Mesenchymal stem cell-derived exosomes stimulates cycling quiescence and early breast cancer dormancy in bone marrow

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Running Title: MSC Exosomes in Cancer Dormancy

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ABSTRACT

Dormant breast cancers (BRCA) resurge as metastatic disease after a long dormancy period in the bone marrow where cancer cells interact with mesenchymal stem cells (MSC). However, the nature of early interactions between BRCA cells and MSC in the bone marrow microenvironment that facilitate adaptation to a quiescent state remain poorly understood. Here we report that BRCA cells prime MSC to release exosomes containing distinct miRNA contents such as miR-222/223, which in turn promotes quiescence in a subset of cancer cells and confers drug resistance. Building on these results, we developed a novel, non-toxic therapeutic strategy to target dormant BRCA cells based on systemic administration of MSC loaded with antagomiR-222/223. In an immune-deficient mouse model of dormant breast cancer, this therapy sensitized BRCA cells to carboplatin-based therapy and increased host survival. Overall, our findings illuminate the nature of the regulatory interactions between BRCA cells and MSC in the evolution of tumor dormancy and resurgence in the micrometastatic microenvironment of the bone marrow.
Introduction

Breast cancer (BC) recurrence continues to pose a major clinical problem, despite significant advancement in early diagnosis and, an aggressive mode of treatment. It is widely accepted that BC recurrence is linked to a prolonged dormancy and successful survival of BC cells (BCCs) in the bone marrow (BM). In such state, the BCCs are in mitotic arrest and resist anti-cycling treatments (1-8). To prevent the recurrence of BC, one potential novel approach includes targeting the dormant BCCs and/or restrain BCCs from establishing dormancy. However, because the region of dormancy is also the home of the endogenous hematopoietic stem cells in the BM, this approach poses a major challenge for successful targeting of the BCCs without untoward effects on the endogenous stem cells (4, 7, 9).

Developing treatments for dormant BCCs in the BM is particularly important because of similar location and non-cycling phase with the hematopoietic stem cells. Therefore, for the next major advance in this area, we must first have a deeper mechanistic insight of events that allow BCCs to adapt a dormant phenotype in the BM milieu. Dormant BCCs have been identified within the stromal compartment close to the endosteum (10). BM stroma establishes gap junctional intercellular communication (GJIC) with BCCs and this partly explains how the latter survives as cycling quiescent cells in the BM (11). The GJIC allows miRNAs to be exchanged between BCCs and stroma (7, 11). Interestingly, mesenchymal stem cells (MSCs) can also communicate with BCCs through GJIC (12). GJIC occurs subsequent to the interaction between membrane-bound CXCR4 and CXCL12 (12, 13). Such intercellular contact creates a functional cellular chaperone that allows the MSCs to provide immune protection for the BCCs (14). In addition, MSCs can also support the survival and growth of cancer cells by their ability to differentiate into cancer-associated fibroblasts (15, 16).
Although much of our current understanding of the BC dormancy is derived from interactions between BCCs and stroma (12-14), the nature of interactions between the BCCs and cells in the BM microenvironment during the early phase of entry into the cavity and how BCCs adapt to cycling quiescence state remain poorly understood. MSCs are proposed to be an early source of cellular interaction with BCCs because of their anatomical location within the cavity. BCCs initially encounter MSCs after traversing the periphery into the BM cavity as MSCs are anatomically located at the abluminal surface of the central vasculature (17, 18). Furthermore, even if the BCCs bypass the MSCs within the perivascular region, they can still encounter MSCs that can be located in contact with the trabeculae and/or close to the endosteum (19, 20). Thus, an understanding of how BCCs and MSCs interact during the early stages of dormancy is highly significant to develop treatment strategies.

Here we investigated the mechanisms by which MSCs communicate with BCCs through exosomes to impart cycling quiescence. We report on BCCs priming of MSCs to release exosomes with a specific profile of miRNAs. We further discovered that the miRNA contents of exosomes are responsible for conferring a cellular quiescence in a subset of BCCs. We next applied these findings to develop a therapeutic strategy to target dormant BCCs in the femurs of immune-deficient mice as a physiological relevant experimental model. To this effect, we present evidence suggesting that MSC-loaded antago-miRNAs and reduced chemotherapy eradicated BCCs in the femurs of mice.

**MATERIALS AND METHODS**

**Cell Lines**

MDA-MB-231 and T47D were purchased from American Type Culture Collection (ATCC) and cultured as per their instructions. All cell lines used in this study were tested by
Genetica DNA Laboratories (Burlington, NC). Both cells were validated as the original cells using ATCC STR database (www.atcc.org/STR_Database.aspx)).

**Anti-miR-222 and -223 transfected MSCs**

MSCs were co-transfected with anti-miR-223 and anti-miR-222 or negative control anti-miR using Lipofectamine RNAiMAX reagent (Life Technologies Invitrogen, Carlsbad, CA). After 24 h, the transfectants were incubated at 37°C for 30 min with serum-free media containing 10μM of Cell Tracker Dye CMTMR. After this, the labeled cells were washed twice with PBS.

**Preparation of BCC-primed MSCs/naïve MSCs/Stimulation of BCCs**

Transwell cultures were established in 6-well plates with 0.4 micron inserts. Equal amounts (6x10⁴) of BCCs and MSCs were added to the outer and inner wells, respectively, in DMEM with 10% exosome-free FCS. After 24 h, the inner wells with BCCs were discarded. The MSCs were washed with PBS and then incubated with DMEM with 2% exosome-free FCS. At 48 h, exosomes were isolated from the MSC media. The exosomes from triplicate wells were added to duplicate naïve BCCs at 2.5x10⁵/well of 6-well plates. The BCCs were cultured in 2% exosome-free FCS. After 48 h, the cells were analyzed for cell cycle phase or protein.

Naïve exosomes were collected in 80% confluent MSCs cultured in 2% exosome-free FCS over a 48 h period.

**Exosome Isolation**

Exosomes were collected by differential centrifugation, as described (21), or using an Exosome Isolation kit from Life Technologies Invitrogen. The average particle size was 96.1 nm at 1/100, resulting in about 2x10⁸ particles/mL media, using the NanoSight.

**Cell cycle analyses**

Cell cycle analyses were performed as described (11): labeling with propidium iodide
(PI) for total cycling status. Go and G1 phases were discerned by co-labeling with 7-AAD and Pyronin Y.

**MiRNA array analyses**

The Human miFinder miRNA PCR Array (Qiagen, Valencia, CA) was run according to manufacturers instructions with RNA isolated using the miRNeasy Mini Kit. RNA (250 ng) was converted to cDNA with the miScript II reverse transcription reaction using HiSpec Buffer. The cDNA was used as templates in real-time PCR using the Human miRNome miScript miRNA PCR Array (miScript). The PCR array used the miScript SYBR Green PCR Kit. The PCR was run on the 7300 Real-Time PCR System using the following thermal-cycling parameters: 94°C for 15 min, 40 cycles at 94°C for 10 sec, 55°C for 30s, 70°C for 30 sec followed by a melting curve analysis.

The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (22) and are accessible through GEO Series accession number GSE85341 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE85341). The data were and were analyzed with the online miScript miRNA PCR Array data analysis tool (http://www.sabiosciences.com/mirnaArrayDataAnalysis.php). To minimize the potential noise introduced by measurements below detection threshold, miRNAs with Ct value greater than 35 in all groups were considered as undetected (23-25). Specifically, the expression levels of miRNAs were evaluated by a comparative Ct method using global median of expressed miRNA on the plate for normalization. The data were only used if the output passed the quality control test with respect to array reproducibility and reverse transcriptase efficiency.

**In vivo Studies**

Female nude BALB/c mice (6 weeks) were obtained from Charles River (Wilmington,
MA) and then housed in an AALAC-accredited facility at Rutgers, New Jersey Medical School (Newark, NJ). The protocol was approved by the Institutional Animal Care and Use Committee, Rutgers School of Biomedical Health Sciences (Newark, NJ).

The method to establish BC dormancy was previously described (7). Briefly, $10^3$ of freshly sorted Oct4$^{\text{hi}}$ BCCs (MDA-MB-231 or T47D) in 0.2 mL PBS were injected in the tail veins of mice. The Oct4$^{\text{hi}}$ BCCs have been shown to have cancer stem cell functions (7). At days 2 and 4, the mice were injected intraperitoneally with low dose of carboplatin (2.5 mg/kg). At day 3, the Oct4$^{\text{hi}}$ BCCs were located within the endosteal regions of femurs (7). At day 6, the mice were injected intravenously with $10^6$ MSCs, transfected with anti-miR-222/-223 or control anti-miRNA. The MSCs were also labeled with the tracker dye CMTMR. At days 9 and 11, the mice were injected with 5 mg/kg carboplatin intraperitoneally. At day 18, the mice were euthanized and the femurs analyzed for the following: Real-time PCR for human PPIB; microscopic examination for fluorescence cells on tissues scraped from the endosteal region; immunohistochemistry for cytokeratin with paraffin-embedded decalcified femurs. Analyses with several house-keeping genes identified PPIB as the most sensitive and consistent with regards to the detection of human cells in mouse. Survival studies were done for 8 weeks with 8 mice.

Dormancy was established with BCCs, stably transfected with pEGFP1-Oct3/4 and Cx43 shRNA, as above. At 72 h later, the mice were injected intraperitoneally with 100 mg/kg AMD3100 and carboplatin using the same schedule as above. After treatment the mice were euthanized and the femurs removed for analyses described below.

**Statistical Analysis**

Statistical data analyses were performed with analysis of variance and Tukey-Kramer
RESULTS

Exosome transfer between MSCs and BCCs

We first characterized the MSC-derived exosomes by transmission electron microscopy (TEM) and Western blotting for two tetraspanin proteins, CD63 and CD81 (Figs 1A and 1B). The presence of the tetraspanins supported an endosomal origin of the secreted vesicles, consistent for exosomes nature of these vesicles (26, 27). We next validated the transfer of exosomes between MSCs and BCCs in a Transwell system separated by 0.4 micron membranes (Fig. 1C). BCCs and MSCs, stably transfected with pCD63-GFP, released green fluorescence exosomes, indicating that the analytical system used here could track exosome transfer (Figs 1D/E, Supplemental Figs 1B/C). The pCD63-GFP transfectants were designated as ‘Donors’ and the untransfected cells, ‘Receivers’. At 24 and 48 h post-culture, the released vesicles showed a sharp peak of mostly 98 nm vesicles, thereby eliminating the release of apoptotic bodies, which range at 500-5000 nm (26) (Fig. 1F, Supplemental Movie 1). Flow cytometry of receiving cells cultured for 48 h showed a right shift (red), validating successful transfer of exosomes from the donor cells (Fig. 1G). A corollary study with the donor cells in the lower chamber resulted in overlap with vector transfectant, indicating little transfer of exosomes against gravity (Fig. 1G). However, confocal images of Z-sections showed some upward transfer of the exosomes (Fig. 1H, Supplemental Movies 2/3). These results suggest that exosomes can be transferred against gravity, although at significantly reduced efficiency.

Cycling effects of naïve MSC-derived exosomes on BCCs

In order to study how MSC-derived exosomes affect the cycling effects of BCCs (7, 13,
we transferred exosomes from naïve MSCs (never exposed to BCCs) and then added them to fresh BCCs. After 48 h, western blots showed increases in cyclin D1 and CDK5 for MDA-MB-231 cells but no change in T47D cells (Fig. 2A; Supplemental Fig. 2). Propidium iodide labeling indicated that the naïve exosomes promoted the ratio of the S-phase for MDA-MB-231 cells and G2-phase for T47D cells (Fig. 2B). Further analyses, based on the DNA and RNA contents, indicated that the exosomes transitioned a subset of BCCs into cycling quiescence (Fig. 2C). T47D cells showed a small population in the G0 phase, based on low to undetectable RNA (red arrow) and another subset with relatively higher RNA content (G1-phase). Together, these findings suggested that exosomes from naïve MSCs enhanced a population of BCCs into cycling phase and a relatively smaller subset in the G0/G1 phase.

**Cycling effects by exosomes from BC-primed MSCs**

We next asked if exposure to BCCs can change the cycling effects of the MSC-derived exosomes (primed exosomes). We primed MSCs in a Transwell system as outlined in Fig. 3A. The exosomes from primed MSCs were added to fresh BCCs for cell cycle analyses. Propidium iodide labeling showed similarities between naïve and primed exosomes (Fig. 3B). Further analyses of the DNA and RNA contents revealed that the primed exosomes caused a significant ($p<0.05$) increase in low DNA in a subset of MDA-MB-231 cells (Fig. 3C). The population with low DNA was in G0 phase (low Pyronin-Y), increasing from 0.75% at baseline (no exosome) to 2.5% for naïve exosomes, then to 3.2% for primed exosomes (Fig. 3C). The effects of the primed exosomes seemed to be cell-specific since there was no marked difference for the less evasive and metastatic T47D cells (Fig. 3C). Western blot analyses of extracts from BCCs treated with primed exosomes showed decreases in CDK4, Cyclin D1 and p21WAF1 (Fig. 3D, Supplemental Fig. 3). Increased CDK6 was expected for the highly proliferating MDA-MB-231 cells since the
subset of cells in the G0 phase was relatively small (Fig. 3C). Together, primed exosomes were more effective in changing the cycling status of a subset of MDA-MB-231 cells but such difference was not seen in T47D cells.

**MiRNA containing exosomes support cycling changes in BCCs**

We investigated the possibility that the miRNA contents of exosomes were responsible for the noted cycling changes in BCCs. To address this, we collected exosomes from primed MSCs in which the pre-miRs could not mature. These studies were conducted with MSCs knocking down the endogenous dicer (Supplemental Fig. 4A). We found that the exosomes from these MSCs failed to impart cycling quiescence; instead, caused the majority of MDA-MB-231 cells to transition into the S phase, relative to control siRNA (Fig. 4A, Supplemental Fig. 4B). These studies revealed an inherent role for miRNAs in the noticed effect of exosomes in cycling quiescence of BCCs. Although pri-miR is nuclear, we nonetheless assess its level in the exosomes. Primers specific for pri-miR222 showed Ct values above the maximum threshold for exosomal RNA whereas similar evaluation in the source MSCs resulted in Ct, 26±0.03.

A heat map of miRNAs from exosomes of naïve and primed MSCs illustrated distinct miRs profiles (Fig. 4B). Scatter plots with the boundaries set at ±1.5 fold-changes indicated similarities as well as distinct differences between two groups (Figs. 4C/4D). Ingenuity pathway analyses of T47D primed/naïve MSCs demonstrated a network with genes linked to the G1/S phase and tumor proliferation (Supplementary Fig. 5A). Similarly, MDA-MB-231 cells primed exosomes narrowed miRNAs involved in the expression of cell cycle genes (Supplementary Fig. 5B). Overall, the predictive analyses were consistent with the functional studies showing a subset of BCCs transitioning into cycling quiescence by exosomes from BC-primed MSCs (Fig. 3).
Reverse dormancy by targeted miR-222/-223

We selected miRs-222 and -223 as potential targets to reverse the cycling quiescence of BCCs for drug sensitivity, based on the following reasoning: their roles in BCC quiescence in BM, increased levels in MSCs and MSC-derived exosomes, increases by 3-5 folds in primed relative to naïve exosomes, and the link of miR-222 to tumor resistance (11, 29) (Figs 4B/5A-5C, Supplemental Figs 6A/6B). Thus, miR222/223 could have a role in early development of cycling quiescence, leading to dormancy. Thus, we targeted these miRs by delivering the respective antagomiRs within MSCs in vivo (30-32). We first tested if the anti-miR can be released from MSCs in a Transwell system. Flow cytometry indicated efficient transfer of Cyanine 5 (Cy5)-tagged anti-miR-222 to ~78% BCCs after 48 h (Fig. 5D). Because the MSCs remained viable and did not migrate through the membrane, we concluded that the observed transfer of miRs was through microvesicles.

The function of the transferred anti-miR was assessed in drug-sensitivity analyses (29, 33). Primed MSCs were transfected with Cy5-anti-miR222 or Cy5-control anti-miR. Exosomes from these MSCs were transferred to fresh BCCs for assessment of total and active P-glycoprotein (P-gp). Using specific antibodies (34), flow cytometry analyses (Fig. 5E) indicated an increase in total P-gp (left) and reduced in the active form (right). These findings were consistent with dye efflux studies, which showed a significant ($p<0.05$) retention of Calcein by anti-miR222 containing exosomes, relative to control anti-miR (Fig. 5F). Together, these findings indicated that anti-miR222 influences the activity of a drug transporter in the BCCs to favor drug sensitivity.

A therapeutic efficacy of anti-miR-222/-223

We next developed a treatment strategy for dormant BCCs with anti-miR-222/-223 and a
lower dose of carboplatin in an *in vivo* model of BC dormancy (Fig. 6A). Mice were injected with CMTMR (orange/red) labeled MSCs, transfected with anti-miR-222/-223 or control anti-miR. After 48 h, the mice were injected intraperitoneally with reduced (<4x, 2.5 mg/kg) carboplatin or vehicle in 0.5 mL, twice at 2-day intervals (7, 13). After one week, the femurs were flushed and then examined for human cells. We expected few to undetectable dormant BCCs in the endosteal region (7). Technical control for murine GAPDH indicated Ct values, ~20 (Fig. 6B). Primers for human PPIB (Cyclophilin B) detected ≥5 BCCs/10^6 murine cells (Supplemental Fig. 7). Human cells were undetectable in the anti-miR/carboplatin-treated mice (Ct >40), as compared to Ct values of ~25 for mice given control anti-miR (Fig. 6B).

Since the injected dormant BCCs were transfected with Oct4^hi^-GFP (7), we examined the endosteal region for green fluorescent cells. Such cells were found in mice given control anti-miR and carboplatin (Fig. 6C, arrows, top/left panel). In contrast, mice given anti-miR-222/-223 and carboplatin were devoid of green cells (Fig. 6C, top/right panel). Since human cells were not detected in the femurs of mice given anti-miR-222/-223/carboplatin (Fig. 6B), we asked if the carboplatin killed the injected MSCs. A dose-response *in vitro* study with carboplatin and MSCs indicated survival at <50 μg/mL carboplatin (Supplemental Fig. 8). An examination of cells scraped from the endosteal region of the femurs identified clusters of CMTMR(+) cells in mice with control anti-miR as compared to few orange cells for anti-miR-222/-223 and carboplatin treatment (Fig. 6C, lower panels). The findings indicated that the MSCs migrate towards the dormant BCCs in the endosteum. Those loaded with antagomiRs became sensitive to carboplatin.

After establishing that anti-miR222/223 facilitated chemosensitivity in the dormant BCCs, we investigated whether this effect can be explained by increased cellular proliferation.
Sections of the decalcified femurs, labeled with anti-Ki67, identified a large number of positive cells in the endosteum of mice with antagomiRs as compared to undetectable Ki67 cells given control anti-miR (Fig. 6D, arrows). Additionally, we confirmed that the BCCs were targeted by labeling with a pan-cytokeratin antibody (brown spots). We observed several brown spots in mice with control anti-miR (arrows) with few spots for mice treated with anti-miR-222/-223 (Fig. 6E).

To determine if the dual treatment improved the survival of the receipt mice, we monitored the mice (n=8/group) for two months. Carboplatin and anti-miR-222/-223 treatment resulted in a total survival during the observational period. However, mice treated with carboplatin and control anti-miR succumbed at about week 3 (Fig. 6F, solid line). Taken together, these results showed an efficient delivery of anti-miR-222/-223 by BM-derived MSCs to femurs and that antagomiRs improved the efficiency of carboplatin by requiring a lower dose to target the BCCs.

**Therapeutic delivery of antagomiR**

Anti-miRs can be transferred from MSCs to BCCs through exosomes, and GJIC (11, 12, 32). We experimentally tested the possibility that MSCs could deliver anti-miR-222/-223 to the dormant BCCs through GJIC-dependent and independent methods using the strategy outlined in Fig. 7A. Dormancy was established with Oct4(hi) (green) BCCs, previously shown to be cancer stem cell, or with similar BCCs knocked down for Cx43 (shRNA/red). This allowed for studies in which GJIC between MSCs and BCCs can be formed or blunted (7). The mice were injected intravenously with MSCs transfected with anti-miR-222/-223 or control anti-miR. In the absence of GJIC, CXCL12 can be increased to enhance the proliferation of BCCs (11, 35) Thus, to maintain cycling quiescence of the CX43 knockdown BCCs, we preserved low activity of
CXCL12 by treating with vehicle or AMD3100. Also, AMD3100 prevented BCCs from interacting with MSCs through membrane CXCL12 and CXCR4 (13).

The close location between the dormant BCCs (green) and Far Red-labeled MSCs was indicated by yellow fluorescence (Fig. 7B, inset in top left panel). One week after treatment, yellow cells (Oct4\textsuperscript{hi} with Cx43 shRNA) were markedly reduced by anti-miR-222/-223/carboplatin/AMD3100 treatment (Fig. 7B, lower right panel). The remaining red/green cells suggested that the antagomiRs may also require GJIC for transfer to the BCCs. These findings were also independently validated when the decalcified femurs were labeled for cytokeratin (Fig. 7C). The arrows showed the alkaline phosphatase (brown) positive BCCs. In brief, we found that MSCs also release anti-miR-223/-222 to the surrounding BCCs. In addition, the noticed transfer of anti-miRs could occur through both GJIC-dependent or -independent manner.

**Discussion**

Findings presented here reveal how MSCs initiate a quiescent phenotype in BCCs, and opened up a new avenue to target miRNA within stem cells for therapeutic gains. We also found that MSCs can be effectively used as a cellular delivery system for antagomiRs to enhance the therapeutic efficacy of chemotherapy. These findings are particularly relevant to targeting dormant BCCs in the BM, which can survive for more than a decade. We provide evidence to demonstrate how BCCs instruct MSCs to release exosomes with distinct miRNAs, resulting in cycling quiescence of a subset of BCCs. These findings are different from other reports that showed BC-derived exosomes preparing metastatic sites (36). The analyses for the cell cycle-linked genes, although seemed inconsistent, can be reconciled because the effects of the exosomes occurred in a relatively small subset of BCC (Figs 2A/3D). Although miR-222/-223 were able to reverse the proliferation of the dormant BCCs (Fig. 6D), it is unclear if these
miRNAs are targets for the cyclins. Hence, our findings highlight the need to further identify the nature of such subsets of BCCs and to dissect how distinct subsets communicate with MSCs (7, 37, 38).

The Transwell model recapitulated early communication between MSCs and BCCs entering the BM, and allowed us to study non-contact interactions between MSCs and BCCs (12, 17). The overall findings on miRNAs, including the distinct profiles within exosomes from naïve and BC-primed MSCs suggested that the BCCs might influence the MSCs to favor their survival (Fig. 4). The results further support a key role for the miRNA content of exosomes in the cycling phase of BCCs (Supplemental Figs. 4 and 5). Both naïve and primed exosomes exerted cycling quiescence in MDA-MB-231, but there was an increase with primed exosomes (Fig. 2). This supported a role for MSCs in the immediate response to facilitate BCC survival. Future studies to dissect the BCC subsets are needed to understand how MSCs might change the heterogeneity of BCCs for drug evasion.

MiR222/223, shown to be involved in GJIC between BCCs and BM stroma, were increased in the primed exosomes (11). In vivo targeting of these miRNAs reversed the quiescent phase of BCCs, into chemosensitive cells (Figs 6 and 8). More importantly, anti-miR222/223 permitted us to use four times less carboplatin, with the mice surviving during the observational period (Figs. 6 and 7). The in vitro studies provided a possible explanation for the positive in vivo outcome with the anti-miRs. Indeed, anti-miR222 was able to retain Calcein in BCCs and decreased the expression of active P-gp, indicating drug sensitivity (Fig. 5).

In vivo studies presented here provide several major insights: Firstly, the MSCs were effective in homing to the BM, suggesting a memory of MSCs to home to their source organ’ Secondly, the effect of antagoniR-loaded MSCs was specific to the release of the anti-miR
rather than other endogenous factors because control anti-miR could not elicit a therapeutic effect (Fig. 6); Thirdly, the antagomiRs increased the sensitivity of the injected MSCs to carboplatin, suggesting a therapeutic strategy to eliminate injected MSCs after their use in drug delivery.

Studies in which Cx43 was knocked down indicated that the ‘cargo’ MSCs can transfer the antagomiRs to BCCs by GJIC and, in this case, to a lesser extent, by a GJIC-independent method (Fig. 7). Cx43 has been shown to be required for GJIC between MSCs and BCCs (12). These findings indicated that we might be able to use the described approach to treat all BCC subsets.

The findings presented here further enhance our understanding of how BCCs influence MSCs to release exosomes with distinct miRNAs. The BCCs could be instructing MSCs through soluble factors such as cytokines as well as exosomes. Studies on such two-way communication also explain how BCCs use BM microenvironmental cells for their survival. Since BM is also the site of hematopoiesis, studies are needed to determine the possible toxicity of such a treatment strategy to the hematopoietic system.

This study adds to the complex path towards BC dormancy. The findings complement results from other studies showing how MSCs protect BCCs from the immune response (14). The findings also show how BCCs, other than the cancer stem cells, might be aided by MSCs to exit dormancy (7, 39). In summary, these findings show BCCs priming MSCs to produce exosomes with distinct miRNA profiles. The exosomes enter BCCs to initiate cycling quiescence. The miRNAs can be targeted using MSCs to deliver antagomiRs to chemosensitize the BCCs and to prevent dormancy. The promising use of antagomiRs in combination with a reduced carboplatin dose was not toxic, since the mice survived. Our therapeutic approach is
consistent with the position taken by the International Society of Microvesicles on exosomes as focal consideration for the development of treatments (40).

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References


FIGURE LEGENDS

Figure 1. Characterization and intercellular transfer of exosomes. A) Representative TEM images of MSC-derived exosomes. B) Western blot for CD63 and CD81 with extracts from MSC-derived exosomes from two different donors. C) A Transwell system with donor cells, stably transfected with pCD63-GFP, and untransfected receiving cells. D) Flow cytometry for GFP with MDA-MB-231 cells, stably transfected with pCD63-GFP. E) Representative 3-dimensional (3-D) image of pCD63-GFP-MDA-MB-231, merged with DAPI images. F) Representative histogram of nanoparticle tracking analysis (NTA) with media from the Transwell cultures in ‘C’. G) Flow cytometry for GFP in the receiving cells in 48-h Transwell cultures from ‘C’. H) Confocal microscopy of >10 fields in ‘C’. Top panel: receiving cells are placed the upper wells; Bottom panel: receiving cells in the lower wells. White arrows: GFP (+) cells; Red arrows: GFP (-) cells.

Figure 2. Cell cycle analyses in BCCs treated with exosomes from naïve MSC. A) BCCs, untreated or treated with exosomes (+exo) from naïve MSCs. After 48 h, whole cell extracts were analyzed by western blot for cyclin-associated proteins and normalized for β-actin. B) BCCs were treated as for ‘A’ and then studied for cell cycle analyses by propidium iodide. The figure represents three experiments with exosomes collected different MSC donors. C) The studies in ‘B’ were repeated, except for co-labeling with 7-AAD and Pyronin-Y. The low DNA content (boxed region, left panels) were stratified for Pyronin-Y incorporation (right panels). The figure represents three experiments with exosomes from different MSC donors.

Figure 3. Cell cycle analyses of BCCs treated with naïve and primed exosomes. A) Shown is the method to prime MSCs with BCCs. The cells were separated by 0.2 micron membranes in exosome-free media. After 24 h, the inner well was discarded and the medium replaced.
Exosomes were collected in the media after 48 h for addition to fresh BCCs. **B)** At 48 h, BCCs treated with primed and naïve exosomes were analyzed for cell cycle by propidium iodide. **C)** The studies in ‘B’ were repeated with 7-AAD and Pyronin Y labeling. **D)** Representative of three experiments in which whole cell extracts from the cells in ‘B’ were analyzed by western blot for cyclin-associated proteins and β-actin.

**Figure 4. Cell cycle with exosomes from dicer knockdown MSCs; MiRNA array analyses with exosomes from naïve and BC-primed MSCs.** **A)** Exosomes from dicer- or control siRNA- transfected MSCs were added to BCCs for cycling analyses with 7-AAD and Pyronin Y. **B)** A cluster map is shown for the output of miRNA arrays from naïve and primed exosomes. **C** & **D)** Scatter plots with cut-offs at 1.5 folds are shown for miRNAs within exosomes of primed/naïve MSCs.

**Figure 5. MiR-222/-223 in cell cycle quiescence and drug resistance.** Real time PCR for miR-222/-223 with RNA from naïve exosomes. The results, are presented relative to HY3, mean±SD, n=4 (**A** & **B**). The level of MiR-222 is presented as primed/naïve exosomes, mean±SD, n=4 (**C**). Co-cultures were established with MSCs, transfected with Cy5-tagged anti-miR222 (anti-miR-222-Cy5) in the upper wells, and the receiving untransfected MDA-MB-231 or T47D cells in the lower wells (**D**, left). After 48 h, the BCCs were analyzed for Cy5 by flow cytometry. Representative figure is shown for MDA-MB-231 cells (**D**, right). MSCs were transfected with anti-miR-222 or control anti-miR and then primed with MDA-MB-231 cells. Exosomes were collected from the primed MSCs and then added to fresh MDA-MB-231 cells. After 48 h, the BCCs were analyzed by flow cytometry for total and active P-gp with specific antibodies (**E**). The studies in ‘E’ were repeated; instead the MDA-MB-231 cells were studied for calcein efflux.
Positive control contained verapamil. The data are presented as the mean fluorescence of retained dye±SD, n=5 (F). *p<0.05 vs. control anti-miR

**Figure 6. Therapeutic delivery of anti-miR.** A) Scheme for *in vivo* treatment of dormant BCCs. The MSCs with CMTMR (red) label were transfected anti-miR-222/-223. B) Real-time PCR for rodent GAPDH and human PPIB with total RNA from cells, flushed from the femurs of mice given control anti-miR or anti-miR-222/-223. The results are presented at the mean Ct values±SD, n=6. ND: not detected (Ct > 40). C) Cells scraped from the femurs of mice given control-anti-miR or anti-miR-222/-223 and carboplatin were examined under the EVOS fl fluorescence imager. Arrows show the presence of GFP cells; organ cells represent CMTMR-labeled MSCs. D) Sections of decalcified femurs from `C’ were labeled with anti-Ki67 and then counterstained with eosin. Arrows show Ki67(+) cells. E) Sections of decalcified femurs of mice given control anti-miR or anti-miR-222/-223 were labeled for human NuMA (brown) and then counterstained with Harris Modified Hematoxylin. Arrows represent NuMA (+) cells. F) Survival of mice (n=8) were followed after the last treatment with carboplatin and control miRNA or anti-miR-222/-223.

**Figure 7. Therapeutic delivery of anti-miR-222/-223 in dormant BCCs knockdown for Cx43.** A) BCCs were stably transfected with Oct4-GFP (green) and also knocked down for Cx43 (bright red). BCCs (10⁶) were injected intravenously in female nude BALB/c and dormancy was ensured as described in the Materials and Method section. The treatment schedule is shown in the diagram. B) At one week after treatment, the femurs were scraped at the endosteal region and then examined with the EVOS fl fluorescence imager. C) The femurs from `B’ were decalcified and the sections were labeled for pan-cytokeratin (brown, arrows) followed by counterstained with Eosin-Hematoxylin. Arrows depict cytokeratin (+) cells.
A. | MDA-MB-231 | T47D
---|---|---
Naive Exosomes: | - | +  + | -  +
| Cyclin D1 | | |
| CDK4 | | |
| CDK6 | | |
| p15 | | |
| β-actin | | |

B. | MDA-MB-231 | T47D
---|---|---
| +Exo | +Exo |

C. | MDA-MB-231 | T47D
---|---|---
| 38% G1/G0 | 37% G1/G0 |
Figure 3

A.

B.

C.

D.

CDK4
Cyclin D1
p21
CDK6
β-actin

T47D
MDA-MB-231
Figure 7

A. BCCs
- phOCT4-EGFP1/CD43 shRNA (red)

Far Red

**MSC**

anti-miR-222/223 or Control anti-miR

**Carboplatin**

Day 0

Days 2 & 4

Day 6

Days 9 and 11

Day 18

**AMD3100**

**Vehicle**

**AMD3100 + Control anti-miR**

**AMD3100 + Control anti-miR**

Survival

B. No treatment

Control anti-miR

Vehicle

Anti-miR222/223/

AMD3100

C. No treatment

AMD3100

Anti-miR-222/223

AMD3100 + anti-miR-222/223

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