed using M-MuLV enzyme RT and reverse specific primers with the following protocol: heating step at 65°C for 5 min, annealing step at 22°C for 5 min, extension at 42°C for 30 min followed by end extension at 48°C for 30 min, and enzyme inactivation at 95°C for 5 min. The products from the RT reaction were PCR amplified with iQ SYBR Green Supermix (Bio-Rad). The protocol was heating step at 95°C for 2 min, followed by 40 cycles of heating at 95°C for 30 s, annealing at 55°C for 30 s, and extending at 60°C for 30 s. A melt curve stage by heating at 60°C–95°C was performed. The amounts of recovered RNA were calculated relative to a standard curve of known RNA input and normalized to the CL4 reference control.

ELONA-based assay

For ELONA, microtiter high binding plate (Nunc MaxiSorp) wells were left uncoated or coated with exosomes (4 μg) by overnight incubation. All of the subsequent steps were performed at room temperature. After incubation, the plate was blocked with 300 μL 3% BSA (AppliChem) in PBS for 2 h and incubated with 100 μL biotinylated aptamers (Tebu-bio) at 200 nM or increasing concentrations (ranging from 0.1 to 200 nM), dissolved in PBS for 2 h. Then, the plate was washed 3 times with 300 μL PBS, and streptavidin-conjugated HRP (Sigma-Aldrich) dissolved in 100 μL PBS at a 1:10,000 dilution was added. After a 1-h incubation, the plate was washed 3 times before the addition of the 3,3,5,5-tetramethylbenzidine substrate solution. The reaction was stopped with 0.16 M sulfuric acid. The signal intensity was analyzed by measuring the absorbance at 450 nm with a microplate reader (Thermo Fisher Scientific).

In order to test aptamer binding on serial exosome dilution, exosomes from MDA-MB-231 were quantified by NTA and serial concentrations (ranging from 0.1 to 100×10^6 particles/mL) were used for plate coating. Following the blocking, the plate was incubated with 500 nM biotinylated aptamer and processed as above. Binding affinity was determined with the GraphPad Prism program.

To check aptamer binding to HSA, plate coating was performed at 30 nM with HSA from Sigma-Aldrich.

Binding assay on exosomes form serum BC samples

Serum samples (Tables S2 and S3) were collected and stored at −80°C on the day of surgical intervention. Exosomes were extracted with the Serum/Plasma Exosome Isolation kit from Norgen Biotek. A total of 13 μg exosomes were coated on 15 μL of CD81 magnetic beads overnight at 4°C. Samples were processed for the binding assay by quantitative real-time PCR as reported in the previous section.

For ELONA, plates were left uncoated or coated with 40 μg serum exosomes overnight at 4°C. The assay was then performed as described above.

Serum stability

To check serum stability, the aptamer was incubated at 4 μM in 85% human serum (Type AB Human Serum, Euroclone) from 1 to 96 h. At each time point, RNA (16 pmol) was recovered, and to remove serum proteins, samples were incubated for 1 h at 37°C with 2 μL of Proteinase K solution (600 mAU/mL). Then, electrophoretic migration into 15% denaturing polyacrylamide gel was performed, the gel was stained with ethidium bromide, and was visualized by UV exposure. The intensity of the bands was quantified with the ImageJ (NIH) program.

Immunofluorescence and FACS analyses

The day before, MDA-MB-231 cells were grown on glass coverslips in 24-well plates in medium supplemented with 10% exosome-free FBS. Exosomes were isolated from MDA-MB-231 cells with ExoQuick-TC Exosome Isolation Reagent (System Biosciences) and then labeled with PKH26, a red fluorescent cell membrane linker (Sigma-Aldrich). Briefly, exosomes were stained for 5 min at room temperature with PKH26 (diluted 1:1,000 in PBS) and then an equal volume of 1% BSA was added to stop the labeling reaction. To recover labeled exosomes, samples were ultracentrifuged for 70 min at 100,000 × *g* at 4°C and washed with 4 mL PBS. The pellet containing labeled exosomes was resuspended in FBS-free culture medium and quantified by standard Bradford (Bio-Rad). Suspended exosomes (40 μg for each experimental point) were incubated in the dark with folded aptamers (400 nM) for 30 min with gentle rotation at room temperature. Cells

were treated for 3 h with exosomes pre-incubated or not with ex-50.T aptamer or CtrlApt.

For immunofluorescence, cells were washed 3 times with PBS and fixed with a freshly prepared ice-cold mix of methanol/acetone (1:1) for 10 min at −20°C. The coverslips were washed twice with PBS and mounted with gold antifade reagent with DAPI (Thermo Fisher Scientific). The cells were visualized by confocal microscopy. The percentage of exosome-positive cells (red signal from PKH26) over total cells (nuclei visualized with DAPI) was calculated considering at least 3 different fields for each slide. Alternatively, cells were recovered and analyzed by FACS (BD Accuri C6).

Wound healing assay

MCF-7 breast cancer cells (2×10^5) were seeded in 24-well plates in DMEM supplemented with 10% exosome-free FBS. Following 24 h, cells were scraped to induce a wound, washed with PBS, and treated with 50 μg MDA-MB-231-derived exosomes pre-incubated or not with ex-50.T aptamer or CtrlApt (200 nM) in serum-free DMEM medium. Pictures were taken immediately after wound creation or following 24 h.

Boyden chamber assay

MCF10A cells (5×10^4 cells/point) were seeded into the upper chamber of a 24-well Transwell plate (Corning) in DMEM/F12 supplemented with 0.5% exosome-free FBS containing 20 μg MDA-MB-231-derived exosomes pre-incubated or not with ex-50.T aptamer or CtrlApt (200 nM). A total of 10% FBS medium was used as a chemoattractant. Following 24 h incubation at 37°C under humidified 5% CO_2, migrated cells were stained with 0.1% Crystal Violet in 25% methanol. The percentage of migrated cells was evaluated by ImageJ.

Cell viability

MCF10A cells (1×10^4 cells/point) were seeded into 96-well plates (Corning) in DMEM/F12 supplemented with 5% exosome-free FBS, EGF (20 ng/mL), hydrocortisone (0.5 μg/mL), cholera toxin (100 ng/mL), and insulin (10 μg/mL) in the absence or presence of 4 μg MDA-MB-231-derived exosomes pre-incubated or not with ex-50.T aptamer or CtrlApt (200 nM). Following 24 or 48 h of incubation, cell viability was measured with CellTiter 96 Proliferation Assay (Promega).

Ethics

The study was conducted according to the criteria set by the Declaration of Helsinki and each subject signed an informed consent form before participating in the study. The study was approved by the research ethics committee of the University of Naples "Federico II" (no. 119/15ES1).

Statistical analysis

Statistics were analyzed with the Student's t test for comparisons between two groups, as indicated. $p < 0.05$ was considered significant. Statistical details of the experiments can be found in the figure legends.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.omtn.2021.01.012.

ACKNOWLEDGMENTS

We wish to thank L. Baraldi, F. Moscato, and F. De Micco for technical assistance. This work was supported by funds from the Associazione Italiana Ricerca sul Cancro (AIRC) (IG 2016 no. 18473 to G.C.); the Friends for an Earlier Breast Cancer Test (Earlier.org) Fundation to G.C.; H2020-MSCA-RISE-2019 cONCReTE (872391), H2020-MSCA-RISE-2019 PRISAR2 (872860), H2020-MSCA-RISE-2017 CANCER (777682), and MSCA-ITN-ETN PAVE (861190) to G.C.; and the Regione Campania Project PO FESR 2014-2020-SATIN to C.L.E. and G.C. The graphical abstract was created with BioRender (https://biorender.com)

AUTHOR CONTRIBUTIONS

C.L.E. performed most of the experiments, designed the study, analyzed the data, and prepared the manuscript. C.Q., F.I., and S.C. performed part of the experiments and participated in editing the manuscript. D.R., G.R., and S.N. provided experimental support. R.T. provided the human BC samples. V.d.F. provided conceptual advice, analyzed the data, and participated in editing the manuscript. G.C. analyzed the data and coordinated the research, was responsible for funding, and revised the manuscript. All of the authors read and approved the final manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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